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Phone: +49 7121 - 51 530 -0 Fax: +49 7121 - 51 530 16; E-Mail: info@nmi.de
Internet: www.nmi.de

Co-organiser
BIOPRO Baden-Württemberg GmbH
Breitscheidstraße 4, 70174 Stuttgart, Germany
Phone: +49 711 90715200; Fax: +49 711 90715202; E-Mail: info@bio-pro.de
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Organisation Team
Ira Digel, Nadja Gugeler, Priscilla Herrmann
E-Mail: meameeting@nmi.de
Internet: www.nmi.de/meameeting2006

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  1 Neuroengineering and Bio-nanoTechnologies Group – NBT, Department of Biophysical and Electronic Engineering - DIBE, University of Genova, Genova, Italy  
  2 Department of Informatics, Systems and Telematics – DIST, University of Genova, Genova, Italy  
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  1 Medical Microbiology, Immunology and Cell Biology,  
  2 Neurology, Southern Illinois University School of Medicine, Springfield, IL, USA  
  3 Bioengineering Department and Beckman Institute, University of Illinois at Urbana-Champaign, Beckman Institute, Urbana, IL, USA

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  1 Department of Neurology, University of Düsseldorf, Germany  
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ISBN 3-938345-02-0  
5th Int. Meeting on Substrate-Integrated Microelectrodes, 2006
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2 Neurobiology and Biophysics, Institute for Biology III, University of Freiburg, Freiburg, Germany
3 INSERM Unité 704, UJF Grenoble, St. Martin d’Hères, France

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2 Institute of Thin Films and Interfaces, Institute 2: Bioelectronics, and CNI – Center of Nanoelectronic Systems for Information Technology, Forschungszentrum Jülich GmbH, Jülich, Germany

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1 Hertie-Institut für klinische Hirnforschung, Zentrum für Neurologie, Universitätsklinikum Tübingen, Tübingen, Germany
2 Faculty of Medicine, Technion, P.O.B. 9649, Haifa 31096, Israel

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1 Biomedical Signal and Systems group, Department of Electrical Engineering, Mathematics and Computer Science, University of Twente, Enschede, The Netherlands

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Joost le Feber1*, Wim Rutten1, Jan Stegenga1, Pieter Wolters2, Ger Ramakers2, Jaap van Pelt2
1 Biomedical Signal and Systems Group, Department of Electrical Engineering, Mathematics and Computer Science, University of Twente, Enschede, The Netherlands
2 Netherlands Institute for Neurosciences, Amsterdam, The Netherlands

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Ricardo Escolá1,*, Christophe Pouzar2, Blaise Yvert3, Isabelle Magnin4, Régis Guillemaud5
1 CEA – LETI, Département de micro Technologies pour la Biologie et la Santé, Grenoble, France
2 Laboratoire de Physiologie Cérébrale (CNRS UMR 8118), UFR biomédicale de l’Université René Descartes, Paris, France
3 Laboratoire de Neurobiologie de Réseaux (CNRS UMR 5816), Université Bordeaux I, Talence, France
4 CREATIS (CNRS UMR 5515), Institut National des Sciences Appliquées de Lyon, Villeurbanne, France

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Antonio Novellino1, Alessandro Maccione2*, Mauro Gandolfo2, Michela Chiappalone2, Paolo Massobrio2, Sergio Martinoia1
1 R&D Lab, ett s.r.l., Genova, Italy
2 Neuroengineering and Bionano Technology Group, Department of Biophysics and Electronic Engineering, University of Genova, Genova, Italy

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Olaf Schröder1,*, Alexandra Gramowski2, Konstantin Jügelt2, Liane Mehnert2, Simone Stüwe2, Dieter G. Weiss2
1 PATTERN EXPERT, Borsdorf, Germany
2 Inst. of Biological Sciences, University of Rostock, Rostock, Germany

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René Schrott1,*, Gerold Bausch1, Klaus-Peter Kirchner1, Carsten Tautorat1, Werner Baumann2, Dieter Weiss3, Helmut Beikirch1
1 University of Rostock, Faculty of Computer Science and Electrical Engineering, Germany
2 University of Rostock, Institute of Biological Science, Biophysics, Germany
3 University of Rostock, Institute of Biological Science, Cell Biology and Biosystems Technology, Germany

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Leslie S. Smith
Department of Computing Science and Mathematics, University of Stirling, Stirling, Scotland, UK.

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Michael J. Berry II1,*, Ronen Segev, Elad Schneidman
Department of Molecular Biology, Princeton University, Princeton, NJ, USA

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Günther M. Zeck12, Richard H. Masland2
1 Max Planck Institute of Neurobiology, 82152 Martinsried, Germany
2 Howard Hughes Medical Institute, Massachusetts General Hospital, Boston, USA

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Darius Schwenger, Alfred Stett1*
Natural and Medical Sciences Institute at the University of Tuebingen, Reutlingen, Germany

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1 University Eye Hospital, Tuebingen, Germany
2 University Eye Hospital, Regensburg, Germany
3 Retina Implant GmbH, Reutlingen, Germany
4 STZ Biomedical Optics, Tuebingen, Germany
5 Natural and Medical Sciences Institute, Reutlingen, Germany
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1 Fachhochschule Kaiserslautern, Zweibrücken, Germany
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1 Experimental Ophthalmology, University Eye Hospital Tübingen, Germany
2 NMI Natural and Medical Sciences Institute, Reutlingen, Germany
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1 National Creative Research Initiative, Center for Neurodynamics and Department of Physics, Korea University, Seoul, Korea
2 Department of Electrical Engineering, Korea University, Seoul, Korea 136-701

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1 Lohmann Neuropharmaceutical Consulting, Castrop-Rauxel, Germany
2 Allg, Zool. & Neurobiol., Ruhr-University, Bochum, Germany
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Frank Sommerhage*, Mathias Schindler, Günter Wrobel, Sven Ingebrandt, Andreas Offenhäusser
Institute of Bio- and Nanosystems, IBN-2: Bioelectronics, and CNI – Center of Nanoelectronic Systems for Information Technology, Forschungszentrum Jülich GmbH, Jülich, Germany

Validation of the possible use of microelectrode arrays in pharmacological studies

Günter Wrobel1*, Chi-Kong Yeung2, Frank Sommerhage1, Mansun Chan1, Andreas Offenhäusser1, Sven Ingebrandt1
1 Institute of Bio- and Nanosystems, IBN-2: Bioelectronics, and CNI – Center of Nanoelectronic Systems for Information Technology, Forschungszentrum Jülich GmbH, Jülich, Germany
2 Department of Electrical and Electronic Engineering, The University of Science and Technology, Hong Kong

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Luc Stoppini1*, Heather Cater1, Rosy Bonfante1, Fay Thomas2, Thelma Biggs2, Janice Davies2 and Lars Sundstrom2
1 Capsant Neurotechnologies, Genenva, Switzerland
2 Capsant Neurotechnologies, Southampton, UK

Detecting neurotoxicity and neuropharmacological potential of compounds through electrical activity changes of neuronal networks on microelectrode arrays

Dieter G. Weiss1*, Alexandra Gramowski1, Simone Stüve1, Konstantin Jügelt1, Liane Mehnert1, Dietmar Schiffmann1, Olaf Schröder1
1 Institute of Biological Sciences, Cell Biology and Biosystems Technology, University of Rostock, Rostock, Germany
2 PATTERN EXPERT, Borsdorf, Germany

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Steffen Kandler and Ulrich Egert1
Neurobiology and Biophysics, Institute of Biology III & Bernstein Center for Computational Neuroscience, Albert-Ludwigs-University Freiburg, Freiburg, Germany

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Francesca Gullo, Enzo Wanke*
Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca, Milano, Italy.

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Guent Werner Gross1*, Sabnam Rijal-Oli2, Maryam Parviz1, Vern Jones1, Dayne Hollmuller1, and Michelle Karg2
1 Center for Network Neuroscience and Department of Biological Sciences, University of North Texas, Denton, TX; 2 Neurobiotex, Galveston, TX.

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Elke Guenther1*, Kathrin Henes1, Sandra Pankow1, Karlheinz Boven2, Thomas Meyer2
1 NMI Natural and Medical Sciences Institute at the University Tübingen, Reutlingen, Germany
2 Multi Channel Systems, Reutlingen, Germany

Remifentanil’s co-factor glycine causes NMDA receptor-dependent increase in murine frontal cortex network activity in vitro

Alexandra Gramowski1*, Frank Kletzin2, Rainer Hofmockel2, Gabriele F.E. Noeldge-Schomburg2 and Dieter G Weiss3
1 Institute for Biological Sciences, Cell Biology and Biosystems Technology, University of Rostock, Germany
2 Anesthesiology and Intensive Care, University of Rostock, Rostock, Germany

Cryopreserved mouse and rat cortical neurons on MEAs: Different electrophysiological properties?

Frauke Otto1*, Wiebke Fleischer1, Philipp Götz2, Anthony Krantis1, Mario Siebler1
1 Heinrich-Heine-University Düsseldorf, Institute of Neurology, Düsseldorf, Germany
2 Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ontario, Canada

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Maryam Parviz1, C.J. Fredericks2, and G.W. Gross2
1 Center for Network Neuroscience and Department of Biological Sciences, University of North Texas, Denton, TX; 2 Neurobiotex, Galveston, TX.

Impact of glutaric acid and 3-hydroxyglutaric acid on neuronal network activity: implications for neurological disturbance in glutaric aciduria type I

Andreas Schröter1, Wiebke Fleischer1, Anne Klusmann2, Mario Siebler1

ISBN 3-938345-02-0
5th Int. Meeting on Substrate-Integrated Microelectrodes, 2006
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Olga Sergeeva1*, Wiebke Fleischer2, Stephan Theiss2, Mario Siebler2, Helmut L. Haas1
1 Institute of Neurophysiology; Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany
2 Department of Neurology, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany

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Timothy J. Shafer1*, Sabnam 0. Rijal2, and Guenter W. Gross2
1 Neurotoxicology Division, U.S. Environmental Protection Agency, Research Triangle Park, NC, USA
2 Center for Network Neuroscience, University of North Texas, Denton, TX, USA

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Peter Fromherz
Department of Membrane and Neurophysics, Max Planck Institute for Biochemistry, Munich, Germany

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Joachim Wegener1*, Charles R. Keese2, Ivar Giaever2
1 Institut für Biochemie, Universität Münster, Germany
2 Applied Biophysics Inc., Troy (NY), USA

Combining the recording of neuronal activity with metabolic parameters: Sensorchips with MEA and integrated sensors for oxygen, pH and temperature

Johann Resslery1*, Helmut Grothe1, Martin Brischwein1, Florian Ichmann1, Michelle Karg1, Guenter W. Gross2, Bernhard Wolf1
1 Heinz Nixdorf-Lehrstuhl für Medizinische Elektronik, Technische Universität München, Deutschland
2 Department of Biological Sciences, University of North Texas, Denton, TX, USA

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Werner Baumann1*, Ralf Ehrel1, Elke Thedinga1, Axel Koflisch1, Andreas Keuer1, Sabine Drechsler1, Angela Podssun2, Philipp Köster2, Carsten Tauton2, René Schrot2, Helmut Beikirch1, Ingo Freund2 and Mirko Lehmann1
1 Bionas GmbH, Rostock, Germany
2 University of Rostock, Biophysics Dept., Rostock, Germany
3 University of Rostock, Faculty of Computer Science and Electrical Engineering, Rostock, Germany
4 Micronas GmbH, Freiburg, Germany

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Claus Burkhardt1, H. Wolburg1, K. Kohler3, H. Schmid3, R. Stoop1, Wilfried Nisch1
1 NMI Natural and Medical Sciences Institute at the University of Tuebingen, Reutlingen, Germany
2 Institute of Pathology, University of Tuebingen, Tuebingen, Germany
3 Experimental Ophthalmology, University of Tuebingen, Tuebingen, Germany

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Sven Ingebrandt1*, Michael Pabst, Günther Wrobel, Frank Sommerhage, Mathias Schindler, Andreas Offenhäusser
Institute of Bio- and Nanosystems, IBN-2: Bioelectronics, and CNI – Center of Nanoelectronic Systems for Information Technology, Forschungszentrum Jülich GmbH, Jülich, Germany

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HL. Khor1, E. Sinner, C. Thielemann, W. Knol1
Max Planck Institute for Polymer Research, Mainz, Germany

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Moritz Voelker1*, Peter Fromherz
Max-Planck-Institut für Biochemie, Martinsried, Germany

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Sang Beom Jun1,2, Matthew R. Hynd1, Natalie Dowell-Messhin1,4, Karen L. Smith1, James Turner1,4, Wiliam Shain1,4, Sung June Kim1,2

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1 Bernstein Center for Computational Neuroscience Freiburg, Albert-Ludwigs University, Freiburg, Germany  
2 Neurobiology and Biophysics, Inst. Biol. III, Albert-Ludwigs University Freiburg, Freiburg, Germany  
3 NMI Natural and Medical Science Institute, Reutlingen, Germany  
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5 Greiner BioOne GmbH, Frickenhausen, Germany

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1 Max Planck Institute for Biochemistry, Department of Membrane and Neurophysics, Martinsried/München, Germany,  
2 Infineon Technologies, Corporate Research, München, Germany

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Alex Lyakhov1, Yevgeny Perelman2, Ran Ginosar2*, Shimon Marom3  
1 Intel Corporation, Haifa, Israel  
2 Technion – Israel Institute of Technology, Faculty of Electrical Engineering, Haifa, Israel  
3 Technion – Israel Institute of Technology, Faculty of Medicine, Haifa, Israel

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1 Laboratory of Biosensors and Bioelectronics, Institute of Biomedical Engineering, ETH Zurich, Switzerland;  
2 Swiss Federal Laboratories for Materials Testing and Research (EMPA), Dübendorf, Switzerland

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Pawel Hottowy, Wladyslaw Dabrowski*, Andrzej Skoczen  
Faculty of Physics and Applied Computer Science, AGH University of Science and Technology, Krakow, Poland

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Jochen F. Meyer1*, Guenter W. Gross2  
1 Department of Medical Electronics, Technical University Munich, Munich, Germany  
2 Department of Biological Sciences, University of North Texas, Denton, TX, USA

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Gregory J. Brewer1,2, Michael D. Boehler1, Bruce C. Wheeler3  
1 Medical Microbiology, Immunology and Cell Biology  
2 Neurology, Southern Illinois University School of Medicine, Springfield, IL, USA  
3 Bioengineering Department and Beckman Institute, University of Illinois at Urbana-Champaign, Beckman Institute, Urbana, IL, USA

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2 Laboratoire Images et Signaux, UMR 5083, Institut National Polytechnique de Grenoble, France  
3 Dynamique des Réseaux Neuronaux, U704 Inserm, Grenoble, France  
4 Physio-pathologie des Réseaux Neuronaux Responsables du Cycle Veille-Sommeil, UMR 5167, Lyon I, France  
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1 Department of Electrical and Computer Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA
2 Beckman Institute, University of Illinois at Urbana-Champaign, Urbana, IL, USA
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2 Institute of Microtechnology (IMT), Université de Neuchâtel, Neuchâtel, Switzerland.
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Ivan S. Uroukov1,*, Mingwen Ma1, Larry Bull2 & Wendy M. Purcell3
1 Faculty of Applied Sciences, University of the West of England, Bristol, U.K.,
2 Faculty of Computing, Engineering, and Mathematical Sciences, University of the West of England, Bristol, U.K.,
3 Faculty of Health and Human Sciences, University of Hertfordshire, Hatfield, Herts, U.K.,

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Foreword

Micro electrode array (MEA) technology has matured: the profusion of recent papers in high-ranking scientific journals reflects the present-day significance of this technology as a valuable electrophysiological tool in basic research and drug discovery.

This year’s conference is the fifth international meeting on substrate-integrated microelectrodes and the fourth being hosted by The Natural and Medical Sciences Institute (NMI) in Reutlingen. The MEA meeting has established itself as an exciting biannual conference and it is an honour and a great pleasure for the NMI to welcome participants from 18 different countries and almost every continent who are joining in the anniversary celebrations. We are particularly happy to greet the familiar faces of those who regularly attend our MEA meetings, and we are delighted that this year’s meeting has attracted very many new participants. We hope that this gathering of researchers, MEA developers and users, students and others interested in MEA technology, stimulates enthusiastic discussions and productive scientific exchange.

From the start in 1998, the MEA meeting served as a forum for scientists working on the development and application of MEAs within a wide spectrum, ranging from neurobiology to the cardiovascular system, from sensory physiology to biotechnology and developmental physiology.

At first, the focus of the MEA meeting was mainly on the development of sensors and hardware; but gradually, great interest developed in exploring a range of applications in basic research and drug discovery. Our present meeting reflects the international MEA community’s growing scientific engagement with and exploration of the functional characteristics of cells, tissues and organs. This is an exciting and challenging development, which proves that the current MEA technology is an appropriate tool for the recording and modification of the functional characteristics of individual cells or systemic properties of tissues.

The present and future significance of MEA technology is entrenched in its capacity to support the analysis and manipulation of systemic properties, thus allowing the continuous recording and investigation of the spatiotemporal activity of electrogenic cells and tissues as well as the non-invasive, multi-focal electrical stimulation of tissue over long periods.

We, at the NMI, sincerely hope that MEA 2006 will be a constructive and rewarding meeting which every participant will enjoy. We also hope you will remember this meeting in the spirit of the on-going World Cup football games - "A time to make friends™ in Germany. We welcome you heartily into the scientific community in Reutlingen and hope that this meeting kicks off new scientific alliances with the aim of achieving common goals in the field of MEA technology.

Hugo Hämmerle
Deputy Managing Director, NMI

Alfred Stett
Head, Biophysics, NMI

Conference Chair

Publisher’s note

In recent years, enormous progress has been made in the field of the electrophysiological application of cellular systems with direct surface electrode coupling, notably in the core areas of medical research, pharmaceutical drug discovery and identification of drug effects and unwanted side-effects. Considering the newness of electrophysiology as a scientific discipline, the interdisciplinary integration of microelectronics - specifically in the field of micro electrode arrays - with modern tissue engineering, has meant the combination of two amazing technologies, creating a hugely exciting variation on current disciplines that is really one of a kind. The products and applications arising from this outstanding combination of technologies will lead to any number of as yet unimagined groundbreaking innovations. Products such as the retinal implant and the automatic testing of unwanted side-effects associated with the inhibition of channel proteins are only the very beginning. Industry is often also largely unaware of the potential of electrophysiology using microelectrode systems. This is why BIOPRO Baden-Württemberg GmbH, as the state agency for the promotion of modern biotechnology and the life sciences, is supporting the publication of the results that will be presented at this year’s MEA meeting, by distributing the findings to Baden-Württemberg companies in the pharmaceutical, biotechnology and medical technology industries. BIOPRO thus hopes to achieve the rapid and targeted dissemination of the potential of this exciting combination of technologies. I wish the participants of the MEA Meeting 2006 a successful conference, leading to many interesting results. In anticipation of the great boost this will give to specific applications of your technology,

Dr. Ralf Kindervater
CEO, BIOPRO Baden-Württemberg GmbH
Neuronal Dynamics and Plasticity
Basal ganglia multiple neurons research with respect to movement disorders

Hagai Bergman\(^1,2\)^* 

[1] Department of Physiology, The Hebrew University – Hadassah medical school, Jerusalem, Israel

* Corresponding author. E-mail address: hagaib@md.huji.ac.il

Keynote address
Recent multiple-neurons and correlation studies of the basal ganglia have enabled neural network analysis of these structures. These studies suggested that the basal ganglia use reinforcement signals and local competitive learning rules to reduce the dimensionality of the more redundant cortical information. These models predict a steady-state situation with diminished efficacy of lateral inhibition and low synchronization between basal ganglia neurons. In this framework, Parkinson's disease can be characterized as persistent state of negative reinforcement, inefficient dimensionality reduction, and abnormally synchronized basal ganglia activity.

1 Introduction
The basal ganglia are a complicated interconnected network of neuronal elements that process motor, cognitive and motivational (limbic) cortical information. The clinical manifestations of neuronal disorders of the basal ganglia include hypokinetic movement disorders such as Parkinson’s disease and hyperkinetic movement disorders such as L-DOPA induced dyskinesia, suggest that the basal ganglia use this multi-dimensional information to generate, or to control, action. Many computational models of the basal ganglia function have been developed (see review in Gurney et al., 2004). These models have generated testable hypotheses, and enable greater insights into the physiology and pathophysiology of the basal ganglia and human diseases. Here we will use this background, as well as the results of multiple-electrode recording in normal and MPTP treated parkinsonian monkeys to construct a better understanding of information processing in the basal ganglia cortical circuits.

2 Actor/Critic model of the Basal Ganglia
Most brain dopamine is generated by midbrain dopaminergic neurons and projected upon the striatum. The central role of dopamine in controlling motivation and learning has been known for many years (Schultz, 1998), however most classical models of the basal ganglia have overlooked the relationships between dopamine and learning in normal basal ganglia function. Recent studies revealed that the dopaminergic signal is best characterized as related to the differences between the animal’s predictions and reality (Fiorillo et al., 2003; Morris et al., 2004). This behavior resembles that of the “critic” in reinforcement temporal delay learning models (Sutton and Barto, 1998). The cortex-basal ganglia-frontal cortex axis is therefore modeled as the “actor” and the dopaminergic (and cholinergic) neurons as the “critic” or the provider of the reinforcement signal.

Actor-critic models predict that the reinforcement signal will modulate synaptic transmission in the actor. Indeed, plastic changes in the morphology of basal ganglia synapses occur after dopamine depletion. Physiological studies show that the dopaminergic signal modulates the access of cortical input to striatal projection neurons (Reynolds et al., 2001). Moreover, as predicted by reinforcement learning models, basal ganglia neurons significantly change their discharge as a function of the prediction of future reward and during different phases of learning (Arkadir et al., 2004).

The mutual inhibition models and the focusing (action-selection) models of the basal ganglia main axis (actor) predict strong lateral interactions and a negative correlation of basal ganglia activity. This prediction, however, has not been borne out by physiological extra-cellular studies. Multiple neuron recordings have failed to reveal correlations between the spiking activity of simultaneously recorded pallidal and substantia nigra reticulata neurons (Raz et al., 2000; Nevet et al., 2004).

The uncorrelated basal ganglia activity can be explained by a model that assumes that the basal ganglia perform efficient dimensionality reduction and decorrelation of the large information space spanned by the activity of the cortical-striatal neurons (Bar-Gad et al., 2003). Theoretical studies demonstrate that neural networks can perform such efficient coding using local cellular competitive learning rules. In the basal ganglia, inter-layer (cortico-striatal and striatal-pallidal) feed-forward connectivity is controlled by
Hebbian rules whereas lateral intra-layer inhibitory connectivity is controlled by anti-Hebbian rules.

According to the reinforcement-driven dimensionality reduction model, the basal ganglia processing is affected not only by the statistical properties of the cortical patterns but also by their behavioral significance. This is achieved by a triple striatal synapse in which the reinforcement (dopaminergic) signal controls the feed-forward cortico-striatal Hebbian learning. Thus, decorrelation of basal ganglia activity is achieved by a dynamic process and not by fixed and sparse inter-nuclei connectivity. Moreover, the reinforcement signal causes the extraction to become discriminative, performing better for reward related inputs but not for unrelated events.

Dopamine depletion (a negative reinforcement signal from the view of the striatal projection neurons) as in Parkinson’s disease substantially impairs the dimensionality reduction process. The consequential modifications of the striato-pallidal synapses results in increased synchronization among basal ganglia neurons (Raz et al., 2000; Raz et al., 2001; Goldberg et al., 2002; Heimer et al., 2002). Conventional dopamine replacement therapy restores the background level of dopamine. However, the intermittent pulsatile nature of the treatment causes inevitable fluctuations in striatal dopamine. These fluctuations are randomly timed relative to the environment and therefore may result in the generation of random encoding and the development of dyskinesia.

3 Summary

Multiple neuron studies and computational models have been instrumental in advancing our understanding of the basal ganglia and normal and pathological behavior. The reinforcement dimensionality reduction model of the basal ganglia circuitry provides insights into some of the mysteries of the basal ganglia. It explains the role of the anatomical numerical reduction and lateral GABAergic connections in the basal ganglia, the tonic (background) level of the neuronal reinforcement signal, and finally the physiological finding of independent and synchronized pallidal activity in the normal and Parkinsonian states, respectively. Further studies of the predictions of this and other models should enable us to better shape realistic models of this neuronal network, and to gain a better understanding of the role of basal ganglia in health and disease.

Acknowledgement

This review was partially support by the “Fighting against Parkinson” fund of the Netherlands friends of the Hebrew university (HUNA). I thank the past and present Hebrew university basal ganglia research group for their day and nights contributions to the experimental and theoretical work presented here.

References


Network plasticity in simulated and living cortical networks: comparison of the Center of Activity Trajectory (CAT) with other metrics

Zenas C. Chao¹, Douglas J. Bakkum¹, Daniel A. Wagenaar², Steve M. Potter¹*

¹ Department of Biomedical Engineering, Georgia Institute of Technology and Emory University School of Medicine, Atlanta, GA, USA
² Division of Biological Science, University of California, San Diego, CA, USA
* Corresponding author. E-mail address: steve.potter@bme.gatech.edu

We study stimulus-induced plasticity in cultured cortical networks as an in vitro model for learning and memory. Lasting changes in functional connectivity have been difficult to detect using standard firing-rate metrics. Here we used a simulated model network to compare the ability of 6 different metrics to quantify network plasticity. We then applied the most successful metric, Center of Activity Trajectory (CAT) and two commonly used ones, to measure functional plasticity in living networks grown on multi-electrode arrays (MEAs).

1 CAT shows the highest sensitivity to changes in network synaptic weights in simulations

We constructed a simulated spiking network model consisting of 1000 “leaky integrate-and fire” (LIF) neurons with spike-timing-dependent plasticity (STDP) and frequency-dependent synaptic depression. The model network was probed with 60 simulated electrodes for recording and stimulation. The simulated model showed a high fidelity of network firing dynamics found in living MEA cultures [1]. We compared six different plasticity metrics in their ability to quantify synaptic changes induced by simulated tetanic stimulation: firing rates (FR), firing rate histogram (FRH), mutual information (MI), shift-predictor corrected cross-correlogram (SCCC), joint peri-stimulus time histogram (JPSTH), and our novel metric, the Center of Activity Trajectory (CAT) [1], were used to decode evoked responses to a random probe stimulation sequence. The CAT was found to be the metric with the highest sensitivity to synaptic plasticity (Fig. 1). This metric summarizes the spatio-temporal propagation of network activity, in response to a probe stimulus, or during a spontaneous burst.
CAT revealed tetanus-induced long-term plasticity significantly better than the other metrics in living networks.

Dense networks of neurons and glia were prepared from embryonic rat cortex, and grown on MEAs (Multichannel systems) as described previously [2, 3]. We compared CAT, the optimal metric obtained from the model, to the FR and SCCC metrics at detecting functional plasticity induced by electrical stimulation in four cultures. Two 2-hour probing periods with random stimulation sequences (randomly distributed to 6 probe electrodes with random inter-pulse-interval at aggregated frequency of 0.5 Hz) were separated by a 15-min tetanus (consisted of 150 trains of pulses, at 6 s intervals, delivered simultaneously to 2 of the 6 probe electrodes; each train consisted of 20 pairs, with 100 ms between pairs). CAT, FR, and SCCC were measured from every 20 stimulus-evoked responses (probe responses), which consisted of the spikes within 50 ms after each stimulus pulse. As in the simulated networks, CAT showed more significant changes in probe responses across the tetanus than FR and SCCC (Fig. 2).

In both simulated and living networks, CAT was found to be the metric that was most capable of showing network plasticity. Additionally, CAT required less computation than MI, SCCC and JPSTH.

Acknowledgement

This work was partially supported by grants NS38628 from NIH/NINDS, EB000786 from NIH/NIBIB and DA18250 from NIH/NIDA, and by the Whitaker Foundation and the NSF Center for Behavioral Neuroscience. We thank Sheri McKinney for technical assistance.

References


Fig. 2 Comparison of the changes in CAT, FR and SCCC across tetanization in MEA cultures. A. An example (from evoked responses to one probe electrode in one MEA culture) is shown. Principal components analysis (PCA) was applied on multi-dimensional metrics. The first component (PC1) represented the ongoing drift (statistics not shown) in most of the cases. The time courses of the second significant component (PC2) were normalized (data was centered by removing its mean and then normalized by dividing through its standard deviation) and plotted. The vertical gray bar represents the tetanus. B. CATs before and after tetanization. CATs from every 20 probes were overlaid (black trajectories), and the average CATs were shown by series of circles (from dark gray to white). C. For each time course of PC2 from each metric, the mean distance of each PC2 in Pre1 (shown in A) to the mean of PC2 in Pre2 was compared to the mean distance between them to their mean. The ratio, ΔDij, was used to quantify the drift before the tetanus (no drift if this ratio~ 1). Similar measure between Pre2 and Post1 was used to quantify the change across the tetanus. For 4 cultures (N= 22 probes), the change across the tetanus was significantly greater than the drift before the tetanus for CAT (**, p< 1e-4, Wilcoxon rank sum test) and SCCC (*, p<0.01). Also, the change across the tetanus for CAT was significantly greater than that for SCCC (*, p<0.01).
Recording long-term potentiation in the CA1 area of the hippocampus using multi-electrode arrays

Maksym Kopanitsa*, Nurudeen Afinowi, Seth Grant

Team 32: Genes to Cognition, The Wellcome Trust Sanger Institute, Hinxton, Cambridge, U.K.
* Corresponding author. E-mail address: mvk@sanger.ac.uk

Models of synaptic plasticity such as long-term potentiation (LTP) are routinely used for studies of proteins and signalling cascades important for learning. Our group aims to create an electrophysiological platform for high-throughput plasticity screening of mouse knock-out strains that will be generated within the Mouse Genome project. Here, we present results of our experiments on the induction of LTP in mouse hippocampal slices using multi-electrode array system (Multi Channel Systems GmbH, Reutlingen, FRG).

1 Introduction

Multi-electrode arrays (MEAs) have become popular tools for recording spontaneous and evoked electrical activity of excitable tissues [1]. Most previous studies of synaptic transmission in brain slices employed MEAs with planar electrodes that had limited ability to detect signals coming from deeper, healthier layers of the slice. To overcome this limitation, we used 3D MEAs with tip-shaped electrodes [2] to probe plasticity of field excitatory synaptic potentials (fEPSPs) in the CA1 area of hippocampal slices of 129S5/SvEvBrd (129S5) and albino C57BL/6J-TyrC-Brd (C57BL/6J) mice.

Fig. 1 Example of a slice placement over 5x13 3D MEAs for an LTP experiment. Principal recording electrode (grey circle) was selected in the proximal part of CA1 stratum radiatum. Control and test stimulation electrodes that excited independent pathways (dashed and solid arrows respectively) are selected at distances ≥ 400 µm from the recording electrode.

2 Methods

Recordings were performed using two MEA-1060BC amplifiers running in parallel and an 8-channel STG-2008 stimulator. Brain slices of 2-3 month old 129 S5 and C57Bl6 c/c mice containing hippocampal regions were placed onto 5x13 3D MEA (Ayanda-Biosystems, Lausanne, Switzerland) and held by a nylon mesh glued onto a platinum wire. Biphasic (100 µs/phase) voltage pulses were used to evoke field responses in the CA1 area by alternate stimulation of test and control pathways at 0.02 Hz. Baseline fEPSPs had amplitudes of ~40% of maximum. LTP was induced by two trains of 100 pulses delivered to the test pathway at 100 Hz with 50 s interval between trains. Theta-burst stimulation consisted of three trains of ten bursts delivered at 5 Hz, each burst consisting of 4 pulses at 100 Hz.

3 Results

An example of the hippocampal slice placement is shown in Fig. 1. Several electrodes in the dendritic region of the CA1 area could usually be used for recording fEPSPs upon stimulation of two independent pathways from the CA3 and subicular sides of the stratum radiatum. In this and other slices, the largest and most consistent potentiation of the fEPSP after tetanus was observed in proximal regions of CA1 apical dendrites. Therefore, we used data recorded by electrodes in that part of the slice for statistical analysis. Two trains of 1 s-long 100 Hz stimulation led to an increase of fEPSP amplitude that remained significantly enhanced 60 min following LTP induction (Fig. 2).
The amplitude of fEPSPs evoked by stimulation of the non-tetanised control pathway remained stable as expected for an independent synaptic input (Fig. 2). As in some recordings, fEPSPs slightly increased by about 10% over the course of the experiment, we routinely normalised test pathway data by the respective values in the control pathway. Fig. 3 shows average normalised amplitudes of the fEPSPs recorded from slices of 129S5 and C57Bl6 mice before and after two trains of 100 Hz stimulation. Pre-incubation with 50 µM D,L-AP5, a competitive NMDA receptor antagonist, largely abolished this type of LTP (104±1%, N=5), confirming its absolute dependence on NMDA receptors. Another stimulation paradigm, theta-burst stimulation, also led to pronounced pathway-specific potentiation of fEPSP amplitude to about 175% of the baseline (Fig. 4).

Our study proved that MEAs could be used to acquire fairly large signals, comparable with fEPSPs recorded by standard extracellular recording techniques. Utilising MEAs, we were able to reliably induce robust LTP in hippocampal slices by established stimulation protocols. Major features of LTP (magnitude, pathway specificity, NMDA receptor dependence) recorded by MEAs were very similar to those seen in conventional glass electrode experiments. Given the relative simplicity of running multiple simultaneous experiments using MEAs, this technology could be extremely useful in large-scale evaluations of plasticity phenotypes and pharmacological screening.
Fig. 4 Summary data on LTP induced by theta-burst stimulation obtained in slices from 129S5 (n = 14) and C57BL/6J (n = 7) mice. Data are presented as mean ± s.d.

Acknowledgements

We would like to thank Paul Charlesworth and Nick Best for their help in setting up MEA suite. This work was supported by the Wellcome Trust Genes to Cognition Programme.

References


Effects of low-frequency stimulation on spontaneous firing dynamics in dissociated cortical cultures on multi-electrode arrays

Jaap van Pelt*, Elly van Galen, Pieter Wolters, Ger J. A. Ramakers, Ildikó Vajda

Netherlands Institute for Neuroscience, Amsterdam, The Netherlands
* Corresponding author. E-mail address: j.van.pelt@nin.knaw.nl

Spontaneous firing rates have been recorded in dissociated rat cortical neuronal networks cultured on multielectrode arrays before and after a period of electrical stimulation. The question was studied whether low-frequency electrical stimulation induces changes in spontaneous firing rates in the period thereafter. It was found that a period of low-frequency stimulation has significant and lasting effects on the spontaneous firing rates at the individual electrodes in the array and, thus, on the total firing rate at all the electrodes. The changes include significant increases and decreases in the spontaneous firing rates, as well as the activation of initially silent neurons, and the silencing of initially active neurons. These findings demonstrate that low-frequency stimulation protocols, as used in the literature for testing the effect of high-frequency tetanic stimulation protocols in plasticity studies, may by themselves induce changes in such cultured cortical neuronal networks.

1 Introduction and background

Plasticity studies in cultured neuronal networks usually include tetanic stimulation protocols of high frequency electrical pulses to induce plastic changes in the network (e.g., Maeda et al., 1998, Jimbo et al., 1998, 1999; Tateno & Jimbo, 1999). The tetanic stimulation is generally preceded and followed by low-frequency, low amplitude test stimuli in order to assess the changes in the stimulus-response characteristics of the network induced by the tetanic stimulation. Ideally, such test stimuli should only probe the input/output characteristics of the network without changing the firing characteristics of the network themselves. In the present study we have investigated whether test-stimuli-like stimulation patterns through planar MEA electrodes induce changes in the spontaneous firing rates of cultured rat cortical neuronal networks.

2 Cell cultures, stimulation protocol and data analysis

Cortical cells from embryonic (E18) rats were dissociated and plated on 60 electrode planar MEAs (100000 cells per plate) (Ramakers et al., 1991, 1998) of the HEXA-MEA type (MCS, Reutlingen, Germany) with electrodes of different diameters (10, 20 and 30 μm, respectively). Experiments were performed on 20 cultures in total (from 7 rats) of ages between 10 and 54 days in vitro. The stimulation protocol (Fig. 1) comprised single pulse trains applied sequentially to 6 different 30 μm diameter electrodes. A pulse train consisted of 40 biphasic rectangular voltage pulses (positive first, 200 microsecond per phase and 1.5V peak-to-peak) at 0.2 Hz. Previous experiments showed that the stimulation voltage threshold for evoking a spike differed from culture to culture (unpublished results). The minimum threshold however was never lower than 1.5 V peak-to-peak which we considered to be a moderate voltage. Spontaneous extracellular spike signals were recorded according to the procedure described in Van Pelt et al. (2004).

Fig. 1

Low-frequency stimulation protocol. A stimulus train of 40 bipolar pulses at 0.2 Hz is given sequentially to 6 different 30 μm diameter electrodes of a MCS-HEXAMEA. Each train and the whole stimulus session is preceded and followed by a period of recording of spontaneous firing activity.
Spontaneous activity was recorded prior to and following each pulse train for about five minutes, and prior to and following the total stimulation session for at least one hour. Firing rates at the individual electrodes and summed over all the electrodes were compared in the periods before and after individual pulse trains for short period effects, and in the periods before and after the total stimulation session for long period effects. Scatter plots were constructed to visualize changes in firing rates on individual sites (Fig. 2).

Fig. 2 Example of a scatter plot of mean firing rates at individual sites for comparison between two periods. The error intervals are drawn assuming Poisson process counting statistics. The data points illustrate sites with Poisson distributed fluctuations (circles), sites with increased firing rates (triangles), and sites with decreased firing rates (squares).

These scatter plots allow visual comparison between the firing rates in the two periods. Equal firing rates in both periods result in data points on the diagonal. However, even under stationary conditions statistical fluctuations result in data points scattering around the diagonal. For the ease of interpretation dashed lines are drawn to mark a region around the diagonal equal to three standard deviations (3SD) based on the assumption that the spikes in the spike train originated from a Poisson process. Then, data points within this region denote mean firing rates that do not differ significantly between both periods. Data points outside this region however denote significant different mean firing rates between the two periods. Data points close to the axes indicate the activation of initially silent sites or the silencing of initially activated sites.

3 Results and conclusions

In addition to the comparison of the mean firing rates in the periods before and after a period of stimulation, comparisons were made to test the stationarity of firing during both periods. To this end both periods were divided into two half periods followed by a comparison of the mean firing rates between first and second half of both periods and a comparison between the second half of the pre-stimulus period with the first half of the post-stimulus period. An example of such a triple comparison is given in Fig. 3C. The left and the right panel in Fig. 3C show that all the data points are within the 3SD area bounded by the dashed lines indicating that the scatter around the diagonal does not exceed the 3SD levels, as expected for a Poisson process generated spike train. The middle panel in Fig 3C illustrates the scatter of mean firing rates when comparing the 2nd half of the pre-stimulus period with the 1st half of the post-stimulus period. This panel shows data points outside the 3SD area illustrating that the period of electrical stimulation has had significant effects on the mean firing rates. The observation that the post-stimulus period again showed stationary firing indicates that the stimulus-induced changes were lasting over the post-stimulus period. The applied low-frequency stimulation thus had significant and lasting effects on the total firing rates of the network. These changes included both significant increases and decreases in total firing rates, which were caused by increases and decreases of firing rates of individual sites, and by activation of initially silent sites, and silencing of initially active sites (see also the spike plot in Fig 3A). Similar results were obtained in all the experiments done.

Fig. 3B displays the firing rates per second during the pre-stimulus and the post-stimulus period. These plots clearly indicate an ongoing alternation between low-level firing and the occurrences of synchronized network bursts. The mean firing rate for such spike trains then depends on the firing rate during low-level and during network burst firing and the frequency of network bursts. Although the assumption of a single Poisson process generated spike train for the scatter estimation is an oversimplification, the observed data points in the left and right panel of Fig. 3C nevertheless obey the scatter area surprisingly precisely.

These findings demonstrate that low-frequency moderate-amplitude stimulation has a major and a lasting impact on the spontaneous firing dynamics in cultured networks. Thus, the assumption that low frequency stimulation for testing network responses does not interfere with network firing properties is not confirmed by our results. These findings point to the necessity of control experiments (e.g. stability of baseline) in plasticity experiments that make use of test stimuli to probe connection strength.
Fig. 3 – Comparison of spontaneous firing activity before and after a period of low-frequency stimulation, (A) the spike traces at the individual sites for one hour period, just preceding (left panel) and following the stimulation period (right panel), (B) the firing rates with 1 sec time bins, and (C) the scatter plots for comparison of the two periods, with the left panel comparing first and second half of the pre-stimulus period, the central panel comparing second half of the pre-stimulus period with the first half of the post-stimulus period, and the right panel comparing first and second half of the post-stimulus period.

Acknowledgement
Funded by EU Neurobit-IST-2001-33564

References
Modification of Evoked Responses Induced by Correlated Stimuli in Culured Cortical Networks

Yuzo Takayama, Yasuhiko Jimbo
Graduate School of Engineering, University of Tokyo, Tokyo, Japan

We tested if repetitive application of spatio-temporally correlated stimuli induced changes in responses of rat cortical neurons cultured on substrates with 64-embedded micro-electrodes. After repetitive application of correlated stimuli, the cultured neurons show significantly different response properties from those in the initial states. Cross-correlation analysis revealed that the spatio-temporal structures of repeatedly elicited activity were highly reflected in the modified response properties at the final states. These results suggested that spatio-temporally correlated inputs can induce modification of synaptic strengths in neuronal networks, which could serve as an underlying mechanism of associative memory.

1 Introduction
To monitor and control the network activity of cultured neurons, micro-electrode array (MEA) based recording and stimulation is a promising tool. The advantages of this method are its noninvasiveness and the capability of multisite, complex stimulation. Applying focal tetanic stimulation via the substrate electrodes, a number of aspects of plasticity in cortical networks have now been studied[1,2].

Recently, spike timing dependent plasticity (STDP) has been reported, which is considered to be a strong candidate governing the modification of synaptic strength[3,4]. This suggested that the temporal structures of the induced firing patterns in neuronal ensembles might affect the response characteristics of networks. Here in this work, we applied spatio-temporally correlated stimulation to cultured cortical networks. The ability to modify a pair of evoked responses through repetitive application of spatio-temporally correlated stimulation was studied.

2 Methods
2.1 Cell culture
Cortical tissue was obtained from E18 Wistar rat embryos and dissociated by trituration after digestion with 0.02% papain (Boehringer). Dissociated cells were plated on substrates. The cultures were maintained in an incubator at 37°C, with a 5% CO₂ and water-saturated atmosphere. Half of the culture medium was exchanged twice a week.

Fig.1 (A) Cultured cortical networks on a MEA. Double stimulation sites A and B are indicated. (B) The protocols for spatio-temporally correlated stimulation. The first pulse was applied from site A followed by the second pulse from site B with 100, 50, 20, 10 and 0 ms time delays.
2.2 Electrical recordings

The MEA used in our experiments comprises 64 indium tin oxide (ITO) electrodes. The stimulation and recording area consists of two blocks of 32 embedded electrodes, separated by 500μm. The size of each terminal was 30μm×30μm and the distance between them was 180μm (center to center). The extracellularly recorded spikes were sampled at 25 kHz with 16 bits, stored on a hard disk.

A recording series consisted of: (1) evoked responses to test stimuli and (2) responses to the correlated stimuli. The test stimuli were composed of 100 single pulse stimuli from site A, followed by 100 from site B, each separated by six seconds. Stimulation sites A and B are shown in Fig. 1 (A). Correlated stimuli consisted of 100 double pulses, one at A closely followed by one at B. A-B time delays of 100, 50, 20, 10, and 0 ms were tested. The total series in each experiment was composed of 6 sets of test stimuli interdigitated with 5 sets of correlated stimuli: test1-A&B100-test2-A&B50-test3-A&B20-test4-A&B10-test5-A&B0-test6, where A&B100 denotes the correlated stimuli set. Each stimulus was a single biphasic pulse (100μs at +0.3 V, followed by 100μs at −0.3 V), throughout the experiment. Responses to individual correlated stimulus pairs at the 5 different delays are shown in Fig. 1 (B) as raster plots.

3 Results and Discussion

Figure 2 (A) shows evoked responses recorded at five selected sites, elicited by test stimuli from site A. Five typical responses are superimposed. The initial responses, responses after the second correlated stimuli A&B50, and the third correlated stimuli A&B20 are illustrated. The evoked activity consisted of early and late components [2,5]. Early components within the first 15 ms were detected for all the three states. At the initial state, almost no late components were recorded. After the correlated stimuli, strong late components appeared. The late components were detected after about 30 ms (After A&B50), and 20 ms (After A&B20), respectively. In contrast, the activity elicited by the stimuli from site B showed no signifi-
cant changes after any correlated stimuli as shown in Fig. 2 (B).

To analyze the relationship between the applied correlated stimuli and induced activity-changes, cross-correlation functions were calculated [1]. Figure 3 shows cross-correlations between site-A-evoked responses and site-B-evoked responses. Three traces, the initial state, after the A&B50 and the A&B20 correlated stimuli are superimposed. After the correlated stimuli, the shoulder of the plot increased and formed almost the second peak. After subsequent correlated stimuli, this second peak moved toward the central peak. These results suggest that the properties of the applied temporally correlated stimuli are reflected in the spatio-temporal structures in the evoked responses.

4 Summary

In this work, the effects of spatio-temporally correlated stimuli on the neuronal activity were studied. After the correlated stimulus application, the cortical neurons showed significantly different response properties from those at the initial states. Correlation analysis revealed that the spatio-temporal structures of the applied repetitive stimuli were highly reflected in the modified responses at the final states. These results suggested that spatio-temporally correlated inputs could systematically induce modification of synaptic strength in neural networks, which could serve as an underlying mechanism of associative memory.

Acknowledgement

This work was funded by Grant-in-Aid Scientific Research (No. 16300150, 17650129) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

A neuro-robotic interface for studying the adaptive properties of cultured neuronal networks

Sergio Martinoia1*, Antonio Novellino1, Michela Chiappalone1, Paolo D’Angelo2, Laura Cozzi2, Vittorio Sanguineti2, Luca Berdondini3, Milena Koudelka-Hep3

1 Neuroengineering and Bio-nanoTechnologies Group – NBT, Department of Biophysical and Electronic Engineering - DIBE, University of Genova, Genova, Italy
2 Department of Informatics, Systems and Telematics – DIST, University of Genova, Genova, Italy
3 Institute of Microtechnology (IMT), University of Neuchatel, Neuchatel, Switzerland
* Corresponding author. E-mail address: martinoia@dibe.unige.it

Embodiment is considered an essential condition for studying mechanisms of sensorimotor integration, control and adaptation in living system. In this context, we developed a novel bi-directional neural interface. We interconnected in-vitro neurons, extracted from rat embryos and plated on a micro-electrode array (MEA), to external devices, thus allowing real-time closed-loop interaction. This hybrid system can be used for studying the computational properties of biological neuronal networks and for investigating the mechanisms underlying complex behaviors like learning at whole network level.

1 Introduction
The research interest on neuro-robotic interfaces is rapidly growing due to plurality and relevance of possible applications, ranging from clinical applications (neuroprostheses, neurorehabilitation), to fundamental research. Innovative experimental models have been recently introduced in cellular, computational and experimental neuroscience (e.g. [1, 2]).

Following this approach, we interfaced a mobile robot with a population of neurons, extracted from rat embryos and cultured on a multi-electrode array (MEA). In this work, we investigated the effect of the stimulus-induced distributed plasticity and functional/structural connectivity on the information processing capabilities of the neuronal network. The task associated to the robot system, exploiting the computational properties of the neuronal network, was obstacle avoidance. Standard MEAs do not induce any specific morphological organization in the neural preparation; therefore synaptic interconnections rearrange randomly within the whole population. In order to study the effect of simple anatomical structuring on the ability of the neuronal network to pursue the desired motor task, we used also a second type of MEA devices. These, featured physical barriers for clustering the network into a number of random sub-networks while preserving a high degree of functional connectivity. Additionally, a specific training protocol (based on revisited tetanic and associative protocols) was used to increase the synaptic connectivity among the sub-neuronal populations. The potentiated efficacy of specific sensory-motor connections was paired by the improvement in the behavioral performances of the robot.

2 Materials and Methods
Cortical neurons extracted from rat embryos (E18) were cultured on two kinds of planar arrays of 60 electrodes: (1) standard MEAs (Multi Channel Systems® - MCS, Reutlingen, Germany), and (2) custom-made cluster MEAs. The main feature of cluster MEAs rely in the possibility of segregating five neuronal sub-populations in separated, although interconnected, clusters [3].

The experimental set-up is based on the MEA60 System, (Multi Channel Systems® - MCS). Details about the methods can be found in [4].

3 Results
Fig. 1 show the functional blocks of the closed-loop system. To establish a bi-directional communication between the neuronal preparation and a mobile robot, the neuronal activity \( \hat{y}(t) \) is converted into motor commands \( o(t) \) for the robot (decoding of neural activity). Conversely, the signals acquired by the proximity sensors of the robot \( s(t) \) are coded into spatio-temporal patterns of stimuli \( x(t) \) that provide a feedback to the preparation. (coding of sensory information).

Here we present an example of application of the closed-loop system for an embodied electrophysiology experiment: a neuro-robotic Braitenberg ‘explorer’ vehicle.
The robot is moving in an arena with some obstacles. The experiment is constituted by different phases in which the robot is driven by the electrophysiological activity which is in turn modulated by the electrical stimulation obtained by the sensory input. Conditioning signals are also applied in some cases to investigate the capability of the system to adapt and improve its performances both in terms of robot indicators and neurophysiological related parameters.

We evaluated different indicators of the robot performance using the two types of MEA (standard vs. clustered) in different experiments. Clustered MEAs resulted in a lower number of hits, and higher fraction of space covered and trajectory length, meaning that the robot explored a wider area of its playground and obstacle avoidance is not a result of the repetition of the same trajectory.

4 Conclusion

In this work we examined the ability of a culture of cortical neurons to process information in a goal-oriented way. In this case the goal was to drive a robot according to a defined motor task. Our experiments show that the presence of a minimal morphological organization is needed for obtaining selectivity in the response of the neural preparation to electrical stimulation. This is a basic requirement for obstacle avoidance.

Acknowledgement

Work partly supported by Neurobit Project (EU, Contract n.: IST-2001-33564 - FET). The authors wish to thank Dr Brunella Tedesco for cell culturing.

References

Rhythm generation in spinal cord cultures

Cédric Yvon, Jürg Streit*

Department of Physiology, University of Bern, Bern, Switzerland
* Corresponding author. E-mail address: streit@pyl.unibe.ch

Spontaneous episodes of oscillatory electrical activity are observed in foetal and neonatal spinal cord. Such activity is believed to play a major role in the development of pattern generating neuronal circuits, which control simple repetitive movements. Here we report on some of our recent findings revealing the mechanisms involved in the generation of rhythmic activity in cultures of spinal neurons.

1 Patterns of spontaneous activity

We have investigated the mechanisms underlying spontaneous electrical activity in organotypic cultures of foetal rat spinal cord slices and in networks of dissociated spinal cord cells. Both were cultured on multi-electrode arrays (MEAs). The MEAs allowed the simultaneous recording of spike-related extra cellular signals from multiple points in the network. Such recordings were combined with intracellular recordings from individual neurons using whole cell patch clamp.

1.1 Slice cultures

In more than 90% of the organotypic slice cultures spontaneous activity was organized into bursts with intra-burst oscillations with frequencies around 4 Hz (Fig.1). In about two third of these cultures, spontaneous activity in the whole slice was followed by oscillations that were restricted to the ventral area of the slices around the central fissure. This suggests a prominent occurrence of oscillatory networks in this area and thus a segregation of networks in the slice.

In order to find out whether such oscillatory networks were also restricted to defined segments of the spinal cord we pooled the slices into three groups, which were roughly corresponding to the cervical, thoracic and lumbar parts. We found no difference in the patterns of activity between the three groups suggesting a ubiquitous occurrence of oscillatory networks along the spinal cord. The patterns of spontaneous activity including oscillations persisted and the oscillations even became more prominent and prolonged when fast synaptic inhibition was blocked by bicusculine and strychnine (disinhibition).

These findings show that synaptic inhibition is not a crucial mechanism for these oscillations, but they don’t exclude a contribution of inhibitory synapses to rhythm generation under control conditions. Indeed such a contribution became evident when comparing the rhythms before and after disinhibition.

On the other hand spontaneous oscillatory activity ceased when fast glutamatergic excitatory synaptic transmission was blocked by CNQX and APV. Together these findings show that spontaneous oscillations are mediated by recurrent excitation in spinal networks and shaped by synaptic inhibition. The latter, however, is not crucial for the generation of oscillations.

1.2 Networks of dissociated cells

In networks of dissociated spinal cells, spontaneous activity is usually not organized into oscillatory bursting. These networks have a much lower density than those in the slice cultures since the cells are plated on much larger areas. Analysis of the size of the spontaneous network events (avalanches, see [1]) revealed that they follow a power law distribution. We have previously shown that disinhibition turns random activity into bursting patterns with persistent activity during the bursts [2]. Similar patterns of spontaneous activity appear even in the presence of functional synaptic inhibition when the density of the networks of dissociated cells is increased by plating the cells on smaller areas. As in slice cultures the typical patterns of spontaneous activity disappear when excitatory glu-
tamatergic synaptic transmission is blocked. However, in contrast to slice cultures, asynchronous activity persists at almost half of the electrodes after synaptic decoupling of the network. This shows that many of the dissociated neurons are intrinsically active. We have confirmed this finding in individual cells using patch clamp [3].

Oscillations of activity as described in slice cultures were rarely seen in networks of dissociated cells. However, such activity reliably appeared when disinhibition was combined with low doses of the sodium channel blocker riluzole. We therefore used such riluzole-induced oscillations in dissociated cultures as a model to investigate the underlying mechanisms.

2 Effects of riluzole

Intrinsic spiking is mainly based on persistent sodium currents $I_{\text{NaP}}$ [4]. Such currents are blocked by riluzole. Since riluzole also blocks spontaneous activity in the cultures, we conclude that intrinsic spiking is a prerequisite for spontaneous activity.

At lower doses, however, riluzole induces oscillations. Synaptic depression and spike frequency adaptation have been proposed as the accommodation mechanism in oscillations after disinhibition. We used riluzole to distinguish between these two possibilities. While riluzole had no effect on synaptic depression, it increased spike frequency adaptation and lead to early depolarization block after few initial spikes. Phenytoin, another sodium channel blocker, which suppresses persistent sodium currents and blocks spontaneous activity in the cultures, had neither an effect on spike frequency adaptation nor on depolarization block. Indeed, phenytoin did not induce oscillations. From these findings we conclude that depolarization block is the major mechanism to terminate activity during riluzole-induced oscillations.

Fig.2 Riluzole induces oscillations in dissociated cultures. Network activity and intracellular recordings.

3 Summary and Conclusions

Our findings revealed intrinsic spiking, recurrent excitation and depolarization block as the major mechanisms underlying oscillatory network activity after synaptic disinhibition. In intact networks synaptic inhibition contributes to the generation of oscillations.

Acknowledgement

We thank Ruth Rubli for preparing and maintaining the cultures. This work was supported by SNF grant No. 3100A0-107641/1

References

Firing patterns in chicken hypothalamic neurons

Ute Abraham, Erik D. Herzog*

Department of Biology, Washington University, St. Louis, MO, USA
* Corresponding author. E-mail address: herzog@wustl.edu

1 Background and rationale

Many behavioral and physiological processes exhibit biological rhythms that are controlled by an endogenous clock. These circadian rhythms are usually synchronized to periodically recurring environmental processes (i.e. the light-dark cycle), but they persist under constant conditions with a period close to 24 hours. In mammals, the central nervous pacemaker driving circadian rhythmicity is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. Individual neurons in slices and SCN dispersed cultures exhibit circadian rhythms in electrical firing rate with an average period that is close to the overall behavioral rhythm of the animal [1]. In birds, two hypothalamic regions (SCN and lateral hypothalamic nucleus, LHN) that are potentially homologous to the mammalian SCN have recently been identified by means of rhythmic clock gene expression [2,3]. However, we still lack evidence that neurons in these regions are able to express functional circadian rhythms. The goal of the present study is to record electrical activity from dispersed neurons from the embryonic chicken SCN and LHN region and from hypothalamic slices containing the avian SCN or LHN for at least 7 days using MEA. We will then identify neurons that express circadian rhythms in electrical activity and compare the percentage of rhythmic cells, their average phasing, as well as the period distribution between slices and dispersals. This will give us insight into whether the putative avian pacemaker regions share similarities with the mammalian SCN besides their location and rhythmic clock gene expression.

2 Methods

Brains were removed from chicken embryos starting at E14 through E20 and sectioned at 300µm. For dispersals, the bilateral avian SCN or LHN were punched out and digested in papain. Dispersed cells were plated on poly-D-lysine/laminin-coated MEA within a 2mm-diameter Silicone gasket in DMEM with 10% neonatal calf serum (NCS). 24 h after plating the medium was changed to Neurobasal with 1% NCS. Slices of the bilateral SCN or unilateral LHN were cultured on membrane insets in DMEM+10% NCS for 2 to 3 days. Subsequently, the slice-on-membrane was inverted on a poly-D-lysine/laminine-coated MEA and cultured in 200µl of medium. Cultures were kept humidified at 37°C with 5% CO2. Medium was changed every 2 (slices) to 4 (dispersals) days. Slices and dispersals were recorded at 2 to 3 weeks after plating.

3 Preliminary results

We were able to record spontaneous firing patterns from chicken SCN and LHN neurons in both dispersals and slices for at least 7 days in vitro. As of now, spontaneous action potentials were recorded as early as embryonic age 14 and as late as E17. Although detailed analysis is still in progress, we find that the cultured hypothalamic neurons tend to fire in bursts and firing patterns change with changes in temperature and medium. Moreover, we observed that in contrast to mammalian SCN neurons, chicken hypothalamic neurons are able to sustain firing rates at 40°C, the brain temperature in these birds, and even at temperatures up to 43°C.

Acknowledgement

Funded by the NSF (grant # IOB-0425445).

References

Neural plasticity in hippocampal neurons by paired-pulse stimulation on MEA

Gregory J. Brewer1,2, C. Nick Fogleman1, Michael D. Boehler1, Bruce C. Wheeler3

1 Medical Microbiology, Immunology and Cell Biology, 2 Neurology, Southern Illinois University School of Medicine, Springfield, IL, USA 3 Bioengineering Department and Beckman Institute, University of Illinois at Urbana-Champaign, Beckman Institute, Urbana, IL, USA
*Corresponding author. Email gbrewer@siumed.edu

Modulation of synaptic strength has been well documented in hippocampal slices, but fewer reports have characterized plasticity with the high throughput possible with dissociated neurons on MEAs. Here we provide examples of different stimulation intervals reproducibly producing either facilitation or depression on MEAs with dissociated hippocampal neurons. In one case, a paired pulse stimulation regimen produces facilitation at a 50 ms interval and depression at a 150 ms interval over the same electrode. Other examples will be provided.

1 Rationale

Paired-pulse facilitation and depression are well-established forms of synaptic plasticity in CNS neurons where certain inter-pulse intervals stimulate either facilitation or depression of the EPSP amplitude [1, 2]. However, tedious patch-clamping studies limit the rate of data acquisition as well as the determinations of the robustness of this plasticity over time. Multi-electrode arrays provide the potential to elicit frequency-dependent responses of a large number of neurons simultaneously, as well as monitoring their long-term persistence in neuronal networks.

2 Interpulse intervals affect the rate of firing action potentials

To avoid desensitization and increase reliability of spike production from stimuli, we have been stimulating uniform cultures of hippocampal neurons plated at 500 cells/mm2 with uniformly low frequencies of 5 to 10 Hz. As a result, we achieve low mean spike rates of only 0.5 Hz. Since an important function of hippocampal neurons is to detect coincident stimuli, we reasoned that a paired-pulse paradigm might be more appropriate. Here, we compared the effect of alternatively stimulating with a 15 µA bipolar pulse at 50 ms to one at 150 ms delay from the previous pulse. Surprisingly, the short 50 ms interpulse interval resulted in a large increase in the number of action potentials elicited from each pulse (Fig. 1). Conversely, the longer interval of 150 ms produced a larger fraction of responses in which spikes that were seen in the first pulse were decreased or missing in the second pulse.

![Fig. 1](image)

Fig. 1 Interstimulus interval is critical for determining spike response rates from hippocampal neurons. Multiple spikes elicited from one stimulus produced responses greater than 100% for the 50 ms interval. Criterion was set at 2 S.D. from the mean amplitude of all spikes on one electrode within 15 ms of stimulus.

3 Interpulse intervals affect the action potential amplitude

The mean amplitude of the second spike was on average 70% larger for interstimulus intervals of 50 ms (Fig. 2). Conversely, the mean amplitude of the second spike was 20% lower with an interstimulus interval of 150 ms.

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The mean amplitude of the second spike was on average 70% larger for interstimulus intervals of 50 ms (Fig. 2). Conversely, the mean amplitude of the second spike was 20% lower with an interstimulus interval of 150 ms.
4 Conclusion

Together, these results suggest that a paired pulse stimulation regimen produces facilitation at a 50 ms interval and depression at a 150 ms interval in one case. Further experiments reveal that these relationships are highly dependent on stimulus amplitude and vary from one electrode to another.

Acknowledgement

Supported in part by NSF EIA 01328 and NIH R01-EB00786, R01-NS052233

References


Stimulus-induced distributed synaptic changes in networks of cortical neurons

Michela Chiappalone1*, Paolo Massobrio1, Mariateresa Tedesco1 and Sergio Martinoia1

1 Neuroengineering and Bio-nanoTechnologies Group – NBT, Department of Biophysical and Electronic Engineering - DIBE, University of Genova, Genova, Italy
* Corresponding author. E-mail address: michela@dibe.unige.it

We developed and applied a modified version of the tetanus protocol on networks of cortical neurons. Both high and low frequency stimulations were delivered simultaneously from two electrodes of a Micro Electrode Arrays and the responses of the network were compared with those obtained by applying a single tetanus. We found that the associative protocol seems to produce more repeatable results and it is able to induce potentiation more than no change or depression in the majority of cultures.

1 Introduction

Experimental investigations on the electrophysiological behavior shown by neural ensembles is a fundamental step towards understanding how the brain works. Dissociated cortical networks maintained in-vitro, coupled to Micro-Electrode Arrays (MEAs), represent a neurobiological model where the strategies employed by the nervous system to represent and process information can be easily approached and basic physiological mechanisms can be quantitatively characterized. After a few days in culture, neurons connect with each other with functionally active synapses, forming a random network and displaying spontaneous activity. The shown patterns of collective activity can be stimulated by means of chemical and electrical inputs [1-3], producing short and/or long-term effects in the synaptic efficacy.

Here we report evidence of induction of synaptic changes in network of cortical neurons coupled to MEAs due to revised protocols of tetanic stimulation. First, the network evoked response, as a function of the stimulating sites, is tested for stability; then, a tetanic stimulus and a reference test stimulus are applied together at two specific electrodes (up to 58 channels). Different trials, based on different features of the reference stimulus coupled to the tetanus (i.e. associative protocol), have been applied to a large set of cultures.

2 Materials and Methods

Cortical neurons extracted from rat embryos (E18) were cultured on planar arrays of 60 TiN/SiN electrodes (Multi Channel Systems® - MCS, Reutlingen, Germany), pre-treated with adhesion factors (Poli-L/D-Lysine and Laminin).

The experimental set-up is based on the MEA60 System (MultiChannelSystems, Reutlingen, Germany). Details about the methods can be found in [4].

2.1 Experimental protocol

We developed an experimental protocol based on four steps of stimulation:
1. Test stimulus 1. Delivery of a test stimulus from six-eight sites serially (50 biphasic pulses, 1.5V pp 250µsec single-phase duration, frequency 0.2Hz).
2. Test stimulus 2. Repetition of point 1 (stability).
3. Tetanic stimulation. Two types:
   a. Single tetanus, delivered from one channel, chosen among the ones used for the test stimulus [1].
   b. Associative tetanus. A single tetanus and a train of pulses at a defined frequency are delivered together from two specific stimulation sites.
4. Test stimulus3. Comparison of the evoke responses for each channel.

2.1 Processing technique

Extracellularly recorded spikes are usually embedded in biological and thermal noise ranging from 10µV up to 40µV peak-to-peak and they can be detected using a threshold based algorithm [5]. To investigate the neural activity evoked by stimulation, we computed the post-stimulus time histogram (i.e. PSTH), which represents the impulse response of each site of the neural preparation to electrical stimulation. The PSTHs were calculated by taking 600-msec time windows from the recordings that follow each stimulus [6].
3 Results

At the beginning of each experiment, the network is tested for stability (i.e. repeatability of the response after two consecutive stimulation trials). Not stable cultures are discarded and not used for the analysis. On stable cultures only, the tetanic stimulation protocol (i.e. single or associative) is applied.

Experimental results, analysed at the network level and based on the PSTH evaluation, have shown remarkable differences in the electrophysiological activity of the network after the tetanus, denoting an induction of potentiation, related to the level of initial spontaneous activity shown by the cultured network itself. In particular, important differences can be appreciated by comparing the responses to a single tetanus (Fig. 1, top) and to the associative protocol (Fig. 1, bottom). The PSTH area is increased in almost all the channels after the application of the second protocol. This results can be appreciated also by looking at the area under the PSTH, which increases from 1 to 4-6 times in the case of the co-activation protocol, and at the mean latency from the stimulus of the first evoked spike, which usually decreases after the application of the associative protocol. The application of the single tetanus, on the contrary, produces no changes or decrease of the response.

As a preliminary result, also changes in functional connectivity and global homeostatic behavior have been reported by using the associative protocol, denoting a distributed equalization in the strength of connections.

4 Conclusion

Stimulation with single tetanus (one site) seems to produces no changes or depression in network of cortical neurons. The associative stimulation (two sites) seems to produce reliable and reproducible results in terms of global potentiation. At the same time, the network “equalizes” its connection strengths to respond to the local increase of the evoked firing rate, showing a kind of homeostatic behavior [7].

Our findings give an experimental evidence testing the feasibility of imposing a kind of “adaptive learning” in a biological non-linear system acting on defined multi-site stimulating protocols.

Acknowledgement

Work partly supported by Neurobit Project (EU, Contract n.: IST-2001-33564 - FET).

References


Striatal neurons develop oscillating neuronal networks in vitro

Wiebke Fleischer¹*, Olga Sergeeva², Mario Siebler¹

1 Department of Neurology, University of Düsseldorf, Germany
2 Institute of Neuro- and Sensory Physiology, University of Düsseldorf, Germany

* Corresponding author. E-mail address: wiebke.fleischer@uni-duesseldorf.de

The morphological and neurophysiological development of cultured embryonic (eStr) and postnatal striatal (pStr) cells of the rat was investigated. Striatal neurons formed dense axonal networks exhibiting oscillating spike bursts only when seeded in very high densities (>1 x 10⁵/cm²). Otherwise, only few and short neurites between cells were observed and spontaneous spike activity was rare and uncorrelated. Correlated spike bursts of striatal networks were framed with single spikes that were turned off after an enhancement of synaptic efficacy due to magnesium withdrawal. Immunostainings revealed a high ratio of GABAergic neurons and the GABA-receptor antagonist bicuculline activated network activity in both cell types by prolonging burst events. The potassium channel inhibitor barium chloride enhanced spike activity in pStr activity but inhibited eStr activity. This may be due to developmental differences in channel expression, mainly the prominent inwardly rectifying potassium channel.

1 Introduction
The striatum is the largest part of the basal ganglia and a key-player in movement control. Selective degeneration of neurons in the striatum or functionally related brain areas results in neurological disorders like Parkinson’s or Huntington’s Disease. Since the striatum is highly interlinked with other brain regions and consists of more than 90% projection neurons it is controversially discussed whether mono-cultured striatal neurons form an intrinsic neuronal network in vitro. Here, we report that striatal cells from embryonic and postnatal rats developed oscillating burst activity when they were seeded in very high densities and that their activity pattern could be modulated by synaptic and ion-channel acting substances.

2 Materials and Methods
Primary dissociated striatal cells were prepared from embryonic (eStr) or postnatal Wistar rats (pStr). Extracellular potentials were recorded on poly-D-lysine and laminin coated MEAs with 60 planar Ti/TiN-microelectrodes (30µm diameter, 100µm spacing; Multi Channel Systems, Reutlingen). We used the rabbit anti-GABA antibody (1:1000) and the monoclonal Anti-MAP2 (microtubule associated protein) antibody (1:500) from Sigma, (Taußkirchen, Germany).

3 Results
Striatal cells developed synchronous bursting only if the cells had been seeded in a very high density (> 1 x 10⁵). Otherwise, only rare and uncorrelated spikes were measured during the whole cultivation time.

Immunofluorescence revealed a correlation between synchronization of the activity pattern and the degree of synaptic integration. A high proportion of GABAergic neurons as observed in striatal cultures did not affect the development of synchronous oscillation.

Incubation with magnesium-free bath solution significantly increased the ratio of spikes that were part of synchronous bursts. Uncorrelated spiking was suppressed.

Application of the GABA_A-antagonist bicuculline (50µM) prolonged burst duration in pStr and eStr. The potassium channel blocker barium (500µM) enhanced burst frequency in pStr and destroyed oscillation in eStr. The presence of synaptic glutamate within striatal cultures could be demonstrated by addition of the NMDA-receptor antagonist APV.

4 Conclusion
Dissociated striatal cells that were prepared from embryonic as well as postnatal rats developed fundamentally different morphological and functional network parameters when seeded in low and high densities. Striatal network activity could be modulated by ion channel and synaptic acting substances revealing developmental state-specific sensitivities.
The formation of a neuronal network by striatal cells strictly depends on the seeding density. a) Embryonic striatal cells seeded in a density of $2.5 \times 10^4$ cells/cm$^2$ after 19 days in vitro (div) show only few and short neurites and hardly any contacts between the cells. b) Embryonic striatal cells in a density of $1 \times 10^6$ cells/cm$^2$ after 26 div developed a neuritic network characterized by cell clusters and thick axon bundles between them. The ratio of GABAergic neurons (light-colored) depends on the network morphology as well.

Fig. 3 Barium chloride in a concentration of 500µM significantly increased spike rate and burst rate in pStr (n=10) but decreased both parameters in eStr (n=15). The spontaneous spike activity of cortical cells (CryoCx) was not significantly altered by the administration of barium chloride (n=10). The ratio of spikes that were part of bursts was reduced in all cell types after administration of barium. Asterisks indicate significant changes (p≤0.05).

Fig. 4 Bicuculline (50 µmol/l) increased the overall spike rate in both striatal cultures (n=11). This activation was due to a higher number of spikes constituting a burst while the number of bursts remained constant (n=11). Spike rate in CryoCortex was unchanged by administration of 50 µmol/l bicuculline (n=12). Neither burst rate nor the number of spikes within a burst was significantly altered.

Acknowledgement

We thank QBM Cell Science for providing Rat Brain Cortex CryoCells and Brigida Ziegler for excellent technical assistance.
Low density hippocampal cultures on MEAs

Eckehard Freitag*, Frank Hofmann, Hilmar Bading

Department of Neurobiology, Interdisciplinary Center for Neurosciences (IZN), University of Heidelberg, Heidelberg, Germany
* Corresponding author. E-mail address: Eckehard.Freitag@urz.uni-heidelberg.de

Synaptic connections are not static but undergo use-dependent modifications leading to strengthening or weakening of their efficacy. This phenomenon, commonly referred to as neuronal plasticity, is thought to play an important role in learning and memory. We used a simplified cell culture system, plating hippocampal neurons on MEA chips to study the molecular events necessary for the maintenance of long-lasting, transcription-dependent changes in network activity.

1 Introduction

The recent years prevailed the view that activity-dependent changes in synaptic connectivity or efficacy are critical for neuronal differentiation, development and long-term plasticity. We have previously shown that culturing hippocampal neurons on MEAs can serve as a simplified system to study the long-lasting changes of neuronal plasticity [1]. After a brief (15 minutes long) stimulation of hippocampal cultures with the GABAA receptor antagonist bicuculline, a random spike pattern that was observed before the stimulation is replaced by rhythmic synchronous bursting that can last far beyond the duration of the inducing stimulus (Fig. 1). These long-lasting changes may represent a physiological correlate of a memory trace.

Furthermore we have shown that these alterations in network activity are initiated by calcium entry through synaptic NMDA receptors and require for its long-lasting maintenance (beyond 2-3 h) calcium-dependent signaling pathways that activate gene transcription in the nucleus [1].

2 Results and Discussion

2.1 Adaption of the cell culture system

To achieve higher infection rates, we established conditions to decrease the cell density because a network that consists of fewer neurons may be more susceptible to disturbances. By plating neurons on a layer of cortical glial cells, we were able to lower the density of neurons necessary to form an interconnected network by about 70 % (Fig. 2). First tests indicate that these low density cultures display the same features and spike patterns as cultures with higher neuronal density.

![Figure 1](image1.jpg) Induction of recurrent synchronous bursting in cultured hippocampal networks. A, before and B, 2 h after exposure to bicuculline.

Our experimental system consists of hippocampal neurons, which after a culturing period of 10 to 14 days, form an elaborate and highly interconnected network with about 90 % excitatory neurons and 10 % inhibitory interneurons. The aim of our study was to identify the molecular mechanisms and intracellular signaling pathways through which synaptic inputs can generate stable and long-lasting changes in firing patterns. We have focused in particular on the role of the nuclear calcium-Calmodulin kinase IV-CREB signaling pathway. MEA recordings were carried out using mouse hippocampal cultures in which components of this signaling pathway were manipulated through viral gene transfer. The results obtained showed little differences between virus infected cultures and non-infected controls. However, infection rates were only about 50 to 70 %, raising the possibility that the rather large fraction of non-infected cells was sufficient to sustain the signal-induced rhythmic firing pattern.

![Figure 2](image2.jpg) Low density mouse hippocampal culture on MEA. Cell density is about 40 % of normal culture (about 400 cells /mm²) (bar: 200µm).
2.2 Manipulating cellular calcium signaling

Calcium plays the crucial role in the translation of neuronal activity pattern into specific gene expression programs. The CREB/CBP transcription factor complex is the principal mediator of activity-dependent gene expression. To interfere with CREB/CBP activation, we infected neurons with recombinant adeno-associated viruses (rAAVs) expressing the CREB inhibitor K-CREB, a negative interfering mutant of CaMKIV [CaMKIV(K75E)], and the calmodulin (CaM) binding-peptide, CaMBP4 [2]. CaMBP4 is a nuclear localized protein that contains 4 repeats of the M13 calmodulin binding peptide derived from the rabbit skeletal muscle myosin light chain kinase; it binds to and inactivates the nuclear calcium/CaM complex [2].

In our experiments we could improve the infection of hippocampal neurons; using the rAAV-CaMBP4 construct about 80 % of viable neurons can be infected (Fig. 3). We are currently investigating whether manipulation of the nuclear calcium – CREB/CBP pathway affects the late phase of network plasticity.

3 Summary

We have previously shown that our simplified cell culture system is a powerful tool to study mechanisms of synaptic plasticity [1]. Methods using viral gene delivery are being established to interfere with nuclear calcium signaling and CREB/CBP-mediated gene expression. This will allow us to dissect the mechanisms underlying the late phase of plasticity.

Fig. 3 Infection of mouse hippocampal culture with rAAV-CaMBP4. A, nucleus and B, CaMBP4 staining reveal that CaMBP4 is nuclear localized and infection rate is about 80 % of viable neurons (bar: 100 µm).

Acknowledgement

This work was supported by the Alexander von Humboldt Foundation.

References


Assessing minimal networks for epileptiform activity

Ute Haeussler1,2,3, Ralph Meier2, Antoine Depaulis3 and Ulrich Egert1

1 Bernstein Center for Computational Neuroscience Freiburg, Freiburg, Germany
2 Neurobiology and Biophysics, Institute for Biology III, University of Freiburg, Freiburg, Germany
3 INSERM Unité 704, UJF Grenoble, St. Martin d’Hères, France

The processes causing the transition from normal brain activity to hypersynchronous spiking during epileptic seizures are still fairly unknown. We ask what kind of network is needed to create such activity. Here we show in a model for Temporal Lobe Epilepsy that the network preserved in slices from the sclerotic part of the affected hippocampus alone is not sufficient to generate epileptiform activity (EA). Apparently healthy brain areas are likely involved in the generation of epileptiform events.

1 Investigation of origin of seizures

1.1 Mesial Temporal Lobe Epilepsy

Mesial Temporal Lobe Epilepsy (MTLE) is one of the most common types of partial epilepsies in humans [Wieser, H. G. Epilepsia 45.6 (2004): 695-714]. Since it responds very poorly to pharmacological therapy and surgical resection of affected brain areas can only take place in a limited number of patients, advances in therapies are needed. Therefore, a detailed understanding of the transition from normal brain activity to hypersynchronous spiking during epileptic seizures is essential. The mechanisms causing this transition are, however, largely unknown. Besides studies in patients, adequate animal models for MTLE are increasingly important to facilitate research in this field.

Fig. 1 Cresyl violet staining of the hippocampi from an epileptic mouse. Unilateral injections of kainate into one hippocampus caused severe and typical changes of hippocampal anatomy. Granule cells dispersed; pyramidal cells and inhibitory cells in CA1 and the hilus died. The contralateral hippocampus, however, remained intact.

1.2 Animal model for MTLE

We used a model for MTLE in which a single unilateral injection of the glutamate agonist kainate into the hippocampus induces structural changes of the hippocampal network reminiscent of hippocampal sclerosis in human MTLE [Bouilleret, V. et al. Neuroscience 89.3 (1999), Riban, V. et al. Neuroscience 112.1 (2002): 101-11]. This includes granule cell dispersion, mossy fiber sprouting with formation of recurrent circuits and loss of excitatory and inhibitory cells in CA1 and the hilus (Fig.1). We are interested in how EA is generated and if the degenerated but highly interconnected network is capable of generating this activity alone or, alternatively, if healthy brain areas are required for this process.

2 Epileptiform activity involves more than sclerotic hippocampal areas

2.1 In-vivo recordings indicate involvement of the contralateral hippocampus in epileptiform activity

Adult mice (N=26) received a unilateral injection of kainate into the right dorsal hippocampus. Electrodes were implanted into the injected and the contralateral hippocampus and in the cortex. The mice were recorded freely moving twice a week during 2 months. EA appeared in the injected hippocampus. Concomitantly, spikes could be observed in the contralateral hippocampus (Fig. 2), whose morphology remained intact. This illustrates that EA also involves apparently healthy brain areas. We now asked, how much and what kind of tissue is needed for the generation of EA by investigating slices of the sclerotic hippocampus with microelectrode-arrays (MEA, Multi Channel Systems, Reutlingen).
2.2 Slices from the sclerotic hippocampus do not contain a network sufficient for the generation of epileptiform activity

Hippocampal slices (0.4 mm thick) from 15 kainate injected mice and 7 saline injected controls were recorded on MEAs (60 electrodes, 0.2 mm pitch). Slices were stimulated at 11 different positions throughout all hippocampal areas with an external electrode. Response patterns in slices from kainate treated (KA, i.e. epileptic) and control (CT) mice were compared. KA slices showed significantly diminished responses in CA1 and CA3, which is compatible with the observed cell loss in these areas. Retrograde stimulation of the mossy fibers elicited presynaptic fiber volleys in KA and CT slices, but only in KA slices were these followed by postsynaptic potentials in the dentate gyrus. This indicates that sprouted mossy fibers form functional synapses on granule cells dendrites. Neither KA slices nor controls showed EA upon stimulation. Elevating K+-levels (10 mM) or the application of Bicuculline (5-20 µM) induced EA in control slices (Fig.3). KA slices cut directly adjacent to the injection position, however, did not show EA with either drug (Fig. 4). This indicates that the network in these slices from highly sclerotic hippocampal areas alone is not sufficient to generate EA and that either adjacent, less sclerotic areas of the injected hippocampus or the contralateral hippocampus need to participate. Latest results indicate a gradual decrease in sclerosis with distance from the injection site and with the aforementioned parameters EA could be induced in those distant slices. Spontaneous EA was, however, also missing in those slices.

3 Summary

We could show that the highly sclerotic hippocampal areas of mice with chemically induced MTLE are not capable of generating EA alone. Our results indicate that less sclerotic areas of the injected hippocampus as well as healthy brain areas, like the contralateral hippocampus, are involved in those processes. This suggests an increased importance of involving these healthy brain areas in modelling studies used to understand transitions from normal brain activity to epileptic seizures, which recommends the investigation of the interaction of sclerotic and healthy brain areas in humans in the pursuit of novel therapeutic strategies.

Acknowledgements

BMBF grants FKZ 01GQ0420 and 16SV1743, Inserm, Fondation pour la Recherche Médicale, DAAD.
Characterisation of local-field-potentials in the auditory brainstem using multielectrode arrays

Martin Haustein, Thomas Reinert, Ivan Milenkovic, Rudolf Rübsamen

Faculty of Biosciences, Pharmacology and Psychology at University of Leipzig, Institute of Biology II, Leipzig, Germany

Multielectrode arrays (MEAs) allow recordings of single-unit spike activities and local field potentials (LFPs) from 60 electrodes simultaneously. Such high yield experimental approach may be useful to scrutinize the properties of the potential „sources“ of inhibition and to investigate their impact on afferent, excitatory stimulation in the brainstem nuclei involved in encoding the auditory signals. In order to investigate spatio-temporal characteristics of the GABAergic and glycinergic inhibition in vitro in our future experiments, the present study focused on establishing the MEA-recordings in acute brainstem slices. Medial nucleus of the trapezoid body (MNTB) is a relay nucleus in the auditory brainstem which plays an important role in the processing of interaural intensity differences, a feature critical for the localization of sound sources. Each principal cell of the MNTB receives a single, large somatic synapse (Calyx of Held) whose activation evokes an excitatory postsynaptic current mediated primarily by AMPA receptors. Previous in vivo studies from our group revealed that, when recorded by a single electrode, a prepotential reflecting the excitatory input via a calyx of Held and a postsynaptic action potential can be simultaneously monitored. Therefore, we took advantage of this well characterized synapse and demonstrate here LFPs recorded from MNTB, and evoked by electrical stimulation via a tungsten electrode in the ventral acoustic stria. The recordings were performed on acute brainstem slices (300µm) containing MNTB and acquired from P18-P24 gerbils (Meriones unguiculatus). Similar to the extracellular in-vivo recordings, obtained LFPs are characterized by a pre- and a postsynaptic component (Fig.1) and their shape is specifically related to MNTB. The postsynaptic component could be abolished by removing the extracellular calcium or by blocking the AMPA/kainate receptors. Additionally, application of TTX was shown to inhibit the residual component of the LFPs, thus supporting the notion of its presynaptic origin (Fig. 2).

Since it was previously shown that the discharge properties of the principle neurons in MNTB are...
shaped by inhibitory inputs, we investigated the impact of GABA and glycine on LFPs. Bath applications of GABA or glycine (both 1mM) evoked a decrease in the LFP amplitude for 22.7% and 15.1%, respectively.

On the other hand, application of strychnine (10µM) had minor effect on the postsynaptic component, suggesting that the stimulation in the ventral acoustic stria does not activate inhibitory inputs and also that they are not spontaneously active in our in vitro preparation. To determine the possible sources of inhibitory inputs further experiments should include an additional electrical stimulation of the putative inhibitory nuclei.

Therefore, the combination of pharmacological applications and MEA-recordings is a promising tool to investigate in-vitro the role of inhibition in brainstem circuits that encode interaural timing of auditory signals.

Fig.2: Pharmacological characterization of LFPs and differentiation in pre- and postsynaptic components.

(A). Blocking the AMPA/Kainate receptors by GYKI (10µM) & CNQX (25µM) respectively, leads to a significant decrease of the postsynaptic component only. (box plot: amplitude [µV] for 8 electrodes/ 3 slices)

(B). After the application of Tetrodotoxin (1µM) both components are blocked indicating a presynaptic provenience of the first response. (box plot: amplitude [µV] for 14 electrodes/ 3 slices)
Synchronized Reverberatory Bursting Activity in Cultured Network of Rat Cortical Neurons

June Hoan Kim¹, Joon Ho Choi¹, James Jungho Pak² Kyoung-Jin Lee¹*

1 CRI Center for Neurodynamics, Department of Physics, Korea University, Seoul, Korea
2 Department of Electrical Engineering, Korea University, Seoul, Korea
* Corresponding author. E-mail address: kyoung@nld.korea.ac.kr

We have investigated spatially distributed dynamics that arises in cultured networks of cortical neurons of rats over two weeks by using a home-built multi-electrode array (MEA) plate recording system. The spiking dynamics detected through the MEA electrodes is typically a mixed signal of random single firing events and reverberating bursting events. The bursting activities seem to arise only when some degree of networking is established. Our analysis also indicates that the bursting activity spreads out in space gradually over days and becomes globally synchronized by the time the culture reaches approximately 10 days in vitro.

1 Materials and Methods

1.1 Cell Culture

High density cultures of dissociated neonatal (1-2 day old) Sprague-Dawley rat cortical neurons were prepared. Cell suspension (8×10⁴ cells in a volume of 20 µl) were plated in the central part of the MEA plate, where electrodes are located. The MEA dish was pre-coated with poly-D-lysine and laminine for the cell attachment. The MEA was filled with 1.5 ml of culture solution and the culture dishes were kept in an incubator (5% CO₂ and 95% air, 37 °C).

1.2 MEA Fabrication

The desired pattern of electrodes is formed on Pyrex glass wafer by photolithographic etching. Polyimide is coated over the metallic tracks except ends of tracks in center of plate for the insulation. After then, the gold electrodes are Pt-black electroplated to reduce their resistive impedance to near 20-30kΩ.

2 Results and Discussion

At a very early stage (e.g., DIV 3-4) of the culture, it is typical to have signals only in a few electrodes (Fig.1 (a)). During this period, the action potentials are detected rarely and irregularly. The number of active channels grows rather steadily over several days, as shown in Fig.1 (a) and Fig.2 (a). The number of firing action potentials detected through each active electrodes also increases as the network becomes mature. Interestingly, however, this increase is rather sudden and its dramatic change is related with the emergence of bursting activity on each channel. One notable thing is that the onset of bursting activity is channel-specific. In other words, some channels show bursting activity much earlier than the others, or vice versa. This is clearly reflected in Fig.3 (b) -- the mean ISI rapidly decreases by a factor of 100. The small ISI sequences driven by bursting activities decrease further as the number of bursting activities increases.

We hypothesize that the increase in the number of bursting events is attributed to the reverberating recurrent activity, initially supported by the local network only but eventually supported by the global network in the end. It is plausible that the network acquires more synaptic connections as it grows, and this renders more frequent recurrent excitations in a form of burst. The sequence of bursting activities can, in turn, strengthen the synaptic coupling, providing a positive feedback. The existence of bursting activity in a cultured network of neurons is a quite well-known phenomenon and seems to be a generic feature of any high-density dissociated neuronal cell cultures.

The most notable feature of the observed bursting activity is the synchronization. Bursts initially appear only in a few channels, and they are not synchronized -- we believe that the initial bursts can be generated by a small local network of neurons. This argument is indirectly supported by the plot given in Fig.2 (b): the onset of bursting activity (i.e., the point of dramatic decrease in the ISI) is quite different from one channel to the others. The localized bursting activities become coherent as the network becomes fully connected. The number of bursts increases accordingly as shown in Fig.1 (a). The recurrent bursts are not identical to each other but similar as shown in Fig.1 (b).

As a first step along this line of investigation to identify and characterize the sequential patterns of synchronized bursts or single-firing neuronal activities, we have analyzed the cross-correlation between the firing activities acquired at two different electrodes. Fig.3 shows that the amplitude of the cross-
correlogram peak increases sharply over a short time span of 12 hours. The cross-correlogram is constructed for a specific pair of channels but its general trend is identical for almost all pairs of channels.

![Fig. 1](image-url) Raster plots of 32 channels marking each action potential at three different stages of development in (a) [from top to bottom, t = 102 h, 162 h, and 263 h, respectively], and a superimposed high-resolution raster plot overlaying two different bursting events in (b).

By comparing the original and the rebuilt cross-correlograms, one can definitely claim that the origin of the central peak in the original correlogram is the synchronized bursting activity. Likewise, one can also claim that single spiking activities are not correlated at all: they may act like a background noise to the synchronized bursts. Another important thing to note here is that the dramatic increase of the peak amplitude in the correlogram arises around 9 DIV only when the cell network is quite matured and strongly connected. In other words, the critical time at which the cross-correlation becomes significant is much later than the onset of bursting activity. Two factors can contribute to the increasing cross-correlogram peak: 1) the number of synchronized bursts increases while there is not much change in the single firing activity, and 2) spatially distributed bursting activities acquire a better coherence. In fact, the increasing activity of bursts in a cultured network of neurons over time is also confirmed in a recent independent study.

At this point, we should also indicate that there are significant short-term as well as long-term fluctuations in the mean ISI, even for a fully grown network. There may be a few different reasons for this.

For instance, the state of the firing cell itself or its various synaptic connections to the neighboring cells may have modified, or, possibly, the active cell (body) may have moved to a different location, losing its electric contact with the once-attached electrode. In fact, each electrode may pick up the mixed signal orchestrated by a small group of cells.

3 Conclusions

We have investigated the spontaneous spatiotemporal dynamics that arises in a cultured network of cortical neurons by using a multi-electrode recording system. It is found that the spontaneous neural dynamics drifts with time, engaging more cells as the system becomes matured and its connectivity becomes strengthened. Initially, single spikes prevail, but they soon yield to bursting dynamics. The bursts become more frequent in time and cover a larger domain. The spatial structure of the recurrent bursts is not identical, but similar. Our experimental results discussed here are only preliminary. In the future, we are going to identify various “synfire chains” and investigate their fidelity and diversity with systematic control over various physical and pharmacological conditions.

Acknowledgement

This work was supported by Creative Research Initiatives (CRI) of the Korean Ministry of Science and Technology.
Late phase LTP recordings in hippocampal slices

Frank Hofmann*, Bettina Buschle, Hilmar Bading

Department of Neurobiology, Interdisciplinary Center for Neurosciences (IZN), University of Heidelberg, Heidelberg, Germany
* Corresponding author. E-mail address: Frank.Hofmann@urz.uni-heidelberg.de

Long term potentiation (LTP) in the hippocampus is one of the most common models for studying learning and memory. Although much interest is focused on the initiation of LTP, the late phase that allows LTP to persist for hours to days might be of even greater importance for the understanding of information storage in the brain. We established stable long term recordings of hippocampal slices on MEAs and stimulation paradigms to induce decaying and long lasting LTP to investigate the different signalling mechanisms involved in these processes.

1 Introduction

Since its discovery, long term potentiation (LTP) in the hippocampus has become one of the most commonly used models for studying synaptic plasticity in the central nervous system. Although much interest is focused on the initiation of LTP, the late phase that allows LTP to persist for hours to days (L-LTP) might be of even greater importance for the understanding of information storage in the brain. This late phase is difficult to access in acute slice preparations, as it is not easy to maintain a stable recording over several hours. Recordings with substrate integrated planar electrodes should be more stable and less sensitive to factors such as vibrations or the death of isolated cells in a slice, as planar electrodes sample activity from a large number of neurons. Despite these advantages, only few publications exist using MEAs for studies of late phase LTP. However, these studies have demonstrated that stable long term recordings on MEAs are possible [1].

2 Material and Methods

We established stable recordings from acute hippocampal slices of rats and mice on MEAs lasting 5 hours and longer. Two different input pathways to CA1 cells were stimulated by biphasic voltage pulses alternately with one stimulus per minute for each pathway. The optimal stimulation strength was determined by an input/output (I/O) curve with pulses ranging from 500mV to 3V. After 40 minutes of baseline recording, different high frequency stimulation paradigms were applied to one pathway. The second pathway was used as an internal control. After the induction of LTP, the responses of the slices were recorded for an additional four hours or longer. After this period, the same I/O curve as before was acquired again. From these two I/O curves, the relation from EPSP slope to population spike amplitudes from electrodes in the CA1 cell layer was determined. Furthermore, potentiated and untreated slices were used for gene expression analysis after recording. MEA data was used to determine the area affected by the LTP inducing stimulus. This area was micro dissected before isolating the RNA to minimize the influence of unstimulated tissue and thereby improve the signal to noise ratio.

3 Results and Discussion

3.1 LTP recordings

We established two stimulation paradigms to induce either a decaying form of LTP that persists only for about 2h, or a long lasting LTP that is stable over the whole recording period (L-LTP). In slices from p21 animals, a single high frequency stimulus (100Hz, 1s) was sufficient to induce a long lasting (>2h) form of LTP (see Fig. 2A). A stimulus of only 0.5 resulted in a decaying LTP (data not shown). Generally, four trains of 100Hz stimulation are needed to induce lasting LTP. Indeed, in animals only two weeks older (p35), a single 100 Hz train was not longer sufficient.
to induce L-LTP. So it seems that the age of the animals, as long as they are still juvenile, plays a critical role in the threshold needed to induce L-LTP.

3.2 E-S potentiation

Additionally, we determined the relationship of EPSP slope to population spike amplitude in the CA1 pyramidal cell layer. The change of this parameter in slices displaying LTP is known as EPSP population-spike potentiation (E-S potentiation), and may represent a measure for the excitability of the cells. In slices with lasting LTP, the E-S curve showed a shift towards higher excitability after LTP induction (Fig. 2B). In slices with only decaying LTP, there was no significant difference between curves determined before and 4h after induction of LTP (data not shown).

Thus, L-LTP and E-S potentiation seem to have a similar threshold, even though both phenomena are thought to depend on different mechanisms and are also known to occur independently from each other.

3.3 Gene regulation

The gene expression analysis was focused on brain derived neurotrophic factor (BDNF) that has been implicated in the regulation of synaptic efficacy. The levels of BDNF mRNA will be determined using quantitative reverse transcriptase (QRT-PCR) that had undergone induction of either lasting or decaying LTP.

4 Summary

We were able to establish LTP recordings from hippocampal slices lasting 5h and longer. Apart from the relatively easy handling, MEA recordings provide additional information that can be used to simultaneously investigate parameters like E-S potentiation or gene regulation. Therefore, our results suggest that acute slices on MEAs are well suited to investigate especially the late phase of synaptic plasticity. In the future, we will use slices from animals in which through viral gene transfer signaling pathways involved in LTP induction and maintenance have been manipulated.

Acknowledgement

Supported by the Alexander von Humboldt Foundation.

Reference

Spatio-temporal dynamics of oscillatory network activity in the neonatal mouse cerebral cortex

Jyh-Jang Sun, Heiko J. Luhmann *

Institute of Physiology and Pathophysiology, University of Mainz, Mainz, Germany
* Corresponding author. E-mail address: luhmann@uni-mainz.de

We have recently demonstrated in the intact cerebral cortex preparation of the newborn mouse that electrical stimulation, activation of muscarinic acetylcholine or metabotropic glutamate receptors elicits transient network oscillations in the beta and gamma frequency range [1]. Here, we use a 60-channel multielectrode arrays (MEAs) to study in thick neocortical slices the spatio-temporal characteristics of this network activity. Beside the previously described carbachol-induced and stimulation-elicited oscillations we also observed spontaneous oscillatory activity in physiological ACSF (3 or 5 mM K\textsuperscript{+}, 1 mM Mg\textsuperscript{2+}, 1.6 mM Ca\textsuperscript{2+}). Locally propagating as well as long-distance propagating spontaneous oscillations could be observed. Long-distance propagating spontaneous and carbachol-induced oscillations were similar in duration (7.3 ± 3.8 s, 9.3 ± 1.4 s), amplitude (136.0 ± 11.1 µV, 161.0 ± 14.0 µV) and frequency (10.2 ± 1.4 Hz, 12.9 ± 0.7 Hz). However, the stimulation-elicited oscillations had a shorter duration (0.5 ± 0.1 s) and higher frequency (17.5 ± 1.2 Hz). And locally propagating spontaneous oscillations had a shorter duration (2.22 ± 0.52 s).

Current source density analyses demonstrated an alternation of current sinks and sources within the cortical plate and subplate. Our data indicate that synchronized and carbachol- or stimulation-induced network oscillations have similar functional properties and may reflect an intrinsic capacity to generate early functional networks.

1 Introduction

Synchronized oscillatory network activity plays an important role in early cortical development. Using different technical approaches, such as calcium imaging [2], single cell recording [3,4], EEG or 16-channel electrode recording [1,3], oscillatory activity patterns have been demonstrated in the cerebral cortex of various species, including humans (for recent review see [5]). However, the spatio-temporal properties of these synchronized oscillations are still unclear. Here, we use a MEA system to study in newborn mouse (P0-P3) neocortical slices the spatio-temporal dynamics of this early cortical network activity.

2 Methods

Acute 700-1000 µm thick coronal or sagittal slices including the primary somatosensory cortex from C57BL/6 mice aged 0-3 days were prepared as described previously [3].

After an incubation period of ca. 1 h, slices were transferred to 3-D MEA (Ayanda Biosystems, Switzerland) on MEA 1060-INV-BC (Multi Channel Systems (MCS), Germany), which was mounted on an inverted microscope (Optika microscopes, Italy), to which ACSF was continuously perfused at a rate of 2-3 ml/min at 32°C by TCO2 and TH01 (MCS, Germany).

Data were recorded at 5 kHz sampling rate via MEA_RACK (MCS, Germany). In some cases, two of the planar microelectrodes were selected for bipolar constant-current pulses (10–80 µA, 10 pulses of 200 µs duration at 50 Hz [1] application via STG 2004, MCS, Germany).

Data were imported to a custom-written program in Matlab (Mathworks, USA) with datastrm.m and nextdata.m (MC_Rack, MCS, Germany). Time-frequency plots were calculated by transforming field potentials using Morlet continuous wavelet (Matlab, cwt.m) [6] and power values were normalized with the formula (value-minimal)/(maximal-minimal). The number of iterations of centre frequency was 8.

1-D CSD analysis was applied to vertical channels with 3-point kernel (1, –2, 1) [7]. 2-D CSD analysis was performed using a 3 X 3-weighted average kernel (0 1/8 0, 1/8 1/2 1/8, and 0 1/8 0) and the result was transformed with a 3 X 3 X 3 Laplacian kernel (0 1 0, 1 -4 1, and 0 1 0) [8] to produce a discrete approximation of the second spatial derivative. All data are expressed as mean ± SEM.
3 Results

Spontaneous oscillatory activities were observed between cortical plate and subplate of somatosensory cortex (n=113 slices). Spontaneous oscillations recorded in normal ACSF (3-5 mM KCl) were analyzed in detail in 16 slices. In 10 of these 16 slices spontaneous oscillations were locally restricted to cortical regions of ca. 0.3 mm in diameter and in the remaining 6 slices oscillations were propagating within the cortical plate and/or subplate over long distances of >1 mm (Fig 1B). The propagating directions were medial to lateral in 3 slices, lateral to medial in 4 slices, posterior to anterior in 1 slice and in 8 slices could not be differentiated. Altering pairs of sinks and sources could be seen with 1-D CSD analysis in the subplate and layer IV (Fig 1D).

Oscillations elicited by local electrical stimulation near subplate were obtained in 10 out of 17 slices (6x subplate, 3x white matter and 1x layer V-VI). These stimulation-induced oscillations had a shorter duration (0.5 ± 0.1 s, n=8) and higher frequency (17.5 ± 1.2 Hz, n=8) as compared to the spontaneous events (4.0 ± 1.4 s, n=15; 11.4 ± 0.9 Hz, n=16).

In 36 slices long-distance propagating oscillations could be elicited by bath application of 30-100 µM carbachol [1]. These carbachol-induced oscillations were similar in frequency, duration and amplitude as compared to the spontaneous events. In 11 slices, carbachol-induced oscillations propagated for more than 700 µm and in 4 slices over >1400 µm. Altering pairs of sinks and sources could be seen with 1-D or 2-D CSD analysis in the subplate and cortical plate.

4 Summary

Here we characterize spontaneous oscillations in newborn mouse somatosensory cortex in vitro. This activity has similar properties as recently described cortical network activity in vivo, both in rodents [9] and humans [5]. Our results indicate that spontaneous, carbachol- or stimulation-induced network oscillations
reveal similar functional properties and may reflect an intrinsic capacity to generate early functional networks.

Acknowledgement
This study was supported by a grant of the Deutsche Forschungsgemeinschaft to HJL (DFG Lu375/4).

References
Individual neurons of the suprachiasmatic nucleus of the hypothalamus cultured on multielectrode arrays as model for the action of phase-shifting stimuli

Christopher Klisch, Sabine Mahr and Hilmar Meissl

Max Planck Institute for Brain Research, Neuroanatomical Department, Frankfurt, Germany

In mammals, the primary circadian pacemaker is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. This pacemaker, entrained in vivo by the daily light-dark cycle, controls (or influences) many physiological, behavioral and endocrine rhythms in the body. The temporal organization of the SCN was analyzed in the last two decades predominantly by single unit recording techniques in hypothalamic brain slice preparations showing that the SCN generates in vitro a 24-hour oscillation in neuronal firing rate with stable mean activity peaking at subjective near midday (circadian time = CT 7) (Gillette, 1991). Although these brain slice preparations could survive only a limited time (up to 2 days), these single unit experiments have provided important insights into the gating mechanisms of the clock, i.e. the external control of the pacemaker.

In the last years, planar microelectrode arrays (MEA) have become a valuable tool for studying the extracellular activity of excitable cells over long time periods, i.e. days, weeks or even several months and were also introduced in circadian research (Welsh et al., 1995; Tousson & Meissl, 2004; Aton et al., 2005). In the present study, we used multi-site electrical recordings with MEA chips to monitor the firing pattern of dispersed SCN neurons for up to several weeks. This was done to investigate the action of phase-shifting stimuli on circadian rhythmicity of individual neurons and to assess the contribution of network formation on possible phase shifting responses.

SCN neurons from newborn rats (P2-P3) were dissociated using papain and plated at low densities (<3000 cells/mm²) on MEAs consisting of sixty electrodes of 30 µm in diameter. Developing neuronal processes were observed a few hours after cell dissociation and the first spontaneously active neurons could be recorded after approximately 24 hours. After one to two weeks in culture, SCN neurons resting on a dense layer of flattened astrocytes had developed small networks of neuronal processes. Despite the formation of networks correlated spike activity was only rarely observed in low density cultures of the SCN. However, dispersals of the target areas of the SCN, the dorsomedial nucleus of the hypothalamus or the arcuate nucleus, frequently displayed synchronized activity pattern or correlated burst activity.

Robust circadian activity rhythms with a period of ca. 24 hours were observed in a minority of cultured SCN cells. Most neurons ceased rhythmic firing after 2 or 3 days, became less stable or started to fire with an irregular pattern. From these data it appears that many neurons require a neuronal network, i.e. an input from other neurons, to maintain their rhythmic discharges.

Spike activity of cultured SCN neurons was modulated by several neuroactive substances causing acute, short-term, excitation or inhibition of the spike rate as well as effects on the circadian phase of the neuron. Phase-shifting effects were observed in cultures of dispersed SCN neurons after stimulation with the neurotransmitters of the retinohypothalamic tract PACAP (Fig. 1), glutamate (Fig. 2) or the pineal hormone melatonin. Application of PACAP (100 nM for 30 minutes) at CT 24 (maximum defined as CT 6) caused a phase delay of 5 hours, whereas application at CT 3 was almost without effect (Fig. 1). Glutamate application at subjective night (CT 17) caused a phase advance of 0.75 hours (Fig. 2). Melatonin applied at different circadian times elicited also phase advances or phase delays in individual SCN neurons.

Because of the higher variability of phase-shifting stimuli in dispersed cultures compared to brain slice preparations it is concluded that network properties play an essential role for the stability of the circadian output of the SCN.

References
**Fig. 1** Circadian rhythm in spontaneous firing of a representative SCN neuron cultured on a MEA. PACAP application (100 nM for 30') caused a phase delay when applied at CT 24, but not at CT 3. Continuous and dotted curves represent cosine functions with a period of 24.4 hours.

**Fig. 2** Spontaneous firing rate of a SCN neuron cultured on a MEA. Glutamate application (10 µM, 30') caused a phase advance when applied at CT 17.
Synaptic potentiation re-organized functional connections in a cultured neuronal network connected to a moving robot

Suguru N. Kudoh¹,², Isao Hayashi³ and Takahisa Taguchi¹

¹ Cell Engineering Research Institute (RICE), National Institute of Advanced Industrial Science and Technology (AIST)
² PRESTO, Japan Science and Technology Agency, Japan
³ Fac. Informatics, Kansai University.
* Corresponding author. E-mail address: taguchi-takahisa@aist.go.jp

Neurons form complex networks and it seems that the living neuronal network can perform certain types of information processing. We are interested in intelligence autonomously formed in vitro. The functional connections between cultured neurons were dynamically modified by synaptic potentiation and this process may be required for reorganization of the functional group of neurons, as such neuron assemblies are critical for information processing in brain. In addition, we carried out the system integration for KheperaII robot and living neuronal network, and we are analyzing the interaction between the robot and neurons. Our goal is reconstruction of the neural network, which can process "thinking." in the dissociated culture system.

1 Functional assemblies in cultured neuronal network

1.1 Connection Map Analysis

We elucidated that dissociated rat hippocampal neurons on a multielectrode array formed heterogeneous networks of functional connections. We estimated the functional connections between neurons of which signals detected by electrodes. Spike trains were subjected to cross correlation analysis. Then connectivity indices were calculated for each pair. The connectivity index is defined as follows:

\[ C = A_{peak} \times \left( \frac{0.01 \times A_{peak}}{A_{total}} + \frac{1}{\Delta t + 1} + \frac{1}{\text{correlation}} \right) \]

Where \( A_{peak} \) is an area within the 2 msec range around the peak of cross-correlolfunction of the pair, \( A_{total} \) is the area of cross-correlolfunction, \( \Delta t \) is the distance of the peak from 0 ms. The connectivity index indicates the relative robustness of the relationship between the pair. The mean and standard error of all connectivity indices were calculated. When the value of the connectivity index exceeded the mean plus standard error, the pair was assumed to be functionally connected. Each neuron is denoted as a small point in maps, and lines between the points represent the connectivity between the neurons indicated by these points. In this study, we generated connection maps from data recorded for 10min. The bin width for calculation of the cross-correlation was 5 msec, and the range of the calculation of cross-correlolfunction was 120 msec. The connection maps indicate that each culture contained some hub-like neurons with many functional connections.

Fig. 1 Functional connections in a cultured neuronal network. An example of a connection map. Each point in the map represents an identified neuron and the lines represent functional connections between these neurons. Red bold circles indicate "hub"-like neurons; each hub neuron has more than ten inputs from other neurons.

1.2 Drastic re-organization of functional assemblies

We already showed in previous reports that long lasting synaptic potentiation in the amplitudes of spontaneous synaptic currents (SSCs) is induced by a Mg²⁺-free condition in dissociated neuronal networks [1]. Our results are consistent with the hypothesis that synaptic potentiation modifies the flow of information in part by reorganizing cell assemblies in living neuronal networks. We have analyzed the spontaneous action potentials in living neuronal networks on a multi-electrode array, and the connection map before
and after the induction of the synaptic potentiation suggest that the functional connectivity between neurons changed drastically by synaptic potentiation. We found that the emergence of new hub-like neurons after the induction of synaptic potentiation by a Mg²⁺-free condition. It implies that additional neuron assemblies formed or that existing neuron assemblies formed new associations with groups of coupled neuron assemblies. Spontaneous activity is enough to construct dynamic functional assemblies of neurons through self-organization even in cultured neuronal networks. Furthermore, synaptic potentiation can induce the re-organization of such assemblies of neurons.

**Fig. 2** Effect of synaptic potentiation on a connection map. Connection map before (upper panel) and after (lower panel) the induction of synaptic potentiation. These maps were obtained from the same culture (E17DIV19).

2 Interaction to outer world in cultured hippocampal neurons interfaced by moving robot.

In addition, we are developing the system in which living neuronal network interacts to outer world by the intermediary of miniature moving robot. We use Khepera II robot (K-Team), Lab View (National instruments) because Khepera II can be programmed easily by LabView environment and MED64 system includes DAQ board of National instruments. We extended the "Hybrot (Hybrid living+robotic)" approach of the Potter group [2] and provided a program which generates "premised control rules" for making a robot avoid obstacles, instead of autonomously generated rules. The premised rules were described by Fuzzy logic. Robot control unit receives 8 inputs from the neuronal network. Stimulation control unit receives outputs of 8 IR sensors of the robot. When Robot control unit detect particular pattern of neuronal activities evoked by the electrical stimulation to "left" labelled electrode, the speed of left actuator is set to be fast. Sometimes the robot seemed to ignore the sensor inputs, but it feckly avoided collision. In our previous reports [3], the pattern of network activities can be modified by a particular pattern of electrical inputs. If we observe modification in the behaviours of this robot by interaction to outer world, can we define that phenomenon as the learning of living neuronal network? Our ultimate goal is to generate intelligence in culture dish.

**Fig. 3** Trajectory of KheperaII robot controlled by cultured neuronal network. Orange dots indicate the sensors located at left side of the robot detect objects. Green dots indicate the right sensors detected objects.

**Acknowledgement**

We thank M.S. Kiyohara for culture preparation and for assist of recording of neuronal activities.

**References**


Developmental switch from LTD to LTP in low frequency-induced plasticity

Fabien Lanté, Mélanie Cavalier, Catherine Cohen-Solal, Janique Guiramand and Michel Vignes*

Laboratory ‘Oxidative stress and Neuroprotection’ University Montpellier II, Montpellier, France
* Corresponding author

We have recently demonstrated that the stimulation of the Schaffer collateral/commissural fibers at low frequency (1Hz) for short durations (3-5 min) could trigger a slow-onset form of long-term potentiation (LFS-LTP) in the CA1 area of the adult rat hippocampus. This phenomenon was observed with MEA recordings. Here we have examined the developmental profile of this form of synaptic plasticity.

In 10 to 15 day-old rats, the application of low-frequency stimulation (1Hz for 5 min) induced long-term depression (LFS-LTD). At later developmental stage, i.e. 17-21 day-old, 1Hz stimulation had no effect when applied for 5 min but mediated LTD when stimulus duration was increased to 15 min. Over 25 day-old, 1Hz stimulation (3-5 min) mediated LFS-LTP, as we previously observed. At earlier developmental stages examined here (10-15 day-old), LFS-LTD was dependent on both N-methyl-D-aspartate (NMDA) and mGlu5 receptor activation as shown by the inhibitory effect of selective antagonists, AP5 and MPEP, respectively. By contrast, antagonists of mGlu1α receptor, LY367385, and cannabinoid receptor type 1 (CB1), AM 251, were ineffective to block LTD induction. LFS-LTD was not associated with a change in paired-pulse facilitation ratio, suggesting a postsynaptic locus of expression of this plasticity. Since the activation of both mGlu5 and NMDA receptors was required to obtain LFS-LTD, we wondered whether a sequence in the activation of these receptors occurred to trigger LFS-LTD. By itself, NMDA induced a LTD which was dependent on mGlu5 receptor activation. By contrast, the direct stimulation of mGlu5 receptors with (RS)-2-Chloro-5-hydroxyphenylglycine (CHPG) mediated a NMDA receptor-independent LTD via a postsynaptic mechanism. In addition, CHPG-LTD was closely related to LFS-LTD since the saturation of LTD with 1Hz stimulation completely occluded the CHPG action.

Taken together, we postulate that LFS-LTD required the sequential activation of NMDA receptor in first and mGlu5 receptor in second. Low-frequency-mediated synaptic plasticity is thus subject to a developmental switch from NMDA- and mGlu5 receptor-dependent LTD to mGlu5 receptor-dependent LTP with a transient period (17-21 day-old) during which LFS is ineffective.
Large Database of Spontaneous and Evoked Activity Patterns in Developing Networks

Daniel A. Wagenaar,1 Jerome Pine,2 and Steve M. Potter,3*

1 University of California, San Diego, Division of Biology
2 California Institute of Technology, Department of Physics
3 Georgia Institute of Technology and Emory University School of Medicine, Coulter Department of Biomedical Engineering
* Corresponding author: steve.potter@gatech.edu

We have made available on the web a comprehensive (>40GB) set of multi-unit spike data on cultures of dissociated embryonic rat cortex. We studied 58 cultures of different densities (3,000 to 50,000 neurons on areas of 30 to 75 mm²) growing on multi-electrode arrays (Multichannel Systems) during the first five weeks of their development (Wagenaar et al., 2006). Half-hour recordings of spontaneous activity were made almost daily, as well as network responses evoked by stimulating each electrode sequentially. The dataset also includes 36 long (overnight) recordings.

1 Spontaneous Population Bursts

We developed an objective classification scheme (detailed in Wagenaar et al., 2006) for the spontaneous bursts that cortical cultures exhibit. Burst examples are shown in Fig. 1, and the development of different classes of bursts is summarized in Fig. 2. We found a clear density-dependence on the timing of burst development, with denser cultures maturing much sooner than sparse ones. Burst sizes in dense cultures were limited to a few electrodes in the first week, and then appeared on most or all of the 59 electrodes (Dish-wide bursts). Cultures plated on the same day showed much more similar bursting behavior than same-age pairs prepared from different donors. Bursting was movement-sensitive: especially at 1-2 weeks in vitro, bursting increased for a few minutes after any slight movement of the MEAs. All recordings were carried out in the incubator housing the cultures, so they could be subjected to as little movement as possible when placed into the preamplifier. Cultures were grown in teflon-sealed MEAs (Potter & DeMarse, 2001), which allows culturing, recording, and stimulating in a non-humid incubator.

2 Responses to Probe Stimuli

We used our custom multi-site stimulator (Wagenaar & Potter, 2004) to apply effective voltage-controlled stimuli (Wagenaar et al., 2004) to each electrode in the cultures sequentially at 0.3 sec intervals, 50 times (15 min total). Stimulus-induced responses usually appeared several days later than spontaneous bursts. Functional projections, as measured by only counting direct, non-synaptic action potentials, reached across the MEA by day 15 in dense cultures, while in sparse cultures such culture-spanning axons may not have been present even by 5 weeks in vitro.

3 Summary

By following the development of a larger number of cultures than any previous report based on MEA recordings, we have found that the range of spiking dynamics exhibited by networks of cortical cells in vitro is much more complex than previous publications suggest. We have only begun to analyze the patterns in our dataset, and invite others to join us. Comparisons of these data with in vivo recordings will help to elucidate the relevance of MEA research to developmental and pathological questions in animals and humans.

Acknowledgements

This work was partially supported by grants NS38628 from NIH/NINDS, EB000786 from NIH/NIBIB and DA18250 from NIH/NIDA, and by the Whitaker Foundation and the NSF Center for Behavioral Neuroscience. We thank Sheri McKinney for technical assistance.

References

Fig. 1 Prototypical examples of burst categories. The Array-wide Spike Detection Rate (ADSR) is plotted for 5-minute recordings of various cultures, with a greyscale rasterplot of spikes across channels depicted below each trace.

Fig. 2 Color- and Pattern-coded depiction of the types of spontaneous bursts observed in 58 rat cortical MEA cultures (plating batch number in parentheses at right) across the first 5 weeks in vitro. Cell densities varied from 2500/mm² (Dense) to 110/mm² (Ultra Sparse), and culture sizes were either 5 mm or 3.1 mm (Small) in diameter.
Extracellular recordings of action potentials in locust neuronal culture with field-effect transistors

Stefan Weigel¹,², Sven Ingebrandt² and Andreas Offenhäusser²*¹

1 Laboratory for Materials-Biology Interactions, Empa, St.Gallen, Switzerland
2 Institute of Thin Films and Interfaces, Institute 2: Bioelectronics, and CNI – Center of Nanoelectronic Systems for Information Technology, Forschungszentrum Jülich GmbH, Jülich, Germany
* Corresponding author. E-mail address: a.offenhaeusser@fz-juelich.de

Insects exhibit a simple nervous system with highly adapted and sensitive sensory and motoric systems. The underlying neuronal circuits e.g. of the locust jump consist of only few neurons, which can be often identified in situ. Investigation of these networks in situ is limited to only one or two neurons with the disadvantage of interactions with surrounding cells and a short recording period. Reconstruction of these networks on non-metallised field-effect transistor or metal multi-electrode arrays will allow long-term extracellular recordings of many neurons in parallel. Here, FET signals recorded of cultured insect neurons are presented and compared to the corresponding patch-clamp signal. In future this non-invasive technique will be used for analysis of signal processing in reconstructed networks of insect neurons.

1 Introduction

Extracellular recording techniques with planar, non-metallised field-effect transistors (FETs) or multi-electrode arrays (MEAs) can be alternatively used for investigation of neuronal signals instead of common electrophysiological recording techniques like patch-clamp or sharp electrode recordings (Fromherz et al., 1991; Offenhäusser et al., 1997; Ingebrandt et al., 2005).

Such sensor arrays allow non-invasive long-term recordings and recordings from many neurons, simultaneously.

In our study we aim to analyse signal processing in cultured neuronal networks. Insects provide many interesting circuits involved in processing of sensory or motor information consisting of only a relatively small number of neurons. We have chosen the neuronal circuit mediating the jump of Locusta migratoria as a model system (Heitler & Burrows, 1976). Neurons involved in this circuit are mostly large in diameter (40 to 120µm) and can be identified individually by a backfill staining technique. Therefore, this circuit is an ideal model for the reconstruction of the in vivo situation on the FETs.

Here, we present electrophysiological data of single neurons recorded with the FET and compared to the corresponding patch-clamp signal.

2 Materials & Methods

2.1 Cell culture

Cell culture conditions were established for primary neurons of Locusta migratoria. The meta- and mesothoracic ganglia of adult locusts were excised, followed by dissociating the neurons mechanically and by enzymatic treatment. Isolated thoracic neurons were seeded on 4x4 FET arrays (Offenhäusser et al., 1997) coated with poly-D-lysine. The cells were cultured in modified Leibovitz medium. Cultures were maintained in 95% relative humidity, at 29°C.

2.2 Electrophysiology

The electrical activity of neurons, which were adhered on top of the FET gates were recorded at 2-6 DIV using FET device and patch-clamp technique (whole-cell mode), simultaneously. We measured action potentials in the current-clamp configuration as well as membrane currents in the voltage-clamp configuration. The resulting ion flux across the membrane led to voltage changes in the small cleft between the cell membrane and the FET gate, which were monitored by the FET. These signals were analysed and compared to the corresponding patch-clamp signals.

3 Results

Here we present extracellular recorded electrical signals of insect neurons measured with FETs. Action potentials resulted in voltage changes $\Delta V_{GS}$ of up to 800µV in the FET (Fig.1). The signals resemble in each case C-type recordings (Schätzthauer and Fromherz, 1998) dominated by high sodium and potassium
conductance in the junction membrane compared to the capacitive current over the membrane. Manipulation of the position relative to the gate resulted in higher signals, if a neuron was moved centric or pressed onto the FET gate. However, changing the distance of the cells to the gate in that way did not affect the signal shape (Fig. 2).

Fig. 1 Simultaneous recording of an action potential with the patch clamp technique (upper trace) and a field-effect transistor (lower trace; data not averaged); \( V_j \) = junction voltage \( V_m \) = membrane voltage.

4 Summary

Extracellular recording systems like field-effect transistor or metal microelectrode arrays provide the possibility to record from networks of cultured insect neurons.

In future, we want to use this powerful tool to investigate the development, synaptogenesis and signal processing of reconstructed neuronal networks in vitro.

Acknowledgement

The authors thank S. Böcker-Meffert, P. Bräunig, R. Helpenstein, S. Schaal, P. Schulte, F. Sommerhage and G. Wrobel for their support.

This work was performed in the frame of CI-CADA, an IST project funded by the Future and Emergent Technologies arm of the IST Programme - FET Keyaction Life-like perception (IST-2001-34718).

Fig. 2 Field-effect transistor signals of action potential during manipulation of the cell position to the FET gate (data not averaged).

References


Sub-Millisecond Latency Closed Loop Set-Up
Enabling the On-line Classification and Modification of Network Activity

Christoph Zrenner¹, Volker Gauck², Shimon Marom², Peter Thier¹

1 Hertie-Institut für klinische Hirnforschung, Zentrum für Neurologie, Universitätsklinikum Tübingen, Tübingen, Germany
2 Faculty of Medicine, Technion, P.O.B. 9649, Haifa 31096, Israel

We present a versatile bi-directional real-time MEA set-up to study the dynamics of neural tissue in closed-loop paradigms with reliable feedback latencies below 1 ms. A custom Mathworks Simulink model compiled to execute in real-time on a standard PC running Mathworks xPC target is used to classify network activity on-the-fly allowing a conditional and precisely timed feedback stimulus to be applied. The real-time target PC is asynchronously polled and controlled over a network connection from a second PC running Matlab. This allows the real-time processing to be separated from the experimental control scripts resulting in a fully automatable and scriptable closed-loop system.

By stimulation at specific points of time during the development of a burst, the modifiability of ongoing network dynamics is investigated. Additionally the effect of different stimulus sites is mapped by automatically selecting different electrodes for stimulation between bursts using the serial interface of the MEA1060-BC head-stage.

Acknowledgement
Supported by the Böhringer-Ingelheim Foundation and the Max-Planck Minerva Stiftung.

Fig. 1 Intra-burst stimulation: Burst onset is defined to occur when the number of action potentials detected across all channels during a sliding window of 25 ms reaches 10 or more. A stimulus (unipolar 30μA current pulse of square form with 200us negative followed by 200us positive flank) is then applied after a set delay of 1ms inside the spontaneously occurring network bursts. This figure shows the statistics of 64 such bursts aligned by their onset. The top left panel is a “peri-burst time histogram” showing the average number of spikes at each electrode in 1 ms time-bins during burst development. The top right pane is similar, but the number of spikes is summed across the 60 electrodes. The bottom left pane shows the overall activity of each of the 64 average bursts separately. The bottom right pane shows the average number of spikes per burst per electrode. The downward slope of the stimulus artefact stems from the blanking circuit of the MEA1060-BC head-stage.
Signal Analysis and Statistics
Identifying functionally interdependent neurons from multielectrode array recordings in local and global network contexts

Karim Oweiss1*, Rong Jin2, Yasir Suhail3, Feilong Chen2
1 Electrical and Computer Engineering Dept., Michigan State University, MI, USA
2 Computer Science and Engineering Dept., Michigan State University, MI, USA
3 Biomedical Engineering Dept., Johns Hopkins University, MD, USA
* Corresponding author. E-mail address: koweiss@msu.edu

Simultaneous recording of large-size neuronal ensembles with high-density microelectrode arrays (MEAs) is becoming a common trend in studying systems neuroscience [1-4]. Despite rapid advances in MEA fabrication technology, the identification of clusters of neurons with correlated spiking activity from the observed spike trains is a nontrivial problem. Classical techniques are limited to the assessment of doublets or triplets of neurons at a fixed temporal bin width. These techniques quickly erode in the face of large ensembles, or when nonstationarity in neural firing is encountered during bursts of neural activity or due to neural plasticity [5-12]. We propose a nonparametric approach for solving this problem that relies on projecting the spike trains onto a nested set of multiresolution spaces which allows the discrete event processes to be mapped onto a ‘scale space’. A new spectral clustering algorithm is applied to identify clusters of correlated firing within distinct behavioral contexts. The technique is able to efficiently identify functionally interdependent neurons independent of the temporal scale from which rate functions are typically estimated. We report the clustering performance of the algorithm applied to a complex synthesized data set and compare it to multiple clustering techniques to illustrate the substantial gain in the performance.

1 Theory

1.1 Problem Formulation

Suppose there are \( P \) neurons recorded in a discrete time interval of length \( N \). These neurons belong to any of \( K \) clusters. Each cluster represents neuronal elements that exhibit functional interdependency across both short and long time periods. In addition, a neuron can belong to more than one cluster, which implies that it can belong to a short rapid synchronized population as well as a slow asynchronous population in response to both local and global inhibition/excitation from other populations. Let the function \( f_p(t) \) denote the firing rate of neuron \( p \), along with its sampled version \( f_p = [f_p(1), f_p(2), f_p(N)] \) within the interval \( T = [t_1, t_2, ..., t_N] \). Let \( F_k \) denote an underlying unknown base rate function of the \( k \)th cluster. For the scope of this paper, we model the firing rate function of neuron \( p \) as a linear combination of base firing rate functions of all \( K \) clusters, i.e.,

\[
f_p = \sum_{k=1}^{K} a_{pk} F_k
\]

where \( a_{pk} \) represents the degree of membership of neuron \( p \) to the \( k \)th cluster. Generalizing to nonlinear combinations is straightforward and is demonstrated elsewhere [13]. The spike train \( s_p \) can then be modeled as the sum of the sampled firing rate \( f_p \) plus an independent error term as

\[
s_p = f_p \delta T + z_p
\]

where \( \delta T \) is the bin width used to sample the spike train for estimating the rate function \( f_p(t) \), and \( z_p \) represents an estimation error incurred in the approximation of the true rate function \( f_p(t) \) by the point process \( s_p \).

1.2 Scale-Space Data Model

Let \( W(j) \) denote the lumped matrix operator of a Haar discrete wavelet transform at time scale \( j \) [14]. The spike train at level \( j \), denoted \( s_p \), can be expressed as

\[
s_p = W^{(j)} s_p = \sum_{k=1}^{K} a_{pk} F_k^j \delta T + W^{(j)} z_p
\]

where \( F_k^j \) represents the \( k \)th cluster base firing rate function of the \( k \)th cluster at the time scale \( j \). The sample cross correlation between neurons \( p_1 \) and \( p_2 \) at time scale \( j \) is determined as

\[\delta T \text{ is selected to account for the refractory period, i.e., the ‘digital’ form of the spike train can accommodate consecutive ‘1’ bins.}\]
\[ c_{p_1,p_2}^j = \frac{1}{N} \sum_{n=1}^{N} s_p^T n s_p^j n \]  

Taking expectations

\[ E[c_{p_1,p_2}^j] = \frac{1}{N} \sum_{n=1}^{N} \sum_{p_1,p_2} \epsilon_{p_1,p_2}^j + \epsilon_{p_1,p_2}^{j, \epsilon} \]  

where \( \epsilon_{p_1,p_2}^j = \frac{1}{N} E[zz^T] \) is the noise covariance. It is shown elsewhere that \( \epsilon_{p_1,p_2}^j \) is zero whenever \( p_1 \neq p_2 \) [13].

1.3 Clustering

Identifying the subset of dominant time scales where the cluster energy is mostly present is of central importance. At any given time scale \( j \), the \( P \times P \) correlation matrix of the spike trains is expressed as

\[ C^{(j)} = \sum_{k=1}^{K} C_k e_k^j + \Delta^j \]  

where the matrix \( C_k \) denotes the pairwise correlation matrix of the neurons in the \( k \)th cluster, and

\[ \Delta^j = \sum_{k=1}^{K} \sum_{p_1,p_2} e_k^{j,p_1,p_2} \]  

Simultaneously diagonalizing the correlation matrices \( C^{(j)} \) across time scales can be achieved by first forming a block diagonal matrix \( R \in \mathbb{R}^{(P \times P)(P \times j)} \) that has the \( j \)th \( P \times P \) block as \( C^{(j)} \), and applying Singular Value Decomposition (SVD) as

\[ R = \begin{bmatrix}
    C^{(0)} \\
    C^{(1)} \\
    \vdots \\
    C^{(j)} \\
\end{bmatrix} = \sum_{q=1}^{Pj} \lambda_q u_q u_q^T \]  

where \( \lambda_q u_q \) denote the eigenvalue/eigenvector pair associated with the \( q \)th dominant mode of the block diagonal correlation \( R \). The presence of large eigenvalues \( \lambda_q \) indicates the presence of strongly correlated neurons in the population. Thus, the pairwise entries of the matrix \( R \) can be expressed as

\[ R[p_1,p_2] = \sum_{q=1}^{Q} \lambda_q \epsilon_{p_1,p_2}^{(q)} \]  

where \( \epsilon_{p_1,p_2}^{(q)} \) denotes the correlation between neurons \( p_1 \) and \( p_2 \) in the direction of the eigenvector \( u_q \). Using the similarity measures computed in (8), neurons are further represented as objects in a graph and connected by an undirected edge whose weight \( w_{p_1,p_2} \) is equal to the corresponding entry in \( R \). With the graph representation, the problem of identifying functionally interdependent neurons reduces to partitioning the graph to maximize cluster aggregation [15]. In this study, a soft membership is used to reduce the computational complexity in which \( a_{pk} \) represents the probability for the \( p \)th neuron to be in the \( k \)th cluster [16]. Then, the graph partitioning problem is solved by finding the set of probabilities \( \{a_{pk}\} \) that maximizes the following objective function:

\[ l = \sum_{k=1}^{K} \sum_{p_1,p_2} a_{pk} w_{p_1,p_2} \]  

The final cluster memberships are derived from \( \{a_{pk}\} \) by assigning each object to the cluster with the largest probability, i.e., \( k_p^* = \arg \max_k a_{pk} \). In scale space, we set the similarity between the two neurons \( w_{p_1,p_2} \) in equation (9) equal to \( R[p_1,p_2] \) as determined from (8).

2 Results

Figure 1 illustrates a conceptual data flow processing for 3 hypothesized spike trains. For neurophysiological data, we used a synthetic data set for which the ground truth was known. The data set was obtained using freely available software tool [17]. The software package is a powerful tool to simulate biological neural networks with a considerably large number of neurons. The user is allowed to build synthetic neurons with multiple pre-built neuronal models. Noise controls can be used to control the variation of each neuron type from a baseline cell model. These
controls mimic the statistical distribution of axonal lengths and synaptic weights within a given cluster. For example, if a large mean and standard deviation are used in a given cluster, this would imply that the network is largely distributed, with a mix of strongly and weakly connected, adjacent and spatially distant neurons. The type of connectivity can also be set between clusters to choose excitatory, inhibitory or random connections from neurons of one cluster to those of another.

We simulated 4 different clusters of functionally interdependent neurons with 30 neurons in each cluster. In each cluster, a distinct pre-built neuronal model was selected; for each model, a set of parameters can be set to determine its behavior. The parameters include firing threshold, resting potential and post-spike potential, noise mean, noise standard deviation, and so on. Table I shows the values of these parameters for the four clusters, and the configuration files which contain settings of all the parameters can be downloaded.

The parameters for cluster 1 and 2 were set to be similar, so are those for cluster 3 and 4, such that the two pairs will behave similarly. The noise parameters were varied to synthesize a variety of within-cluster distributions. For example, noise parameters were set to small values for cluster 1. This means that the neurons belonging to these two clusters behave similarly. In contrast, the noise parameters for cluster 2 were set to a much larger value, so as to induce more stochastic behavior in the neuronal firing. The network editor of the software allows selecting the type of connectivity. Clusters 1 and 2, cluster 3 and cluster 4 were bi-directionally linked, while there was no connection among the two pairs. The data file obtained contains a timed-sequence of records for all the neurons; at each iteration, the record for a neuron includes its identification, membrane potential, firing threshold and post-spike resting potential. In particular, slow variations of resting potential was shown to be of crucial important in characterizing cross correlations between spike trains since it can yield artefactually fast cross-correlations.

Figure 2 illustrates a snapshot of the 120 spike trains obtained as a 4-band structure. In particular, neurons in clusters 3-4 tend to be more synchronized, while neurons in clusters 1-2 tend to be more stochastic. Figure 3 illustrates the performance of the algorithm compared to that of the $k$-means, he Bayesian and the soft-cut clustering as a function of the number of clusters given. It is clear that the proposed technique outperforms the other techniques irrespective of the choice of the number of clusters. In addition, it achieves an error of ~20% at the correct number of clusters.
3 Summary

We proposed a new technique that exploits a scale space representation to capture “causal” dynamics of neuronal firing. It allows mapping to single cluster neurons in “local” circuits that may possess time-locked or phase-locked temporal synchrony, as well as neurons in “global” circuits that exhibit slower temporal dependency arising later in the response. The objective of the technique is to cluster larger populations while reducing the dimension of the search space. It can be further applied to neurons within each cluster to increase the accuracy and identify sub-clusters. Generalization of the technique using other similarity measures depending on the application at hand such as causal entropy, mutual information and many others (as opposed to the Pearson correlation used in this study) is straightforward. It can be further extended to include nonlinear relationship between neurons and is demonstrated elsewhere [13].

Acknowledgement

This work was supported in part by NIH/NINDS grant number NS047516-01A2.

References

Intra-burst firing characteristics as network state parameters

Jan Stegenga1*, Joost leFeber1, Wim Rutten1, Enrico Marani1

1 Biomedical Signal and Systems group, Department of Electrical Engineering, Mathematics and Computer Science, University of Twente, Enschede, The Netherlands
* Corresponding author. E-mail address: j.stegenga@utwente.nl

Network bursts are the dominant type of activity patterns that neuronal networks in culture spontaneously produce. We show that the spatio-temporal structure of bursts, while not deterministic, is statistically stable over a period of several hours. We use the instantaneous array-wide spiking rate during bursts to compare bursts with each other. The statistical structure is thought to reflect the functional connectivity, or state, of the network. The analysis presented can be useful to study (induced) plasticity.

1 Introduction

In our group we are aiming to demonstrate learning and memory capabilities of cultured networks of cortical neurons. A first step is to identify parameters that accurately describe changes in the network due to learning. Usually, such parameters are calculated from the responses to test-stimuli before and after a learning experiment [1,2,3]. We propose that parameters should be calculated from the spontaneous activity before and after a learning experiment, as the applying of test-stimuli itself may alter the network (see also [4]).

Spontaneous activity is generally dominated by network bursts; periods in which the spiking activity is very high, as compared to the nominal level. Consequently, most of the data collected from spontaneous activity originates from bursts. Bursts are present throughout the period that we measured (from 7 to 61 DIV), although the make-up of bursts change with age [5,6]. Due to the fact that the burst appearance changes with age but is quite stable over a period of hours, any parameters extracted from bursts should have a natural time-base that would be useful to observe changes due to stimulation algorithms.

Spontaneous activity patterns, and particularly network bursting events, are closely related to the development of synaptic connections. In early stages of development (DIV4 to DIV14), this can be attributed to new outgrowth of synapses. After this period, a decline in number of synapses occurs as the culture enters a mature state. The spontaneous activity associated with each stage maturation is markedly different, thus showing the dependence of spontaneous activity on the physical network structure. However, the formation and elimination of synapses is also dependent on the electrical activity, as shown in [7].

2 Materials & methods

2.1 Culturing

Cortical neurons are obtained from newborn E18 Wistar rats by trituration and chemical dissociation using trypsin. The cells were plated in a concentration of 1M cells/ml, and allowed to adhere for 4 hours. The MEA’s were coated with polyethylene-imine (PEI) to increase adhesion. The resulting monolayer had a density of ~5000 cells/mm². The cultures were stored in an incubator at 37 ºC, at a CO2 concentration of 5 % and near 100% humidity, in R12 medium (DMEM/HAM’s F12, Gibco) without serum. The medium was entirely changed 2 times a week.

2.2 Measurement setup

We use commercially available MEA’s from MultiChannel Systems (MCS) with 60 Titanium-Nitride electrodes in a square grid. The inter-electrode distance is 100 μm, and the diameter of the electrodes is 10 μm.

Signals were measured using the MC1060BC pre-amplifier and FA60s filter amplifier (both MCS) to prepare the signals for AD-conversion. Amplification is 1000 times in a range from 100 Hz to 6000 Hz. A 6024E data-acquisition card (National Instruments, Austin, TX) is used to record all 60 channels at 16 kHz. Custom-made Labview (National instruments, Austin, TX) programs are used to control the data acquisition. During the experiments, the temperature was controlled at 36.0 ºC, using a TC01 (MCS) temperature controller.

2.3 Data analysis

Spikes were detected online using a threshold detection algorithm. The threshold was set at 5 to 6 times the RMS noise level, which was continually
monitored and was typically in the range of 3 to 5 μV_RMS. The spikes were validated offline using the
algorithm described by [8].

Network bursts were detected by analysing the Array-Wide Spiking Rate (AWSR, the sum of activity
over all electrodes) in bins of 100 ms. If a threshold was crossed, adjacent bins were added if it had more
spikes than 20% of the maximum. The threshold was set at 2 times the number of active electrodes (i.e. hav-
ing a firing rate > 0.1 Hz), with a minimum of 10.

The instantaneous AWSR during a burst was esti-
minated by convolving spike-occurrences with a Gaus-
sian function. A standard deviation of 5 ms was wide
enough in most cases to obtain a smooth function near
the peak AWSR. This is necesserary for a good esti-
mation of the location of the peak AWSR.

We investigated the changes in these burst pro-
files over time by aligning them to their peak AWSR.
To this end, the profiles were grouped over 1 hour of
measurement. Differences in and between these sets
are calculated using the following equation:

\[ E(a, b) = \frac{\sigma^2_{a-b}}{\sigma^2_{b}} \]

where \( a \) is an individual profile and \( b \) is an aver-
age taken over an hour of measurement. The normali-
sation is only to \( \sigma_{b} \), so that the measure is not symmet-
rical.

In addition, a sufficient amount of aligned bursts
yielded enough data to calculate the contribution of
each recording site. The instantaneous spike rate for
each site was also calculated by convolving all spikes
with a gaussian.

3 Results

3.1 Hour-to-hour variation

The burst profiles, calculated over a period of 1
hour, generally show little variation (figure 1).

The differences in subsequent hours are shown in
figures 2 and 3. Figure 2 shows that in the last two
hours of measurement the peak AWSR is lower than
in the 6 hours before which are very similar to each
other.

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each recording site. The instantaneous spike rate for
each site was also calculated by convolving all spikes
with a gaussian.

3 Results

3.2 Day-to day variation

Over a period of days, the shapes are distinguish-
bly different (figure 5). On DIV11, the bursts have a
single peak and a long tail.
Fig. 4 Profiles of the 10 most active sites. The average burst profile is shown in grey.

Fig. 5 Average burst profiles per day of measurement.

By DIV13, this has changed into bursts with a distinct second phase of firing. Over the next days the second phase declines and on DIV16 it has disappeared entirely.

The differences in shape are also visible in figure 6, where the normalised errors are shown for the same measurements as shown in figure 5. Here, 2 hours of each measurement was divided in 4 blocks of 30 minutes. The partitioning in 4x4 blocks indicates that the correlation between blocks on a particular day are correlated among themselves more than to blocks on any other day.

4 Discussion

The apparent structure in the burst profiles result from the relationships between individual recording sites, and thus also on the connectivity in the neural network. The burst profiles prove to be stable over a period of one hour, and gradually change their shape over several hours, as has also been suggested in [5]. The day-to-day changes in burst profiles may be the result of these gradual changes, thereby suggesting an intrinsically changing network.

However, they can also be the result of putting the cultures back in the stove. The spike envelopes per recording site offer more detailed descriptions of the network state than the burst profiles. This may however be the amount of detail required to reveal the changes made during learning experiments. A subsequent refinement can be made by identifying distinct subgroups of bursts, as has been suggested in [9]. This may be necessary for the complex firing patterns seen in cultures in a mature state.

References

Cultured cortical networks described by conditional firing probabilities

Joost le Feber¹*, Wim Rutten¹, Jan Stegenga¹, Pieter Wolters², Ger Ramakers², Jaap van Pelt²

¹ Biomedical Signal and Systems Group, Department of Electrical Engineering, Mathematics and Computer Science, University of Twente, Enschede, The Netherlands
² Netherlands Institute for Neurosciences, Amsterdam, The Netherlands
* Corresponding author. E-mail address: j.lefeber@utwente.nl

Networks of cortical neurons were grown over multi electrode arrays to enable simultaneous measurement of action potentials from 60 electrodes. All possible pairs of electrodes \((i,j)\) were tested for synchronized activity. We calculated conditional firing probability (CFP\(_{i,j}[\tau]\)) as the probability of an action potential at electrode \(j\) at \(t=\tau\), given that a spike was detected at \(i\) at \(t=0\). If a CFP\(_{i,j}[\tau]\) distribution clearly deviated from flat, electrodes \(i\) and \(j\) were considered related. A function was fitted to each CFP-curve to obtain parameters for strength and delay.

In young cultures the set of identified relationships changed rather quickly. At 16 days in vitro (DIV) 50% of the set changed within one day. Beyond 25 DIV this set stabilized: during a period of a week more than 50% of the set remained intact. Most individual relationships developed rather gradually. Moreover, beyond 25 DIV relational strength appeared quite stable during periods of \(\approx 10\) hours, with coefficients of variation \((100\times \text{SD/mean})\) of \(\approx 25\%\) on average.

CFP analysis provides a robust method to describe the stable underlying probabilistic structure of highly varying spontaneous activity in cultured cortical networks. It may offer a suitable basis for plasticity studies, in which induced changes should exceed spontaneous fluctuations. CFP analysis is likely to describe the network in sufficient detail to detect subtle changes in individual relationships. Analysis of data continuously recorded for \(\approx 6\) weeks, showed that highest stability is reached after \(\approx 25\) DIV, suggesting the 4th and 5th week as a suitable period for plasticity studies.

1 Introduction

To demonstrate learning or memory in cultured neuronal networks one needs to monitor connections between neurons. Most learning studies used electrical stimulation to induce connectivity changes in networks [1, 2]. One of the problems is that spontaneous network activity may mask, or even cancel out induced alterations [3].

Networks may be characterized by ‘functional connections’ between pairs of electrodes: abstract representations of possibly parallel neuronal pathways between attached neurons [4, 5]. Various techniques have been developed to identify such connections, most of which are based on or related to cross-correlation analysis [4-6]. We developed a method to describe functional connections between all pairs of active electrodes in neuronal networks. The method estimates conditional firing probabilities to calculate parameters for strength and delay in relationships between electrodes. These relationships will serve to provide a stable underlying probabilistic structure in widely varying patterns of spontaneous activity, which may facilitate demonstration of learning or memory.

2 Methods

Networks of cortical neurons (cells obtained from 9 fetal or newborn Wistar rats) were grown over multi electrode arrays to enable simultaneous measurement of action potentials from 60 electrodes. All possible pairs of electrodes \((i,j)\) were tested for synchronized activity. We calculated the conditional firing probability (CFP\(_{i,j}[\tau]\)) as the incidence of an action potential at electrode \(j\) at delay \(\tau\) \((0 \leq \tau \leq 500\) ms) after a spike at electrode \(i\), divided by the total number of action potentials at electrode \(i\):

\[
N_{\text{follow}_{i,j}}[\tau] = \sum_{t=0}^{\tau} X_i[t] \cdot X_j[t+\tau]
\]  

Equation 1 holds because it is applied to binary arrays \(X_{i,n}\), with \(X_{i,n} \in \{0,1\}\) for all \(n\). CFP\([\tau]\) can be calculated by dividing \(N_{\text{follow}_{i,j}}[\tau]\) by the total number of action potentials at electrode \(i\) \((N_i)\):
It may be noted that $\text{CFP}_{i,j}[\tau]$ is a measure related to cross-correlation ($R_{i,j}[\tau]$):

$$R_{i,j}[\tau] = \frac{1}{N} \sum_{r=1}^{N} X_i[r]X_j[r+\tau]$$  \hspace{1cm} (3)

If $\text{CFP}_{i,j}[\tau]$ showed a distribution that clearly deviated from flat, electrodes $i$ and $j$ were considered related. Figure 1 shows an example.

![Figure 1](image1.png)

**Fig. 1** Example of a conditional firing probability curve, calculated using Equation 2. Fitting Equation 4 yields maximum CFP ($M_{i,j}=4.5\times10^{-3}$) and delay until this maximum ($T_{i,j}=29\text{ms}$). Means $\pm$ SD of 10 consecutive values from 0.5 ms bins are shown, data was recorded at 10 DIV. The maximum probability may seem extremely small. However, this is the probability to record a spike in a 0.5 ms interval. In the above figure, the probability to record a spike at electrode $j$ within 50 ms after an action potential at electrode $i$ can be estimated as $\approx 2\times50\times0.004=0.4$

Equation 4 was fitted to the shape of the CFP curve.

$$\text{CFP}_{i,j}(\tau) = \frac{M_{i,j}}{1+(\frac{\tau-T_{i,j}}{\text{offset}_{i,j}})^2} + \text{offset}_{i,j}$$  \hspace{1cm} (4)

$M_{i,j}$ and $T_{i,j}$ were interpreted as measures for strength and delay of the relationships. We constructed 2 matrices M and T, containing all parameters $M_{i,j}$ and $T_{i,j}$ to describe the whole network. We investigated the stability of the set of relationships in a network and the stability of strength and delay of individual relationships.

To assess the stability of the set of relationships, we divided long term recordings into data blocks with a fixed number of action potentials (33000) and we calculated M matrices for each data block. All non-zero elements in M represented an identified relationship. Similarity between two data blocks ($S_i$) was calculated as the number of relationships that were found in both blocks, divided by the product of the number of relationships in data block A and data block B. To obtain a balanced expression the denominator was squared. Finally, we took the square root of this fraction:

$$S_i = \sqrt{\frac{\left|M_{A} \neq 0 \cap M_{B} \neq 0\right|^2}{\left|M_{A} \neq 0\right| \cdot \left|M_{B} \neq 0\right|}}$$  \hspace{1cm} (5)

Consecutively, we calculated similarity indices between each possible reference block and all data blocks. Three examples of thus obtained curves are shown in Figure 2. All curves showed a maximum at the location of the reference block, and decreased in both directions. We defined Int50 as the interval around a reference block with >50% of the set of relations intact. Figure 2B shows the development over time of the length of this interval.

Additionally, we grouped the data blocks into series of 15 consecutive blocks. For all relationships that were found in more than 50% of the blocks in a series, we calculated the coefficients of variation (100×SD/mean) of $M_{i,j}$ and $T_{i,j}$.

**Fig. 2** Development of similarity indices. A: typical example of a long term recording, divided into 772 data blocks. Successively, similarity indices ($S_i$, Eq. 5) were calculated for all blocks, using three reference blocks: 16 DIV ($\Delta$), 27 DIV ($\circ$), and 35 DIV ($\square$). Similar graphs were constructed using all other data blocks as a reference. Curves were smoothed using a 5th order moving average filter (not shown). Then, we determined Int50: the duration of the interval around each reference point, in which 50% of the set of relations remained intact ($S_i$ remained above 0.5). For each culture Int50 was averaged per day. B: Averaged graph for all cultures. Standard deviations refer to differences between cultures. Beyond 35 DIV, in two or more cultures the 50% intact interval could not be determined because the end of the long term recording was reached before $S_i$ dropped below 0.5. The increase beyond 25 DIV was significant (ANOVA, p<0.01).

In each series these coefficients of variation were averaged to obtain a measure for stability in that series: $CV_M$ and $CV_T$.

### 3 Results

**CFP curves**
The vast majority of the non-flat relationships in all 9 cultures could be adequately described by Equation 4. Relationships differed widely in both strength and delay. \( M_{ij} \) ranged from \( 6 \times 10^{-6} \) to \( 6.8 \times 10^{-2} \) (approximately following a negative exponential distribution and averaging \( (1.0 \pm 1.1) \times 10^{-3} \)). We found \( T_{ij} \) values between 0 and 250 ms, more than 98% of which were below 100ms. Figure 1 shows an example.

**Stability**

Long term recordings from four cultures were investigated for stability. In young cultures the set of identified relationships changed rather quickly. At 16 days in vitro (DIV) 50% of the set changed within one day on average. Beyond 25 DIV this set stabilized: during a period of a week more than 50% of the set remained unchanged (See Figure 2B).

![Fig. 3](image-url) Examples of development of strength and delay of relations throughout long term recordings. 61 relations that were found in the last 50 data blocks of long term recordings from 4 cultures were selected. The development of \( M_{ij} \) (upper panels) and \( T_{ij} \) (lower panels) of these relations between pairs of electrodes \( (i,j) \) was traced back throughout the long term recording. The figure shows examples of two basic types of development: I) shows a relation with increasing \( M_{ij} \) (39% of the relations); in II) \( M_{ij} \) increased first and then decreased (28%). In the other 33%, \( M_{ij} \) fluctuated around a horizontal line.

Most individual relationships showed a rather gradual development on a time scale of days. Figure 3 shows two examples. Moreover, beyond 25 DIV relational strength appeared quite stable during measurement series of 15 data blocks (≈ 10 hours), with coefficients of variation around 25% on average (see Figure 4).

![Fig. 4](image-url) Development of the average coefficient of variation of relational strength in 4 long term recordings. A long term recording was divided into data blocks. In all data blocks M matrices were determined. Next, data blocks were grouped into series of 15 consecutive blocks. For the set of relationships that were found in at least 50% of the 15 blocks in a series, means and standard deviations were calculated for all \( M_{ij} \). Then coefficients of variation (100×SD/mean) were calculated for each relation in the selected set. The figure shows mean coefficients of variation (\( CV_M \)). ♦: culture I, ●: culture II, Δ: culture III, and □: culture IV. Pooled data of all cultures yielded a correlation coefficient \( \rho = 0.32 \). This correlation was significant (\( p<0.01 \)).

\( CV_M \) was calculated from the set of relations that were found in at least 50% of the data blocks in a series. The size of this set averaged 54% of the total number of relationships that could be analysed.

\( CV_T \) showed much larger values than \( CV_M \), often more than 100%. \( CV_T \) also tended to decrease with aging of the culture, but this decrease was not significant.

**4 Discussion**

CFP analysis provides a robust method to describe the stable underlying probabilistic structure of highly varying spontaneous activity in cultured networks of cortical neurons. The set of identified relationships appeared quite stable. Furthermore, these relationships were quite stable in terms of strength and delay on a timescale of several hours to several days, while development in strength and delay on this time scale were rather gradual. Stability increased with aging of the culture. The high values of \( CV_T \) were caused by relationships that had (almost) zero delay in the major part of a series, with one or a few outliers that led to a relatively large standard deviation and thus to a high \( CV_T \).

It is probable that relationships are single abstract representations of multiple pathways between electrodes. This was illustrated by an occasional CFP curve that showed two distinct peaks (<<1%). Our fit
algorithm reduced these to a representation with a single peak and thus one strength and delay.

Besides the pathways between electrodes, relationships were also influenced by the surrounding network. Many of the recorded neurons were in excitatory loops, leading to autocorrelations with extra peaks at certain (non-zero) delays. In a linear approach, it has been suggested to deconvolve the autocorrelation out of the CFP curve to obtain a ‘synaptic response function’ [7]. In this study we did not perform such a deconvolution. However, if a relationship is identified, this does indicate the existence of a neuronal pathway between a pair of electrodes.

CFP analysis may offer a suitable basis for plasticity studies, in which induced changes should exceed spontaneous fluctuations. Furthermore, the analysis is likely to describe the network in sufficient detail to detect subtle changes in individual relationships.

Analysis of data continuously recorded for ≈ 6 weeks, showed that highest stability is reached after ≈ 25 DIV, suggesting the 4th and 5th week as a suitable period for plasticity studies.

References
SIMONE: A new simulation engine of realistic MEA-based extracellular measurements

Ricardo Escolá1*, Christophe Pouzat2, Blaise Yvert3, Isabelle Magnin4, Régis Guillemaud1

1 CEA – LETI, Département de micro Technologies pour la Biologie et la Santé, Grenoble, France
2 Laboratoire de Physiologie Cérébrale (CNRS UMR 8118), UFR biomédicale de l’Université René Descartes, Paris, France
3 Laboratoire de Neurobiologie de Réseaux (CNRS UMR 5816), Université Bordeaux I, Talence, France
4 CREATIS (CNRS UMR 5515), Institut National des Sciences Appliquées de Lyon, Villeurbanne, France
* Corresponding author. E-mail address: ricardo.escola@cea.fr

We have developed an elegant tool to simulate user-defined scenarios of extracellular neural data recorded with multi-electrodes arrays (MEA). Since most of the parameters might be defined by a probabilistic distribution, tens of simulations may be obtained from the same scenario. This should provide an enormous help in the validation process of algorithms to be embedded into an ASIC close to the MEA.

1 Introduction

One of current challenges in MEA development is to build compact high-density devices with integrated electronics [5]. The next step in these developments will be to also embed signal processing strategies into these dedicated integrated circuits (ASICs). When developing new algorithmic embedded procedures to treat extracellular measurements obtained by a MEA, one important concern is the set of signals used to validate such methods. It is necessary to rely on different sets of experimental data that depict all representative states of a neural network. However, such an approach presents uncertainty regarding the exact spiking activity for every single neuron in the tissue.

For this reason, we have developed a simulation engine to accurately depict the spiking activity for a statistically defined network, while preserving individual cells activity waveforms. Because accurate simulations (at a network level) using Hodgkin-Huxley equations are highly resource demanding, we chose to build a stand-alone network simulation based on integrate-and-fire models, which focuses primarily in reproducing network dynamics and neural waveforms at the same time. We have worked under the R environment [2], using a reproducible research approach. This tool is named SIMONE: Statistical sIMulation Of Neuronal networks Engine.

2 Simulation model

A biological neural network is automatically defined from user-defined parameters, as detailed in section 2.3: neurons can be positioned spatially (in the xy-plane) and characterized, as well as their synaptic connections.

A 2D MEA is automatically generated in a plane parallel to the xy-plane. A 3D array may be eventually created by hand by the user.

Simulation step is fixed at 100 μsec. This way we are simulating a 10 KHz sampling frequency, which is widely accepted as the minimum cadence at which neuro-signals should be acquired.

2.1 Intracellular simulation

Intracellular simulation is based on the leaky integrate-and-fire model with active conductance [3]. It is statistically parameterized as described in 2.3.1. We have put a strong emphasis in the modelling of input currents: pre-synaptic (excitatory and inhibitory), artificial (pre-synaptic stimulation), membrane, noise and self-induced currents [1] are considered. As a result we achieve a very accurate sub-threshold voltage simulation describing the dynamics of the network. This approach will allow us to depict more coherent behaviors. For spikes to be realistic as well, we will use a unique intracellular spike template assigned to each neuron, modulated (attenuated) according to previous firing history in order to determine the spike shape that should be used at each threshold crossing. The output of this stage will be the intracellular potential evolution of each neuron.

2.2 Extracellular simulation

The goal of this step is to reproduce the extracellular voltage measured by each electrode (see Fig. 1).

This level of simulation is based on a pseudo monopole of current within a homogeneous environment (the neural tissue). We consider each neuron as being a small spherical source of (membrane) current. The extracellular voltage will be inversely proportional to a power of the distance between the neuron and the theoretical electrode (in the ideal monopole model \(x\) equals 1) and directly proportional to the membrane current. For any given electrode \(j\), the contributions of each neuron will be summed at its tip.
This model is consistent with the notion that extracellular voltage is approximately proportional to the first derivative of membrane potential [4].

Electrodes are assumed to be identical and quasi-ideal: no capacitive, but only resistive component is taken into account.

2.3 User-defined scenario

The simulation scenario describes a MEA and a neural network. Once these elements have been configured, the duration of the simulation may be entered.

Neural Network

Neural network features must be quantified in terms of three basic biological parameters: size, synaptic propagation speed and characteristic conductivity. Several statistical distributions may also be entered by the user (otherwise, standard distributions are considered) defining the neurons spatial distribution; the ratio between excitatory and inhibitory neurons (type); the probability of a synapse being established between any pair of neurons (generally distance and type-dependent distributions) and its synaptic weight; the statistical distributions of the resistance and capacitance value in the membrane model; the threshold, inverse and reset voltages in the spiking model.

Five functions control the dynamics of each neuron: 1) a kernel modulating the synaptic weight decay between two connected neurons; 2) a kernel modulating spikes shape according to previous spiking history; 3) a self-interaction filter of the neuron [1]; 4) synaptic noise reproduction; 5) a time-dependant function allowing the user to introduce an artificial stimulation into the model. By default, the stimulation current is set to increase exponentially when neuron spiking activity risks ceasing.

A gallery of spike templates is provided and may be fully re-designed by the user. If the number of templates is smaller than the total number of neurons, new templates are automatically created using a random linear combination of a random-chosen pair of spikes.

MEA

Three acquisition functions are available to accurately depict the electrode’s behaviour: a time-dependant gain that may reproduce degradation of electrodes gain or gain noise; a range of visibility of the tip (typically around 100-140 µm) (see Fig. 1); and the time-dependant extracellular noise. These functions may evolve during simulation runtime.

An automatic placement of the electrodes is possible thanks to three parameters: number of electrodes per dimension (2D or 3D), the distance between them and the coordinates of the first one.

3 Conclusion

We have developed an elegant way to simulate coherent MEA-based acquisitions from a fully defined neuronal network. Since the individual activity for each cell is known, this environment will be a useful tool to test potential prospects for embedded algorithms in the domain of MEA acquisition (such as the detection, the alignment and the sorting of spikes).

Most of the parameters might be defined by a probabilistic distribution; then tens of simulations may be obtained from the same scenario. This should provide an enormous help in the validation process of embedded architectures.

References

A novel spike detection algorithm for real-time applications

Antonio Novellino¹, Alessandro Maccione²*, Mauro Gandolfo², Michela Chiappalone², Paolo Massobrio², Sergio Martin oia²

¹ R&D Lab, ett s.r.l., Genova, Italy
² Neuroengineering and Bionano Technology Group, Department of Biophysics and Electronic Engineering, University of Genova, Genova, Italy
* Corresponding author. E-mail address: alessandro.maccione@dibe.unige.it

Neurons exchange information producing trains of action potentials or spikes with an apparently random delay between each other. A correct identification of spike signals embedded in biological noise is a fundamental pre-requisite for producing reliable spike trains that can be processed with high level algorithms. In this work we present a new simple, fast and reliable spike detection algorithm that could be conveniently utilized for real-time applications.

1 Introduction

Investigations in networks of neurons by using Micro-Electrode Arrays (MEAs) differ from classical electrophysiological methods, mainly devoted to study distinct neurons or small networks dynamics. In the first case the interest is not aimed at measuring the single membrane potential or ionic current, but rather at the detection that a spike (i.e. an action potential) occurred. Experimental results show that the recorded extra-cellular action potentials increase in amplitude and sometimes even resemble the shape of the intracellular potential. Furthermore, not always it is possible to find a perfect one-to-one correspondence between a single cell and a microelectrode of the array, since a single microelectrode can pick up the signals of a few neurons. For these reasons, the detection of neural spike activity is a technical challenge that is a prerequisite for a correct post-processing session.

Commercial systems utilize specific spike-detection algorithms capable of individuating spike events. Usually, automatic spike-detection algorithms range from simple hard-threshold to more sophisticated signal classifications [1-4].

In this work we present a new simple and fast spike detection algorithm that could be conveniently employed in real-time applications (RTSD). We compared the proposed method to some other most-popular spike detection and spike sorting technique.

2 Materials and Methods

2.1 Algorithm description

By using the proposed algorithm, three parameters have to be defined in order to detect spikes: (1) a differential threshold (DT), (2) a peak lifetime period (PLP) and (3) a refractory period (RP). The threshold, defined separately for each recording channel, is set according to the standard deviation of the biological and thermal noise of the signal. The peak lifetime and the refractory period are related to the minimum gap between two consecutive spike events.

The algorithm computes the Relative Maximum/Minimum (RMM) by using an inspection buffer. When the RMM is a Minimum, the algorithm looks for the nearest Maximum within the peak lifetime window, and vice versa. If the difference between the two found RMM (differential value) overcomes the absolute threshold and there is not any other RMM within the refractory period, the spike is identified and its timestamp is stored.

2.2 Performance Evaluation

To evaluate the performances of each spike detection method, we asked three experienced researchers to identify the spikes in a stream of electrophysiological signal. The recognized peaks have been used as reference sequence to be compared with the output of the automatic detection methods.

The parameters [5] used for evaluating the performances of the tested algorithms are:
- False Negative (FN = X-Z; FN% = 100*FN/X);
- False Positive, (FP = Y-Z; FP% = 100*FP/Y);
- Efficiency, $\eta = Z/(FP+FN)$;
- Final Efficiency, $\eta^2 = \eta/(\eta+1)$;

where X is the amount of spikes recognized by the experimenter; Y is the amount of the automatically detected spikes; and Z is the amount of corresponding spikes between the two considered sequences (i.e., one reference sequence given by the experimenter and the other sequence being the output of an automatic spike detection method).
2.3 Data set

We analyzed a raw data sample of 20 sec (10kHz sampling) of spontaneous electrophysiological activity recorded by means of a standard MEA. We then tested these following spike detection methods:
- Our novel spike detection method (RTSD);
- Hard Threshold (HT), as implemented by the McRack MCS system;
- differential threshold (DTSW) (see ref. [6] for details)
- Offline Sorter Hard Threshold (OSHT);
- Offline Sorter Signed Energy (OSE).
(see Offline Sorter user’s manual for further details, http://www.plexoninc.com).

3 Results

We evaluated the performances of each method by changing its input parameters. In particular, for the proposed RTSD method, we computed the performances obtained by using a differential threshold (DT) ranged from 5 to 8 (step 0.5), a peak lifetime period (PLP) and a refractory period (RP) alternatively of 1ms or 1.5ms. For the other methods, we changed the parameters related to the threshold definition and to the other possible variables (data not reported).

<table>
<thead>
<tr>
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<th>FN%</th>
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<td>OSE</td>
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<td>1.75</td>
<td>0.637</td>
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</table>

TABLE 1 Performances of the tested algorithms. The proposed method (RTSD) is highlighted.

We then compared the best performances obtained by each peak-detection algorithm. Table 1 reports a comparison of the proposed algorithm with already available methods. As it can be clearly noticed, the proposed algorithm (RTSD) is the one giving the best performances (i.e. the best trade-off between false positives and false negatives). Fig. 1 shows the different spike detections in function of the compared algorithms on the same short raw data sample.

Because of the good accuracy and the low computational cost, it could be conveniently utilized for reliable real-time applications.

Fig. 1 Comparison among the different spike detections methods tested.

Acknowledgement

This work was partially supported by IDEA project (NEST – No 516432).

References

Data Analysis of Microelectrode Array (MEA) Experiments with Pattern Recognition Methods

Olaf Schröder*, Alexandra Gramowski1, Konstantin Jügelt2, Liane Mehnert2, Simone Stüwe2, Dieter G. Weiss2

1 PATTERN EXPERT, Borsdorf, Germany
2 Inst. of Biological Sciences, University of Rostock, Rostock, Germany
* Corresponding author. E-mail address: olaf@pattern-expert.de

MEA experiment data analysis is performed with spike train analysis methods. There exists a wide range of proposals for spike train analysing algorithms. We computed more than 200 different spike train features. With the application of feature scores known from pattern recognition theory we were able to identify for 21 substances their individual spike train features that are most relevant in each of five general categories (regularity of oscillations, burst shape, synchronisation, general activity in bursts and general spiking activity).

1 Classification Approach

MEA experiments with neuronal network cultures are an exciting tool for investigating neuronal networks on the level of microcircuits. These microcircuits show a new quality of features which are very difficult to analyse with conventional statistical methods. In particular, emergent features which are a consequence of complex behaviour patterns require adequate analysis methods. Neuronal networks show a high variation in behaviour. It is very difficult to distinguish between behaviour that is unaffected, i.e. still varies within normal ranges, and behaviour that exhibits a first reaction to e.g. toxic substance which is possibly too subtle to be noticed with the naked eye.

Pattern recognition methods combine spike train analysis methods with classification or regression algorithms known from statistical machine learning theory. We have developed a method of spike train analysis and classification experiments for analysing MEA experiments. This method can help to answer a wide range of questions. At first, reproducibility can be proven with classification or regression experiments in a very stringent manner. Proof of reproducibility by classification experiments could be established as a “gold standard” for quality assurance.

Furthermore there is a wide choice of spike train analysis methods. It is difficult to prove the relevance of the different spike train methods. However, by performing classification experiments their usefulness for classifying MEA experiments can be assessed. Because a high significance in data classification correlates with a high relevance of these spike train analysis methods.

In our research group, we have established a standard protocol for experiments which is suited for data analysis with pattern recognition methods. We have performed 21 experiment series with chemical stimulation according to this protocol using 21 different substances.

Each experiment was performed with increasing substance concentration in an accumulative manner. After each addition of substance we recorded 30 min of stabilised spike train activity following the response. For each of these time periods we computed more than 200 feature values which describe the general spike train activity, the activity in bursts, the burst shape, the regularity of oscillation and the network’s synchronicity.

2 Best describing features

For those five general categories, we found the following best describing parameters with the highest relevance:

- Regularity of oscillations: The standard deviation of the maximal spike rate in burst.
- Burst shape: The average duration of plateaus in bursts.
- Synchronisation: The number of coordinated spikes within 1 msec.
- General activity in bursts: Mean burst amplitude.
- General spiking activity: Mean spike rate.
3 Summary
We found new relevant describing spike train features. On the basis of these findings new assessment of results is possible. GABA-B receptor involvement (Baclofen) seems to cause trends in the selected spike train features significantly different from those of GABA-A and other receptor ligands.

Acknowledgement
This work was supported by the State Ministries of Education and of Economy Mecklenburg-Vorpommern and the European Community (ERDF) through the State Priority Research Program "Biosystems Technology".

Reference

Fig. 1 Spike train features for Baclofen, Clonazepam, Fluvoxamine and Levetiracetam in columns.
Evaluation System for Neural Data Acquisition and Analysis

René Schrott¹*, Gerold Bausch¹, Klaus-Peter Kirchner¹, Carsten Tautorat¹, Werner Baumann², Dieter Weiss³, Helmut Beikirch¹

1 University of Rostock, Faculty of Computer Science and Electrical Engineering, Germany
2 University of Rostock, Institute of Biological Science, Biophysics, Germany
3 University of Rostock, Institute of Biological Science, Cell Biology and Biosystems Technology, Germany
* Corresponding author. E-mail address: rene.schrott@uni-rostock.de

With the help of silicon micro sensors with integrated multi-electrode arrays (MEA) the cell monitoring system CMS® allows the measurement of cell network responses to certain external stimuli. This poster will report on the strategies to increase the level of automation during MEA measurements with the CMS®. The applied methods include approaches for adaptive action potential detection, signal conditioning and template generation for subsequent spike sorting. Based on the calculated templates online classification will be performed.

1 Methods

The combined development efforts are aimed at the latest micro sensor generation with on-chip electronics. In addition to glass-based MEA [1, 2], this approach is able to measure several network parameters in parallel [3, 4, 5]. Apart from power supply and temperature control units also external analogue hardware components are designed for the silicon sensor chip to aid the conditioning of recorded neuronal signals prior digitalisation. Once the signal is in the digital domain, a field programmable gate array (FPGA) is used to process the recordings of up to 64 channels in parallel. The processing includes discrete wavelet transform (DWT). This multiresolution analysis permits noise analysis, filtering and action potential detection.

The FPGA acts as a pre-processing module, which analyses and captures the sensor signals prior data transfer to a host personal computer (PC). Two different operating modes are considered. First, continuous data streaming transfers the entire recording onto a storage device of the host. Apart from offline sorting this data can be used for template generation during a learning phase to allow online unit separation. Second, during normal operation only detected action potentials are evaluated and transferred, which greatly reduces required bandwidth and storage resources.

Further extensions will allow additional classification based on the provided templates in the latter mode. Currently appropriate algorithms for feature extraction, clustering and classification are evaluated. For this purposes a dedicated digital signal processor (DSP) allows the implementation of previously simulated approaches. The primary aim is the development of a system that adaptively detects and classifies action potentials according the characteristics of the measurement. Semi-automatic approaches are expected to reduce the level of supervision during the measurements with up to 64 channels of a multi-electrode array (MEA).

2 Results

For the external sensor chip support, analogue signal conditioning, data digitization and transfer several hardware evaluation designs have been developed and tested. Signal conditioning, channel cross-talk as well as bandwidth requirements and timing constraints have been scrutinized. The results are taken into account for the latest redesign.

The streaming and detection modules could be implemented successfully on a Virtex-II FPGA. Cur-
rently the performance of the DWT for different wavelets is tested. The setup configuration of the pre-processing system and the data transfer to the host are carried out via universal serial bus (USB2.0) and inter-integrated circuit bus (I2C). Feature extraction and clustering approaches are still mainly simulated. The results of wavelet coefficients processing methods and principal component analysis (PCA) approaches are compared to identify their classification abilities with respect to our recording conditions [6].

3 Summary

Together with industrial partners the University of Rostock develops cell monitoring systems for the measurement of neural network responses with the help of silicon based sensors. Modular hardware and software developments for a new generation of micro sensor chips are performed to reduce manual interactions during recording experiments. Hardware components have been tested successfully to optimise their performance. Algorithms for action potential detection and processing have been implemented and tested. Approaches for feature extraction, clustering and classification are presently simulated and evaluated with regard to the actual recording conditions.

Acknowledgement

Supported by the state Mecklenburg-Vorpommern and the European Community (EFRE).

References

Generating noisy spike trains for testing spike detection and sorting algorithms

Leslie S. Smith

Department of Computing Science and Mathematics, University of Stirling, Stirling, Scotland, UK.
E-mail address: lss@cs.stir.ac.uk

Software for generating spike trains with realistic interference is presented. The interference is derived from a biophysically realistic model. The aim is to permit the assessment of different spike detection and sorting algorithms.

1 The Problem

Neural signals recorded by extracellular electrodes (and in particular signals recorded from multi-electrode arrays at the bottom of culture dishes) often suffer from low signal:noise ratios. This makes spike detection and sorting particularly difficult. There are many algorithms (and a considerable amount of software) for detecting and sorting spikes in noise [1]. These generally follow a particular structure, namely spike detection, spike signal segmentation and preprocessing (usually including dimensionality reduction) then clustering and cluster labelling. However, assessing the quality of different spike detection and sorting systems is difficult because one does not normally know the “ground truth”: that is, when the spikes from each neuron actually occurred.

2 The Solution

We have developed a noisy spike generator which can generate signals for which the “ground truth” is known. This is based on an equivalent circuit for the transfer of charge from neurons to an electrode (figure 1).

We simulate three sets of spiking neurons: (1) target neurons, namely those for which we hope to detect and sort spikes, (2) correlated interfering neurons, namely neurons which generate spikes which are correlated with one of the target neurons, and (3) uncorrelated interfering neurons, namely neurons which generate spike trains uncorrelated with the target neurons. We can emulate variations in the spike train shape and size (as recorded at a single electrode) caused by (i) variations in the distance of the different parts of the spiking surface from the neuron, (ii) variations in the mode of transfer of the signal to the electrode, and (iii) shape of intracellular spike.

Signal transfer from neuron to electrode during spikes is based on analysis of the circuit model in figure 1. We compute the effect of the transfer path from patch of spiking surface to electrode as a weighted sum of the intracellular spike and its first and second derivatives (figure 2). We then compute the effect of the spatiotemporal distribution of the spike over the spiking part of the neuron membrane by modelling the integral as a weighted sum of delayed spikes. This permits variation in electrode/neuron distance between the electrode and the neuron’s spiking surface to be modelled. This is described in more detail in [2].

The weights used in signal generation are user specified, permitting considerable variation of the generated spike shape as well as user determination of the relative strengths of the different components. The user can thus alter the shapes of the target neural signals, and the relative strengths of all the different signals. Clearly, care needs to be taken, since inappropriate weight selection will result in unrealistic spike shapes. For an appropriate parameter selection, the signal generated looks like a real electrode signal (figure 2B). The actual spike times for all target neurons are recorded (and may be re-used), so that the results from different spike detection and sorting algorithms may be compared with the ground truth. The experimenter may vary the strengths of the target signals and the interference.

Fig. 1 Equivalent circuit for transfer of charge from a patch of neural membrane to an extracellular electrode. Two extracellular electrode locations are illustrated: the first is situated in the extracellular fluid, and the second is (for example) situated at the bottom of a culture dish, (partially) covered by glial cells.
Fig. 2 (A) shows original, 1st and 2nd derivative of signal from a realistic spike (based on [3]). (B) shows model spike recorded. Y axis is arbitrary; X axis is samples, at 24,000 Samples/second so that duration of graph is 2.4 ms.

3 The Software

The software is a set of MATLAB .m files, currently version 1.0 (note: it needs the Statistics Toolbox), and is freely available from http://www.cs.stir.ac.uk/~lss/noisyspikes. It consists of a set of .m files, and some detailed documentation, including a user manual. It is still currently under test, but is believed to be usable.

Acknowledgement

This work was funded by EPSRC grant GR/R65602/02.

References


Retinal Signaling
Studying the Population Code of the Retina

Michael J. Berry II*, Ronen Segev, Elad Schneidman

Department of Molecular Biology, Princeton University, Princeton, NJ, USA
* Corresponding author. E-mail address: berry@princeton.edu

Keynote address
Understanding the collective code used by populations of neurons to encode sensory stimuli or direct motor output involves both experimental challenges in our ability to record simultaneously from many of neurons, as well as conceptual challenges in our approach to analyzing and interpreting multi-neuronal data. We have made significant progress in both of these areas, using MCS technology to record from all or nearly all of the ganglion cells in a patch of the retina and new methods of information theoretic analysis to quantify the patterns of correlation and redundancy in the neural population.

1 Introduction
While much of our understanding of the brain is derived from studies of single neurons, it is clear that neural systems generally rely on populations of neurons to represent stimuli and to direct motor outputs. Therefore, a fundamental issue that we must address in order to understand how the brain works is: what is the difference between population codes and single neuron codes? The key concepts are synergy, redundancy, and independence. In a synergistic code, multi-neuronal firing patterns represent information that is not accessible by observing the activity of individual neurons. In a redundant code, the individual neurons encode some of the same messages, and the brain can improve its estimates of the stimulus by recognizing this redundancy. In an independent code, neurons pay attention to different stimulus features; this code is completely understandable from single neuron studies.

Here, we present new experimental techniques along with information theoretic analyses of correlation among populations of retinal ganglion cells. We find extensive overlap of receptive fields, with a total coverage of ~60 cells for each point in visual space. We also find mixing of visual information between different functional types of ganglion cells, indicating that the retina contains perhaps only ~4 independent parallel channels of visual information. Finally, we show that the pattern of correlations in a larger population can be understood using only the measurement of pairwise correlations.

2 Results
2.1 New Multi-Electrode Recording Methods
We have developed a new method of multi-electrode recording and spike sorting that allows us to record from every single ganglion cell in a small patch of the retina [1]. This technical tour-de-force is the first instance in which neuroscientists have been able to record from every relevant neuron in a neural circuit in a vertebrate animal. Previous techniques only allowed recording from up to 15% of the retinal ganglion cells over the electrode array. This method

![Multi-Electrode Recording with a Dense Array. A. A patch of the salamander retina placed over a hexagonal multi-electrode array. Ganglion cells and axon bundles are fluorescently stained with rhodamine dextran (green); electrodes and leads of the array are black. B. Electron micrograph of a cross-section of the optic nerve. C. Example of the spike sorting method. Three templates (left; red) are separately time shifted and superimposed to achieve a very close fit (right; red) to a 3.2 ms segment of raw data (right; blue). D. Number of recorded ganglion cells plotted versus the number of fluorescently labeled cells for 7 retinal patches; 3 patches were recorded with the hexagonal array (diamonds), 4 with the rectangular array (squares). E. Distribution of the fraction of refractory violations for 164 ganglion cells; 90% of the cells have less than 0.1% refractory violations.](image-url)
now affords unprecedented access to the population code of retinal ganglion cells.

Our method relies on two important elements. First, we record with a dense multi-electrode, where every ganglion cell spike is sensed on many channels (~10) and hence produces a unique pattern of activity on the array (Fig. 1). Such dense arrays were not commercially available, so we custom designed them in collaboration with MCS for our purpose. Second, we have developed a new spike sorting algorithm that combines signals from all of the electrodes, allowing us to unambiguously resolve contributions from overlapping spikes. This algorithm first identifies the average electrical pattern produced on the array when each cell fires an action potential, then iteratively matches these spike templates to the raw data.

To verify that we had recorded from all of the cells over the array, we simultaneously stained the ganglion cells with a fluorescent, retrograde label: rhodamine dextran. This allowed us to count the number of ganglion cells over array and compare to numbers from overlapping spikes. This algorithm first identifies the average electrical pattern produced on the array when each cell fires an action potential, then iteratively matches these spike templates to the raw data.

To verify that we had recorded from all of the cells over the array, we simultaneously stained the ganglion cells with a fluorescent, retrograde label: rhodamine dextran. This allowed us to count the number of ganglion cells over array and compare to the number of cells we were able to identify with multi-channel spike sorting. We concluded that 80-100% of the cells over the array are accounted for [1].

2.2 Receptive Field Organization

Using our new recording techniques, we have systematically studied the receptive field organization of ganglion cells in the salamander and guinea pig retinas. Receptive fields were mapped by stimulating the retina with checkerboard random flicker and finding the average stimulus pattern preceding a spike for all ganglion cells, a process known as reverse correlation [1]. The stimulus used small square regions (55 µm) and a fast frame rate (33 Hz) in order to probe the limits of the spatial and temporal sensitivity of ganglion cells. This method of mapping receptive fields is especially convenient for multi-electrode recording, as one can simultaneously characterize all of the recorded ganglion cells.

Our measurements reveal that the retinal code has extensive spatial overlap (Fig. 2). If we multiply the average area of the receptive field center (derived from the 1-sigma ellipse) times the ganglion cell density, we find a coverage factor of 59 ± 5 in the salamander [1]. This means that roughly 60 ganglion cell receptive fields look at every point in visual space. Similarly, we find a total coverage factor of 60-70 in the guinea pig at 1-2 mm retinal eccentricity. These numbers are in rough agreement with the coverage of ganglion cell dendrites. The total coverage factor is a very basic property of ganglion cell organization that has important implications for the retina’s population code.

Contrary to the view that ganglion cells of a single functional type ‘tile’ or just barely cover visual space, we found that using a variety of classification schemes, cells of the same type overlap extensively in many cases [2]. We can distinguish ‘fine’ functional types for ganglion cells recorded in a single retinal patch. While cells of the same fine type typically exhibit a territorial organization, where their receptive field avoid direct overlap, they still cover visual space several times over. For instance, local edge detectors have a coverage of ~4, and OFF brisk sustained have a coverage of ~3 in the guinea pig (Fig. 2).
code. However, sampling the full variety of natural scenes is very difficult.

Our approach was to use our intuition about what properties of natural scenes could lead to qualitative differences in the retinal response. Since retinal ganglion cells primarily fire when the light intensity on their receptive field changes, we considered natural scenes with qualitatively different kinds of motion.

Fig. 3 Shared Information. The shared information is plotted as a function of the distance between ganglion cells in the guinea pig. This quantity is the information between the cell’s responses, $I(R_a; R_b)$, normalized by the minimum single cell entropy, so that the value runs from zero (independent cells) to one (identical cells). A. Independence is present for broadly different cell types: ON-OFF pairs (blue), fast OFF-slow OFF (pink), and fast ON-slow ON (aqua). B. Cell pairs of the same type are shown in color; mixed type are shown in grey. Notice that there are many mixed-type pairs with high degrees of shared information, while some same-type pairs share less information.

We considered five major categories of motion in this paper: intrinsic motion, where the camera is held steady and objects in the world move; optic flow, generated by forward translation of the camera; smooth pursuit eye movements, simulated by slow camera pans; saccadic eye movements, simulated by rapid shifts of camera direction; fixational eye movements, generated by jittering a still image using computer software. Our movie clips were filmed in both natural and artificial environments, including trees, plants, and streams as well as cars, people, and buildings.

When we recorded retinal spike trains under natural stimulus conditions, we found that most ganglion cells were sparse and precise, as found in white noise experiments. Surprisingly, there was relatively little difference in sparseness, precision, or information content for different kinds of movie clips [3]. Fig. 4 shows an example of 10 ganglion cells simultaneously recorded during a movie of optic flow in the woods.

Fig. 4 Retinal Responses to Natural Movie Clips. A. Single frames from four natural movie clips having different categories of motion. B. Examples of spike rasters from ten cells recorded simultaneously during the forest walk movie clip. Each dot represents the time of a spike; vertical dimension shows 120 repeated stimulus trials for each cell, and thin black lines divide cells.

We next calculated the redundancy between pairs of ganglion cells, which is defined as the sum of the visual information conveyed by each individual ganglion cell minus the information conveyed by the joint responses of both cells: $A_{ab} = I_a + I_b - I_{ab}$. Significant redundancy was found both among cells of the same functional type and between cells of different functional types. The redundancy ranged up to 50% of a cell’s information, and significant values were found for cells up to 500 μm apart (Fig. 5). The average pairwise redundancy was ~10%, but a single ganglion cell shared such correlations with ~100 neighbors, indicating a widely distributed population code [3].
When one measures the correlation between pairs of retinal ganglion cells, one often observes an excess of synchronous spikes, which leads to redundancy in their information content. However, for most cell pairs this excess is somewhat small. For instance, if we count spikes in a 20 ms window for a population of ganglion cells recorded under natural stimulus conditions and calculate the correlation coefficient, we find that 90% of all pairs have values in the much smaller range of [-0.02, +0.1].

One might be tempted to ignore these correlations for small groups of ganglion cells. But this turns out to be a surprisingly bad approximation. If we count the rate at which 10 out of 40 neurons fire a spike in a 20 ms time window, we find a rate of ~0.05 events/second. If we instead assume that all cells fire independently and estimate this rate from the firing rate of each cell, we get a rate of ~0.002 events/hour, which is ~100,000 times smaller [4]! The discrepancy is even greater if we consider specific combinations of spiking and silence. For 10 ganglion cells, there are $2^{10} = 1024$ possible responses. At the extreme, some of these specific firing patterns occur a million times more frequently than predicted from the assumption of independence (Fig. 6). Even common events, such as patterns with only one neuron spiking have errors of an order of magnitude.

Clearly, the correlations are very important. But how big a contribution is made by pairwise correlations, as opposed to higher order correlations? We used the framework described above to find the maximum entropy distribution consistent with all observed firing rates and pairwise correlation coefficients, $P_2$; this is the unique probability distribution that assumes no correlation beyond pairs [4]. When we plotted the predictions from $P_2$ versus the observed event rates, we got excellent agreement (Fig. 6). The deviations between the actual probability and our predicted probability were typically ~10%! Thus, pairwise correlations alone not only imply very strongly correlated states in groups of 10 or more neurons, but they also provide an excellent approximation to the entire correlation structure in the network. This maximum entropy model is mathematically identical to the Ising model in statistical physics, allowing us to make predictions about error-correcting properties in even larger populations [4].

![Fig. 5](image.png)

**Fig. 5** Pairwise Redundancy. Fractional redundancy between pairs of ganglion cells plotted as a function of the distance between their receptive fields. Color indicates the kind of natural movie clip. No systematic differences between movie clips were found.

![Fig. 6](image.png)

**Fig. 6** Effect of Pairwise Correlations on the Population. The rate of occurrence of all specific firing patterns of one group of 10 ganglion cells plotted agains against the expected rate if cells were independent (blue) or predicted from the maximum entropy model $P_2$ that takes into account all pairwise correlation between the cells (red). Each dot stands for one of the $2^{10} = 1024$ possible binary activity patterns cells (spiking and no spiking combinations). Black line shows equality.

3 Summary

We found that the retina uses a large population code with hundreds of ganglion cells responding to even the smallest features of the visual image. Due to high receptive field overlap, this code is extensively redundant. This result challenges previous ideas about coding efficiency and suggests that robustness is a major goal of the retinal population code.

Acknowledgement

We thank the NEI and the E. Mathilda Ziegler Foundation for grant support.

References


Patterns in the spike train of individual retinal ganglion cells from the rabbit retina contain cell-type specific coding sequences. These patterns are defined by the timing of action potentials in successive interspike intervals in brief coding epochs. They can be identified within the ongoing spike train by a simple timing algorithm. Because they are largely independent of particular stimulus sets, these patterns will be a useful tool for distinguishing the many unidentified ganglion cell types that exist in all mammalian retinas.

1 Introduction

High frequency coding events followed by periods of silence are a generic feature of many sensory systems [1]. In extracellular recordings using multielectrode arrays we confirm that "burstiness" is a generic feature of every retinal ganglion cells' activity in the rabbit (n = 83) and mouse retina (n = 19).

We address a new question, the cell type specificity of the bursting pattern. It is generally assumed that the mammalian retina deconstructs the visual world using more than a dozen different neural channels, embodied in morphological and physiological ganglion cell “types”. Each of these parallel pathways reports upon a specific aspect of the visual input.

2 Materials and Methods

Whole mount retinal patches from dark adapted adult animals were placed ganglion cell side down on a multielectrode array (Multi Channel Systems) with dense electrodes (30 µm spacing between electrodes). This allowed better identification of cells and better discrimination of optimal recorded signals.

Ganglion cells were recorded for hours while stimulated by visual stimuli ranging from artificial flashed spots to more naturalistic movie sequences. Spontaneous activity under dim light was recorded as well.

The recorded cells were classified into the following cell types: On brisk transient; Uniformity detectors; On direction selective; Local edge detectors and On - Off direction selective cell subtypes. These classifications were made on the basis of established criteria as receptive field size, discharge rate and direction selective indices.

3 Results

We define in analogy to a thalamic burst a discrete “coding event” of a ganglion cell. An action potential can be classified as the first (cardinal) spike of a coding event if it is preceded by a period of silence longer than an arbitrary time T and if the following spike occurs within the time interval T. Once the interspike interval (ISI) exceeds the threshold T we define the coding event as completed. A convenient way to visualize the coding events is to plot a two-dimensional joint interspike interval map (Fig. 1) where each spike is represented as a function of both the preceding and the following interspike interval.

Starting from the joint interspike interval maps we found spike train patterns which were specific for every cell-type and largely independent of the tested visual inputs.

The cell-type specific signatures do not prevent the cell from signaling to the brain the strength of its response to a particular stimulus, which is expressed in the number of spikes in each coding event. Because they are independent of particular stimulus sets, analysis of these patterns should be useful as a tool for distinguishing the many unidentified ganglion cell types that exist in all mammalian retinas. In addition, the spike train patterns could plausibly be used by the brain to identify the type of stimulus to which an individual ganglion cell is tuned, because each ganglion cell axon carries a physiological signature of its particular functional type.

References

Fig. 1  Retinal ganglion cell activity can be described by burst-like coding events (a) Schematic spike train with coding events and solitary spikes. We define a coding event when a spike is preceded by a period of silence > T and followed by another spike in the interval T. The assignment of individual spikes relates to (b). The burst-like activity is visible in joint interspike interval plots for spike trains recorded from rabbit and mouse ganglion cells. For each spike a point is plotted displaying the duration of the preceding interspike interval and the duration of the subsequent interspike interval. The joint ISI maps display four distinguishable areas, which are used to define coding events: cardinal spikes in the lower right quadrant (red) represent the first spikes in a coding event, the spikes in the lower left quadrant the intermediate spikes (black dots) and the spikes in the upper left corner the last spikes in an event (blue). Spikes in the upper right quadrant represent solitary spikes, which are preceded by a long interval and also followed by a long interval. Figure adapted from a submitted manuscript (Zeck & Masland [2]).
Electrical stimulation of the receptive field of ganglion cells in degenerated retina of RCS rats

Darius Schwenger, Alfred Stett*

Natural and Medical Sciences Institute at the University of Tuebingen, Reutlingen, Germany
* Corresponding author. E-mail address: stett@nmi.de

Subretinal implants aim at restoring vision to blinds by excitation of the neuronal network in the degenerated retina by electrical stimulation. In order to investigate electrically evoked spatio-temporal activity patterns of degenerated retinas we stimulated explanted retinas from blind RCS rats with microelectrode arrays (MEA) containing line-like electrodes. Retinas with completely degenerated photoreceptors (RCS rats, 90 days and older) responded with a complex spatio-temporal activity indicating that in degenerated retinas the receptive-field-organization is accessible to electrical stimulation.

1 Introduction

The neurotechnological approach to restore vision to blinds suffering from photoreceptor degeneration is to implant electrode arrays into the retina for multifocal electrical stimulation [1]. Subretinal implants aim at excitation of the neuronal network in degenerated retinas by electrical stimulation of the distal retina.

In order to investigate whether degenerated retinas can be activated adequately by electrical stimulation, we performed ex vivo experiments with explanted retinas from blind Royal College of Surgeon (RCS) rats, which suffer from an inherited retinal dystrophy.

We showed in an earlier work that application of different spatial voltage patterns resulted in distinctive temporal patterns of ganglion cell activity. The response depended on the strength and pattern (points, lines, edges) of stimuli and on the lateral distance between stimulation and recording [2].

We now investigated the receptive field of ganglion cells that is sensitive to spatial extended electrical stimulation.

2 Methods

Retinas were explanted from the enucleated eyes from RCS rats (age: 90 days and older) and adhered to microelectrode arrays (MEA) with the photoreceptor side facing the array (Fig. 1). The retinas were continuously superfused at 1 ml/min with standard medium solution (in mM: 120 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 30 Na-HCO₃, 15 glucose, 0.2 l-glutamat) at 35 °C, equilibrated with 95% O₂ / 5% CO₂ gas mixture.

The MEA contained 30 line-like TiN electrodes (LINE MEA, Fig. 2) of length 1 mm, width 10 µm, interelectrode distance 10 µm. For electrical stimulation we applied anodic voltage impulses (duration: 500 µs, amplitude up to 3 V) to selected electrodes. The retinal response was measured from individual ganglion cell bodies with a glass pipette [3] and analyzed with respect to the lateral distance of the stimulated line electrode to the recorded ganglion cell. To identify electrically activated intraretinal signal paths we added dopamine (1 mM) and high concentrations of Mg²⁺ (10 mM) to the bath ringer to de-couple amacrine cells and to suppress synaptic transmission, respectively.
3 Results

Ganglion cells responded to subretinal electrical stimulation with a complex spatio-temporal spike pattern. Temporal ON and OFF responses (Fig. 3) could be measured when the stimulating line electrode ran through a well-described field surrounding the ganglion cell body (Fig. 4). These receptive fields had sharp borders with a transition from no response to half-maximal response within 40 µm. The width of the corresponding spatial sensitivity profile (Fig. 5) within this borders was in the range between 100 µm and 400 µm (median of full width at half maximum: 200 µm, n = 9). Administration of dopamine led to a significant decrease of the diameter of the receptive field and slowed down spontaneous activity of ON ganglion cells.

4 Conclusion

Adequate spatial resolution and distinguishable encoding of different electric stimuli patterns by the retinal network can be obtained in vitro with degenerated retinae. In the partly degenerated neuronal network of the RCS rat retina capabilities for lateral signal processing are present and can be activated by electrical input with an accuracy of less than 0.2° visual angle. We conclude that in blind retinas the receptive-field-organization is accessible to electrical stimulation which is an important prerequisite for restoring vision by multifocal electrical stimulation.

References

Subretinal chronic multi-electrode arrays in blind patients: function testing and pattern recognition

Eberhart Zrenner1*, Dorothea Besch1, Karl Ulrich Bartz-Schmidt1, Florian Gekeler1, Veit-Peter Gabel2, Carolin Kutenkeuler1, Wilfried Nisch3, Helmut Sachs2, Heiko Sailer3, Alfred Stett5, Barbara Wilhelm4, Robert Wilke1, Subretinal Implant Study Group

1 University Eye Hospital, Tuebingen, Germany
2 University Eye Hospital, Regensburg, Germany
3 Retina Implant GmbH, Reutlingen, Germany
4 STZ Biomedical Optics, Tuebingen, Germany
5 Natural and Medical Sciences Institute, Reutlingen, Germany
* Corresponding author. E-mail address: ezrenner@uni-tuebingen.de

1 Objective

Assessment of function of subretinal implants, consisting of a chip (3x3x0.1mm, 1540 microphotodiodes, amplifiers and electrodes of 50x50 micrometers, spaced 70 micrometers) and a 4x4 array of identical electrodes, spaced 200 micrometers, for direct stimulation (DS), chronically implanted next to the foveal rim of 2 blind RP patients.

2 Method

Chip and DS array are positioned on a small subretinal polyimide foil powered via a subretinal transchoroidal, retroauricular transdermal line. The charge injection delivered by each DS electrode was controlled by a wireless, software-driven stimulator device modulating the amplitude, shape (monophasic, biphasic), duration, frequency, and interstimulus intervals by simultaneous or successive addressing of individual electrodes in order to present temporal or spatial patterns (for surgical details see Sachs et al. 2006).

3 Results

The implants were well tolerated without adverse events. OCT showed stable and intricate connection of the retina (200 – 230 micrometers thickness) with the electrode arrays during the initial four weeks as well as during the subsequent observation period (see Voelker et al 2006). According to the study plan, the implant was removed in Pat. H-P.R. after 4 weeks. Pat. L.W. decided to keep the implant. Here, the results of chronic subretinal direct stimulation are reported for the first time in patients.

The charge injection delivered by 16 DS electrodes was simultaneous or successive in order to present temporal or spatial patterns. Patients reported homogeneously appearing, small, yellowish or greyish phosphenes (e.g. “peanut at arm’s length”) for individual electrode stimulation. They were able to differentiate spatial patterns, such as lines, angles or a bright square with round corners if all electrodes were switched on. Simultaneous stimulation of four electrodes in a line or a row was reported by patient W.L. as “bright yellowish rod with round corners in the size of a match with four slight indentations on both sides.” The patient was able to clearly distinguish horizontal from vertical lines and to correctly describe the alignment and direction of dot movement, if three or four neighbouring electrodes were switched on sequentially at one second intervals. Electrical thresholds assessed by chronaxy measurements of each individual electrode and perceptual correlates remained relatively stable.

4 Conclusions

Chronic subretinal stimulation via small electrodes with a distance less than 1° allows the discrimination of patterns consisting of small, bright, steadily appearing dots that can be individually discriminated as well as combined into lines and figures, reliably repeated over weeks via a well-tolerated, still intact subretinal micro-electrode array in blind RP patients.

Acknowledgement

Supported by BMBF 01KP0008 and Retina Implant GmbH
Spatial frequency and contrast sensitivity tuning of chicken retinal ganglion cells, as recorded with the Micro-Electrode Array (MEA)

Erich Diedrich, Frank Schaeffel

Section Neurobiology of the Eye, University Eye Hospital Tübingen, Tübingen, Germany

1 Purpose
The chicken is one of the most frequently used animal models in myopia research, but little is known about the response characteristics of its retinal ganglion cells. We have used the MEA to study their responses to phase-reversing checkerboards that were projected on the retina. These checkerboards had different angular field sizes and contrast. "Visual acuity" in vitro was compared to published data on visual acuity in behavioral studies. The final goal of these studies is to find out (1) whether ganglion cells can distinguish optical defocus from a global drop in contrast and (2) whether they can distinguish positive from negative defocus.

2 Methods
A punch of retina with adherent RPE (about 4 mm diameter) was cut from the fundus of freshly enucleated chick eyes (age range 1-6 days) and was glued onto the MEA by nitro cellulose with the ganglion cell layer down. The preparation was perfused with 400 μl/min with modified Hanke-buffer. Computer-generated spatial stimuli were displayed on a CRT-monitor and projected onto the retina from below via a 6 mm lens. Stimuli were checkerboard patterns with variable field sizes and contrasts but isoluminance was preserved by reducing or increasing the brightness of the individual fields from a baseline in the middle of the gray level scale (Michelson contrast). Phase-reversal frequency was 0.5 Hertz. The term "spatial frequency of the checkerboard" refers to the number of bright and dark fields per one degree of visual angle of the retina, assuming that the fundamental of the checkerboard pattern determined the responses of the cells.

3 Results
The frequency of the spikes, following a phase reversal of the checkerboard (referred to as "responses" below), were dependent on both spatial frequency and contrast of the fields. Responses declined with increasing spatial frequency. Examples are shown from 3 individual ganglion cells in Figure 1. At a spatial frequency of 6.94 cyc/deg, the responses of the ganglion cells were no longer significantly different from their baseline (spontaneous) activity.

To evaluate the average contrast sensitivity of the ganglion cells, spike frequencies of all 60 ganglion cells were added while the contrast of the checkerboard was varied (Figure 2). The threshold contrast was found to be at around 0.3, but there were not much data available between 0.1 and 0.5.

4 Conclusions
The spatial acuity of 3 selected ganglion cells was at about 7 cyc/deg which is in the range of the optomotor grating acuity of the chick (Schmid KL, Wildsoet CF, Assessment of visual acuity and contrast sensitivity in the chick using an optokinetic nystagmus paradigm. Vision Res. 1998, 38, 2629-2634). Other studies provided similar results: 6.0-7.7 cyc/deg (DeMello LR, Foster TM, Temple W., The effect of increased response requirements on discriminative performance of the domestic hen in a visual acuity task. J Exp Anal Behav. 1993, 60, 595-609), 4.6 cyc/deg (Johnson HM, Visual pattern-discrimination in the vertebrates-II. Comparative visual acuity in the dog, the monkey and the chick. J. Animal Behav, 1914, 4, 340-361). It should be noted that the 3 ganglion cells were selected based on their responses to the spatial features of the checkerboard pattern and were probably the ones with the smallest receptive field sizes (analogue to the mammalian P cells).

A contrast sensitivity of 3 (defined as 1/contrast threshold) is surprisingly low, compared to human contrast sensitivity, which can be as high as 200 at 5 cyc/deg. At least, this result is similar to previous findings by the same authors (Diedrich E, Schaeffel F, Invest Ophthalmol Vis Sci 46, #1976, ARVO abstract 2005). In behavioral studies, Schmid & Wildsoet (1998) found a slightly higher contrast sensitivity of 5 to 10 in the chick, but still much lower than in humans. It could also be that the chick visual system is not very responsive to repetitive patterns like checkerboards, and that other test stimuli might reveal higher contrast sensitivities.
In summary, these initial results provide confidence that the targeted questions can be answered with the MEA approach in the future.

Acknowledgement

Funded by the fortüne program of the medical faculty of the University of Tübingen F.1222694.1

Fig. 1 Spike frequencies (ordinate) observed in three ganglion cells of a single chick retina that was exposed to phase-reversing checkerboards with varying fundamental spatial frequency (abscissa). Beyond about 7 cyc/deg, the spike frequencies were no longer different from spontaneous activity. The three ganglion cells are not individually denoted.

Fig. 2 Sum of all spikes of the about 60 ganglion cells that were recorded by the MEA (abscissa), while the checkerboard field contrast was varied (Michelson contrast, ordinate). The responses of the ganglion cells were no longer different from baseline activity when the contrast declined below about 0.3. Checkerboard fundamental spatial frequency was 1.7 cyc/deg.
Simulation of extracellular stimulation of bipolar cells with monopolar and dipolar electrode configurations

Matthias Gerhardt¹*, Alfred Stett²

¹ University Eye Hospital, University of Tuebingen, Germany
² NMI Natural and Medical Sciences Institute at the University of Tuebingen, Reutlingen, Germany
* Corresponding author. E-mail address: matthias.gerhardt@nmi.de

We calculated the membranpotential of the pre-synaptic terminals of the bipolar cells in the bipolar cell layer in response to stimulation with monopolar and bipolar electrode arrangement, respectively. We find out, that both concepts of stimulation are be able to depolarize the pre synaptic terminals of bipolar cells. In addition we find out, that stimulation with the dipolar electrode arrangement results in a hyperpolarisation of the pre-synaptic terminal of bipolar cells on the top of non stimulating electrodes.

1 Introduction

Electrical stimulation of a distal retina site causes stimulus-correlated ganglion cell activity within a well defined area surrounding the stimulating electrode [1]. Animal experiments showed that this focal excitation is projected retinotopically correct to the visual cortex. Due to this fundamental result it is supposed that multi-site subretinal stimulation evokes patterned phosphene perception that is helpful to restore vision to blind people suffering from photoreceptor degeneration [2].

The quality of seeing will depend on the extension of the focal excitation and the superposition of the excitation foci after multi-site stimulation of the retina. Since the targets for subretinal stimulation after photoreceptor degeneration are bipolar cells, we simulated the polarisation of the pre-synaptic membrane of bipolar cells due to multi-site stimulation with monopolar and dipolar electrodes.

Fig. 1 Simulation of the electrical field distribution caused by stimulation with dipolar and monopolar electrodes.

2 Model

The simulated model consisted of a 3x3 array of 9 monopolar and 9 dipolar electrodes (distance 50 µm, electrode diameter 13 µm), respectively, contacting a dense layer of bipolar cells (Fig. 2). The ground electrode was placed in far distance in case of monopolar stimulation and concentrically around the stimulation electrodes in case of dipolar stimulation.

Using Finite elemente (FE) modulation the electrical potential caused in the bipolar layer by positive
voltage applied to the stimulating electrodes was simulated. The bipolar cells were described by an electrical cable (length 70 µm) consisting of a passive RC network which is determined by the membrane-resistance: $R_m=1.9\times10^4 \ \Omega/m$, the membrane-capacity: $C_m=10^{-2} \ \text{F/m}$ and the intra-cellular and extra-cellular media-resistance: $R_e = R_i = 4.54\times10^{12} \ \Omega/m$. The response of the bipolar cell membrane to external stimulation was calculated in the frequency domain and the step response in the time domain was obtained by numerical inverse Laplace transformation.

3 Result

The FE simulation offered an additive superposition of electrical fields of neighbouring monopolar electrodes, in contrast to dipolar electrodes which showed no superposition (Fig. 1). This effect was passed to the bipolar cell layer resulting in a spatial modulation of membrane polarisation that was more pronounced with dipolar stimulation than with monopolar stimulation (Fig. 3). With monopolar electrodes, all bipolar cells were more or less depolarized, whereas with dipolar stimulation the cell terminal was depolarized in cells located above the stimulating electrodes and hyperpolarized in cells located in between.

4 Discussion

We simulated the extracellular stimulation of cable-like cells with monopolar and dipolar electrode configurations. In terms of spatial contrast modulation multi-site stimulation with dipolar electrode configurations seems to be superior to monopolar configurations.

References

Single-unit activity and visual response characteristics in retinal ganglion cells of ex vivo retina recorded using 3D MEA microelectrode array recordings in mice

Kolomiets B1,2*, Sahel JA1,2,3 and Picaud S1,2,3

1 INSERM U592, Paris, F-75012, France
2 Université Pierre et Marie Curie-Paris 6, Paris, France
3 Fondation Ophtalmologique A de Rothschild, Paris, France
* Corresponding author. E-mail address: Kolo@st-antoine.inserm.fr

Extracellular microelectrode array recordings using MEA60 setup (Multi Channel Systems) were carried out in a large population of retinal ganglion cells of isolated mouse retina. This study enabled us to characterize their viability, typical patterns of spontaneous spiking activity and visual responses as well as to evaluate the effects of pharmacological agents application on retinal information processing.

1 Introduction
The retinal ganglion cells (RGCs) provide the retinal output signal to the brain and, therefore, readout of the retinal information processing. The multi-electrode recording technique more recently permitted to obtain simultaneous single-unit recordings from large cell group and thus provide a powerful, non-invasive tool that holds great promise both for physiology of perception and clinical neuropharmacology. We therefore applied MEA60 technique (MCS, Multichannel Systems) to investigate the information processing via the ON- and OFF-pathways in mouse ex vivo retina which preserved the cellular organization.

2 Methods
Dark-adapted (1h) retina was isolated under dim red light and placed with RGC layer face down into 3D MEA recording chamber (Ayanda Biosystems). The tissue was continuously superfused at 37°C with NaHCO3-buffered Ames medium mixed with 95%O2/5%CO2. Extracellular activity was filtered on-line, band-passed and sampled at 25 kHz by means of MEA60 setup and MC Rack data acquisition software. Since each electrode typically recorded waveforms from more than one RGC, it was necessary to perform further spike sorting using template match algorithm and statistical analysis off-line. Spatially uniform monochromatic visual stimuli of different light intensities and durations were generated by light-emitting diodes (LED) positioned underneath the 3D MEA.

3 Results
Stable recordings from a large population (15-40) of RGCs could be simultaneously monitored with a good signal/noise ratio for periods lasting over 6 h. In wild type animals RGCs displayed different patterns of spontaneous activity typically encountered in other brain areas at rates frequently ranging from 0.01 Hz to 30 Hz in dark (Fig1) and sometimes exceeding 60 Hz during light stimulation.

![Fig. 1 Spontaneous activity of RGCs in mouse isolated retina. ISI – interspike interval histogram, ACG –autocorrelogram.](image-url)
Fig. 2 Visual responses in ex vivo rodent retina. Upper panel – overall view of the multichannel recordings using MEA60 system. Below - peristimulus time histograms of typical responses to light stimulus (5 s duration) presentation in different RGCs.

Frequently, in young animals (<2 weeks), synchronous waves of spike discharges at minute intervals were often prevailing as is well demonstrated in different mammalian species [1]. At the same time certain RGCs in mature rodent retina may present oscillations at long intervals (Fig. 1C). Some RGCs displayed no signs of spontaneous activity unless the light stimulus was applied. Cross-correlation analysis of single-unit activity revealed relationships among neighboring RGCs (Fig. 1E), predominantly synchronous firings due to the shared input activation or in odd cases – mutual excitatory/inhibitory influences. Application of 3D MEA array with 0.2 mm interelectrode spacing significantly reduced the probability of simultaneously recording the same cell by adjacent electrodes compared to planar MEA biochips. Post-stimulus time histograms revealed that full field monochromatic light LED stimulation evoked characteristic ON (sustained or transient), ON-OFF and OFF responses occurring at latencies ranging from 45 to 250 ms which were sensitive to stimulus intensity, duration or rate (Fig. 2).

The RGCs viability was verified also using molecules with well-known “classical” effects on synaptic activity such as glutamate, GABA or their receptor agonists and antagonists as well as second messenger cascade blockers. Experiments performed with these drugs gave the similar results to those described for in vivo RGCs.

4 Conclusion
These results indicate that classic physiological light responses can be recorded simultaneously in large populations of RGCs in isolated retina preparation. This technique therefore offers a valuable approach to investigate the pharmacology and information processing in normal and mutant animals with altered ERG.

Acknowledgement
This work was supported by INSERM, EVI-GENORET LSHG-CT-2005-512036, Fondation Ophtalmologique A de Rothschild, Fédération des Aveugles de France. We wish to thank Dr. Michael Roux and Manuel Simonutti for technical support and advices during preparation of this study.

Reference
Electroretinogram recordings with MEAs from explanted rat retina (rat µERG)

Andreas Kraft¹, Thoralf Herrmann², Alfred Stett²*

¹ Fachhochschule Kaiserslautern, Zweibrücken, Germany
² NMI Natural and Medical Sciences Institute, Reutlingen, Germany
* Corresponding author. E-mail address: stett@nmi.de

Retinal function and its modulation by diseases and drug effects can be analyzed by measuring of electroretinograms (ERG). In order to develop ex vitro ERG recording by microelectrode arrays (µERG) as a drug screening tool we standardised the preparation of and recording from rat retina samples.

1 Introduction
Retinal activity evoked by standardized light flashes is reflected in the electroretinogram (ERG). Injuries, intoxications, retinal diseases and drug effects affects the retinal function and therefor alters the shape and amplitude of an ERG. For easily assessing effects of pharmacological compounds on retinal activity we use microelectrode arrays for recording ERGs from explanted retinas [1]. In order to develop ERG recording by Microelectrode arrays as a drug screening tool we standardised the preparation of and recording from rat retina samples.

2 Methods
Retinal segments with the pigment epithelium attached, dissected from explanted rat retinas (Long evans, age 4-6 weeks), were placed ganglion cell site down on the translucent surface of a microelectrode array (Fig. 1).

Experiments were started after a 20 min. recovery period under stationary perfusion and temperature controlled ringer solution. The preparation and handling of rat retina for MEA recordings is described in an application note and can be downloaded from http://www.multichannelsystems.com/applications/applicationnotes/applicationnotes.htm.

Full-field stimulation with light impulses of defined strength was carried out with a halogen lamp and electro-magnetic shutter. The light was projected through the objectives of an inverted microscope homogeneously onto the retinal sample. Intensity and wavelength was controlled by colour and neutral filter. The full-field light intensity on the retina level without filter (white light) was 100 mW/cm² (500 kLux), 20 mW/cm² (blue light) and 40 mW/cm² (red light), respectively. For separating rod and cone responses a twin-flash stimulation paradigm was applied [2].

3 Results
ERG recordings from explanted rat retina could be obtained reproducible at different wavelengths (Fig. 2) and intensities (Fig. 3). However, only at high intensities (8200 lx) the b-wave amplitude remained stable up to 60 min, whereas at low intensities (< 1000 lx) a strong run-down was observable (Fig. 4). Twin-pulse stimulation resulted in a clear separation of the cone responses from the mixed rod and cone response (Fig. 5). This allowed to separate the dose-dependend
effect of AP-4 on the ERG components resulting from rod and cone stimulation (Fig. 6).

![Graph showing the effect of AP-4 on the ERG components]

**Fig. 3** b-wave amplitude in dependence of the intensity and colour of the light

![Graph showing the stability of b-wave amplitude as a function of time and light intensity (blue light)]

**Fig. 4** Stability of the b-wave amplitude as a function of time and light intensity (blue light)

![Graph showing the separation of a-wave of rods (pulse 1) and cones (pulse 2) by twin-pulse stimulation and AP-4 (line marked by arrows: AP4). Stimulatio with blue light, 9200 lx.]

**Fig. 5** Separation of the a-wave of the rods (pulse 1) and cones (pulse 2) by twin-pulse stimulation and AP-4 (line marked by arrows: AP4). Stimulatio with blue light, 9200 lx.

4 Summary

Under well defined conditions µERG recording with reproducible results from explaned rat retina is feasible. At high intensities reliable dose-dependent effects on the ERG components can be obtained up to 60 minutes after preparation without remarkable influence of the run-down of the sample on the amplitude of the a- and b-wave.

![Graph showing the dose-dependency of a- and b-wave amplitude in the twin-pulse experiment]

**Fig. 6** Dose-dependency of a-and b-wave amplitude in the twin-pulse experiment

**Acknowledgement**

Funded by BMBF grant

**References**


ISBN 3-938345-02-0

5th Int. Meeting on Substrate-Integrated Microelectrodes, 2006
Organotypic retinal cultures on Micro Electrode Arrays: Validation of the stability of the in vitro system and cellular alterations induced by prolonged electrical stimulation

Heiko Schmid¹, Thoralf Herrmann², Matthias Gerhardt², Heiko Sailer³, Alfred Stett²*, Konrad Kohler¹

¹ Experimental Ophthalmology, University Eye Hospital Tübingen, Germany
² NMI Natural and Medical Sciences Institute, Reutlingen, Germany
³ Retina Implant AG, Reutlingen, Germany
* Corresponding author. E-mail address: stett@nmi.de

During recent years electrical devices have been developed that can be implanted into the eye and directly attached to the neuronal retina in an attempt to restore vision to patients suffering from retinal degeneration. A major challenge of this approach is to experimentally determine stimulation paradigms that allow optimal signal transfer from the technical implant to the neuronal network without inducing pathological alterations within the remaining retina. To explore effects of continuous electrical stimulation on retinal tissue we developed an organotypic retina culture that allows us to keep segments of adult RCS rat retina over six days on an array of iridium (Ir) microelectrodes.

1 Electrical long-term stimulation of neuronal tissue

Electrical stimulation of neuronal tissue has to be effective and safe on a long-term time scale. Stimulation parameters have to be adjusted within a multidimensional window that guarantees for stable suprathreshold activation of a certain physiological effect and prevents for tissue injury.

It is well known that prolonged current injection can cause tissue injury or depression of neuronal excitability due to electrochemical processes occurring at the interface between electrode and tissue, and due to metabolic stress by prolonged excitation [1].

Several aspects related to prolonged electrical stimulation can be investigated in vitro. We have therefore established an ex vivo model using explanted retinas from adult Royal College of Surgeons (RCS) rats and microelectrode array (MEA) technology [2,3]. RCS rats became blind after postnatal day 60 due to inherited retinal degeneration. The model allows for study effects that develop during the first few days of continuous micro stimulation.

2 Methods

Organotypic retina cultures on MEA. Segments from retinas from adult RCS rats (postnatal day 90) were mounted onto MEAs (Fig. 1A,B) with nitrocellulose, with the photoreceptor side facing the surface of the device (Fig. 1 C). The culture was kept for six days in a purpose-developed tilting cell-culture system [3] in Leibowitz L15 medium with 10% FCS at 37°C in natural atmosphere. The stimulation period (up to 5 days) was preceded by a one-day equilibration period.

Electrical stimulation. MEAs containing 59 substrate-integrated electrodes (sputtered nanocolumnar Iridium, size 50x50µm², spacing 200 µm) in a 8x8 array and one large return electrode were used. Up to 8 groups of MEAs (group size max. 4) can be connected to a 8–channel stimulation generator (STG, Multi Channel Systems). Rectangular monophasic voltage pulses with variable amplitude (max. 3 V, pulse duration 500 µs, frequency 20 Hz) were delivered to 30 electrodes of each MEA. The others remained unstimulated for internal control. The stimulation current was measured by a 8-channel current-to-voltage converter (Fig. 1C) and integrated to obtain the charge injected per pulse.

Immunohistochemistry. Cryostat sections of 12µm were collected on silane-coated slices. OX-42 were used to stain resident peritoneal and activated
macrophages in the retina. Apoptotic cells were detected by TUNEL reaction performed on slices as well as on flat-mounted retinas directly on MEA.

3 Results

3.1 Validation of the stability of MEA-based organotypic retinal cultures

To determine the quality of the retina culture, apoptotic cell death was quantified by TUNEL-assays. During the first 3 days in vitro (DIV) apoptosis remained low with only 7% of all cells showing a TUNEL positive signals, followed by a pronounced increase of the apoptosis rate to 28% at DIV 4 and further to 34% at DIV 6 (Fig.2).

Fig. 2 A: TUNEL-staining at different in vitro stages; staining marks apoptotic cells in green (arrows) and cell nuclei (arrow heads). B: Quantification of apoptotic cells during different stages of non-stimulated MEA-based retinal cell culture.

3.2 Electrical stimuli induced alterations

To determine thresholds for electrically evoked alterations of retinal morphology, voltage impulses up to 3 Volts were continuously applied to the cultured retinas for 2, 3 or 6 days (N ≥ 6 for each set of parameter). A significant difference in the number of light-microscopically “dark” appearing cells was observed in the stimulated area compared to the non-stimulated area after 3 days in culture and a minimum stimulus of 2 Volt (Figs.3, 4). These cells were identified immunohistochemically as microglial cells and macrophages by the use of specific antibodies (ED1 and OX-42).

Fig. 3 Comparison between stimulated retinal area (left, 2V) and non-stimulated area (right). The stimulated area has less dark appearing cells than the non-stimulated. A MEA electrode (E) is visible out of focus in the centre of each picture.

Fig. 4 Counting of macrophages/ microglial cells; cell counts are normalized against the non-stimulated side of the retina. After 3 days in culture and a 2 Volt stimulation a reduction of dark appearing microglia and macrophages was observed in those areas of the retina overlying the stimulated side of MEA.

4 Summary

Our results indicate that electrical stimulation of the degenerating retina has an effect on the appearance and distribution of phagocytotic glia cells.

Acknowledgement

The project is funded by a BioProfile grant by the Bundesministerium für Bildung und Forschung (BMBF) to the Retina Implant AG.

References

Comparative multielectrode recording studies on retinal development in the chick

Tanja Ugniwenko*, Christiane Ziegler, Axel Blau

Department of Physics and Biophysics, University of Kaiserslautern, Kaiserslautern, Germany
* Corresponding author. E-mail address: ugniwenko@physik.uni-kl.de

Much is known about the maturation of the vertebrate, especially the mammalian retinal network, including developmentally regulated activity waves as well as the progression of photosensitivity. In contrast, information on developmental features of the chick primary visual system is less consistent and there is little information on robust strategies for directly probing the electrophysiology of the retinal layers during development. We present a comparative multielectrode recording approach based on acutely dissected retinas of the embryonic chicken at different developmental stages to examine and characterize the light perceiving structures as well as spontaneous network activity in more detail.

1 Purpose

The maturation of the vertebrate retina comprises the refinement of retinal connectivity, provided by periodic waves of spontaneous activity, and the acquirement of photosensitivity, born by image forming as well as non-image-forming photoreceptor cells [1-4]. We applied multielectrode recordings on acute retinal slices of the embryonic chicken to investigate the progression of these events, especially the photoreceptivity of non-image-forming neurons that is based on the photopigment melanopsin [4,5].

2 Experimental procedures

Retinas from embryonic chicken were dissected at different embryonic stages. 4-5mm squares from the central region were prepared. The retinal slices were placed ganglion cell layer down onto planar microelectrode arrays (30/200 MEAs, Multichannelsystems, Reutlingen, Germany) and held in place by a platinum grid, allowing the recording of retinal ganglion cell activity. Retinas were permanently held in carbogen gassed Ringer solution. An adequate perfusion set-up was established to ensure constant conditions during recording experiments. Thus, it was possible to record from one slice for up to 8 hours. Drugs were applied via the perfusion system to test various synapse blockers. For closer examination of non image forming light responses, kynurenic acid was applied to silence potential rod or cone photoreceptor actions as well as the periodical burst waves that inhibited light responses. MEA recordings were performed using a MEA60-1System (Multichannelsystems, Reutlingen, Germany). Light pulses were applied by a white LED that was positioned over the centre of the array delivering diffused light.

3 Results and discussion

Using MEA technology, early maturation of the so-called intrinsically photosensitive retinal ganglion cells (ipRGCs) has already been described for the mammalian retina [6]. Alike, periodical retinal waves as well as light evoked activity could be recorded from several regions of the retinal network of embryonic chicken. Non-image forming photosensitive ganglion cells were found to display light evoked activity from the first embryonic stage (E) tested (E13). That is much earlier than E18, when their image forming complements start to respond to light [7,8]. The characteristics of the light responses varied between cells as well as between different developmental stages (Fig.1). Periodic burst activity of the retinal network could be observed for all examined stages (E13 to E17). Application of drugs delivered obvious variations in wave characteristics for the different drugs and developmental stages, indicating the driving connections responsible for wave generation, expansion and modulation that are shifting during retinal network maturation (Fig. 2) [9-11].

Acknowledgement

We are grateful to the Landesgraduiertenförderung for financial support.

References


Fig. 1 Perievent histograms from 6 photosensitive ganglion cells stimulated by 1 sec at 0.1 Hz frequency light pulses indicated by light bars. The histograms on the left display light responses from an E13, on the right from an E17 retina. The number of evoked spikes per 25 ms bin was summed for 30 successive light responses.

Fig. 2 Mean firing rate histograms displaying periodical wave activity of the E13/14 (top) and the E16 retina (bottom) after application of kynurenic acid, a non-selective antagonist for NMDA and AMPA/kainate receptors, and dihydro-β-erythroidine (DHβE), a nicotinic receptor antagonist. The number of evoked spikes was summed within 500 ms bins.
Comparison of Retinal Spikes Recorded with Multielectrode Array in Normal Mouse vs. Retinal Degenerate Mouse

Jang Hee Ye¹, Je Hoon Seo², Yong Sook Goo¹*

¹ Department of Physiology, Chungbuk National University School of Medicine, Cheongju, Korea
² Department of Anatomy, Chungbuk National University School of Medicine, Cheongju, Korea
* Corresponding author. E-mail address: ysgoo@chungbuk.ac.kr

For the goal of restoring vision for the blind, Korean retinal prosthesis project launched. As part of this project, we investigated the differences of the retinal waveforms in normal and degenerate retina. Multielectrode recordings were performed in the isolated mouse retina. In normal mouse of 4 weeks old, only short duration (< 2 ms) ganglion cell spikes were recorded. In rd/rd mouse, besides normal spikes, waveforms with long duration (~ 100 ms) were recorded. We tried to understand the circuit of visual signaling in degenerate retina using many different synaptic blockers.

1 Introduction

In retinal degenerative diseases, such as retinitis pigmentosa (RP) and age related macular degeneration (ARMD), the majority of cell death occurs in the outer nuclear layer (ONL) containing the photoreceptors (PRs) which transform the light signal to electrical signal [1].

A number of animal models of retinal degeneration have been developed, and the most extensively studied animal is the rd/rd mouse. In this mouse, similar to some human patients with autosomal recessive RP, defects in the β-subunit of the rod cGMP-phosphodiesterase gene [2] result in a rapid loss of rod photoreceptors with a subsequent slow loss of cones [3].

It has been proposed that rod signals can travel via several different routes to reach ganglion cells [4] (Fig. 1). In this paper we focus on the differences of visual signaling in degenerate retina using many different synaptic blockers.

2 Material and Methods

2.1 Recording of retinal ganglion cell waveform

We used C57/BL6J strain as control mouse and C3H/HeJ strain (rd/rd mouse) as retinal degeneration model. We modified the method of Stett et al [5] for retinal preparation. Briefly, from isolated mouse retina, retinal segment (~ 5X5 mm) was attached with the ganglion cell side on to the surface of microelectrode arrays (MEA) and retinal waveforms were recorded.

2.2 Electrode and data recording system

We purchased MEA from Multichannelsystems GmbH, Germany. The MEAs we used have diameter of 30 um and interelectrode distance of 200 um. The impedance level of MEA is 50 kohm at 1 kHz. Raw data from the MEA is amplified by MEA 1060 amplifiers (amplification: ×1200, sampling frequency: 25 kHz/channel). We applied photopic (1.45 uW/cm²) light intensities. For synchronization of light stimulus and data recording, we used RS232C output.

3. Results

3.1 Histological findings and retinal recordings

Whole mount sections of retina were done. In one month old normal mouse (n=5), all the layers were intact (Fig. 2A top). In 5 weeks old rd/rd mouse (n=4), outer nuclear layer was disappeared while ganglion cell layer remained intact (Fig. 2B top). In control mouse, only short duration (< 2 ms) ganglion cell spikes were recorded reproducibly in 3 of 5 mice (Fig. 2A bottom). But in 4 of 4 rd/rd mouse, besides normal spikes, abnormal waveform with long duration (~200 ms) were reproducibly recorded (Fig. 2B bottom).

3.2 Effects of synaptic blockers

Since the glutamatergic pathway is major synapse in retina (Fig. 1), we used specific AMPA/kainate receptor blocker, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and specific NMDA receptor blocker, 2-amino-phosphonoheptanonic acid (AP-7) together. With this co-treatment, the abnormal waveforms were
blocked (Fig. 3). We tested the effects of strychnine, which interferes with the glycinergic synapse from AII amacrine cell. The amplitudes of abnormal waveforms were enhanced with strychnine. With additional treatment of CNQX, and AP-7, the abnormal waveforms were blocked while normal ganglion cell spikes last like in no strychnine (Fig. 4). The Hi-Di (high Ca$^{2+}$, Mg$^{2+}$) solution, which blocks the polysynaptic component of postsynaptic potential [6] completely blocked all the waveforms (Fig. 5).

4 Summary

Deciding from the long duration (~100 ms) and blockade with Hi-Di treatment, the abnormal waveforms of rd/rd mouse are not action potential of ganglion cell origin but it seems to be postsynaptic potential of polysynaptic component. Because strychnine induced enhancement of abnormal waveforms was blocked by CNQX and AP-7, the effect of CNQX and AP-7 should be down-stream of AII amacrine input to cone OFF bipolar cell. We identified that outer nuclear layer cells were disappeared in rd/rd mouse in histology. Therefore, the glutamatergic synapse blocked by CNQX and AP-7 is more likely to be the cone bipolar to ganglion cell input with photopic light stimulus. With this glutamatergic synapse blocked, all the abnormal waveforms disappeared, suggesting that stronger glutamatergic input from bipolar to ganglion cell in rd/rd mouse probably than normal mouse contributes most to these abnormal waveforms. We should investigate possible mechanism for this stronger glutamatergic input.

Acknowledgements

Supported by grants of Ministry of Health & Welfare (A050251), ERC Program of MOST/ KOSEF (R11-2000-075-01002-0), and KRF (KRF-2004-202-E00034).

References

Heart
Electrophysiological Assessment of Human Embryonic Stem Cell–Derived Cardiomyocytes Using Microelectrode arrays

Izhak Kehat

Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel
E-mail address: ikehat@tx.technion.ac.il

Keynote address
Microelectrode array provide a powerful tool for the in-vitro assessment of conduction in cardiac cultures. The combination of this technology with the human embryonic stem cell derived cardiomyocyte cultures is a novel in vitro model that can be used for drug testing on human cardiac tissue and for the assessment of the function and integration of cell grafts.

1 Introduction

1.1 Models of Propagation
Impulse propagation in cardiac tissue is a complex process governed by the excitable properties of the tissue and its macroscopic and microscopic architecture. Although the spread of electrical excitation has been studied extensively in the native heart, only a few in vitro models are currently available for studying structure-function relationships. Although important experimental findings were gathered from these models, they may be hampered by their relatively short life span, by the inability to genetically manipulate the cells directly, and by the lack of a human model.

1.2 Human Embryonic stem cells
Human embryonic stem (hES) cells are pluripotent cell lines recently derived from human blastocysts [1]. Cultured as three-dimensional embryoid bodies (EBs), hES were demonstrated to differentiate into derivatives of all three germ layers including spontaneously contracting tissue. We have recently demonstrated that dispersed cells isolated from these beating areas have ultrastructural, gene expression, and functional properties of early-stage cardiac phenotype [2]. Not only isolated cells, but rather cardiomyocyte aggregates can be found inside the embryoid bodies. Therefore, we next assessed whether some higher form of two or three dimensional organization existed [3]. Initially we attempted to define the tissue’s structural properties. This analysis identified an isotropic tissue with the cardiomyocytes arranged in various orientations. The cells were relatively small and round-, triangular-, or rod-shaped. Next, we determined the presence and properties of gap junctions within the contracting areas because their number, size, and distribution are important determinants of conduction during physiological and pathological conditions [4]. Gap junctions were relatively small and distributed homogeneously along the cell circumference with no preferential polar orientation. This pattern is similar to the one observed by Peters et al [5] in human fetal and neonatal tissue. We also identified a predominance of connexin45 in the gap junction connecting the human ES derived cardiomyocytes. The significance of Cx45 in this model is not surprising. Although almost absent in adult ventricular myocardium, Cx45 has been shown to play a major role in early cardiac embryonic development [6].

1.3 Electrophysiological Assessment
Next we microdissected the spontaneously contracting areas and plated them of the Micro-electrode array plates (MEA). This allowed long-term, high-resolution electrophysiological recordings form the EBs. These measurements demonstrated the presence of a functional syncytium with stable spontaneous pacemaking activity and synchronous action potential propagation. Both the site of earliest focal activation and the conduction properties within each EB were relatively reproducible during short-term (3 hours) and long-term (10±5 days) recordings. The conduction-velocity values observed by us are lower than those reported in the intact human heart and in the neonatal rat and mouse monolayers [7]. This slower conduction may stem from the small cell dimensions, the isotropic nature of the tissue, the heterogeneous distribution and presence of nonmyocytes within the cell network (which may electrically couple with cardiomyocytes and thereby slow conduction), the lower gap junction size and density, the significant presence of Cx45, and possibly by the less developed ionic channel machinery in early-stage cardiomyocytes.

The spontaneous activity and the relatively rapid conduction in these studies suggested the presence of a high-density inward current via voltage-activated
channels. Therefore, we next tested the effects of Na⁺ channel and Ca²⁺ channel blockers on spontaneous activity and conduction [8]. The Na channel blocker TTX caused a significant slowing of the conduction. In contrast to TTX, the Ca²⁺ channel blocker diltiazem, or nifedipine neither slowed nor blocked action potential conduction and had no effect on automaticity. These results suggested that Na⁺ channels, but not Ca²⁺ channels, are essential for initiation and efficient conduction of action potentials through the human ES cardiomyocyte syncytium. The ionic mechanism responsible for the automaticity of the human ES derived cardiomyocytes was further explored in our lab [8]. A prominent Na⁺ current, and a hyperpolarization-activated current (HCN), but no inward rectifier K⁺ current was found in isolated cardiomyocytes. The absence of background K⁺ current creates conditions for spontaneous activity that is sensitive to TTX in the same range of partial block of the Na channel; thus, this channel is important for initiating spontaneous excitability in hES-derived heart cells. We have shown that this model may serve the first long-term in vitro model for human cardiac tissue. Such a model can be used to further explore conduction in vitro in human cardiomyocytes. Moreover, this model can be used as a platform to study the effect of drugs on the conduction properties in vitro on human cardiomyocytes as demonstrated for the Na⁺ and Ca²⁺ channel blockers.

2 Graft Cell Integration

We also used this system to assess the ability of the hES cardiomyocytes to integrate in vitro with primary cultures of neonatal rat ventricular myocytes. The contracting areas within the embryoid bodies were dissected and added to the cardiomyocyte cultures. Within 24 h after grafting we could already detect microscopically, in all 22 cocultures studied, synchronous mechanical activity. To further characterize the functional interactions within the cocultures, we mapped their electrical activity with a high-resolution MEA mapping technique. By recording extracellular potentials simultaneously from 60 electrodes, we were able to generate high-resolution activation maps that characterize impulse initiation and conduction within the cocultures. We next carried out pacing studies in which either the human or rat tissues were stimulated through one of the MEA electrodes. Synchronous activity was maintained in the cocultures during both conditions. To assess electromechanical coupling between the two tissues, we correlated the mechanical contractions in the hES cardiomyocytes, as detected by a photodiode, with the electrical activity. The mechanical contractions of the embryoid bodies were time-locked with the electrical activity in both human and rat tissues.

Our results demonstrate long-term electromechanical integration between host and donor tissues at several levels. Electromechanical coupling was initially demonstrated in vitro by the presence of positive Cx43 immunostaining at the interface between the hES and rat cardiomyocytes and by the appearance of synchronized electrical and mechanical activities in these cocultures. The high degree of coupling was evident by the lack of local conduction delay at the tissues’ junction, by the continuous long-term coupling and by the persistent coupling during altered pacemaker position, adrenergic stimulation and partial (but not total) gap-junction uncoupling.

3 Summary

In conclusion, the combination of human ES derived cardiomyocytes with the MEA high resolution mapping technique provides a powerful tool for the in-vitro assessment of conduction in human cardiomyocytes. This tool can be used for drug testing and to assess the function and integration of cell grafts.

References

Long-term observations on the development of HL-1 mouse atrium cardio-myocytes using a MEA system

Joon Ho Choi¹, Jin Hee Hong¹, Tae Yun Kim¹, Byung Wook Bae¹, James Jungho Pak² and Kyoung J. Lee¹*

¹ National Creative Research Initiative, Center for Neurodynamics and Department of Physics, Korea University, Seoul, Korea
² Department of Electrical Engineering, Korea University, Seoul, Korea 136-701
* Corresponding author. E-mail address: kyoung@nld.korea.ac.kr

1. Spiral wave activities in populations of cardiac cells

Many cardiac arrhythmias are driven by the reentrant spiral waves. (See Fig. 1) Many studies probing the nature of reentrant spiral waves have used cultured layers of primary cardiac cells because they have many advantages over several restrictions in in-situ experiments. Here, we use HL-1 atrial myocytes cultures in particular to investigate some properties of cardiac spiral waves. Their activities are monitored through a home-built microelectrode array recording system and their properties are compared with those of primary cultures of atrium cells of mouse.

Fig. 1 A spontaneous spiral wave from primary culture of mouse atria is captured by high speed CCD camera

2. Methods

A MEA dish was built from 3-inch diameter Pyrex wafer with a sputtered 2500 Å gold layer. The desired pattern of electrodes and track was formed by photolithographic etching and covered by polyimide (PI2771) which provided electric insulation and cell friendly surface except electrode openings and connection pads. A culture ring was attached on the plate and Pt-black was electroplated on the electrodes to attain a proper value of impedance.

HL-1 myocytes (passage No.38) were obtained from Dr. William Claycomb (Louisiana State Univ.) 2 × 10⁵ cells were plated onto the MEA plate which was coated with 0.1% fibronectin and cultured in Claycomb medium (JRH Bioscience) supplemented with norepinephrine, L-glutamine and 10% FBS. The MEA dish was kept in an incubator (37°C, 5% CO₂ and water vapor saturated) throughout the whole experiment. A peristaltic pump delivered fresh medium to culture at 1.17 μl/min. The MEA holder was connected to 32 channel pre- and filter-amplifiers (Multi Channel Systems) with SCSI connector. The amplifiers were connected to ADC card (DAQ-2204, Adlink) installed in a PC running Linux operating system. Custom written softwares were used for data recording and processing.

3. HL-1 cardio-myocytes vs. Primary atrium culture

Cultured HL-1 myocytes show a more or less regular spontaneous beating and it is usually associated with spiral wave. The inter beat intervals (IBIs) of HL-1 myocytes increase gradually. [Fig.2 (a)] In a control experiment, contrary to the HL-1 myocytes, IBIs of cultured primary atrial cells decrease. [Fig.2 (b)] This indicates that period of spiral wave in HL-1 culture medium is getting bigger while that in primary atrium culture medium is getting reduced. Immunostaining cells with connexin43 (CX-43) antibodies show that the distribution of gap junction in cultured HL-1 myocytes is similarly increase to that in primary atrial cultures. This provides that cell to cell connection is the same for both cultures. Therefore, we can assume that increasing cell density from proliferation of HL-1 myocyte is the biggest contribution of opposite trend between two cell groups.
Fig. 2 Mean IBI vs. DIV in HL-1 myocytes culture (a) and in a primary culture of atria cells of mouse (b).
Characterization of cardiac micro-electrode array field potentials

Halbach, M.1, Banach, K.1,3, Egert, U.4, Pillekamp, F.1,2, Reppel, M.1, Hescheler, J.1

Institute of Neurophysiology, University of Cologne, Germany
Department of Pediatric Cardiology, University of Cologne, Germany
Department of Physiology, Loyola University Chicago, USA
Neurobiology and Biophysics, Institute of Biology III, Albert-Ludwigs University Freiburg, Germany

For multifocal in vitro recordings of cardiac field potentials (FPs) the micro-electrode array (MEA) system has become popular in recent years. While the MEA system enables the evaluation of excitation spread by calculating the delay of FPs at neighboring electrodes, little is known about the information single FPs contain.

1. Methods
To further characterize single FPs and the electro-technical properties of the MEA system, we performed synchronous MEA and current-clamp measurements in a monolayer of murine embryonic cardiac myocytes (n=47 paired recordings from 15 preparations) and applied rectangular stimuli to MEA electrodes not covered by cells via a patch-clamp setup.

2. Results
(i) The FP amplitude showed no correlation with the amplitude of the local action potential (n=47). (ii) The relative magnitude of the positive prespike FP PRE (i.e. amplitude of FP PRE divided by the amplitude of the first FP minimum, FP MIN), which occurred solely in non-pacemaker areas, could be related to conduction speed (n=34). (iii) The decline from baseline to FP MIN took 1.6±0.3 ms if FP MIN was caused by fast Na⁺ currents (n=11) and 18.1±1.5 ms if it was evoked by L-type Ca²⁺ currents (n=12). In the former case, usually a second minimum, FP SLOW, existed, which was caused by L-type Ca²⁺ currents and possessed a decline time of 14.3±1.9 ms (n=9). (iv) Application of rectangular stimuli to MEA electrodes revealed that the input signal is not only filtered by the band-pass filter of the MEA system but also by an additional low-pass filter depending on the distance between stimulating and recording electrode. (v) Besides filter properties this distance strongly influences the amplitude of the output signal; however even in a distance of >1 mm (more than fivefold inter-electrode distance) the influence is noticeable.

3. Conclusion
Single MEA FPs contain information about local transmembrane currents, local cell differentiation and local conduction speed, but may also be influenced by distant cardiac myocytes.
MEA recordings from acute heart slices from adult rats and guinea pigs

H. Lohmann¹, A. Bussek², M. Meier¹, M. Schmidt², E. Wettwer³, U. Ravens³

¹ Lohmann Neuropharmacological Consulting, Castrop-Rauxel, Germany
² Allg. Zool. & Neurobiol., Ruhr-University, Bochum, Germany

Acute tissue slices from the brain have been extensively studied with the multielectrode array technique in neuroscience research. Comparable studies on tissue slices from the heart, however, are only rarely encountered. Here we describe the preparation, maintenance of slices from heart tissue of adult rats and guinea pigs and the evaluation of signal propagation on standard planar MEAs.

All experiments were carried out according to the guidelines for animal welfare. The heart was carefully removed under deep anaesthesia. After perfusion with oxygenated physiological solution containing 10-15 mM 2,3-butanedione monoxime BDM), tissue blocks (4 mm x 6-8 mm) were prepared from the left ventricle. A block of tissue was glued to the cutting stage of a precision vibratome (Integraslice, Campden, UK) with cyanoacrylate glue. The cutting stage was mounted in the bath chamber of the vibratome filled with oxygenated physiological solution plus BDM. 300 µm thick transmural longitudinal, transverse slices as well as sagittal slices could be prepared from the tissue blocks by use of ceramic blades. Slices were maintained in a preincubation chamber at room temperature until they were used in the experiment.

Slices were positioned on standard planar MEAs with 200 µm interelectrode distance and 30 µm electrode diameter by use of a harp shape specimen holder (Harvard App.). An external stimulation electrode was advanced onto the tissue by means of a manual micromanipulator. Biphasic pulses could be applied over the stimulation electrodes with a STG2004 stimulator. Extracellular field potentials and its propagation throughout the heart slice were recorded with a MEA60 upright system with artifact blanking circuit. Control experiments with intracellular recordings from ventricular cells were performed in order to verify the viability of the heart cells.

Extracellular action potentials (AP) after electrical stimulation correspond well to the first derivative of the intracellular AP and could be recorded from any region of the heart slice for up to 30 hours. The AP size and form was dependent on the heart subregion and animal used. Ventricular AP from rats were much shorter as those from guinea pigs. Pharmacological effects of standard drugs were observed corresponding to the ion current components affected. Application of the IKr specific blocker E4031 revealed AP prolongation in accordance with previous results.

Multielectrode experiments demonstrated AP conduction velocities in transverse and sagittal direction in the range of 0.1-0.4 m/s. In order to calculate the spatio-temporal distribution of single AP peaks, time slices were determined and plotted in grey scale in a 8x8 grid. Results from this analysis showed a clear non-isotropic spatial distribution indicating a re-entry path of the signal conduction.

Our results show that standardized acute heart slices with normal physiology and pharmacology can be prepared from adult rat and guinea pig heart and maintained for up to 30 hours. Therefore, the native heart slice will be a valuable new tool in heart research including AP propagation studies for evaluations of heart arrhythmia and risk assessment of QT prolongation.
Fig. 1: A. Intracellular AP from guinea pig ventricular cells; black line: control; grey line: after 1 µm E4031. B. Extracellular AP of guinea pig ventricular tissue slices after electrical stimulation at 2 Hz (upper trace) and 0.5 Hz (lower trace) demonstrating the strong frequency dependence of AP duration. C. MEA recording of extracellular potentials from guinea pig ventricular heart slice after electrical stimulation (planar electrodes 200 µm electrode distance). D. MEA recording of extracellular potentials from rat ventricular heart slices after electrical double pulse stimulation (planar electrodes 200 µm electrode distance). E. Spatio-temporal distribution of neg. AP deflection in guinea pig heart slice (MEA recording) after electrical stimulation; grey scale display: bright: early responses, dark: late responses; propagation of activity grouped into 4 time slices; note the non isotropic distribution of activity and its non-linear propagation throughout the heart tissue.
Simulation of extracellular recorded cardiac action potentials

Frank Sommerhage*, Mathias Schindler, Günter Wrobel, Sven Ingebrandt, Andreas Offenhäusser

Institute of Bio- and Nanosystems, IBN-2: Bioelectronics and CNI – Center of Nanoelectronic Systems for Information Technology, Forschungszentrum Jülich GmbH, Jülich, Germany
*Corresponding author. E-mail address: f.sommerhage@fz-juelich.de

Arrays of metal microelectrodes are commonly used to get qualitative information about extracellularly recorded signals from electrogenic cells. Due to the contribution of small-sized electrodes and our improved amplifier set-up we are able to obtain extracellular recordings from single cells. In further processing we fit the different transmembranal input currents of a heart cell model to the extracellularly recorded signals. So we can even get quantitative information about different ion currents of beating heart cells on multielectrode arrays.

1 Introduction

Extracellular recording of the electrical activity of cardiac myocyte cultures with microelectrode arrays (MEA) enables non-invasive, long-term monitoring of action potentials (AP). To characterise not only the excitation size or the velocity of excitation from multiple-site AP recordings, a more exact analysis of the individual signal shape is necessary. In the present study we obtained quantitative information about the contribution of different ion currents in the respective extracellular recording for electrophysiological experiments. Therefore we used the HL-1 cell line as a standard signal source and simulate the extracellular recorded APs with an equivalent electrical circuit model.

2 Materials & Methods

2.1 The cell line system

Primary cardiac myocytes from embryonic or adult rats show a broad variety of different signal shapes on the MEAs, as they were obtained from the entire heart. However, in a cell line are always the same cells and accordingly they produce constant signal shapes. In our current study, we used the HL-1 cell line [1] (Fig 1) to obtain a standard extracellular signal shape as a simplified model system for our signal simulations. Our aim is to constitute from the extracellular signal shapes, which ion channels are expressed in the HL-1 cell line.
2.2 The 64-channel MEA

For extracellular recordings of APs, a MEA systems were used, consisting of 64 planar gold electrodes with diameters of 6, 8 or 10 µm, respectively (Fig 2). With our small-sized microelectrodes we increase the possibility to record signals of only one cell located on the electrode compared to the ‘field-potentials’ recorded by other systems [2]. The influence of our amplifier set-up has been checked with lock-in measurements and with signal shape studies, when an additional shunt resistor is inserted into the amplifier system [3].

![Fig. 3 The standard Point-Contact-Model for cardiac myocytes](image_url)

3 Results

3.1 Signal modelling and analysis software

We use the basic Point-Contact-Model (PCM) [4] to simulate spontaneous cardiac myocyte signals (Fig. 3) because this model can distinguish inputs for different ion currents. The PCM was implemented in Matlab and Simulink [5] and used simulated APs and ion currents from LabHEART [6] as input data. Furthermore, the current contributions of different ion channels have been approximated progressively to fit our simulated APs to the real recordings (Fig. 4).

![Fig. 4 Comparison of recorded (gray trace) and with typical currents simulated (black trace) AP of HL-1 cells.](image_url)

3.2 Patch clamp recordings of cardiac HL-1 cells located on an electrode

In addition to extracellular recordings, we performed electrophysiological experiments to obtain statistical information about typical electrical cell parameters, e.g. the seal resistance (Fig. 3) between cell, electrode and bath. The collected experimental data can then be used to simulate the influence of the ion currents to the signal shapes in more detail.

4 Conclusion and outlook

With our approach we try to set the stage for better analysis of cardiac tissues on MEAs. Further work will focus on the exact back-simulation of transmembranal ion currents from extracellular recorded APs of HL-1 cells and we might be able to distinguish between different cardiac cell types from primary myocyte cultures.

References


Validation of the possible use of microelectrode arrays in pharmacological studies

Günter Wrobel¹*, Chi-Kong Yeung², Frank Sommerhage¹, Mansun Chan², Andreas Offenhausser¹, Sven Ingebrandt

¹ Institute of Bio- and NanoSystems, IBN-2: Bioelectronics, and CNI – Center of Nanoelectronic Systems for Information Technology, Forschungszentrum Jülich GmbH, Jülich, Germany
² Department of Electrical and Electronic Engineering, The University of Science and Technology, Hong Kong
* Corresponding author. E-mail address: g.wrobel@fz-juelich.de

We developed an improved MEA amplifier set-up with a broad frequency bandwidth and a high signal-to-noise ratio. Here we determined the effects of potassium channel openers (KCOs) on cardiac myocytes cultured on the microelectrode arrays.

1 Introduction

Recording extracellular signals from electrogenic cells using planar metal microelectrodes has already been presented more than 30 years ago [1]. Recently, the interest on this non-invasive technology has grown, because the microelectrode arrays (MEAs) are able to measure extracellular signals at about 256 sites simultaneously [2]. Consequently, MEAs are poised to become a fundamental tool in long-term monitoring of electrogenic cells in culture. Here we present extracellular recordings of action potentials (AP) from rat embryonic cardiac myocytes using arrays of small gold microelectrodes (ME) with high input impedance. We developed an improved MEA amplifier set-up with a broad frequency bandwidth and a high signal-to-noise ratio [3]. The major improvement is, that there is no need for an additional surface-modification of the gold microelectrodes. With our system we are able to record both, the high- and the low-frequency signal components of myocyte APs, which coincide with the membrane depolarisation and the activation of distinct types of ion-channels [4-6]. Here we show the suitability of our MEA system for pharmacological studies.

2 Materials and methods

The MEAs were manufactured using standard silicon technology [7]. The gold interconnecting lanes, microelectrodes (ME) and bond pads were fabricated on glass wafers (Borofloat 33, SCHOTT GLAS, Germany). The chips consisted of 64 ME arranged in an 8 × 8 array with a diameter of 10 µm. The surfaces of all the different chips were passivated by an ONO stack (SiO₂/Si₃N₄/SiO₂), which allowed multiple uses of the chips. For further details of the chip fabrication and the encapsulation process see [7, 8].

The embryonic cardiac myocyte culture (Wistar rats, E17, Charles River GmbH, Sulzbach, Germany) was adapted from previously published protocols [9, 10]. The myocytes were plated onto the MEAs at densities of 60000 cells per chip (effective surface: 38.5 mm²). The chips were incubated for 5-6 days at 37 °C, and 5% CO₂. After 5-6 days in culture, the extracellular signals of the cardiac myocytes were recorded.

We determined the effect of different cardiac-active drugs (e.g. norepinephrine [NOR], pinacidil [PIN], cromakalim [CROM], or SDZ PCO400 [SDZ]) on the myocytes.

3 Results

In our recordings, we can clearly distinguish between different signal shapes of myocytes of the same preparation on the same MEA (Fig. 1). With our small-sized microelectrodes we increase the possibility to record signals of only one cell located on the electrode compared to the ‘field-potentials’ recorded by other systems [11].

![Fig. 1 Comparison of different extracellular signal shapes of myocyte action potentials. These signals were recorded with myocytes of the same preparation on different electrodes of the same MEA.](image)

We found a propagation waveform pattern of the APs travelling through the electrode array and localized the pacemaker region (Fig. 2). Once a certain drug is applied, the activity of distinct ion-channels in the membrane is either enhanced or reduced, and we detected changes in the signal shape (Fig. 3). The catecholamine NOR results in a positive chronotropic effect, with an increased Ca²⁺ influx and an increased AP steepness. In fact, we recorded an increase in the beat frequency and the slow negative component,

*Corresponding author. E-mail address: g.wrobel@fz-juelich.de
which coincides with the activation of the L-type Ca\textsuperscript{2+}-channels (Fig. 3A, NOR). The potassium channel openers PIN, CROM and SDZ cause a negative chronotropic effect. In the experiment, these drugs caused a concentration-dependent reduction of the beat frequency and significant changes in the signal shape. The respective signal components of the depolarisation, and Na\textsuperscript{+}-influx became smaller and had a substantially slower time course (Fig. 3B, PIN).

4 Summary
This study shows the suitability of our MEA system for pharmacological studies and provided a quick and simple means of determining the relative effects of some well known KCOs on cardiac myocytes cultured on the MEA. Based on the correlation between the signal shapes components of a cardiac myocyte AP and the extracellular MEA recordings, it is possible to obtain detailed information of the cellular processes resulting from the drug application. In future it may be possible to identify these characteristic changes in the signal shape as 'signatures' of drugs.

Acknowledgements
We thank R. Helpenstein (IBN-2) and N. Hersch (IBN-4) for the cell culture. We also thank R. Otto, N. Wolters and Y. Zhang (IBN-2) for the technical support with the amplifier systems.

References
Pharmacology, Toxicology, Drug Screening
Organotypic in-vitro systems for compound testing for CNS disorders

Luc Stoppini1*, Heather Cater1, Rosy Bonfante1, Fay Thomas2, Thelma Biggs2, Janice Davies2 and Lars Sundstrom2

1 Capsant Neurotechnologies, Genenva, Switzerland
2 Capsant Neurotechnologies, Southampton, UK
* Corresponding author. E-mail address: luc.stoppini@capsant.com

Keynote address
The pharmaceutical industry is striving to improve the effectiveness of its drug discovery programmes (i.e. improving drug discovery rates whilst decreasing clinical attrition rates) by increasing the biological relevance of the assays through which it identifies and screens its drug candidates. Over the last decade this strategy has driven substantial growth in cell-based assays and in high-content screening. In the last few years there has also been an increasing recognition that cells grown in standard 2-dimensional culture conditions, exhibit different phenotypes and drug responses to cells within intact tissues, and thus often at risk of providing misleading data. This disconnection cannot be addressed by increased use of animal models due to throughput, cost and societal pressures. A strong drive now exists for 3D tissue-based in-vitro systems that reproduce the biochemical and physiological properties of intact tissue, but which can be used in formats compatible with drug discovery. Capsant Neurotechnologies has developed a new 3D tissue culture system, ‘Hi Spots’, where cells from tissues have been first dissociated, then “reconstituted” into tissue spots of standardised size and cell number, and grown on an air-liquid interface. Hi Spots offer a new opportunity for the screening of compounds under tissue like conditions and addresses the functional pharmacology bottleneck which currently exists between high content in-vitro systems and animal models for safety and efficacy testing of new compounds.

1 Introduction

1.1 From 2D to 3D cell cultures
In general, drugs and chemicals are first screened in cell free systems to profile initial biological effects. As the compounds are selected for further development, more sophisticated systems are employed to more closely mimic conditions in man. Prior to testing in humans (clinical trials) a number of animal experiments are conducted for both efficacy and toxicity. However large numbers of molecules continue to be terminated both at this animal stage and later in human trials, as it is difficult to predict ultimate effects. Therefore, two developments are needed. Firstly a more sensitive screen should be employed to screen out compounds before they are introduced into animal systems thereby increasing the chance of ultimate success. Secondly, a more technically sophisticated system that more closely mimics human conditions would be useful in profiling compounds.

Cell based assays (from cultured cells lines or freshly harvested tissues or primary cells) are known as two dimensional (2D) as they demonstrate drug effects on individual cells existing in solution or as a monolayer. Drug and chemical research is evolving to exploring three dimensional (3D) tissue like models [1]. These offer not only the effects on an individual cell, but cells in a tissue like environment, where they behave in a more “natural” manner. The most sophisticated models are based on tissue explants harvested from animals and maintained in a buffered solution for two to three weeks. These offer “in-vivo-like” characteristics as they maintain the anatomical structure but are limited by the number of tissues one can prepare and the time the tissue can be maintained in a viable state.

1.2 Hi-Spot Technology
Currently 3D organotypic culture has required either the use of biocompatible scaffold systems or...
spheroid aggregation in rotating or spinning culture systems [3]. Dynamic spheroid culture systems have so far proved to be highly useful for generating organotypic cultures for drug screening [4,5], however, they are inherently difficult to utilise in standard liquid handling formats by the very fact that they are not static. Over the past decade we have introduced static organotypic culture systems for CNS tissue based on an air liquid interface [2] that have now become adopted as a standard format for explant cultures. While these have made inroads into drug screening, and are able to reduce the need for animal experiments, they suffer from low throughput due to the need to manually dissect the tissue prior to culture and thus tend to be used largely for lead optimisation rather than drug discovery [1].

Recently, we have developed a new technology (Hi-Spots) which overcomes many of the limitations found while attempting to adapt 3D tissue culture systems to industry standard formats. Hi-spots can be generated from embryonic or early post natal tissue and involves dissociating tissues then re-aggregating these onto a semi-porous membrane in a static air liquid interface system. So far tissue has been obtained mainly from rodent brains, but proof of concept studies have indicated that it is applicable to a wide range of other tissues (including heart, liver muscle and skin) and across several species including man. We have also demonstrated to proof of concept level, that it is possible to cryopreserve Hi-spots, offering the possibility of supplying tissues preformed on suitable industry standard formats such as 96 or 384 well plates to be used in a variety of high content assay systems. One of the major advantages of the Hi-spot technique is that it does not require external scaffolds as the tissue acts as its own scaffold. Key advantages of this technology are:

- Ease of manufacture and maintenance
- Uniformity of tissue size and cell number
- Cell-cell interactions and paracrine mechanisms
- Consistency of cell type and ratios of cell mix
- Advance preparation and long term storage – spots can be prepared in advanced and frozen
- Compatibility with automation of liquid handling and high through put systems.
- Applicable to most tissues
- Applicable to human tissues where available (including stem cells)
- Reduced tissue use (100s to 1000s can be prepared from a single donor)
- Minimal staff time and expertise required

2 Characterisation and Validation of the CNS Hi-Spots

The method involves the compaction and the culture of many dissociated cells isolated for example, from the central nervous system. These compacted nervous system cells will spontaneously reorganize into a complex 3-D functional parenchyma over time leading to the formation of a tissue-like structure in which much of the appropriate synaptic circuitry, physiology and neurotransmitter receptor distribution of the intact central nervous system region are present. Since these high-density mixed cultures are grown on porous membranes, the resulting relatively thick tissues remain at the interface of the culture medium and the air, thus ensuring good survival for several weeks or even months.

We are using different approaches to validate and characterise the model:

- Histology
- Electrophysiology
- Transgenic animals
- Transfection and transduction
- Biochemical analyses.
Electrophysiological monitoring of the neural activity of Hi-Spots were performed using MEAs designed on porous membranes. These types of arrays (BioCell-Interface cartridges) allow good survival of thick preparations in static conditions. Excitatory as well as inhibitory pathways were found to be similar to those observed with other neural networks like hippocampal slice cultures (Fig. 4). Since CNS Hi-spots are compact 3D neural networks, only a few electrodes are needed to monitor the complete network allowing us to upscale the number of Hi-spots that can be tested simultaneously with a single MEA.

This system was designed primarily to perform pharmacological studies on the effects of compounds on excitability and synaptic transmission when applied to models that require long-term monitoring of activity. An important condition therefore is to be able to apply drugs to the tissue through an efficient perfusion system that does not alter stability. At the same time, the amount of medium to be added has to remain as small as possible as the quantities of substances to be tested are usually limited. A compromise has therefore to be found for each condition so as to be able to exchange the medium in the chamber, but without excessive perfusion volumes and while maintaining the stability of the recording. Even with a small amount of medium, and no constant perfusion, the Hi Spots remain synaptically active over a prolonged period of time (Fig. 5).

Classical pharmacological experiments are currently being undertaken using drugs that affect the excitability or synaptic transmission. Preliminary studies have shown that application of the sodium channel blocker, TTX, leads to a concentration-dependent decrease in the amplitude of the evoked field response (Fig. 6). This effect was obtained within a few minutes of drug application, which is equivalent to the speed of drug diffusion seen in regular submerged or interface chambers using tissue slices. Furthermore, application of low concentrations of TTX did not result in a degradation of the response, indicating an efficient perfusion system that does not affect the stability of the recording despite the fact that the array is built onto a porous membrane.

Recordings from the CNS-derived Hi Spots have demonstrated the existence of paired pulse inhibition, at paired pulse intervals less than 40ms (Fig. 7). Experimental analysis has yet to determine whether this paired pulse inhibition is due to a reorganised inhibitory circuit within the Hi Spots or whether it is related to an unusually long refractory period.
3 Summary

By offering the opportunity of higher through put while maintaining many of the advantages of whole animal systems, Hi Spots are a new opportunity in screening compounds which require tissue like conditions in which to demonstrate its effects, both efficacious and potentially toxic.

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Fig. 7 Representative evoked field recordings obtained from a Hi
Spot that illustrate the paired pulse inhibition that is typically seen.
As the paired pulse interval is increased from 15ms (A) to 20ms (B)
and then to 50ms (C), the second pulse increases in amplitude. *
indicates stimulus artefact.

The ability to transport and store Hi Spots is the key to the ease of handling with this tissue system. Synaptically active Hi Spots can be generated from frozen cells rather than from fresh tissue. Furthermore, the functional activity of the Hi Spots is unaffected by transportation, allowing for shipping of the Hi Spots for instance to specialist laboratories. The flexibility of using this tissue is thus vastly increased and supports recent calls from industry to supply tissues on demand, similar to reagents for screening purposes.
Detecting neurotoxicity and neuropharmacological potential of compounds through electrical activity changes of neuronal networks on microelectrode arrays

Dieter G. Weiss¹*, Alexandra Gramowski¹, Simone Stüwe¹, Konstantin Jügelt¹, Liane Mehnert¹, Dietmar Schiffmann¹, Olaf Schröder²

¹ Institute of Biological Sciences, Cell Biology and Biosystems Technology, University of Rostock, Rostock, Germany
² PATTERN EXPERT, Borsdorf, Germany
* Corresponding author. E-mail address: dieter.weiss@uni-rostock.de

Neuronal network cultures respond to transmitters, their blockers, and other neurotoxic compounds in a substance-specific manner. Networks grown on 64-microelectrode arrays remain spontaneously electrically active and stable for many months providing a suitable test platform for High-Content Screening and for detecting undesired or desirable effects of test compounds on neuronal activity at various phases of drug development. Changes in spatio-temporal electrical activity were quantified and subjected to pattern analysis for a detailed study of the effects of selected neurotoxic, sedative, narcotic and other neuro-active compounds. The results underline the suitability of this broadband biosensor system for functional neurotoxicity testing, pharmacology and safety pharmacology.

1 Introduction

Spontaneously active networks in culture have been proposed as sensitive and efficient model system to study the neurotoxic properties of chemicals, as biosensors, and to accelerate drug development [1]. Neuronal networks represent the functional units of information processing in the brain. Due to their high level the complexity they reflect major aspects of neuronal function in mammals. In contrast to patch-clamp and imaging methods, the microelectrode array technology allows online and real time analysis of up to 256 neurons in short and long-term studies. Therefore, they offer the potential to obtain data on neuro-active compounds' properties that are technically difficult or impossible to obtain in vivo. In vitro-studies can replace animal experiments that would often deliver only qualitative data.

2 Methods

2.1 Advanced analysis of spike train data

Microelectrode arrays were provided by the Center for Network Neuroscience (CNNS) at the University of North Texas. The fabrication techniques and the culture methods have been described previously [2]. We extracted a total of 67 (in more recent analyses 200) activity describing parameters to quantify the substance specific activity changes: 31 parameters derived from spike train data, their derived 31 normalized values to compensate for the variability of the activity in different cultures, and five parameters derived from the dose-response curves. Bursts were determined by spike train analysis based on spike intervals with the software NeuroExplorer (Plexon Inc., Dallas, TX, USA). Spike rate and burst rate values were derived from 60s bin data. The dose response curves were fitted to the Hill equation, determining the effective concentration causing 50% of the maximal response (EC₅₀). The spike and burst parameters were additionally processed to derive sets of the temporal and network coefficients of variation (CV_TIME and CV_NETWORK). These CVs were used to describe the spatiotemporal behavior of the network activity [3,4]. CV_TIME reflects the periodic behavior of a single neuron’s activity pattern. CV_NETWORK is a measure of synchronicity. Results are expressed as series means ± SEM. The features’ distributions were tested for normality. The level of significance after compound application was assessed using Student’s paired t-test.

3 Results

3.1 In vivo-like tissue specificity

We compared the spiking and bursting activity of spinal cord and frontal cortex networks to demonstrate that in vitro networks are pharmacologically histotypic representations of their parent tissues. Neurotoxins with known site of action, namely the inhibitory GABA_A and glycine receptor systems were used. Disinhibition of the electrical activity was achieved by blocking the GABA_A receptors with 40 µM bicuculline or the glycine receptors with 1 µM strychnine,
while complete disinhibition was reached with both compounds (Fig. 1).

Fig. 1 Tissue specific responses of spinal cord and frontal cortex networks. Spiking activity increases after blockade of the inhibitory GABA<sub>A</sub> receptors with 40µM bicuculline (BCC) and/or glycine receptors with 1µM strychnine (STR). There is a stronger influence of GABA in the frontal cortex in contrast to a stronger influence of glycine in the spinal cord. (* p < 0.05, ** p < 0.01).

Blocking the inhibitory circuits with bicuculline and/or strychnine elicited rhythmic and synchronized activity with clear-cut differences between both tissues. Strychnine increased the burst rate of spinal cord networks to 815% of native activity, while in frontal cortex networks it increased only to 127%. Bicuculline induced an increase in spike rate to 457% for spinal cord networks, compared to 204% in the case of frontal cortex networks (Fig. 1). Thus, inhibition in frontal cortex cultures is mainly mediated by GABA<sub>A</sub> receptors, but in spinal cord cultures by glycine receptors. This reflects the in vivo situation and demonstrates that important aspects of tissue specificity are retained in vitro. The 7fold increase in spike rate in spinal cord networks after complete blockade of inhibitory receptors indicates that spinal cord activity is more mediated by inhibitory circuits than frontal cortex with a twofold increase.

3.2 Tissue-specific neurotoxicity

Experiments with the neurotoxic antifungal and antifouling compound trimethyltin chloride show that spinal cord and auditory cortex cultures exhibit characteristic and dose-dependent changes of their electrical firing patterns [5]. Spinal cord networks began to respond at 1–2 µM, and shut off activity at 4–7 µM TMT. Auditory cortex cultures first responded at 2–3 µM; shut-off occurred at 7–8 µM TMT. Repeated applications of low doses of TMT always influenced the electrical activity in a reversible manner, with no overt cytotoxic effects. This analysis demonstrates also the relatively low variability among different networks of one tissue type (Fig. 2). The non-overlapping EC<sub>50</sub> range for cortical and spinal cord cultures suggests tissue specificity for network responses to TMT. Shut-off concentrations are within a factor of two of the lethal concentration ranges reported for mice in vivo [6].

Fig. 2 Dose response curves of trimethyltin chloride on 5 spinal cord and 3 auditory cortex networks. EC<sub>50</sub> values are 1.5 ± 0.5 µM for spinal cord and 4.3 ± 0.9 µM for auditory cortex, demonstrating tissue-specific sensitivity to this neurotoxicant.

3.3 Mixtures of compounds

"Dirty drugs" acting at several receptors or ion channels or mixtures of compounds cannot easily be studied by patch clamp techniques. Pharmacologically active plant extracts usually contain numerous potentially active compounds. The assessment of overall electrical activity changes caused by sedative and mild antidepressive herbal extracts from Hypericum, Passiflora and Valeriana and various combinations thereof revealed a receptor-specific and concentration-dependent inhibition of the firing patterns [7]. The effects of specific receptor blockades on the inhibitory responses provided positive evidence that the herbal extracts act on GABA<sub>A</sub> and serotonin receptors, which are recognized targets of pharmacological antidepressant treatment (Fig. 3). This demonstrates the sensitivity, selectivity and robustness of electrode array recordings for high content screening even of complex mixtures of neuro-active substances and for providing multiparametric information on neuronal activity changes for the assessment of the therapeutic potential of psychoactive substances and mixtures thereof.

3.4 Pharmacology

Employing refined pattern recognition analyses we demonstrate that it is possible to ascribe the network impairment to different receptors and ion channels. This is shown for a number of agonists and antagonists of the various binding sites of the GABA<sub>A</sub> but also for GABA<sub>B</sub> and other receptors when a larger number of parameters is used to characterize the action profiles in detail (Mehnert et al. in prep.) (Fig. 4). Data derived from dose-response curves for the anesthetic ketamine, remifentanil and other anesthetics confirm its strong receptor-specific effects on the electrical activity (Gramowski et al., in prep., see also Gramowski et al., this Volume).

Furthermore, a comparison of 9 anticonvulsants has shown that they all have slightly different modes of action. While they have similar overall effects such...
as reducing the spiking activity and synchronicity but not the bursting activity, there are clear differences in some parameters that make their action spectra unique (Schröder et al. in prep., see also Schröder et al., this Volume).

Fig. 3 Comparison of the influences of GABA\textsubscript{A} and serotonin 5-HT\textsubscript{1,2,7} receptor blockers on the burst rate changes caused by Hypericum (St. John’s wort) extract. Application of the extract alone (left curve), together with 5-HT\textsubscript{1,2,7} receptor blocker (middle curve), or with GABA\textsubscript{A} receptor blocker shows a shift to higher concentrations.

4 Conclusion and Outlook

Nervous tissues express patterns of electrical activity as part of their normal function. Any major interference with these patterns can generate behavioral and/or autonomic malfunctions in response to a toxic agent. The cessation of electrical activity (functional neurotoxicity), even if not associated with cell death (cytotoxicity), can lead to the death of the organism. Also, major changes in pattern generation can severely alter the performance of organisms without necessarily threatening survival. Our studies primarily deal with acute functional neurotoxicity. The detected similarity in concentration ranges for the \textit{in vivo} and \textit{in vitro} situation suggests that neuronal networks \textit{in vitro} allow a quantitative assessment of functional neurotoxicity. Whole network responses yielding a global information on the interaction of all receptors present in the network are closer to the animal situation than data from single cell or single ion channel studies.

We use a refined approach for data analysis with methods of pattern recognition, which allows the analysis of the significance and relevance of the multitude of computed features (up to 200) that are used to quantitatively describe the activity patterns (Schröder et al. 2006, this volume). This way a better understanding which features are significant to describe toxicity is possible by using feature score and feature selection methods well known from pattern recognition. In this manner evidence of several burst features such as shut-off, changes in bursting patterns, in rhythmicity, overexcitation, EC\textsubscript{50} values, or reversibility were assigned to known toxins for characterizing significant changes. A quantitative database of neuro-active effects in specific nerve tissues \textit{in vitro} provides a tool to classify known and unknown neuro-active compounds. In a second step the toxicity of substances is classified by their spike train responses and used to judge the toxic potential of unknown substances.

Fig. 4 Representative dose responses for a set of 30 of the electrical network features for a test compound (y-axis, % changes caused by the compound; nat, untreated, set to 100%; z-axis, concentration range $10^{-8}$ – $10^{-4}$ M).
Different from patch clamp and brain slice techniques this approach allows long-term studies as well as the testing of complex mixtures and unknown compounds. It is closer to the real situation in the nervous system, because it makes accessible the complexity level of multicellular functional ensembles, and not only that of single cells or single channels. It is also therefore, that this technique is expected to give fewer false negative results than other in vitro-techniques.

Detailed knowledge of the in vitro effects will greatly contribute to risk assessment, although it is difficult to predict exactly which specific symptoms in whole animals may be correlated with the different features of network activity. However, we have previously discussed how this system can be used to generate databases of well-characterized substance “fingerprints” which allow detailed comparisons of the activity spectra of neurotoxic substances [4].

Our results demonstrate that neuronal networks retain tissue-specificity and respond to transmitter receptor blockers and other neurotoxic compounds in a substance-specific, dose-dependent manner and when analyzed by advanced multi-parametric data analysis and pattern analysis, they provide a powerful tool for functional neurotoxicity testing, pharmacology and safety pharmacology. These sensitive and quantitative [4, Schröder et al., this Volume] responses have made such platforms very useful broadband biosensors for high content screening.

We see the main applications of our approach in:
- the testing of complex mixtures
- target identification
- long-term studies
- target validation
- drug profiling
- decision making between different lead compounds of similar quality, by comparison with related drugs
- safety pharmacology (exclusion of adverse neuronal effects of non-nervous system-directed drugs)

In addition, a new generation of multi-sensor neurochips in silicon CMOS (Complementary Metal Oxide Semiconductor) technology will provide a further considerable improvement of this approach. We are presently comparing the results of the two multielectrode arrays [8]. It allows also electrical recording online monitoring of the metabolic state of the network by integrated physiological sensors for temperature, oxygen consumption and pH value [8,9].

Acknowledgements
This work was supported by the DFG Innovationskolleg “Komplexe und Zelluläre Sensorsysteme” (INK 27), by the State Ministries of Education and of Economy of Mecklenburg-Vorpommern, and the European Community (ERDF) through the State Priority Research Program “Biosystems Technology”. We thank Guenter W. Center for Network Neuroscience, University of North Texas, Denton, USA for his help and support and Kristine Gürtler for excellent technical assistance.

References
Functional classification of neuroleptics based on population response profiles

Steffen Kandler and Ulrich Egert*

Neurobiology and Biophysics, Institute of Biology III & Bernstein Center for Computational Neuroscience, Albert-Ludwigs-University Freiburg, Freiburg, Germany
* Corresponding author. E-mail address: egert@biologie.uni-freiburg.de

The characterization of a novel pharmacological compound in a screening assay or animal test is a challenging undertaking in terms of assessing its potential physiological effects within the organism. Here, we present an effective method for the functional classification of substances in native tissue. Extracellular microelectrode array recordings of modulations through dopamine receptor (DAR) ligands of spontaneous spiking activity in acute cerebellar slices were evaluated using data mining techniques.

1 Dopamine and Neuronal disorders
Several neuronal disorders such as schizophrenia, psychosis, or Parkinson, are characterized by changes within the dopaminergic system. Major symptoms arise from altered dopaminergic signaling which, in turn, up- and/or down-regulates neuronal activity. Consequently, most therapeutic approaches of, e.g. schizophrenia focus on the dopamine receptor (DAR) subtypes D2 and D3 that are dysregulated in this disease [1]. These DARs are the key targets of typical (D2-selective) and atypical (D3-selective) neuroleptics. In recent years, D3-mediated treatments were established that elicit fewer side effects compared to the severe extrapyramidal side effects in D2-based therapy [2] due to slightly different signaling mechanisms and an improved dose dependency at the D3 receptor.

2 Cerebellar slices as model system for neuroleptic drug action
To adequately assess possible side effects of novel substances it is crucial to distinguish the influence of signal transduction mediated through D2 and D3 receptors in neuronal activity in native tissue. Due to the mixed expression of DARs in different brain areas, the neuronal responses to specific ligands are very heterogeneous and the contribution of either subtype of DARs is difficult to assess. In the rat cerebellum, however, D2 receptors are selectively expressed in lobes I-VIII and D3 receptors in lobes IX-X (Fig. 1) [3, 4]. In this study, we introduce an approach for a functional classification of dopaminergic ligands based on the modulation of the spontaneous spike activity in acute cerebellar slices.

Fig.1 Localization of D2 (lobe I-VIII) and D3 (lobe IX-X) receptors in a parasagittal slice of the rat cerebellum.

Taking advantage of this segregation of D2 and D3 receptors, the responses to specific ligands of neuron populations in tissue regions expressing either one DAR subtype were recorded simultaneously with microelectrode arrays (MEA, Multi Channel Systems, Reutlingen; Fig. 2). Spontaneous spike activity was modulated by dopaminergic, serotonergic, and GABA-ergic receptor ligands (Fig. 3), which were applied to the perfusion solution in three phases of 30 min with stepwise increased concentration after an initial reference phase of 30 min. In each of the phases, changes of spike rate, rate variability, temporal structure within autocorrelations, and bursting behavior were analyzed. Drug-induced changes of these parameters at one electrode site constituted a dataset used to build a response database. Each experiment yielded a set of responses from different lobes and layers of the cerebellar cortex.
show that responses to novel ligands can be characterized by their assignment to clusters in a pre-trained SOM.

In summary, our approach thus facilitates the comparison of ligand-receptor interactions in native tissue and constitutes a promising screening tool for neuroleptic drug research.

**Fig. 3** Sample recording protocol and typical rate modulation of spontaneous spike activity for the D3 agonist PD128907 (PD). After a reference phase, PD was applied in three ascending concentration steps (0.2-0.4mM), terminated by a wash-out phase that served as cross-reference for the initial phase. Application phases usually lasted 30min. Activity properties were evaluated for the final 400s of the test phases (colored horizontal bars) to minimize the effect of transients after drug application. All parameters were normalized to the reference phase.

**3 Data mining for ligand-induced activity modulation**

To identify substance-specific features of the responses in this database, we applied a data mining approach based on self-organizing maps (SOM) [5]. This technique assigns the datasets of the response database to prototype vectors of a neuronal network which are updated in subsequent training steps leading to a reordering of data according to their similarities. In a trained SOM similarities are visualized as proximity in 2D maps. SOMs are thus useful tools to exploit the structure and interdependencies of complex datasets. Our results revealed similarities of neuronal responses through clustering of the neuronal responses depending on ligand type, drug concentration, and tissue position of an electrode (Fig. 4). We could

**Fig. 4** Data mining of dopaminergic and control datasets in the response database using SOMs. A clustering of ligand classes based on similarities in the modulation pattern of spontaneous spike activity becomes visible by ligand labels on adjacent map units. CON control; DPAT 7-OH-DPAT, a D3 agonist; PD PD128908, a D3 agonist; QUIN quinpirole, a D2 and D3 antagonist; U99 U-99194A, a D3 antagonist.

**Acknowledgements**

We thank R. Meier for his help on SOMs, and B. Jost and S. Reinartz for their contributions to the experimental data and analyses. Supported by BMBF grants FKZ 01GQ0420 and 16SV1743.

**References**

Spontaneously Active Cultured Neocortical Networks: Heterogeneity under Pharmacological Treatments

Francesca Gullo, Enzo Wanke*

Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca, Milano, Italy. *Corresponding author. E-mail address: enzo.wanke@unimib.it

Networks of cultured cortical neurons are characterized by spontaneous and persistent rhythmic activity that resembles observations in brain areas in vivo. Previously, the multielectrode recordings (MEA) technique suggested the presence of balanced excitatory/inhibitory sets of homogeneously distributed synapses. On the contrary, we show here, by power spectra analysis of action potentials firing, that the spontaneous activity of small groups of neurons can be increased or strongly depressed by antagonists of the cholinergic and dopaminergic synapses, suggesting a mature and coherent interplay of “cell assemblies” as proposed by Lorente de Nó and Hebb. If applied to knock-in mice models of human diseases such as Alzheimer’s and channelopathies should allow to discern the effects of CNS degeneration or single point mutations.

1 Introduction

Precise neural circuitry development is traditionally thought to involve two separate phases. In the early activity-independent phase, neurons develop distinct phenotypes and establish an initial set of connections. In the later, activity-dependent phase, neural activity is thought to refine connectivity [1-3].

We have investigated the spontaneous properties of mouse neocortical networks in culture from 10 to 20 days-in-vitro (div), by using a novel non invasive technology that allows simultaneous extracellular recordings from 60 electrodes (MEA) [4, 6]. Previous pharmacological manipulations of the inhibitory (GABA) and excitatory (glutamate) synapses suggested that such networks were homogenous [5]. On the contrary, application of this technique to intact retinas, to study retinal ganglion cells, revealed responses characterized by an unexpected heterogeneity [7, 3]. We here show that in cultured neuronal networks, a strong heterogeneity is present as well.

2 Materials and Methods

Cell culture. Primary cultures of cortical neurons are prepared as reported by others [8-9], with some modification. The postnatal mice (P1-P3) were decapitated and the entire cerebral cortices were removed excluding the hippocampus. Cortices were cut into 1 mm³ pieces and digested by tripsin (0.15%) and DNAse (10μg/ml) at 37°C for 30min. After enzymatic digestion the cells were mechanically dissociated by trituration, and plated on the MEAs (Multichannels system, Germany) precoated with polyethyleneimine 0.1% (wt/vol) and laminin 20μg/ml. After 3h of incubation, plating medium was replaced with Neurobasal medium (NB) with B27 (Invitrogen, Italy), glutamine 1mM, bFGF 10ng/ml. The culture was maintained at 37°C, 5% CO₂. One-half of the medium volume was substituted every 3 days. Cultures were covered with gas permeable covers (MEA-MEM, Ala Scientific instruments, Inc. USA). Preliminary immunocytochemistry for GABA, acetylcholinesterase, synaptophysin and GFAP showed that the distribution of all these markers is normal but heterogeneously expressed. Atropine, DHβE (dihydro-β-erythroidine), GBR-12909 ((1-(2-bis(4-fluorophenyl)-methoxy)-ethyl)-4-(3-phenyl-propyl)piperazine) and eticlopride were purchased from Sigma, (Italy). Quinpirole, and SCH23390 (7-cloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydroxy-1H-3-benzazepine) were purchased from Tocris (UK).

Perfusion of the MEA. All experiments were done in an incubator with 5% CO₂, at 36°C, under ambient humidity. Solutions exchange was performed by means of a ValveBank8-II (AutoMate Scientific, Inc. USA) perfusing system located inside the incubator. Continuous flow (2-4 µl/s) to and out of the MEA dish was obtained by means of inlet and outlet tubings connected to a peristaltic pump. A complete pharmacological experiment duration lasted about 1-2 h (continuous flow). All drugs were diluted in the standard NB+B27 culture medium and MEAs were allowed to reach a steady-state condition (control) for 20 min before applying the different drugs. All changes were completely reversible within ±8% of the average activity. We were able to repeat similar results also by perfusing completely artificial saline solutions instead of using the NB+B27 media, but the network activity strongly declined and long experiments became unattainable. Several experiments in few days, when performed with the NB+B27 media, were possible in the same MEA dish.
Recording and analysis. Raw analog data, sampled at 40 KHz, were recorded from a MEA-1060BC preamplifier (bandwidth 1-8000 Hz, Multichannel Systems, Germany) connected simultaneously to a MEAWorkstation (bandwidth 120-8000 Hz, Plexon Inc. USA). Analog signals were sorted to timestamp files by the MEAWorkstation Sorter software and successively cleaned (from artifacts) using the OFFLine Sorter (Plexon Inc. USA). From each electrode, the sorting procedure never sorted more than three different classes of action potentials (extracellular action potentials peaks, well exceeding noise, whose peaks ranged from -30 to -450 µV). In a typical experiment such as that shown in Fig. 1, a mean of 54.3±13.5% of the 60 electrodes were active and a mean of 21±4.3% of these had multiple units. Electrodes responding irregularly during the whole experiments were disregarded from the analysis. The maximal firing frequency observed in 10 ms bin histograms was around 300 Hz. Mean firing frequency over the whole experiment was 2.2±0.27 Hz (range from 0.15 to 12.1 Hz). NeuroExplorer (ver. 3.2, Nex Technologies, USA) was used to perform various types of analysis such as activity histograms (spikes/s) and power spectral densities. Origin 7 (Microcal Inc, USA) software was routinely used for further analysis.

3 Results

The spontaneous activity in MEA dishes is characterized by synchronous reverberating bursts. In Fig. 1A are shown exemplary timestamps rasters from two electrodes. Each timestamp originates from single action potentials distributed into, or between bursts as indicated in the insets. Several evidences [10] indicate that the propagation velocity in axons of CNS neurons is of the order of 2 mm/ms, suggesting in the MEA network (1.4 x 1.4 mm), a maximum delay of less than 1 ms. On the contrary, crosscorrelation analysis indicates that peak activity between distant electrodes can be delayed by several tens of ms and that it is influenced by neurotransmitters [4]. This implies a strong multi-synaptic network connection among the cultured cells. Previous data have shown that in neuronal networks such as those we use, the GABAergic and glutamatergic systems are always present [11], and we confirm these findings, pointing out also that at least 90% of the responding neurons receive mature inhibitory GABAergic synapses, contrary to the concept that, at early stages of development, the GABA response is excitatory because of the high intracellular Cl⁻ concentration [12]. We here show that also other synaptic systems are well represented. The data presented here have been analyzed as the power spectral density of the action potential activity, because, according to our opinion, this type of statistical analysis extracts a considerable amount of properties from the recorded data (PSD, see Methods)

![Fig. 1 A) Typical timestamp rasters recorded from two electrodes, "82" and 26" separated by 1440 µm. Insets (duration: 1300 µs, amplitude: ±50/-100 µV): superimposed traces of the extracellularly recorded action potentials during an inter-bursts and a burst. In this example action potentials are smaller during bursting in electrode 26.](image)

3.1 The cholinergic synapse: muscarinic and nicotinic receptors.

We wanted to study the cholinergic system. First we tested our network with agonists and antagonists of the muscarinic acetylcholine receptors (mAChR). Fig. 2A shows the spectra of two electrodes in control and during perfusion with both oxotremorine (oxo, 1 µM) and atropine (atr, 10 or 20 nM). Under these condition, the activity is clearly potentiated by activation, and inhibited by blockade of the muscarinic receptors. On average (n=7 MEAs), the potentiation and the depression were 8.98±0.15 and -9.7±0.07 PSD units, respectively. We cannot exclude that the use of more selective agonists and antagonists could change these observations [14-15]. Muscarinic receptors are considered important in the Ca²⁺ channel-mediated presynaptic regulation of Ach release, but it is also known [16-17] that network oscillations, in slices, can be induced by muscarinic induction, as we found in our networks.

In order to investigate if a specific nicotinic receptor (nACHRs) is present, we used a specific blocker of the αβ; nAChR, such as DHβE [18]. The experiment started with a control recording (CON), a perfusion with atropine to remove the effects of the muscarinic receptors, and a subsequent blockade of the αβ; nACHRs by DHβE (100 nM), in the continuous presence of atropine. Fig. 2B left shows the responses obtained from two electrodes, analyzed as PSD. The spectra showed a marked depression during atropine perfusion and a complete recovery during the supplementary and specific blockade of the αβ; receptors. Some electrodes did not respond to the drug
We repeated the experiments shown in Fig. 2A and Fig. 2B on the same MEA dish. One of these experiments (out of 3) is shown in Fig. 2C left (for atr and atr+DHβE), and in Fig. 2C right (for oxotremorine, same electrode). Also in this case we reproduced the results shown in A and B.

![Fig. 2 Functional cholinergic synapse data. A) PSD plots of the activity (200 s, frequency range 0.08-20 Hz) recorded during control or application of either 5 µM oxotremorine (oxo) or 20 nM atropine (atr). Same two electrodes in both experiments. B) PSD plots of the activity (200 s) recorded during control and application of 20 nM atr or atr+100 nM DHβE. C) PSD plots of the activity (240 s, same electrode, frequency range 0.02-20 Hz) recorded during control, application of atr or atr+DHβE (left panel) or oxotremorine (right panel). In the region 1-20 Hz data were smoothed by adjacent averaging.](image1)

On the whole, since the nicotinic receptors are excitatory, and their blockade (after mAChRs block) results in a re-activation of the network activity, we suggest, following others [19], that the inhibitory neurons receive the majority of the nicotinic α4β2 synapses which are almost silent because of the strong presynaptic inhibition.

3.2 The homeostasis of the dopaminergic synapse.

We tested also the dopaminergic synapse by using the D1 and D2 receptors antagonists SCH23390 (SCH, 10 µM) and eticlopride (eticl, 1 µM). Moreover, we tested quinpirole (quin, 10 µM), a D2 agonist, and one drug affecting the dopamine homeostasis, GBR-12909 (GBR, 50 nM), a specific blocker of DAT1 transporter.

![Fig. 3 Effects of antagonists and agonist of the dopaminergic synapse. A-E) PSD plots of the activity (200-400 s) recorded, from the same electrode, during control or application of eticlopride, SCH, quinpirole, GBR-12909, and amphetamine. The control curves have small differences because experiment lasted 90 min. Washout spectra differed by less than ±2 PSD units. Similar activity was recorded also from other 5 electrodes of the 28 electrodes showing activity. MEA7983-9div.](image2)

The results of a representative experiment (out of 6) are shown in Fig. 3A-B. In all panels the control (CON) spectra are shown as continuous lines and during the drugs perfusion the spectra are shown as symbols. The receptor antagonists strongly modified the activity of the network, but their effect was different. While the D2 antagonist potently reduced both the low and high frequency regions of the spectra, the D1 antagonist (Fig. 3B) mostly increased the continuous firing (see the frequency peak at about 9 Hz). Consistently, the application of the D2 agonist quinpirole, also added the continuous firing at 8 Hz and a similar action was produced by the DAT1 antagonist GBR-12909, suggesting that the oscillation at 9 Hz, not present in control (0.9 Hz), and after D2 blockade, is produced through different mechanisms, but all activating the role of the D2 receptor. On the whole, these data suggest that, during spontaneous activity, these defined cortical networks have a mixed control exerted by dopaminergic synapses through D1 and D2 receptors. Moreover, the D2 agonist and drugs known to increase extracellular dopamine concentration, produce parallel effects consisting in an increased firing activity. It remains to be determined, under more strict conditions, if our data are consistent with the evidence...
and suggestions that dopamine is controlling pyramidal neurons in the cortex by means of GABAergic neurons [21].

4 Discussion.

This study shows that a spatiotemporal activity is spontaneously present in neuronal networks with no preexisting anatomical specialization and spontaneously assembled from dissociated cells. Contrary to previous reports, not only classical glutamatergic and GABAergic systems are fully active and influence the reverberation and firing, but the same applies to the cholinergic and dopaminergic systems. To test if the different synapses coexisted in the same network, we repeated the above results with drugs specific to the homeostasis (ionotropic and metabotropic receptors and transporters) of two different neurotransmitters in single MEA dishes (n=5) and found that our results are superimposable also in the same dish.

We think that our data, obtained from hundreds of neurons and thousands of synapses, different from the classical single cell electrophysiology, could open a variety of research areas. This method will not only allow to discover and test new drugs, but also, by using knock-in mice models, to study the physiological roles of many mutations observed in human CNS channelopathies [22-24].

Acknowledgement

We acknowledge the help of Dr. Emanuele Schiavon during the initial phase of this work. This study was partially supported by grants from the Italian Ministero dell’Università e della Ricerca Scientifica e Tecnologica (MIUR-PRIN2003-2005-2001055320, 2003052919, MIUR-FIRB2001-RBNE01XMP4-002, MIUR-FISR2001 0300179), the Università di Milano-Bicocca to EW. FG was supported by Telethon Research Service for electrophysiology (GTF 03007) and is now a post-doc student at the Department of Biotechnologies and Biosciences of the University of Milano-Bicocca.

References

High-throughput microelectrode array platforms for quantitative pharmacology and toxicology

Guenter W. Gross¹*, Sabnam Rijal-Oli¹, Maryam Parviz¹, Vern Jones¹, Dayne Hollmuller¹, and Michelle Karg²

¹ Center for Network Neuroscience and Department of Biological Sciences, University of North Texas, 2 Department of Medical Electronics, Technical University of Munich, Germany
* Corresponding author. E-mail address gwgross@cnns.org

Extensive recent pharmacological surveys of primary cultures on microelectrode arrays have validated such monolayer networks as histiotypic. This validation effort has opened the way for realistic applications in the fields of industrial and environmental toxicology, drug development, biosensors, and basic research in network dynamics. However, industrial applications of MEA technology necessitate the development of high throughput assay platforms that allow the utilization of many neuronal networks in parallel. In view of the fact that a litter of 12 mouse embryos can generate over 1000 separate, spontaneously active networks, and that such cell suspensions are well suited for automated dispensing, primary cell culture will emerge as the dominant core methodology. This report summarizes recent results in quantitative pharmacology and describes efforts toward the development of multinet-work platforms using primary neuronal cultures.

1 Introduction

Normalized 50% network response concentration values (EC₅₀) have been obtained for a diverse group of neuroactive and toxic compounds including ethanol, fluoxetine, quinine, GABA, muscimol, tetrodotoxin, botulinum toxin A, anandamide, and trimethyltin chloride. The EC₅₀’s agree closely with published values from animal models, allowing the conclusion that mammalian primary cultures are at least pharmacologically histiotypic (i.e. similar to the parent tissue [1, 2]). An example of a network titration (frontal cortex tissue) with muscimol is given in Fig. 1, which provides information on the interculture repeatability and the shift of mean CRCs with increasing concentrations of bicuculline.

Fig. 1 (A) Muscimol titration of a frontal cortex culture under 40 μM bicuculline, showing step-wise reductions of activity in response to successive muscimol applications. Muscimol is a GABA analog. Data points represent averaged activity for spike (S) and burst (B) activity per minute. Double horizontal lines indicate the level response plateau used for CRC calculations. (B) CRCs from 10 networks; mean EC₅₀: 19.2±3.5μM. (C) Summaries for muscimol titration in the presence of four different concentrations of bicuculline (from Oli-Rijal; MS Thesis, [3]).
1.1 Dissociation Constants

In order to expand further the quantitative pharmacological explorations, we extended the use of primary cultures to the determination of dissociation constants for the GABA<sub>A</sub> antagonists bicuculline, gabazine, and TMPP (trimethylol propane phosphate). Using stepwise muscimol titrations of frontal cortex network activity, we established a series of dose-response curves in the presence of increasing antagonist concentrations. Such EC<sub>50</sub> values allow the generation of Schild plots [4, Fig. 2] in which the x-intercepts represent dissociation constants (K<sub>b</sub>) and a slope of unity defines the interaction between agonist and antagonist to be competitive. K<sub>b</sub> of 0.63 μM for bicuculline (n=36 cultures) and 0.23 μM for gabazine (n=8) agree well with published data, and a value of 1.90 μM for TMPP (n=7) is a new contribution.

Table 1  Summary of Schild Plot Values

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>pA&lt;sub&gt;2&lt;/sub&gt;</th>
<th>K&lt;sub&gt;b&lt;/sub&gt; (μM)</th>
<th>Slope ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gabazine</td>
<td>6.63</td>
<td>0.23</td>
<td>1.29 ± 0.23</td>
</tr>
<tr>
<td>Bicuculline</td>
<td>6.21</td>
<td>0.62</td>
<td>1.18 ± 0.18</td>
</tr>
<tr>
<td>TMPP</td>
<td>5.4</td>
<td>3.98</td>
<td>1.05 ± 0.03</td>
</tr>
</tbody>
</table>

1.2 Quantification of zinc toxicity

Zinc ions in micromolar concentrations (above 20 μM zinc acetate added to the medium) are highly toxic. After a period of excitation (Fig. 3), neurons swell and enter necrosis. At 200 μM cell lysing has been seen. The temporal evolution of electrophysiologic responses has not yet been examined in the literature. MEA methods are well suited for such analyses and provide quantitative information on activity decay that is irreversible after a suppression of 80% spike activity is reached. Fig. 3B shows the high degree of repeatability for 50% and 90% activity decay. For the 90% decay, the time can be represented by the equation:

\[
\log t = 3.68 - 0.82\log C
\]

where t is expressed in minutes and C in μM/L.

Fig. 2. Schild plots [4] of log (dose ratio-1) vs log antagonists concentration. A linear regression without slope constraint reveals a clear potency series of gabazine > bicuculline > TMPP.

Fig. 3 (A) Typical biphasic response profile of a frontal cortex network to 200 μM zinc. Excitation is followed by irreversible activity loss. (B) Time required for activity decay to 50 and 90% between 20 and 1000μM linearizes in log/log plots.

2 New Approaches to High Throughput

To provide matched control cultures and to enhance throughput, two network arrays are already in routine use at the CNNS, and 8 network modules (32 microelectrodes per network; 256 total) are in the testing phase. A robotic liquid handling platform has been integrated with the recording hardware to add test compounds and compensate for medium evaporation. The robot platform, eight network module, and first stage electronics are housed in a sterile, temperature controlled enclosure at 70% humidity and 10% CO<sub>2</sub>. Fig. 4 shows the robot (BioTek 2000) in a sterile enclosure with a 2-network testplate and an experimental preamp board (Plexon Inc., Dallas) for 64 channels.

An 8-network array plate with 32 cruciform microelectrodes has been fabricated and successfully tested. Amplifier contacts fingers (128 for each side) are 0.3 mm wide with a 0.3 activity loss.mm separation. Zebra strips successfully couple with these contacts. The array plate desing is shown in Fig. 5.

Successful recordings have been obtained from the 8-network array, although electronics have not been installed to allow recording from all networks simultaneously. Fig. 6 shows the test arrangement on an in-
verted microscope stage with two Omnetics preamp modules docked to the circuit board.

Fig. 4 (A) Environmental chamber enclosing liquid handling robot. Chamber maintains sterility, 10% CO2 for pH control, relative humidity of 70%, and temperature at 37 ºC. (B) Robot serving two-network module connected to a Plexon 64-channel preamplifier board. (C) Robotic muscimol titration (CNNS NACTAN display). (D) CRCs from six robotic titrations.

Fig. 5 Glass array plate with 256 indium-tin oxide conductors (128 per side). Contact fingers are 0.3 mm wide with a pitch of 0.3 mm. Large circles represent the position lines for O-rings in the chamber block; small circles represent the approximate area occupied by the neuronal networks. Panel to the right shows the arrangement of 32 cruciform electrodes in each recording matrix.
Four computers will process data from the 256 channels with each computer handling 64 channels and the normal Plexon data acquisition software. A fifth computer is programmed to gather the data for all eight graphical displays. It is anticipated that these platforms will allow quantitative acute and chronic neurotoxicological studies, and rapid evaluation of compound efficacy, secondary binding, and general neuro-physiological effects. Their use as rapid screening platforms in the void between biochemistry and animal experiments is of special significance. These systems may also become key elements in homeland security and biowarfare counter-measures as they react to any known or unknown compound that alters the function of the nervous system or kills cells, without requiring recognition of predetermined molecular signatures.

**Acknowledgment**

Supported by the Texas Advanced Technology Program, Plexon Inc., Dallas, and the Charles Bowen Endowment to the CNNS.

**References**


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**Fig. 6** Initial tests of 8-network module on a microscope stage. The pH is maintained with a stream of 10% CO2 in air under a heated ITO glass plate. The right panels shows bursting activity from two networks in a typical Plexon display.
Assessment of drug effects on myocyte function in vitro

Elke Guenther1*, Kathrin Henes1, Sandra Pankow1, Karlheinz Boven2, Thomas Meyer2

1 NMI Natural and Medical Sciences Institute at the University Tübingen, Reutlingen, Germany
2 Multi Channel Systems, Reutlingen, Germany
* Corresponding author. E-mail address: guenther@nmi.de

The aim of the present study was to validate an automated in vitro approach based on parallel recordings of cardiomyocyte extracellular field potentials in a 96 well plate as a valuable tool for drug screening in safety pharmacology.

1 Introduction
Pharmacological intervention, often for the purpose of treating syndromes unrelated to cardiac diseases, can increase the vulnerability of some patients to life-threatening rhythm disturbances. An important indicator that can cause cardiac arrhythmia is the prolongation of the QT interval of the electrocardiogram (ECG), which reflects the time from the beginning excitation of the ventricle to its peak repolarization. Directly corresponding to this parameter is the ventricular action potential duration, or as measured by micro-electrode arrays (MEAs), the duration of the extracellular field potential (fAP). According to the latest guidelines from the “International Conference on Harmonisation” (ICH S7B), safety pharmacology for human pharmaceuticals is supposed to include in vitro assays assessing the potential for QT interval prolongation [1].

Most of the electrophysiological approaches used to date in the process of drug development and screening are cell-based, assessing drug effects on ion channel function on the single cell level. Conclusions on drug effects on cellular network function are, however, limited in these approaches since the complex interplay of different ion channel types within a tissue cannot be analysed. Organotypic approaches such as the Langendorff heart or isolated Purkinje fibres are rather tedious and costly, requiring sophisticated preparation procedures and highly trained staff for the electrophysiological recordings.

To overcome these problems, we here describe an in vitro assay based on fAP recordings with planar microelectrode arrays that has been developed for drug discovery and safety pharmacology in the field of cardiac research. With the automated QT-Screen approach, alterations in the cellular network activity of cardiomyocytes in response to drug application can be monitored easily and efficiently in a 96 well plate.

2 Methods

2.1 Cell culture and preparation
Cardiac myocytes were prepared from chicken embryos at embryonic day 13 (E13). The ventricles were isolated and collected in Dulbecco's modified Eagle’s medium/nutrient mixture F-12 HAM (D8062, Sigma Aldrich, Deisenhofen, Germany) containing 20% fetal calf serum. After dissection, the ventricles were minced and transferred into phosphate-buffered saline without calcium and magnesium (Ca/Mg-free). Multiple digestion cycles of 8 min each in 0.05% trypsin (T 7409, Sigma)/phosphate-buffered saline (Ca/Mg-free) at 37°C resulted in isolated cardiomyocytes. Cells were collected by centrifugation and plated in a density of approximately 400,000 cells/well. The twelve eggs and yielded in sufficient cell material for one 96-well plate. The total time for preparation was about 70 min. After PEI coating, the cells were cultured directly on QT-Well plates (see below). Medium was exchanged every second day.

After 2 days in a standard incubator at 37°C, the embryonic cells were coupled via gap junctions and formed a syncytium of spontaneously and synchronously beating myocytes.

2.2 Drug preparation
All drugs were obtained from Sigma Aldrich (Deisenhofen Germany). For all lipophilic drugs, a stock solution of 100 mM was prepared with Dimethylsulfoxide (DMSO). The maximum DMSO concentration was 0.1%. DMSO controls did not show any effect compared to control conditions without DMSO (Data not shown).

2.3 Recording procedure
Extracellular field potentials were recorded from cardiomyocytes cultured at day 5 after plating on a so-called QT-Well plate (Multi Channel Systems) that contained a gold recording electrode with a diameter

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of 100 µm in the center of each well, surrounded by a large reference electrode (Fig. 1). The reference electrodes in all 96 wells were interconnected to provide a stable baseline. For compound application the well lid was opened only shortly to ensure stable environmental conditions.

Cells were first recorded for 2 min under control conditions. After a wash-in time of two minutes for each compound and concentration, an additional recording of 2 min was performed. The sampling frequency was 10 000 Hz, the gain was set to 1200, and amplifier bandwidth was 0.6 Hz to 3 kHz. Recording and analysis were performed with the QT-Screen system (Multi Channel Systems).

Dose response relationships were recorded cumulatively and a full dose response relationship was obtained from each well.

A typical signal is shown in Fig. 1C. The recorded fAPs are proportional to the first derivative of the cardiac action potential [2] and consist of three components reflecting ion channel activations during a cardiac action potential. Due to the similarity of fAP signals to an electrocardiogram (ECG) recorded in vivo, the fAP duration, i.e. the time period between the peak depolarization and repolarization caused by the activation of sodium and potassium channels respectively, is referred to in the following as the QT-interval in vitro.

3 Results

In order to validate our in vitro approach, different substances known to prolong the QT interval target the human ether-à-go-go related gene (hERG) potassium channel, the present study focused on substances with a hERG blocking effect.

We here show exemplarily the effects of quinidine. Quinidine is clinically used as a class IA anti-arrhythmic and exerts inhibiting effects on both the hERG channel and cardiac sodium channel. Application of quinidine to our preparation of embryonic ventricular cardiomyocytes resulted in a substantial, dose-dependent prolongation of the ventricular field potential as can be seen in Fig. 2A. In order to demonstrate the quinidine effect on sodium channels, the initial fAP component is displayed on a larger time scale in Fig. 2B. Quinidine application resulted in a dose-dependent decrease in sodium current amplitude and a slowing in sodium current kinetics.

![Fig. 1](image1.png)

**Fig. 1** A) Embryonic cardiomyocytes after two days in culture (right). B) Close-up view onto a QT-plate with one recording and one surrounding reference electrode in each well. C) Typical fAP potential recorded from embryonic cardiomyocytes in culture showing the underlying ionic currents. The fAP duration or QT-interval is indicated by the red bar.

![Fig. 2](image2.png)

**Fig. 2** A) fAP recording under control condition and with increasing concentrations of quinidine. The repolarising T-wave is shifted to the right and the QT-interval is prolonged in presence of the drug. B) The initial depolarizing component of the fAP, reflecting the activation of sodium channels is shown in higher magnification. Increasing concentrations of quinidine resulted in a marked decrease of the sodium current amplitude and a slowing in its kinetics.
Fig. 3  Spontaneous beating activity of ventricular cardiomyocytes in vitro without and in presence of quinidine. As can be seen in the middle and lower graph, quinidine clearly induced arrhythmias. Please note the different x- and y-axes of the graphs that reflect the quinidine-induced prolongation of the fAP duration and the block of the sodium current amplitude.

In addition to its effect on the fAP course, quinidine induced a distortion in the rhythmic activity of the ventricular myocytes which was recorded for 2 min. before and after the application of quinidine. The pro-arrhythmogenic effect was already observed in presence of 10 µM quinidine and increased in strength with increasing concentrations (Fig. 3). Higher concentrations often resulted in a complete block of the spontaneous beating activity (data not shown).

Dose-response curves were recorded for all drugs tested and the concentration that caused a 20% prolongation of the QT-interval was determined. This is a common methodology to assess drug-induced QT-prolongation effects in vivo [3].

Values obtained with the QT-Screen system were plotted against literature data obtained with other assays assessing drug effects on cardiac function: a) QT prolongation data obtained from patients (Fig. 4, red circles) and b) patch clamp analysis performed on hERG currents in HEK cells (Fig. 4, black squares). Since for the latter, drug effects were studied on an isolated ion channel, black squares in Fig. 4 reflect IC50 values and not QT-prolongation values as for the other two assays.

Despite the different experimental approaches, the comparison shows that our data are well in the range of other test systems, especially with data obtained in the in vivo situation in humans.

4 Discussion
The present study shows that cardiac field potential data recorded from embryonic cardiac myocytes in vitro allow an estimation of the duration of the ventricular action potential and that drug induced disturbances of ventricular repolarization are a reliable in vitro indicator for QT prolongation in vivo. As shown above, standard reference compounds evoke field potential prolongations in the same concentration range as observed with standard electrophysiological recording techniques both invasive and non-invasive and on the single cell and systemic level.

The use of native cardiomyocytes ensures that all cardiac ion channels contribute to the recorded signals in their native environment. As there are high structural homologies between the pore domains of various ionic channels, the risk that a drug affects more than one channel type is reasonably high. Multiple drug effects cannot be assessed in pure single channel approaches as for example in hERG patch-clamp assays. Our approach, the recording of extracellular fAP from
native cardiomyocytes offers the advantage of a close
to in vivo situation and thus greatly reduces the risk of
false positive and false negative hits in safety pharma-
cology.

5 Summary
The QT-Screen system is an easy to handle and
reliable in vitro screening tool to assess drug effects
on cardiac function in a throughput scale that is sig-
nificantly higher than other electrophysiological as-
says used in cardiac pharmacology.

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QT interval prolongation and torsade de pointes for a broad
range of drugs: evidence for a provisional safety margin in
Remifentanil’s co-factor glycine causes NMDA receptor-dependent increase in murine frontal cortex network activity in vitro

Alexandra Gramowski1*, Frank Kletzin2, Rainer Hofmockel2, Gabriele F.E. Noeldge-Schomburg2 and Dieter G Weiss1

1 Institute for Biological Sciences, Cell Biology and Biosystems Technology, University of Rostock, Germany
2 Anesthesiology and Intensive Care, University of Rostock, Rostock, Germany

*Corresponding author. E-mail address: alexandra.gramowski@uni-rostock.de

The anesthetics Fentanyl and Remifentanil were found to cause a significant reduction of frontal cortex activity. In higher doses Remifentanil, but not Fentanyl, caused a NMDA-receptor-dependent increase followed by a total loss of network activity. We conclude from these results that this increase is caused by glycine, a common additive or component of Remifentanil formulations, and is possibly caused by NMDA receptor activation followed by excitotoxicity. These results underline the suitability of MEAs as broadband biosensor system for evaluating neurotoxic and neuropharmacological properties and mechanisms of neuro-active compounds.

1 Introduction

We used primary murine frontal cortex networks on microelectrode arrays (MEAs) to study the electrophysiological effects of Fentanyl and Remifentanil. The multiparametric description of the electrical activity changes of a multicellular neuronal network allows a sophisticated approach to quantify the complex effects of neuroactive agents, unknown compounds, and complex mixtures [1,2]. The anesthetics Fentanyl and Remifentanil are agonists at the µ-opioid receptor. Lately activation of the NMDA-receptor by Remifentanil was reported [3,4].

2 Methods

Cells on coated MEAs (CNNS, Denton, Texas) were incubated at 37°C with constant pH at 7.4 in a 10% CO2 atmosphere until ready for use. The MEA fabrication techniques and the culture methods have been described previously [5,6]. We extracted a total of 67 (in more recent analyses 200) activity describing parameters to quantify the substance specific activity changes: 31 parameters derived from spike train data, their derived 31 normalized values to compensate for the variability of the activity in different cultures, and five parameters derived from the dose-response curves. Bursts were determined by spike train analysis based on spike intervals with the software NeuroExplorer (Plexon Inc., Dallas, TX, USA). Spike rate and burst rate values were derived from 60s bin data. The dose response curves were fitted to the Hill equation, determining the effective concentration causing 50% of the maximal response (EC50). The features’ distributions were tested for normality. The level of significance after compound application was assessed using Student’s paired t-test.

Effects of Fentanyl (n=9) and Remifentanil (n=12) application on native activity and Remifentanil application to activity after blockage of the NMDA receptor (n=11) were recorded.

3 Results

Fentanyl and Remifentanil caused a significant reduction of network activity at ≥30nM (SR: 84.1 ± 6.5%, p=0.04) and ≥0.01pM (SR: 98.3 ± 0.7 %, p=0.04), respectively. Further application of Fentanyl up to 24µM caused no complete loss of activity. In higher concentrations Remifentanil showed a biphasic behavior with a significant increase in activity at 20µM (SR: 165.8 ± 7.3 %, p=0.003) followed by a complete loss of activity at 100µM. After NMDA receptor blockage no increase of activity occurs (Fig.1). However, pre-treatment with 3mM of glycine enhanced the reference activity (SR: 163.0 ± 16.5%, p=0.03) and prevented the latter activity increase (n=10).
4 Conclusion

Fentanyl and Remifentanil caused a significant reduction of frontal cortex activity. In higher doses Remifentanil, but not Fentanyl, caused a NMDA-receptor-dependent increase followed by a total loss of network activity. We conclude from these results that this increase is caused by glycine, a common additive or component of Remifentanil formulations, and is possibly caused by NMDA receptor activation followed by excitotoxicity.

Acknowledgement

We thank Kristine Gürtler for excellent technical assistance.

References


Fig. 1 Dose-Response-Curve of the anesthetic Remifentanil (Ultiva®) under different reference conditions: untreated, with preceding NMDA receptor blockade with 10µM MK801, and preceding application 3mM glycine.
Cryopreserved mouse and rat cortical neurons on MEAs: Different electrophysiological properties?

Frauke Otto1*, Wiebke Fleischer1, Philipp Görtz1, Anthony Krantis2, Mario Siebler1

1 Heinrich-Heine-University Düsseldorf, Institute of Neurology, Düsseldorf, Germany
2 Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ontario, Canada
* Corresponding author. E-mail address: frauke.otto@uni-duesseldorf.de

In this study we developed a MEA-biosensor based on mouse cryopreserved murine neurons from embryonic mice and characterized it electrophysiologically and immunohistochemically. In comparison to previously published data from rat cryopreserved neurons the results revealed some statistically significant differences in electrophysiological but not in immunohistochemical properties.

1 Introduction

Cultured neurons on microelectrode arrays can be used as biosensors for pharmacological and diagnostic research [1]. At present, neuron-based MEAs are mainly run with freshly dissociated rat or mouse cortical and spinal cord cells. Our previous work has demonstrated that rat cryopreserved cortical neurons on MEAs don’t differ from freshly prepared rat neurons in electrophysiological and immunohistochemical properties, but have advantages like a homogenous cell pool and longer survival time on chip [2]. Mice and dissociated mouse neurons are widely used in research especially in the field of immunology or genetics as animal models for human diseases, e.g. with the use of transgenic mice. Therefore, the aim of this study was to create and characterize a MEA-biosensor based on murine neurons for species-specific experiments combined with the advantage that cryopreserved cells have to offer.

2 Methods

Commercially offered ready to use cryopreserved neurons from CD-1 mice (embryonic day 14-15; QBM Cell Science, Canada) were plated onto poly-D-lysine- and laminin-coated MEAs (Multi Channel Systems, Reutlingen, Germany) and then cultured in a humidified atmosphere at 37 °C. No mitotic inhibitors were used. Dose response curves were based on the change of spontaneous spike rate (SSR) relative to the first control recording (SSRSubstance / SSRcontrol 1). Parallel to cultivation on MEAs, cells were plated on coated cover slips for immunocytochemical staining with antibodies against neuronal and glial markers. Cryopreserved mouse neurons began neurite outgrowth within several hours after plating and formed a neuritic network within a few days with highly synchronous spike and burst activity.

Parameters analyzed included spike rate, burst rate, spike amplitude, burst duration, number of spikes per burst and inter-burst interval.

3 Results

Mouse and rat cryopreserved cortical cells expressed the same neuronal markes (neurofilament, synaptophysin and MAP-2) in the same extent. Staining for the inhibitory neurotransmitter GABA was also positive. Co-staining with antibodies against neurofila-
ment and GFAP revealed a high proportion of glial cells in both cultures. However, this study revealed some differences in electropysiological properties. Mouse neurons exhibited shorter bursts with fewer spikes per burst, shorter inter-burst intervals and shorter inter-spike intervals inside a burst. For mouse neurons recordings revealed a sensitivity to the neuroactive substances GABA, TTX and NMDA with IC₅₀ values of 4.3 µM ± 1.4 µM, 3.9 nM ± 0.97 nM and 2.3 µM ± 0.3 µM, respectively. Compared to our previously published data for cryopreserved rat cortical neurons, cryopreserved mouse neurons were significantly less sensitive to TTX (p < 0.001) while there was now significant difference in sensitivity to NMDA and GABA (p=0.07 and p=0.3 respectively).

4 Summary

This study demonstrates that extracellular recordings can be achieved from previously cryopreserved dissociated primary murine cortical neurons. The sensitivity of these cells to TTX, GABA and NMDA and the immunocytochemical staining indicate the physiologic basis for the recorded activity and the expression of functional corresponding receptors. Compared to rat cryopreserved cortex cells there were some differences in electrophysiological but not in immunohistochemical properties. Further application of this novel neurochip might include the use of genetically modified mouse neurons e.g. from knock-out mice or animal models as a tool for evaluation of neurological diseases.

Acknowledgement

We thank QBM Cell Science, Canada, for providing Mouse and Rat Brain Cortex Cryo Cells.

References


Fig. 3 Dose response curves: Change of spontaneous spike rate plotted versus substance concentration (log scale). IC₅₀ values are derived from a fit to the Hill equation. We measured an IC₅₀ of 3.9 nM for TTX and of 2.3 µM for NMDA (mouse cortex neurons).
Electrophysiological Quantification of Zinc Toxicity Using Neuronal Networks on Microelectrode Arrays

Maryam Parviz$^1$, C.J.Frederickson$^2$, and G.W. Gross$^1$

1 Center for Network Neuroscience and Department of Biological Sciences, University of North Texas, Denton, TX; 2 Neurobiotex, Galveston, TX.

Murine neuronal networks, derived from embryonic frontal cortex (FC) and spinal cord (SC) tissues and grown on microelectrode arrays, were used to investigate zinc toxicity at different concentrations (25 to 2000 microM total zinc acetate additions to culture medium).

The cultures were grown in serum-containing medium but were switched to serum-free and albumin-free medium one to two hours before zinc applications. Continual multi-channel recording of spontaneous action potential generation allowed a quantitative analysis of the temporal evolution of network spike activity at specific zinc acetate concentrations.

Cultures responded with excitation (4-15 min, SC; 30-70 min, FC) followed by a gradual irreversible activity decay. The time to 50% and 90% activity loss was concentration dependent. In FC cultures, 250 μM zinc acetate resulted in a 50% activity loss in 65 min (n= 2). At 200 μM, a 50% activity loss was achieved in 73 +/- 7 min (n= 3). At concentrations above 50 μM total zinc, the activity loss was associated with massive cell swelling, blebbing, and even vigorous neuronal cell lysing. Glia showed stress, but did not participate in the extensive cell swelling. Network activity loss generally preceded the morphological changes and could be described by a power function for both the FC and SC tissues. The SC responses (n=10) were significantly more sensitive at all concentrations. The level of spontaneous activity in the native, pre-experimental state had no effect on the degree of toxicity. In fact, a total inhibition of activity with muscimol prior to 300 μM zinc application lead to irreversible activity loss and cell death, suggesting that the entry pathways for zinc are not strongly activity dependent (n=4). Measurement of free zinc in serum-free and albumin-free medium was 2 nM. In serum-free medium containing 200 μM zinc acetate, the free zinc concentrations ranged from 1 – 5 μM.

These results suggest that very low levels of free zinc can trigger a total, irreversible loss of neuronal activity and extensive cell death, even under conditions when all initial spontaneous activity in the network is blocked.
Impact of glutaric acid and 3-hydroxyglutaric acid on neuronal network activity: implications for neurological disturbance in glutaric aciduria type I

Andreas Schröter¹, Wiebke Fleischer¹, Anne Klusmann², Mario Siebler¹

¹ Heinrich-Heine-University Düsseldorf, Department of Neurology, Düsseldorf, Germany
² Heinrich-Heine-University Düsseldorf, Department of General Pediatrics, Düsseldorf, Germany

The organic acids glutaric (GA) and 3-hydroxyglutaric acid (3HGA) accumulate in body tissues and fluids of patients with glutaric aciduria type I (GA I). Affected children suffer a clinically significant acute encephalopathic crisis and subsequent striatal damage. We investigated the neuropharmacological effects of GA and 3HGA on neuronal network function with microelectrode arrays. Both substances dose-dependently and reversibly inhibited spontaneous neuronal network activity only at ranges that caused a pH decrease. Adjustment of pH to similar values without substance application had a congruent impact on the spontaneous spike rate. Although a NMDA receptor-mediated mechanism is discussed for the striatal degeneration in GA I the addition of the selective NMDA antagonist APV did not antagonize GA- as well as 3HGA- induced inhibition of the neuronal network activity. We conclude that the observed effect on neuronal network function on neurochips is likely due to a pH-dependent rather than a receptor-mediated mechanism.

1 Introduction

The inherited deficiency of glutaryl-CoA dehydrogenase leads to glutaric aciduria type I – a recessive metabolic disease, characterized by a specific age- and brain region-dependent neuropathology. If untreated, acute striatal degeneration is often triggered by febrile illnesses during a vulnerable period of brain development in infancy or early childhood. The clinical manifestation is a dystonic dyskinetic movement disorder. In glutaric aciduria type I patients, levels of glutaric acid (GA) and 3-hydroxyglutaric acid (3HGA) are elevated in urine, serum and cerebrospinal fluid. So far, it is not known exactly which substance is responsible for the disturbance of central nervous function and which mechanism is underlying. GA and 3HGA both show structural similarities to the neurotransmitter glutamate. It is assumed that mainly 3HGA but also GA lead to excitotoxic cell degradation via NMDA receptors [1,2,3]. In this study we investigated the impact of GA and 3HGA on cortical and striatal network activity.

2 Materials and methods

The dose-response curves of GA, 3HGA and a combination of both were acquired by recording the spontaneous neuronal spike rate before and after substance application. We used cortical and striatal networks grown on MEAs with 60 planar Ti/TiN-microelectrodes (30 µm diameter, 100 µm spacing; MCS, Reutlingen). Primary dissociated striatal cells were prepared from embryonic Wistar rats. Cryopreserved cortical cells from embryonic Sprague-Dawley rats were purchased from QBM Cell Science (Ottawa, Canada). All measurements were performed using a Mg²⁺-free, Hepes-buffered bath solution (pH 7.4 in absence of test-substances).

3 Results

GA and 3HGA both inhibited the spontaneous spike rate of the neuronal networks at comparatively high concentrations with an IC₅₀ of 954 µM (GA), 1.07 mM (3HGA) and 1.09 mM (GA+3HGA in com-
combination, 50% each) for cortical cells and an IC$_{50}$ of 1.56 mM (GA), 1.33 mM (3HGA) and 1.16 mM (GA+3HGA) for striatal cells (Fig. 1). We additionally acquired pH response curves in absence of test substances with an IC$_{50}$ at a pH of 6.88 for cortical cells and an IC$_{50}$ of 6.87 for striatal cells (Fig. 2). The observed effects concerning all substances or pH values were reversible after wash out for all types of cells.

**Fig. 2** PH response curve: Change of relative spike rate plotted versus concentration of H$^+$-ions (-pH). We measured an IC$_{50}$ value at pH 6.87 on striatal cells. The different pH values were adjusted by titration with NaOH.

The addition of the selective NMDA antagonist 2-amino-5-phosphonovaleric acid (APV) did not reduce GA- as well as 3HGA-induced inhibition of the neuronal network activity on cortical and striatal cell cultures (Fig. 3., right column). If pH values were adjusted to a physiological level, the tested substances did not alter network activity (Fig. 3., left column).

### 4 Discussion

In our in vitro model, both examined substances showed effects on the SSR only in comparatively high concentrations that are not reached in blood or cerebrospinal fluid of GA I patients. The two acids presented similar IC$_{50}$ values on both cell types. We compared the IC$_{50}$ values and the pattern changes of the GA and 3HGA measurements to those acquired by the pH response measurements. The effects seemed to be caused mainly by the acidity of the substances and the consequentially lowered pH. If the pH was constantly kept at a value of 7.4, GA and 3HGA did not have any impact on the neuronal network activity of cortical as well as striatal cell cultures even at a concentration above the IC$_{50}$ value (1.75mM). APV did not abolish GA- or 3HGA-induced inhibition. We conclude that in our model there is no specific (NMDA) receptor mediated effect of the investigated metabolites on the function of neuronal networks. Further investigations of possible triggering factors in the pathogenesis of striatal degeneration in glutaric aciduria type I are required.

### Acknowledgement

We thank QBM Cell Science, Canada, for providing Rat Brain Cortex Cryo Cells. We also thank Brigida Ziegler for technical support and Dr. Maurice Laryea for material support.

### References


Pharmacological properties of identified histaminergic neurons versus whole-MEA responses in primary posterior hypothalamic cultures

Olga Sergeeva¹*, Wiebke Fleischer², Stephan Theiss², Mario Siebler², Helmut L. Haas¹

¹ Institute of Neurophysiology, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany
² Department of Neurology, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany
*Corresponding author. E-mail address: olga.sergeeva@uni-duesseldorf.de

Histaminergic neurons (HA) from the tuberomamillary nucleus (TMN) of the posterior hypothalamus control arousal and attention. Our aim was the identification of HA-neurons in primary cultures of posterior hypothalamus grown on MEAs by their pharmacological properties. Data were correlated with the post-recording stainings. Application of α-methylhistamine, an H3 receptor agonist, inhibited neurons recorded through some of the channels, which were later found to be in contact with HA processes. These “HA” channels, in contrast to the whole MEA, were activated by orexin and ATP. Although pure "HA" channels were not found (processes of non-HA neurons were intermingled with HA endings in all cases) we conclude that heterogeneous cellular populations can be detected and separately analysed in MEA recordings.

1 Introduction

MEAs represent a potent screening technique. Analysis is usually done on the total cellular population activity without considering the cellular heterogeneity. Here, we demonstrate that cell-type specific properties can be examined by a combination of pharmacological tools and modified signal analysis. Histaminergic neurons located in the tuberomamillary nucleus (TMN) of the posterior hypothalamus represent the major waking center of the brain, which controls arousal and attention. TMN neurons can be identified in vivo and in vitro (slice recordings) by their broad action potentials and pacemaker-like activity, which is inhibited by H3-receptor agonists (Haas and Panula, 2003). We applied similar criteria to identify HA-positive MEA channels.

2 Materials and Methods

Primary dissociated cultures were made from the posterior hypothalamus of newborn rats (see Sergeeva et al., 2005). Extracellular potentials were recorded on polyethylenimine-coated MEAs with a square grid of 60 planar Ti/TiN-microelectrodes (30 µm diameter). For the histamine and MAP2 stainings MEAs and parallel cultures were fixed in 4% 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide in 0.1M sodium phosphate buffer (pH 7.4). Rabbit-anti-histamine antibody (1:1000) from Chemicon International, Germany), 1:500 diluted mouse anti-MAP2 (microtubule associated protein 2) antibody (Sigma, Germany)

Fig. 1 Immunocytochemical identification of histaminergic (HA) neurons. (a) Double staining for the mouse MAP2 and rabbit-anti-histamine antibodies in hypothalamic cultures grown on glass coverslips parallel to the MEAs. (b) MEA staining demonstrates strong branching of processes of HA neurons (indicated with arrows), which contact the recording surface of MEA electrode. Scale bars 20µm.
3 Results

Primary posterior hypothalamic cultures contained about 7% histaminergic neurons (calculated as a ratio: histamine-positive cells number / MAP2 positive cells detected with immunocytochemistry (Fig1)).

a)

About 14% of the recorded MEA channels were identified as “histaminergic cell (HA) channels”: The neurons fired action potentials with a triphasic action potential reflecting a broad spike and were inhibited by application of α-methylhistamin, an H3 receptor agonist on autoreceptors. Activity of these identified channels was further analysed separately. Orexin A (10-100nM) and ATP (100µM) enhanced activity on “HA channels” in accordance with our single cell recordings in slices (Eriksson et al., 2001; Sergeeva et al., 2006), whereas overall network activity was reduced (Fig.2).

4 Conclusions

Thus, heterogeneous cellular populations in primary cultures show different pharmacological responses that can be detected and analysed with MEA recordings.

References


Fig.2 ATP reduces activity of a whole MEA, but increases activity recorded from the channel 36, identified as a “HA channel” through inhibition of its activity by the H3 receptor agonist α-methylhistamine.

a) The spike raster plots of MEA recordings illustrate action of ATP and non-correlated pattern of activity of hypothalamic neurons.

b) Examples of field spikes recorded from 2 MEA channels and pharmacological analysis of activity recorded from channel #36 (right).
Deltamethrin and permethrin decrease spontaneous activity in neuronal networks in vitro

Timothy J. Shafer1*, Sabnam 0. Rijal2, and Guenter. W. Gross2

1 Neurotoxicology Division, U.S. Environmental Protection Agency, Research Triangle Park, NC, USA
2 Center for Network Neuroscience, University of North Texas, Denton, TX, USA
* Corresponding author. E-mail address: shafer.tim@epa.gov

Effects of pyrethroid insecticides on spontaneous electrical activity were investigated in primary cultures of cortical or spinal cord neurons grown on microelectrode arrays. Bicuculline (40 μM) was utilized to block fast GABAergic transmission, and concentration-dependent effects of deltamethrin (1 nM - 5 μM) and permethrin (10 nM - 50 μM) were examined. Both compounds caused a concentration-dependent reduction in spike frequency (#spikes/min), as well as the number of bursts/minute. Deltamethrin was more potent than permethrin, with respective IC₅₀ values of ~200 nM and ~4 μM for spike frequency inhibition in both tissues, respectively. Onset of effects was rapid and reductions in spike rate could only be partially reversed with successive complete media replacements. These results demonstrate that both deltamethrin and permethrin are potent inhibitors of network activity in cortical and spinal cord cultures in vitro, and suggests that despite differences in the clinical signs of poisoning, similar modes of action at the network level may contribute to effects of these compounds.

1 Introduction
Pyrethroid insecticides are utilized in a variety of agricultural, industrial and residential products. Pyrethroid compounds appear to exert their insecticidal and toxicological effects via disrupting voltage-sensitive sodium channel (VSSC) function in neurons [1]. VSSC are crucial for control of electrical excitability, and are involved in the initiation and propagation of action potentials. Pyrethroids alter both the activation and inactivation of VSSC.

Differences in effects on VSSC inactivation rates are thought to produce differences in the effects of pyrethroids on neuronal firing patterns, with some compounds causing repetitive firing, while others cause repetitive firing with depolarization of the neuronal membrane, leading ultimately to depolarization-dependent block of action potentials. However, following these modifications in firing patterns, the sequence of events that ultimately gives rise to the different poisoning syndromes is not well understood. In particular, it is unclear how pyrethroids alter the firing patterns of networks of interconnected neurons. Therefore, cultures of cortical or spinal cord neurons plated on microelectrode arrays were exposed to deltamethrin and permethrin, and changes in network activity were recorded.

2 Methods

2.1 Solutions
Technical grade deltamethrin (99% 1R, 3R-(S) isomer, Lot # 325-58A) and permethrin (41% cis/58% trans-isomers, Lot # 328-78A) were obtained from Chem Services (West Chester, PA). Stock solutions (10 mM) of each compound were prepared daily in DMSO, from which serial dilutions in DMSO were prepared. Total DMSO did not exceed 0.05% (v/v) at any pyrethroid concentration and was without effect on network activity. All recordings were made in culture medium that contained 5 and 10 % serum for the spinal cord and cortical neurons, respectively.

2.2 Tissue culture
All animal protocols were approved by the University of North Texas Institutional Animal Use and Care Committee and were designed to minimize pain and distress. For cortical cultures, embryonic 16-17 day mice were removed from the uterus of pregnant HSd:ICR mice and cultures of frontal cortex and/or spinal cord neurons were prepared as described previously [2] and plated on multielectrode arrays prepared by the Center for Network Neuroscience at the University of North Texas.

2.3 Recordings
Extracellular recording of action potentials were made as previously described using hardware and software from Plexon Inc (Dallas TX). All recordings were made in the presence of 40 μM bicuculline. Out-
put was sent to an oscilloscope, audio speakers, and personal computer for recording and analysis.

3 Results

Addition of deltamethrin (1 nM -5 μM) or permethrin (10 nM - 50 μM) resulted in concentration-dependent decreases in spontaneous spiking and bursting activity. However, there were differences in the potency of the two compounds, with deltamethrin being more potent than permethrin in both cortical and spinal cord cultures (Figure 1). In frontal cortex cultures, deltamethrin and permethrin inhibited spike frequency with IC₅₀ values of 0.13 and 3.6 μM, respectively. Similar results were obtained in spinal cord cultures, where IC₅₀ values were 0.32 and 17 μM for deltamethrin and permethrin, respectively. It should be noted that differences in the serum concentrations between the spinal and cortical cultures prevent direct comparisons of potency between the two tissue types.

Fig 1 Concentration response for deltamethrin (top) and permethrin (bottom) effects on spike rate. Spike rate is the number of spikes per minute. Data are the means and SEMs of 3-6 arrays per tissue per concentration.

Several observations indicate that the decrease in network activity is not the result of cytotoxicity of DLM or PTM to the cultures. Even after spontaneous spiking and bursting activity is inhibited by >90%, spontaneous events were still recorded. The decrease in activity, although not easily reversible, can be reversed. With deltamethrin, 42 out of 105 units in frontal cortex and 12/18 units in spinal cord cultures recovered some level of activity following removal of deltamethrin containing medium. Recovery of activity ranged from minimal to complete recovery.

4 Summary

The neurotoxicity of pyrethroid insecticides has been widely studied at the whole animal level as well as at the cellular and subcellular level. However, to date, no study has examined the temporal evolution of functional toxicity induced by pyrethroids in networks of interconnected neurons. The data demonstrate that pyrethroids potently inhibit network activity in cultures of frontal cortex and spinal cord neurons. Deltamethrin is more potent than permethrin, an observation that is consistent with in vivo potencies of these two compounds [3].

Acknowledgement

We thank Ms Jennifer McAnally, University of North Texas, Denton, TX. Supported by the Texas Advanced Technology Program and the U.S. Environmental Protection Agency.

Disclaimer

The information in this document has been funded in part by the U.S. Environmental Protection Agency. It has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

References

Cellular sensors
The Mechanism of Neuroelectronic Interfacing with Capacitors and Transistors

Peter Fromherz

Department of Membrane and Neurophysics, Max Planck Institute for Biochemistry, Munich, Germany
Email: fromherz@biochem.mpg.de

Keynote address

The electrical interfacing of nerve cells with semiconductors and metal electrodes relies on a dissipative mechanism. In the case of stimulation, capacitive current flows across the solid/electrolyte interface and along the seal resistance between cell and substrate. The resulting extracellular voltage gives rise to a polarization of the cell that may elicit an action potential. In the case of recording, action potentials lead to capacitive and ionic current flow across the attached cell membrane and along the seal resistance. The concomitant extracellular voltage displaces the inner electrical potential in the substrate that is recorded by an electronic device. Silicon chips with an insulating oxide are particularly useful to study the details of the mechanism, because no Faradayic current flows across the solid/electrolyte interface in the case of stimulation, because the change of inner electrical potential in the substrate is locally determined with a transistor in the case of recording, and because the chips provide a chemically homogeneous substrate for cell adhesion.

Crucial for the strength of cell-substrate coupling is the seal resistance of cell adhesion. We measure the width of the extracellular cleft between the insulating lipid bilayer of the cell membrane and the insulating oxide of a chip by fluorescence interference contrast microscopy. The sheet resistance of that cleft is determined with voltage-sensitive fluorescence dyes.

The fundamentals of electrical interfacing are studied with recombinant sodium and potassium channels on capacitors and transistors. On one hand, Nav1.4 and Kv1.3 channels in HEK293 cells, that are open by intracellular depolarization, are detected with electrolyte-oxide-silicon transistors. On the other hand, Nav1.4 and Kv1.3 channels are opened under intracellular voltage-clamp by falling voltage-ramps applied to electrolyte-oxide-silicon capacitors with TiO2 and HfO2 as insulating oxides with high dielectrical constant. Special care must be taken to control or avoid the electroporation of the attached cell membrane by the capacitive stimulus.

Extracellular recording of action potentials in nerve cells leads to a spectrum of waveforms, depending on the type of current across the attached cell membrane. The dominant current – capacitive, sodium, potassium – depends on the position of the recording site if the ion channels are not homogeneously distributed. The nature of recording is elucidated with large identified neurons from Lymnaea stagnalis on a 2D array of transistors as implemented with extended CMOS technology. A spatiotemporal pattern of the recording is observed that reflects the segregation of ion channels.

Capacitive stimulation of nerve cells is also studied with large identified neurons from Lymnaea stagnalis. First, the gating of ion channels in the attached and free membrane is investigated by conventional intracellular voltage-clamp and by quasi-extracellular voltage-clamp as achieved by falling voltage-ramps applied to a capacitor. Under current clamp, action potentials are elicited by activation of the upper free membrane as well as by depolarization of the attached membrane with concomitant inward flow of sodium ions that activates the upper membrane.

Publications


I. Schoen, P. Fromherz, Mechanism of Extracellular Stimulation of Neurons on an Electrolyte-Oxide-Silicon Capacitor. In preparation
Electric Cell-Substrate Impedance Sensing: A versatile approach to study animal cells in vitro

Joachim Wegener¹*, Charles R. Keese², Ivar Giaever²

¹ Institut für Biochemie, Universität Münster, Germany
² Applied BioPhysics Inc., Troy (NY), USA
* Corresponding author. E-mail address: wegenej@uni-muenster.de

Invited paper

In Electric Cell-Substrate Impedance Sensing (ECIS) animal cells are grown on planar gold-film electrodes (d = 250 µm) that are deposited on the bottom of a cell culture dish. The frequency dependent impedance of these electrodes is measured and followed with time as an analytical indicator for the cellular behavior in vitro. From impedance readings it is possible to monitor the coverage of the electrode with cells, for instance during cell attachment and spreading, as well as any changes in cell shape that occur in a confluent cell monolayer in response to chemical or biological stimuli. Single frequency measurements with high time resolution report on the motility of the cells on the surface and mirror their metabolic activity. Applications of ECIS comprise: cytotoxicity testing, stimulation of cell surface receptors, monitoring of correlated cell motions or heterologous cell-cell interactions.

1 Substrate-Integrated Electrodes as Transducers in Cell-Based Assays

Experimental studies based on animal cells as the sensory elements are receiving more and more attention in applied as well as fundamental biomedical research [1]. Besides ethical and economic considerations, working with isolated cells instead of test animals provides many advantages like well-defined experimental conditions in a laboratory environment compared to the complexity and individuality of an intact organism. However, to conduct biomedical or biotechnological experiments with animal cells it is not sufficient to have the proper cells available, there is also a strong demand for appropriate transducers that convert the cell response preferably into an electrical signal. Throughout the last years many different approaches have been described that are all based on integrating a technical component like noble metal electrodes or transistors into the growth substrate of the cells and thereby provide a close proximity to the cell body. Integrating the sensor directly into the growth substrate holds further practical advantages and allows to combine several sensor units in one experimental setup.

Very different technical concepts have been proposed to monitor the functional response of living cells in a given experimental setting. Many approaches address changes in the cells’ energy metabolism when exposed to a certain stimulus [2-4]. Since energy metabolism is a general phenomenon in all cells, biosensors of this kind can be realized with a huge variety of different cell types. Furthermore changes in the rate of energy metabolism were proven to be very sensitive to a broad range of physiological alterations [5]. When it comes to electrically excitable cells as sensory elements, extracellular recordings of action potentials by means of field effect transistors or noble metal electrodes serve as the analytical parameter [6,7]. Most recently, Woolley et al. [8] have introduced a new technology by growing adherent (non-excitable) cells on the surface of gold-film electrodes and measuring the electrical potential difference between the substrate and a reference electrode. The recorded voltages are dependent on the cell type, report on cellular alterations in response to chemical stimuli and vanish when the cells leave the electrode surface. Very different from the aforementioned devices is the use of shear wave resonators as growth substrates for adherent cells [9]. Here the mechanical interactions of the cell with the surface are measured and followed when the cells changes its shape or visco-elastic properties in the course of an experiment.

2 Electric Cell-Substrate Impedance Sensing

In Electric Cell-Substrate Impedance Sensing or short ECIS the cells are grown on the surface of thin (100 nm) gold-film electrodes that are deposited on the bottom of a cell culture dish. When cells attach and spread on the electrode surface, the overall impedance of the system increases due to the dielectric nature of the cell bodies that block current flow from the electrode. Each measurement unit contains two coplanar electrodes, one working electrode and one counter electrode (two probe measurement). Figure 1 sketches the experimental setup. The only difference between working and counter electrode is the surface area exposed to the bulk electrolyte. The counter elec-
trode is roughly 1000 times bigger in surface area than the working electrode. Due to this difference in surface area the measured impedance is dominated by the impedance of the working electrode. However, the entire bottom of the dish is covered by the cells.

Frequency dependent impedance data are recorded within a frequency range of 10 Hz to 100 kHz using sinusoidal voltages of 20 mV amplitude (rms). Dependent on the frequency of the driving voltage the current will either flow around the cell bodies (see arrows in the insert of figure 1) or couple through the cells by means of displacement current via the plasma membranes (broken arrows in the insert of figure 1).

As soon as the cells attach and spread on the electrode surface the impedance starts to increase with a time course that strongly depends on the AC frequency. The immediate increase in impedance at high frequencies ($f > 10$ kHz) is due to the attachment and spreading of the cells on the electrode surface. The biphasic time course at intermediate frequency also mirrors the establishment of cell-substrate contacts (first rise) followed by the subsequent expression of cell-cell contacts (second rise). Thus, dependent on the AC frequency different aspects of the cells can be monitored.

2.1 Modeling Impedance Data of Cell-Covered Electrodes

When the cell layer is confluent, the frequency dependent impedance of the cell-covered electrode can be analyzed by means of a physical model that is illustrated in figure 3. The original model is described in detail elsewhere [10].

According to the proposed model the cells are considered as cylindrical disks with radius $r_{cell}$ that are an average distance $d$ separated from the surface of the electrode. The resistance due to the cell-cell junctions is simply accounted for by an ohmic resistance $R_b$ while the capacitance of the membrane is denoted as $C_m$. Describing the cell-electrode junction (blow up in fig.3) is more involved. The narrow channel between cell membrane and electrode surface behaves as 2D core-coat conductor. The electrolyte in the channel is the conducting core while the electrode/electrolyte interface ($Z_{el}$) and the plasma membrane ($C_m$) are the insulating coat on either side.

Gold electrodes in contact to an electrolyte behave almost like ideally polarized electrodes so that the associated interface impedance is very close to that of a capacitor.
Fig. 3 Schematic illustrating the model to analyze the frequency-dependent impedance of cell-covered ECIS electrodes.

More detailed analysis has, however, revealed that the interface impedance $Z_{el}$ of the gold electrode is even better described by a constant-phase-element (CPE) which is an empiric impedance element that follows the equation:

$$Z_{CPE} = [(i \cdot \omega)^n \cdot A]^{-1}$$

The CPE is characterized by a constant and frequency independent phase shift that is determined by the value of the parameter $n$ ($0 \leq n \leq 1$). CPE behavior has been found to describe the interface impedance of many noble metal electrodes in contact to electrolyte solutions accurately [11].

The resistance of the electrolyte-filled cleft between membrane and electrode surface (cell-electrode junction) is accounted for by the cleft resistance $r_{cleft}$. The lower part of figure 3 illustrates the model in a notation with finite elements. Data analysis with the complete transfer function returns a global cleft resistance $R_{cleft}$ that is defined as

$$R_{cleft} = r_{cleft}^2 \cdot (\rho / d).$$

$\rho$ denotes the specific resistivity of the electrolyte in the cell-electrode junction while $d$ is the height of the channel.

Thus, time dependent changes in $R_b$ along the course of an experiment report on changes in the cell-cell junctions while changes in the resistance of the cleft $R_{cleft}$ indicate alterations in the cell-electrode junction.

2.2 Stimulation of Cell Surface Receptors

In order to monitor the stimulation of cell surface receptors by ECIS readings we have grown CHO-M1T cells to confluence on the surface of the electrodes. These cells are genetically engineered to over-express the muscarinic acetylcholin receptor that belongs to the family of G-protein coupled receptors (GPCRs) that are particularly relevant from a pharmacological point of view. GPCRs transmit the signal induced by ligand binding to the extracellular binding site of the receptor by activating an intracellular G-protein that itself triggers a signal transduction cascade. The M1T receptor can be activated by well-known agonist like carbachol.

Figure 4A shows the time course of the impedance magnitude at a sampling frequency of 4 kHz when confluent CHO-M1T cells are stimulated by 100 µM carbachol whereas another population of cells was treated with the solvent only (control).
Upon stimulation with 100 µM Carbachol the CHO cells show a strong increase in impedance within one hour whereas the corresponding controls do not show any significant effect. In order to learn about the changes that occur on the cellular level we have processed the raw data by the ECIS model in order to obtain the time course of $R_\text{b}$, $R_\text{clef}$ and $C_m$. The outcome of this data analysis is shown in figure 4B. As apparent from the figure, it is mostly $R_\text{b}$ that shows any significant changes and these resemble strongly the character of the curve of the raw data (fig. 4A). Accordingly carbachol induces predominantly changes at the sites of cell-cell junctions that lead to a collapse of the intercellular cleft and a corresponding increase in the resistance $R_\text{b}$. Electron microscopic studies on the very same system provided experimental proof that this conclusion from the recorded ECIS data was correct.

The data shown above have been recorded with cells that had been genetically modified to overexpress the receptor of interest. However, the same kind of screening is also possible with wild-type cells that are derived from the tissue of interest. When these cells are grown on the electrodes ECIS can also serve as a transducer for physiological or pharmaceutical studies [12].

2.3 Other ECIS applications

ECIS has been first described in 1984 [13]. During those years after its first notion many applications have been evolved. Besides monitoring the stimulation of cell-surface receptors as described above, the system has also been used to study cytotoxicity in general [14] and chemically induced apoptosis in particular [15]. ECIS was furthermore applied to study (a) the attachment and spreading of cells to the electrode surface that had been pre-coated with different proteins before [16]; (b) the interactions of metastatic tumor cells with endothelial cell monolayers [17]; (c) the time course of wound healing [18] or (d) the cell response after in situ electroporation [19].

In general, ECIS is capable of recording the changes that occur within an ensemble of cells as long as the cells grow adherently and the shape of the cells changes during the experiment. Only in those rare cases in which the cell does not alter its 3D shape upon exposure to a certain stimulus, the ECIS technique is blind.

References

Combining the recording of neuronal activity with metabolic parameters: Sensorchips with MEA and integrated sensors for oxygen, pH and temperature

Johann Ressler1*, Helmut Grothe1, Martin Brischwein1, Florian Ilchmann1, Michelle Karg1, Guenter W. Gross2, Bernhard Wolf2

1 Heinz Nixdorf-Lehrstuhl für Medizinische Elektronik, Technische Universität München, Deutschland
2 Department of Biological Sciences, University of North Texas, Denton, TX, USA
* Corresponding author. E-mail address: ressler@tum.de

The utilization of cultured neuronal networks grown on sensorchips with micro-electrode-arrays (MEA) for reliable detection of toxic compounds enables broadband fields of application, e.g. drug-screening or basic research. However, on the one hand stable culturing of neuronal networks for extended culturing periods requires a strong control of the environmental parameters. On the other hand the correlation of electrophysiological activity with metabolic activity of neuronal networks is a novel field of research. Therefore glass-based sensorchips are described, including a MEA and sensors for oxygen, pH and temperature. Transparent chip-substrates allow all applications of microscope imaging methods.

1 Introduction
The utilization of cultured neuronal networks grown on sensorchips with MEAs for reliable detection of toxic compounds and the evaluation of bio-electronic signal processing is well tried and enables broadband fields of application, e.g. drug-screening or basic research. However, on the one hand stable culturing of neuronal networks over long culturing periods requires strong control of environmental parameters. On the other hand correlating electrophysiological activity with metabolic activity of neuronal networks is an interesting and encouraging field of research. For this reason glass-based sensorchips have been developed, including MEA and sensors for oxygen, pH and temperature. Due to the transparence substrate, all kinds of microscopic monitoring are possible, making these chips powerful tools for high content screening and neuron-bioinformatics.

2 Material and Methods

2.1 Sensors

2.1.1 MEA
For detection of electrophysiological activity, a microelectrode array is placed in the centre of the sensorchip. It consists of 64 electrodes arranged in an 8x8 array. Each electrode is 23µm in diameter with a grid spacing of 150µm. The tracks are 8µm in width and open out in two 32 contact strips on either side of the sensorchip for connection to electronic equipment [1]. Fig. 1 shows the arrangement of the 64 electrodes.

2.1.2 Oxygen-sensor
With the oxygen-sensor it is possible to detect the oxygen consumption of the cells. This allows information on cellular respiration and metabolic effects. A planar oxygen sensor, fabricated of a platinum layer by thin film technology is used. The sensor consists of three electrodes (working-, reference- and auxiliary-electrode) and realizes an amperometric measurement method. A constant potential is applied between reference- and working-electrode. Additionally, an auxiliary-electrode is used to keep the reference-electrode without current [2]. The resulting current can be calibrated to the concentration of the oxygen in the culture medium. The relatice surface areas of working-, reference- and auxiliary-electrodes are about 1 : 1000 : 3000. The relatively small surface of the working-electrode minimizes oxygen self-consumption of the sensor itself and ensures that all measured changes in the electrochemical current are due to changes in the oxygen concentration caused by the cells [3].
main electrochemical reaction taking place at the working-electrode and causing the measured current:

\[ \text{O}_2 + 4e^- + 4\text{H}^+ \rightarrow 2\text{H}_2\text{O} \]

Fig. 2 shows the three-electrode configuration of the used planar oxygen-sensor:

2.1.3 pH-sensor

Acidification of the culture medium is measured by pH-sensors. This acidification provides information on the general cellular metabolism. For planar pH-sensor usually ion sensitive field effect transistors (ISFETs) are used [4]. To avoid the cost expensive and with cross sensitivity to sodium-ions afflicted silicon technology, we have integrated a metaloxide-based pH-sensor. Together with a reference-electrode which is integrated in the fluidic-system, a ruthenium-oxide (RuO\textsubscript{2})-spot allows potentiometric measurements of the pH-value of the culture medium [5]. The sensor is performed as a RuO\textsubscript{2}-spot, which is deposited on a platinum electrode by sputtering [6]. Fig. 3 shows a microscope image of the RuO\textsubscript{2}-spot with the platinum electrode beneath. In Fig. 4 the sensor-response to several pH-values is outlined.

Fig. 2 Amperometric oxygen-sensor

2.1.4 Temperature-sensor

For temperature-measurements a Pt100 platinum resistor is integrated in addition to the metabolical and morphological sensors. With this sensor the temperature of the culture medium can be monitored and facilitate the control of micro-environmental parameters.

2.1 Sensorchip and technology

The electrodes for the MEA, the oxygen- and temperature-sensor and all tracks are fabricated from a platinum layer on a 0.55mm thick glass-based substrate by thin film technologies. The chip dimension is 5cm x 5cm. Good adhesion of the metallization is ensured by a thin layer of titanium. The RuO\textsubscript{2} for the pH-Sensor is deposited by sputter-processes. The sensorchip (Fig. 5) is insulated by a layer of SU-8 and is opened on the active sensor surface and the contact strips. Fig. 6 shows the layout of the sensorchip.

Fig. 3 RuO\textsubscript{2} sensor-spot for measuring the pH-value

Fig. 4 pH-sensor response for switches of the pH-value (pH 6, 7, 8)

Fig. 5 RuO\textsubscript{2} sensor-spot for measuring the pH-value
3 Conclusions

The glass-based sensorchip with MEA and metabolic sensors described above can deliver information on electrophysiological and metabolical parameters of living cells. For this reason there are many possible fields of application in biomedical and pharmaceutical areas, environmental engineering and basic research. Due to the transparency of glass-based chip substrate, this sensorchip can also be employed for all kinds of fluorescence applications such as live cell microscopy. The combination of multiple sensor data and data from microscope imaging frequently makes this chip a powerful tool in neurobiological research. Together with an appropriate analyzing system, which provide the measurement electronic equipment for sensor control and data acquisition for all described sensor-types and an adequate liquid handling system, this sensorchip enables a wide range of possible applications. Fig. 8 shows an example of an analyzing system, which has been developed together with the sensorchip.

4 Outlook

Presently our group is working on the design of a 24-well-plate with a sensorchip with MEA and sensors for pH, oxygen and temperature under each well. Therefore already a sensorchip with dimensions of 8.1mm x 8.1mm has been fabricated (Fig 9) [7].

Acknowledgement

The authors would like to thank the current staff of the Heinz Nixdorf-Lehrstuhl für Medizinische Elektronik involved in this work.
References


Cell monitoring systems with multiparametric CMOS biosensorchips

Werner Baumann2*, Ralf Ehret1*, Elke Thedinga1, Axel Kob1, Andreas Keuer1, Sabine Drechsler1, Angela Podssun2, Philipp Köster2, Carsten Tautorat2, René Schrott3, Helmut Beikirch3, Ingo Freund4 and Mirko Lehmann4

1 Bionas GmbH, Rostock, Germany
2 University of Rostock, Biophysics Dept., Rostock, Germany
3 University of Rostock, Faculty of Computer Science and Electrical Engineering, Rostock, Germany
4 Micronas GmbH, Freiburg, Germany
* Corresponding authors. E-mail address: ralf.ehret@bionas.de or werner.baumann@uni-rostock.de

For on-line monitoring of cellular reactions under well controlled experimental conditions we develop(ed) different Cell Monitoring Systems (CMS®). They allow a parallel, label-free and non-invasive measurement of different parameters of cellular systems by use of CMOS silicon microsensors. Main application for our systems is in the field of high content screening in the drug development process.

1 Introduction

Biochemical substances are sensitively recognized and processed by living cells, either to provide energy or to trigger an adequate cell-type specific response. In the pharmaceutical industry it is important to acquire as much information as possible about e.g. the metabolic effects of an active substance. Most classical pre-clinical studies are very expensive and time-consuming. Often they are so-called end-point tests which require many individual tests before approximate statements can be made about how an effect takes its course.

2 Cell monitoring systems

For monitoring of metabolically relevant data including extracellular oxygen consumption, extracellular acidification rate and the adhesion (cell impedance) of cells Bionas GmbH has developed a new screening system (Bionas® 2500 analyzing system) to allow a continuous recording of how an active substance acts (Fig. 1)[1-4]. Living cells are directly cultured on the sensorchip (Fig. 2 & 3). Using ion-sensitive field effect-transistors (ISFET) and electrode structures (Fig. 4) it is possible to observe metabolic parameters both continuously, label-free and non-invasively over longer periods. The system has already been established for several cell models, cell lines as well as primary cells.

Fig. 1 Bionas® 2500 analyzing system

Fig. 2 SEM picture of a neuronal network at beginning of growth after 3 days on chip.
For monitoring the activity of neuronal networks in the Biophysics Dept. at the University of Rostock a CMOS-sensorchip based system has been realized for measurement of electrical as well as acidification rate (Fig. 5) [5]. In cooperation with Bionas an external stimulation unit for the neuro sensorchip was realized. In present a CMOS sensorchip with integrated electronics (filters, preamplifiers, multiplexer, I2C-bus, Fig. 6) is evaluated. With the integrated electronics more sensors can be integrated using the same chip carrier and additional features like PC controlled switching (on chip!) between stimulation and recording has been realized.

To manage the high amount of data from the measurement of the electrical activity of a neuronal network we also developed algorithms for automated spike detection and unit separation.

3 Results

System setup, sensorchips and measurements of metabolic as well as electrical activity of different cell types with CMOS sensorchips will be presented at the MEA 2006.

The more parameters we can study in parallel the better we will understand for example cellular reactions in e.g. drug screening applications.

Acknowledgement

The research effort is sponsored by the European Regional Development Fund Community (ERDF), the state Mecklenburg-Vorpommern and Micronas GmbH.

References

In-situ preparation of bio-technical interfaces by FIB-SEM microscopy

Claus Burkhardt\(^1\), H. Wolburg\(^2\), K. Kohler\(^3\), H. Schmid\(^3\), R. Stoop\(^4\), Wilfried Nisch\(^1\)

\(^1\) NMI Natural and Medical Sciences Institute at the University of Tuebingen, Reutlingen, Germany  
\(^2\) Institute of Pathology, University of Tuebingen, Tuebingen, Germany  
\(^3\) Experimental Ophthalmology, University of Tuebingen, Tuebingen, Germany  
\(^*\) Corresponding author. E-mail address: burkhardt@nmi.de

FIB-SEM (Focused Ion Beam – Scanning Electron Microscope) is a new tool for the preparation and imaging of surfaces and interfaces of various materials. The focused ion beam allows precise milling of cross sections at defined areas between soft biological and hard technical materials with minimized artefacts. Results on cell monolayers on different substrates are presented.

1 FIB-SEM Microscopy

1.1 Introduction

A close contact between living cells and tissue and surfaces of bioelectronic devices like MEAs and neuroprostheses is a crucial parameter for the signal transduction from electrodes to cells and vice versa. Great efforts have been made in the past to investigate ultrastructural details of this bio-technical interface. Ultra microtomy of araldite replicas of thin micro-structured metal surfaces was used to investigate cell adhesion by transmission electron microscopy [1]. Preparation of cells and tissue on solid substrates was done by sawing and polishing after embedding [2]. Sophisticated imaging methods have been applied to probe the electrical characteristic of the interface [3]. However, ultrastructural details of the interface are not accessible by this methods. Additionally, this methods are time-consuming and require in the case of optical interference microscopy special microstructures on the technical surface.

To obtain high-resolution images from both details of the technical substrate, the biological material and the interface in between we combined conventional fixation and embedding techniques, Focused Ion Beam (FIB) technology and low voltage Field Emission Scanning Electron Microscopy (FESEM).

1.2 CrossBeam FIB-SEM Microscope

FIB preparation and SEM imaging was performed in a CrossBeam\(^\text{®}\) 1540 XB microscope (Carl Zeiss, Oberkochen). This FIB-SEM microscope allows an easy selection of the region of interest, fast milling at selected areas and SEM imaging of the FIB prepared micro block face [4]. By serial sectioning at this micro-block face, layers with a thickness down to 20 nm are removed by the FIB and the new block face is imaged with the SEM (live milling). The recorded image stack may be used for 3D reconstruction of sample volumes with dimensions up to several 100 \(\mu\)m\(^3\).

1.3 Sample preparation

Cells are chemically fixed “on-chip” in 2.5% Glutaraldehyd, stained in 1% OsO\(_4\) and embedded in Araldit. To get access to the interface a protocol for flat embedding was developed. After embedding, the samples are mounted on a SEM sample carrier and metalised with a thin layer of gold or carbon to get good electrical conductivity for SEM and FIB imaging.

2 Results

We processed two kind of samples to demonstrate the method. Monolayers of fibroblast cells have been grown on silicon substrates coated with a thin layer of titanium.

To investigate the influence of the microstructure on cell adhesion, we performed experiments on flat substrates (Fig. 1). In the SEM micrograph details of the cell membrane and nuclear pore as well as a thin layer on the substrate are resolved.

In a second experiment cells were cultivated and prepared on surfaces with additional nano structure [5] with a periodicity of 200 nm (Fig. 2). On the right site the outer cell membrane is in close contact to the top site of the nano pillars, on the left the distance between cell membrane and substrate is up to one micrometer.
3 Summary

In combination with conventional fixation, flat embedding and staining methods, the 3D FIB-SEM Microscopy allows to obtain high-resolution images of ultrastructural details of bio-technical interfaces. Cell membranes and the contact region between cell surface and solid substrates are clearly resolved. Therefore this new technique has the potential to investigate the influence of different surface coatings on cell adhesion on real implants or micro electrode arrays.

Acknowledgement
We thank Alfred Stett for stimulating and helpful discussions.

References
Cell-sensor contact model including electrodiffusion

Sven Ingebrandt*, Michael Pabst, Günter Wrobel, Frank Sommerhage, Mathias Schindler, Andreas Offenhäusser

Institute of Bio- and Nanosystems, IBN-2: Bioelectronics, and CNI – Center of Nanoelectronic Systems for Information Technology, Forschungszentrum Jülich GmbH, Jülich, Germany

* Corresponding author. E-mail address: s.ingebrandt@fz-juelich.de

The extracellular recording from electrogenic cells is of growing interest. A theoretical description of the electronic contact between the cells and extracellular devices is still not fully available. Usually, an electronic equivalent circuit is used to model the extracellular recorded signal shapes. These models are based on a pure electronic circuit description. In this contribution we present analytical calculations of the electrochemical properties in the cleft based on a PNP equation system.

1 Introduction

In general, the amplitudes of extracellular recorded signals are significantly smaller than the corresponding transmembrane voltage changes. However, due to a large improvement of the recording systems today it is possible to record these extracellular signals either using arrays of metallized microelectrode arrays (MEAs) [1], open-gate field-effect transistors (FETs) [2], or metallized, floating-gate FETs [3].

All these devices are able to register the electrical activity of cells which have been grown on the respective sensor surface. These extracellular recordings can be correlated to the transmembrane currents. For a correct interpretation of the signal shapes, however, it is clear that the understanding of the physical processes in the cleft – the interface between cell and sensor – is mandatory. A simple model, like the point-contact model, describes the electrical coupling between cell and sensor by a single point of contact [4]. Replacing the point contact by a two-dimensional circular contact area and incorporating specific properties of the cell membrane are further improvements of this model [5]. The models so far deal with electrical effects only and no electrochemical properties of the contact region are considered. Recent publications try to include electrochemistry utilizing the Nernst equation [6] or a spacial integration of the Poisson-Nernst-Planck equation [7]. However, the Nernst equation describes situations only, where a membrane separates different ionic reservoirs and no current is flowing. An improvement of these models would be based on a consequent solution of the Poisson and Nernst-Planck (PNP) equation system.

In this contribution we present analytical calculations of the electrochemical properties of the cleft based on a PNP equation system. In the stationary state near equilibrium it is possible to derive analytical expressions for the potential and the ion charge densities in the cleft, as functions of external excitation parameters, cell properties and cleft geometry. Theoretical results are compared quantitatively with experimental data received by simultaneous membrane current and FET recordings of cells.

2 Results

2.1 Contact Model

For the theoretical description of the electrical contact of single cells to single sensor spots it is clear that the shape and the time course of the extracellular signals should be independent on the device type (MEA or FET) used for recordings (Fig. 1).

Fig. 1 Schematics of the experimental condition, which is used for the theoretical coupling model. A cell, which is located on top of a sensor surface (either FET or MEA) is attached by a patch pipette. The contact region between cell and sensor is modelled as a thin disk, in which electrodiffusion of ions occur.

In this contribution we used the PNP equation system, which couples the continuity equations, the Nernst-Planck equations and the Poisson equation. Based on a circular coordinate system, we solve the equation system in an analytical way. We are able to derive analytical expressions for the potential and the ion charge densities in the cleft, as functions of external excitation parameters, cell properties and cleft geometry [8].
The PNP equation system describes the electro-chemistry of a system with different ion species. For each ion, indexed by \( i \), let \( \rho_i(r,t) \) and \( \mathbf{j}_i(r,t) \) present the electrical charge densities and the electrical current densities, respectively. The densities are functions of the position \( r \) and the time \( t \). If \( \lambda_i \) defines the source rate of the different ions, the continuity equations are given by

\[
\nabla \cdot \mathbf{j}_i(r,t) + \frac{\partial \rho_i(r,t)}{\partial t} = \lambda_i.
\]

(1)

Current densities, charge densities and the electrical potential \( \psi(r,t) \) are coupled via the Nernst-Planck equations

\[
\mathbf{j}_i(r,t) = -D_i \nabla \rho_i(r,t) + \frac{z_i \epsilon_i}{k_B T} \rho_i(r,t) \nabla \psi(r,t)
\]

(2)

with the diffusion constants \( D_i \) and the electric charge number \( z_i \) of ion \( i \). The potential is coupled to the total charge density \( \rho_{\text{tot}}(r,t) = \sum \rho_i(r,t) \) by the Poisson equation

\[
\Delta \psi(r,t) = -\frac{\rho_{\text{tot}}(r,t)}{\epsilon_0 \epsilon_{r}}
\]

(3)

with the dielectric constants \( \epsilon_0 \) and \( \epsilon_{r} \).

2.2 Results

The seal resistance \( R_s \) in the cleft can be described by the following equation:

\[
R_s = \frac{k_T}{4\pi D_k \epsilon_0 n_w^0 h}
\]

(4)

which is mainly dependent on the ionic strength of the bulk \( n_w^0 \) solution and on the height of the cleft \( h \). For the total particle density inside the cleft we derive the following equation:

\[
n_{\text{tot}}(r) = n_w^0 + \frac{\lambda_i}{4D_k \epsilon_0} a^2 \left(1 - \frac{a}{r}ight)
\]

(5)

with \( a \) being the radius of the radius of the contact area and \( D_k \) being the diffusion constant of potassium. Usually, a total electro-neutrality is postulated, which confirms an analytical solution of the equation systems. In our case we do not need to postulate electro-neutrality. An estimation of the remaining net-charge, however, leads to a ratio of \( \sim 10^{-9} \), which justifies the usual postulation. Nevertheless, for the calculation of the correct potential in the cleft the knowledge of the total charge density is essential, even if it is very small.

A test against experimental data, as e.g. depicted in Fig. 2, shows good agreement with the postulated model. Remaining signal shape differences between the different configurations (FET and MEA) can be explained by surface effects at the sensor inputs or by differences in the amplifier circuits.

3 Summary

We present a theoretical model for the electronic contact between a cell and a sensor surface. In contrast to the point-contact model, which relies on a pure electrical circuit description, the model here includes electrical and diffusive properties of the participating ions in a consistent way. Our model is based on the Poisson-Nernst-Planck theory of electrodiffusion and was solved analytically for a stationary state close to equilibrium. As a result, analytical equations for the potential, the single charge ion densities, and the seal resistance were derived. Comparison with experimental data showed good agreement. Therefore, the analytical expression, which relates the seal resistance to the thickness of the cleft, can be used to evaluate the thickness when experimental data are given. Future work will focus on the solution of the time-dependent PNP equations for the cell-sensor contact.

Acknowledgements

We are grateful to A. Baumann (IBI-1, Forschungszentrum Jülich) for providing us with the HEK293 cells.

References

Extracellular recording of neurons derived from P19 embryonic carcinoma cells: characterisation and application

HL. Khor*, E. Sinner, C. Thielemann, W. Knoll
Max Planck Institute for Polymer Research, Mainz, Germany
* Corresponding author. E-mail address: khor@mpip-mainz.mpg.de

The electrophysiology of P19–derived neurons was investigated. They were exposed to different concentrations of GABA antagonist, Bicuculline and were found to display synchronised burst activity. The P19-derived neurons were found to display neural network properties.

1 Aim
P19 is a mouse-derived embryonal carcinoma cell line capable of differentiation toward ectodermal, mesodermal and endodermal lineages [1]. It had been used as an in vitro model system for the study of neuronal phenotype acquisition [2]. Cell lines also offer advantages of reproducibility, unlimited availability, homogeneity, and pliability to genetic manipulation. In this study, we investigated the electrophysiology of neurons derived from P19 embryonic carcinoma cells. The response of the P19-derived neurons to GABA antagonist Bicuculline was investigated.

2 Methods
MEA were purchased from MultiChannel Systems. P19 embryonic carcinoma cells were differentiated into neurons by treating with retinoic acid and cultured up to a month on the MEA. The cells were tested with different concentration of Bicuculline and recordings were done with MC rack software and analysed.

3 Results and Discussion
Spontaneous neuronal activity was detected after 5 days in vitro. We found that P19-derived neurons developed network activity with synchronised burst activity after 18 days in vitro. After treatment with bicuculline, a GABA antagonist, it was found that there was enhanced synchronisation of their burst activity.

4 Outlook
P19-derived neurons displayed neural network properties. Experiments are ongoing to record the activity of P19-derived neurons in response to other neurotransmitters and as well as the activity of transfected P19-derived neurons.

References
Cell adhesion and the thermodynamic limit of extracellular recording

Moritz Voelker*, Peter Fromherz

Max-Planck-Institut für Biochemie, Martinsried, Germany
* Corresponding author. E-mail address: voelker@biochem.mpg.de

The junction between cultured cells and substrates is filled with electrolyte. The aqueous cleft between membrane and solid has a width of typically 50 nm and a sheet resistance in the order of several MOhm per square. As every electrical resistance, the junction resistance must exhibit Johnson noise in the form of fluctuations of the local voltage with respect to the bulk electrolyte.

We present a measurement of these voltage fluctuations for rat hippocampus neurons on silicon dioxide, using electrolyte-oxide-silicon field-effect-transistors with a particularly low intrinsic noise. We evaluate the spectral power density of the junction noise by subtracting the noise of open transistors from the total noise of covered transistors. In a first approximation, the resulting net power spectrum is fitted with a Lorentz spectrum for an RC equivalent circuit of the cell-chip junction.

The novel technique is non-invasive, does not rely on molecular probes and does not require any intra or extracellular stimulation.

In addition to the observation of cell adhesion, the adhesion noise has a general relevance for extracellular recording, as for frequencies above 1.5 kHz the observed adhesion noise is larger than all other noise sources combined, which means that the thermodynamic limit of extracellular recording has been reached.
Advances in culture, recording and stimulation techniques
Identification of synaptic activities in microelectrode array-based neural networks

Sang Beom Jun¹², Matthew R. Hynd³, Natalie Dowell-Mesfin³⁴, Karen L. Smith³, James Turner³⁴, Wiliam Shain³⁴, Sung June Kim¹²*

¹ Nano-Bioelectronics & Systems Research Center, Seoul, Korea
² School of Electrical Engineering, Seoul National University, Seoul, Korea
³ Wadsworth Center, New York State Department of Health, Albany NY, USA
⁴ Department of Biomedical Science, School of Public Health, University at Albany, Albany NY, USA
*
Corresponding author. E-mail address: kimsj@snu.ac.kr

The Microelectrode Arrays (MEAs) have been used for several decades to investigate neuronal networks in vitro. In most of the studies, the neuronal networks have been studied statistically due to complexity of cultured neuronal networks. However, in order to understand the behaviours of neuronal networks dynamically, the identification of synaptic activities of individual neurons is crucial. In this study, we observed individual synaptic activities by utilizing low density neuronal networks arranged orthogonally on MEA’s.

1 Aligned microcontact printing
Rat hippocampal neurons were finely patterned by soft lithography technique on microelectrode array fabricated as previously reported. The polydimethylsiloxane (PDMS) stamp was moulded from the stamp master designed to match the patterns of MEAs as depicted in Fig. 1. The fabricated PDMS stamp has the pattern of 2μm-wide line and 20μm circles at the crossing points. The circles were intended to induce the attachment of neuronal cell bodies for recording neural activity through the underlying electrodes. Flourescein isothiocyanate (FITC) labeled poly-L-lysine (PLL) was inked to the PDMS stamp for 1 hour after dissolved at 1mg/1ml in borate buffer solution. After blowing off PLL, the PDMS stamp was mounted at the custom-made alignment tool and made to contact with the surface of the MEA for more than 1 hour.

2 Electrophysiology
From rat hippocampal neural networks cultured on PLL-printed MEAs, signal propagations were identified as a proof of the synaptic connectivity, for the first time to our knowledge, from MEA-based neuronal networks. When cells were plated at 200cells/cm², spontaneous activity could be recorded as early as 7 days in culture. Recordings were made for as long as 71 days. Electrical stimulation (200μA current step, 50μs pulse width) was also used to evoke activity (Fig. 2). It was also shown that activities were evoked by chemical stimulations of high-K⁺ solution.

3 Immunocytochemistry and Scanning Electron Microscopy
After a series of electrophysiological experiments, the neurons were exposed to a mixture of rabbit polyclonal anti-MAP2 and mouse monoclonal anti-synaptophysin. In order to identify these primary anti-
bodies, Texas Red goat anti-rabbit and Alexa 488 goat anti-mouse were used as secondary antibodies. MAP-2 is a maker for the identification of the neuronal cell bodies and dendrites, and synaptophysin is one of the presynaptic proteins which show the locations of the synapses. After the immunostaining, cells were applied with DAPI for staining nuclei. Presynaptic proteins (Fig.3b) were located along neurites and near cell bodies (Fig.3c). It shows that synapses have been developed between neurons successfully in this patterned neuronal network. After critical point drying process, scanning electron microscopy was followed to identify the cells directly.

![Immunohistochemistry images](image)

**Fig. 3** Immunohistochemistry images for (a) dendrites, (b) presynaptic proteins, (c) nuclei, and (d) merged image

### 4 Summary

Neurons were successfully cultured in low density for long term survival. From the neuronal networks cultured in low density, propagation of signal along the network was recorded for the first time. The evoked potentials were also observed following electrical current stimulations. The formation of synapses and the location of cell bodies were identified by immunocytostaining.

![SEM image](image)

**Fig. 4** SEM image of a patterned neural network

### Acknowledgement

This work was supported by the International Collaboration Program, NBS-ERC (Nano Bioelectronics and Systems Engineering Research Center)/KOSEF (Korea Science and Engineering Foundation) and NIH, NS-044287, NSF, ECS-9876771.

### References

Added astroglia promote greater synapse density and higher activity

Gregory J. Brewer1,2*, Michael D. Boehler1, Bruce C. Wheeler3

1 Medical Microbiology, Immunology and Cell Biology, 2 Neurology, Southern Illinois University School of Medicine, Springfield, IL, USA 3 Bioengineering Department and Beckman Institute, University of Illinois at Urbana-Champaign, Beckman Institute, Urbana, IL, USA

*Corresponding author. Email gbrewer@siumed.edu

Dissociated neurons grown in serum-free Neurobasal/B27 medium have lower numbers of astroglia and lower spike rates than cultures in serum-containing media. Since astroglia from serum media tend to occlude electrodes on an MEA, we developed techniques for adding astroglia to established neuron cultures. Added astroglia increased synapse density and facilitated higher spike rates for many elements in the network.

1 Rationale

Astroglia are known to potentiate individual synapses, but their contribution to networks is unclear. Here we examined the effect of added astroglia on entire networks of cultured rat hippocampal neurons on microelectrode arrays in serum-free Neurobasal/B27.

2 Effects of added astroglia on spike rates

Added astroglia increased spontaneous spike rates nearly two-fold and glutamate-stimulated spiking by 6-fold (Fig. 1A), with desensitization eliminated for bath addition of 25 μM glutamate (Fig. 1B). Addition of the GABA antagonist bicuculline also increased spike rates but with no difference between networks without or with added astroglia, which suggests that networks without added astroglia were greatly inhibited. The number of active electrodes was also increased by 50% with addition of astroglia. In all conditions, the log-log distribution of spike rates fit well to linear Levy distributions over 3 orders of magnitude (Fig. 2). Networks with added astroglia were consistently shifted toward higher spike rates.

3 Effects of added astroglia on cell numbers

Immunostaining for GFAP revealed a linear increase with added astroglia (unable to show color). Addition of astroglia also increased neuronal survival. Without addition of astroglia, after 22 days in culture, the number of astroglia nearly equalled that of neurons.

4 Effects of added astroglia on synaptophysin puncta

By counting synaptophysin immunoreactive puncta, an increase of 70% was observed with added astroglia (Fig. 3), which correlated with an increase in immunoreactive synaptophysin puncta.

5 Conclusion

Together, these results suggest that added astroglia increase synapse density and facilitate higher spike rates for many elements in the network.

Acknowledgement

Supported in part by NSF EIA 01328, NIH R01-EB00786 and RO1 NS052233.
Fig. 2  A) Spike frequency distribution shows a non-Gaussian peak with only one tail.  B,C,D) Log-log (Levy) distributions of networks without added astroglia (o) and with added astroglia (■).  (B) Levy distributions for initial condition,  (C) after bicuculline and  (D) after glutamate.  In all cases, cultures with added astroglia show a distribution shift to higher frequencies.

Fig. 3 The concentration of synaptophysin puncta increased more than 70% as more astroglia were added (p=0.008).
Optimized Oxygen Availability and Signal-to-Noise Ratio in Brain Slice Recordings with Perforated Microelectrode Arrays

Ulrich Egert1,2*, Samora Okujeni1, Wilfried Nisch3, Karl-Heinz Boven4, Ralf Rudorf4, Norbert Gottschlich5, Alfred Stett1

1 Bernstein Center for Computational Neuroscience Freiburg, Albert-Ludwigs University, Freiburg, Germany
2 Neurobiology and Biophysics, Inst. Biol. III, Albert-Ludwigs University Freiburg, Freiburg, Germany
3 NMI Natural and Medical Science Institute, Reutlingen, Germany
4 Multi Channel Systems GmbH, Reutlingen, Germany
5 Greiner BioOne GmbH, Frickenhausen, Germany
* Corresponding author. E-mail address: egert@biologie.uni-freiburg.de

Acute brain slices with accessible and well-preserved neuronal microcircuitry have become the most widespread preparation that can also be recorded with microelectrode arrays (MEA) for spike activity and local field potentials. The supply of O2 and nutrients is, however, considered limiting for tissue stability and viability and slice thickness. MEAs record on the face of the slice not directly exposed to the stream of buffer, which could become critical. We therefore developed perforated MEAs (pMEA) for recording and stimulation to provide a second exchange surface, and compared local O2-partial pressure (pO2) and pH in cerebellar (CB) brain slices on these pMEAs to standard MEAs. On impermeable MEAs, pO2 decreased linearly with depth in the tissue. Added diffusion through the perforated MEA surface decreased the slope of the pO2 gradient as well as the minimum level reached within the tissue. This led to U-shaped pO2 and pH profiles. Improved supply thus allows the preservation of larger networks in thicker slices, i.e. more complex and in-vivo-like networks.

1 Introduction

In the neurosciences, acute brain slices with accessible and well-preserved neuronal microcircuitry have become the most widespread preparation for in vitro studies seeking to investigate cellular and circuit properties, as well as their modulation by neuroactive drugs. They are used to study the dynamics of activity in neuronal networks, synaptic plasticity, pathological conditions such as epilepsy and for high-content drug assays in pharmaceutical research.

Typically, brain slices are prepared freshly for each experiment at 300-450 μm thickness and require efficient superfusion of buffer to supply O2, CO2, and stabilize pH in the tissue. They can be maintained in a submerged configuration, i.e. with buffer flowing above the slice, or in an interface configuration, with buffer supply from below only and exposure to humidified carbogen gas (95% O2/5% CO2) from above. Submerged slices can be homogeneously exposed to the buffer stream, and to drugs supplied via the buffer. They are thus more widespread for pharmaceutical testing. In either preparation, O2 and nutrients are, however, supplied by diffusion only (usually from one side) and are therefore considered limiting for tissue stability, viability, and slice thickness. Furthermore, a steep gradient of O2 etc. availability is assumed. Data on O2-availability within the tissue were, however, not available until now.

Since MEAs record on the face of the slice not directly exposed to the continuous stream of buffer, diffusion rates and slice thickness are even more critical than in single electrode configurations. Increasing the exchange surface would stabilize the recording conditions, in particular for tissues with high metabolic activity, e.g. under elevated temperatures or the application of excitatory drugs. Wash-in and wash-out times for drugs under test should decrease.

One previous report indicated extended tissue survival on pMEAs [1], though without supplying further data on the limiting factors. Following this lead, we developed new pMEAs and compared supply and tissue viability for impermeable and pMEAs.

2 Methods

We developed three types of MEAs and investigated the O2-supply: (1) no perforation (60 TiN electrodes on glass, Fig. 1A) [2, 3], (2) with 4% perforation area (60 TiN electrodes on polyimide, Fig. 1B) and (3) perforation with 28% exchange surface (20 TiN electrodes on polyimide, Fig. 1C). For each array we determined the depth profile of the local pO2 in CB and hippocampal (HC) brain slices under various perfusion conditions.
A

B

C

Fig. 1 MEAs with 60 (A, B) resp. 20 (C) recording electrodes (Ø 30 µm, TiN). Electrodes and leads (Au) are bright; the perforation of the polyimide foil in B and C is dark. The perforation comprises 4% (B) resp. 28% (C) of the relevant area. Electrode pitch in the recording region is 200 µm (A, B) and 90x200 µm (left frame in C). Separate low-impedance stimulation electrodes were added in C (right frame).

pMEAs were produced with thin-film photolithography on polyimide membranes (6 µm thick), which were glued to a glass or polycarbonate carrier with Sylgard (Dow Corning). pMEAs with 4% exchange surface (MEA type 2) had the same electrode type and layout as impermeable MEAs. pMEAs with 28% exchange surface (MEA type 3) with Ø 30 µm electrodes for recording and Ø 50 µm electrodes for stimulation. All electrodes were coated with columnar TiN to increase the electrode capacitance.

CB and HC brain slices were prepared as described previously [2]. Briefly, slices of 300 µm thickness (400 µm for HC slices) were prepared from P18-P23 rats and maintained in artificial cerebrospinal fluid. The slices were mounted onto MEAs (rec. electrode Ø 30 µm; Multi Channel Systems, Reutlingen) coated with cellulose nitrate and were superfused with 8 exchanges of carbogen-saturated buffer in the recording chamber per minute (4 ml/min). Spontaneous spike activity was recorded with an MEA1060 system (Multi Channel Systems) at 25 kS/s (room temperature). CB slices were placed onto the MEA such that all layers of the CB lobes could be monitored for spontaneous spike activity. HC slices were positioned to record local field potentials in CA1 in response to and electrical stimulation of the Schaffer collaterals via the MEA electrodes. pO2 and pH were determined with fiber-optic probes with a polymeric sensor (tip diameter Ø 30/50 µm; presens, Regensburg; Fig. 2A) inserted into the tissue.

3 Results

Spontaneous neuronal activity was recorded in CB slices with all MEA types without significant differences with respect to spike shape and firing rate. O2 availability within the slice, however, differed considerably and depended on MEA type, temperature, perfusion configuration, firing rate and tissue type.

On impermeable MEAs (MEA type 1) pO2 decreased linearly from approx. 800 hPa to 200-400 hPa at 300 µm (electrode level) within the tissue (Fig. 4A). Slope and minimal pO2 depended on the superfusion rate, tissue type (influencing metabolic O2 consumption) and neuronal activity level (Fig. 2B). Blocking spike activity with tetrodotoxin and thereby reducing the energy needs of the neurons increased pO2.

A second buffer stream (termed subfusion for convenience) through the perforation was driven by a low vacuum applied to a chamber below the recording area. The subfusion rate increased with the vacuum applied.

With 4% perforation surface in which the tissue was exposed to the subfusion stream in the recording area (MEA type 2), the pO2 gradient did not change significantly. SNR of single neurons spikes, however, increased greatly to levels rarely found with type 1 MEAs even when only slight suction was applied (Fig. 2C), presumably because of decreasing distances between cells and electrodes, and thus increasing seal resistance between the recording site and the reference electrode. This exchange surface was sufficient to
permit chemical stimulation of neuronal activity by increasing extracellular K\(^+\) to 6 mM.

In contrast, the pO\(_2\) gradient decreased by >50% with 28% perforation area (MEA type 3) and superfusion through a micromachined channel beneath the recording area. Concurrently, the minimal pO\(_2\) increased considerably, absolute values depending on perfusion rate (Fig. 4B). In slices 400 µm thick the position of the minimum moved upward and a U-shape pO\(_2\)-depth profile became apparent. Overall pO\(_2\) was much more homogeneous.

Concurrent with the pO\(_2\) the pH in the tissue decreased with depth. Absolute decrease and slope diminished with improving perfusion through the perforation. On normal MEAs pH dropped up to 0.5-1 units. On type 3 pMEAs this was reduced by approx. 50%.

The electrode arrangement of type 3 MEAs is designed to optimally match the structure of slices from the HC of rats and mice at age P8-P28. Using this array we could effectively stimulate Schaffer collaterals and elicit synaptic field potential responses in CA1 at SNRs comparable to or better than in recordings with type 1 MEAs. Using slight suction also simplified positioning and holding the slice onto the electrodes. Furthermore, compared to standard MEAs this added perfusion extended the useful lifespan of HC slices under recording conditions with limiting O\(_2\) supply by up to 250%.

`Fig. 3` Overall layout of the type 3 MEA prototype in Fig. 1C. The dimensions of the carrier plate conform to standard microscope slides. A polyethylene injection molded component glued on the electrode foil forms the fluidics connector and the recording chamber. The perfusion channel covered by the PI film is visible below the PE plate and is supplied via the connectors to the left. A fluid guide is inserted into the recording chamber to optimize perfusion across the slice. A reference electrode is integrated into the PI foil.

### 3 Discussion

pMEAs improve the recording conditions in two respects: they can improve the SNR and with suitable layout greatly optimize the O\(_2\)-supply in slice recordings. Superfusion from one side only creates a linear decrease of the O\(_2\)-availability that further decreases with increasing temperature. Sparsely perforated arrays facilitate the recording itself by increasing the SNR when suction is applied to the subfusion channels, which also helps to position the slice accurately. Though suitable to allow chemical stimulation, perfusion via these exchange areas did not contribute significantly to pO\(_2\).
The saturation, minimal level and slope of the pO₂ depth profile of type 3 MEAs with optimized exchange area, however, clearly shows an added supply via the perforation, a more homogeneous distribution of O₂ and pH, as well as an optimized supply in the recording region.

pMEAs thus open new venues for network studies and industrial applications: The increased stability of the tissue facilitates long-term studies, including tissue culture work with closed sterile chambers. Improved supply allows thicker slices and thus the preservation of larger networks, i.e. more complex and in-vivo-like networks. Alternatively, with thin slices a homogeneous pO₂ profile is achieved. Finally, drug accessibility to the recorded cells is improved, accelerating dose-response studies. MEAs with 28% perforation area and HC conformal layout are produced in a compact coverslip format (Fig. 3). Since hippocampal slices are widely used to monitor synaptic plasticity this will increase the throughput in drug screening studies.

Acknowledgements

This project was supported by the BMBF FKZ 16SV1743.

References
Direct electrical interfacing of semiconductor chips with neuronal tissue may lead to novel experimental approaches in brain research and also give rise to hybrid computational devices. Here we report on a time-resolved imaging of the electrical activity in organotypic brain slices from rat hippocampus by multi-transistor-array (MTA) recording on an area of 1 mm² at a resolution of 7.8 µm and 0.5 ms. Brain slices were cultured on the inert titanium dioxide surface of silicon chips fabricated by an extended CMOS process. Upon stimulation in the CA3 region we observed fast propagating waves of negative field potentials which we assign to orthodromic and antidromic action potentials in the mossy fibers and slower transient field potentials of postsynaptic activity in CA3 and CA1 with negative sign in stratum radiatum and positive sign in stratum pyramidale. The transistor signals matched local micropipette recordings of electrical field potentials in amplitude and shape. Direct interfacing of an MTA chip provides a complete observation of neuronal signaling in an extended area of brain tissue. This technique is suitable to elucidate the functionality of planar neuronal systems at a high resolution.
Low cost CMOS multi-electrode arrays

Alex Lyakhov¹, Yevgeny Perelman², Ran Ginosar²*, Shimon Marom³

¹ Intel Corporation, Haifa, Israel
² Technion – Israel Institute of Technology, Faculty of Electrical Engineering, Haifa, Israel
³ Technion – Israel Institute of Technology, Physiology, Faculty of Medicine, Haifa, Israel

* Corresponding author. E-mail address: ran@ee.technion.ac.il

Glass-substrate Multi-electrode Arrays (MEAs) have become a valuable tool in neurophysiological research and drug screening. However, they are typically limited to a small number of electrodes. CMOS technology is a promising platform for the development of next generation MEAs, enabling orders of magnitude more electrodes. Fabrication of CMOS microelectrode arrays reported so far employed standard CMOS processes followed by custom processing steps. We have examined the feasibility of fabricating MEAs using a standard CMOS process with no post-processing steps. It was established that neurons can be successfully cultured on a standard CMOS die with no electrode coating. Stimulating neuronal tissue with current, however, did not succeed, due to metal corrosion. We have also investigated simple post processing steps without photolithography to facilitate stimulation.

1 CMOS Multielectrode Arrays with and without post-processing

Glass-substrate Multi-electrode Arrays (MEAs) have become a valuable tool in neurophysiological research and drug screening [1-4]. However, they are typically limited to a small number of electrodes. CMOS technology is a promising platform for the development of next generation MEAs, enabling many thousands and potentially millions of electrodes. This can enable a substantial increase of the spatial density to over a million electrodes. Tight integration of the recording probes and the amplifying circuitry can also enhance noise immunity in CMOS MEAs. Finally, entire data-processing algorithms can be integrated on the same die with the recording electrodes.

Fabrication of CMOS microelectrode arrays reported so far employed a standard CMOS process followed by custom processing steps to protect the recording electrodes (typically aluminium) from corrosion in the physiological solution [4-8]. While fabrication of CMOS is widely available, post-processing steps make CMOS MEAs proprietary and expensive: A special microelectronic processing facility is required, and lithographic mask sets impact the cost.

We have examined the feasibility of fabricating MEAs using a standard CMOS process with no post-processing steps [9]. It was established that neurons can be successfully cultured, with firing activity recorded for several weeks on a standard CMOS die with no electrode coating. Stimulating neuronal tissue with current, however, has not succeeded, due to the high corrosion rate of aluminium under electric stress. To facilitate stimulation in low cost MEAs, we investigate simple post-processing steps that do not require lithography.

2 CMOS Multielectrode Array Test Chip

2.1 Test Chip Design, Fabrication and Packaging

A CMOS MEA test chip was fabricated with a standard 0.35μm mixed signal CMOS process (Fig. 1). It was encapsulated in a 120-pin ceramic pin-grid-array package, with a round glass bath mounted on top of the die (Fig. 2).
The chip included circuitry for DC stabilization of the electrode potentials, analog amplification of the recorded signals, analog buffering of the amplified signals out of the chip (Fig. 3) and temperature control. The analog circuits follow those presented in [10]. Bonding wires were isolated with Sylgard-184, a transparent silicone elastomer (Fig. 4). EpoTek epoxy isolation was also tried, but resulted in cracks that developed during curing (Fig. 5) and damaged the bond wires.

2.2 Coating and Electrical Stress

The chip was tested electrically and found completely functional. Dielectric electrode coatings (not requiring lithography) of various combinations of Al₂O₃, TiO₂ and HfO₂ were applied with an electron beam gun evaporator to several dies. Electrical stress tests were carried out on the dies in a biological solution to verify immunity to electrode corrosion (Fig. 6).

Coated electrodes have shown significantly better corrosion immunity under stress. However, several tested electrodes have performed very unevenly. This was related to the non-uniformity of the evaporated dielectric layer (Fig. 7, 8).

2.3 Culturing Neurons and Recording

Rat cortical neurons were cultured on top of the uncoated test chip for six weeks (Fig. 9). Neuronal signals have been successfully recorded once every two weeks (Fig. 10, 11). After about six weeks some electrodes have deteriorated.
3 Summary

In conclusion, it may be feasible to conduct short term neurophysiological experiments on low cost uncoated CMOS MEAs. Further study is required for low cost non-lithographic thin isolation coating.

Acknowledgement

We thank the staff of Neurophysiology lab, Faculty of Medicine, Technion for helping us with MEA experiments.

References

Electronically Switchable Surfaces for Microelectrode Array Applications

J. Vörös*, C.S. Tang1,2, B. Städler1, M. Halter1, M. Bally1, D. Grieshaber1, M. Gabi1, B. Keller2

1 Laboratory of Biosensors and Bioelectronics, Institute of Biomedical Engineering, ETH Zurich, Switzerland;
2 Swiss Federal Laboratories for Materials Testing and Research (EMPA), Dübendorf, Switzerland
* Corresponding author. E-mail: janos.voros@biomed.ee.ethz.ch

Motivations
The success of biological sensor systems, such as microelectrode arrays (MEA) critically depends on the ability to interact with the biological environment. The bioreponse is often determined by the properties of the biointerface (i.e. the outermost layer of the biosensor) which requires the precise control of this interface on the micron- and nanometer scale.

1 Switchable Surface Functionalization of Selected MEA Electrodes
We have developed electrically responsive, “smart” surfaces based on self-assembled monolayers, poly(ethylene glycol) grafted polyelectrolytes and polyelectrolyte multilayers that can be used to interface biology in a novel, dynamic way. For both molecular systems, functional groups are directly introduced into the molecule. Such functional groups are shown to have three advantages: a) they enable production of well-defined and stable surfaces; b) they can be functionalized with biologically relevant reactive groups such as cell-specific peptides, lipid bilayers, drugs, or growth factors; and c) they can be combined with (nano)lithography techniques producing patterns with different surface chemistries on the submicron scale.[1] Recently, we have put a lot of efforts into achieving not only spatial but also a dynamic control over the surface biochemistry of selected individual microelectrodes. Such surfaces that change upon external stimuli provide us with new research tools for studying complex biological systems and to overcome difficulties in producing heterogeneous microarrays of fragile biomolecules. Highlights for the use of novel, electronically [2] - or photo-active [3] surfaces for MEA applications will be presented. (See Figure 1.)

2 Local Drug Delivery with Electronic Control
We also report a novel method for the electrochemical dissolution of polyelectrolyte multilayers from the surface of an electrode for applications in controlled drug delivery.[4] Biodegradable and bio-compatible multilayers films based on poly(L-lysine) and heparin were selected as model system and built on an indium tin oxide semi-conductor substrate. We followed the buildup and the dissolution process of the multilayers by electrochemical optical waveguide lightmode spectroscopy. (See Figure 2) The formation and stability of the polyelectrolyte multilayers were found to depend on the applied potential and the ionic strength of the buffer. The application of potentials above a threshold of 1.8V induced dissolution of the polyelectrolyte multilayer film following a single exponential kinetics. The rate of this process could be varied by an on-off profile of the potential leading to a controlled release of heparin into the bulk. Atomic force microscopy investigations showed that the electro-dissolution of the polyelectrolyte multilayers is a local phenomenon which leads to the formation of nanoporous films.

We intent to use this novel electronically controlled drug releasing mechanism for the in situ chemical stimulation of cell cultures on a MEA.

References
Fig. 1 Principles of the use of electronically controllable surfaces for microarray functionalization: a) Commercially available ITO micro-electrode array (MEA) from Ayanda Biosystems. (The SEM image shows the 1x1mm2 active area of the chip.) b) The loss of fluorescent intensity, due to the electronic removal of a protein resistant, fluorescently labeled graft copolymer (PLL-g-PEG633) upon a 1.8V applied potential, follows exponential kinetics with a 8.2s time constant. c) CLSM images of the electrically activated spot of the microelectrode array before and after the removal of the PLL-g-PEG633. d) Schematics of the surface architecture: the ca. 5μm thick SU8 film surrounds the spots and insulates the connecting ITO wires. e) Snapshots of a movie demonstrating the use of the MEA: 1) Two bottom electrodes are already functionalized with green-labeled lipid vesicles. 2) The protecting PLL-g-PEG633 coating is removed from the electrode in the top right corner. 3) The electrode is backfilled with the labeled vesicles. f) Schematics of the top right electrode at step 2 and 3. The corresponding CV curves show the presence of an insulating barrier layer of vesicles and/or a bilayer.

Fig. 2 Build-up and electrochemical dissolution of a poly-electrolyte multilayer monitored by an in situ optical biosensor. Heparin was used as model drug in order to show the feasibility of precise drug delivery under electronic control.
Stimchip - a multichannel ASIC for programmable in real time stimulation of neural cells using MEAs

Pawel Hottowy, Wladyslaw Dabrowski*, Andrzej Skoczen

Faculty of Physics and Applied Computer Science, AGH University of Science and Technology, Krakow, Poland
* Corresponding author. E-mail address: W.Dabrowski@ftj.agh.edu.pl

We present a 64-channel Application Specific Integrated Circuit (ASIC) for stimulation of neural tissue. The chip is capable to generate independent stimulation waveforms in all channels with time resolution of 10 μs. The stimulation signals of amplitudes variable over a wide range can be applied either in current or in voltage mode. Each channel is equipped with an artefact suppression circuit.

1 The Stimchip design

1.1 Specification requirements

Investigations of some aspects of information processing in alive neural networks requires simultaneous and independent stimulation and recording of multiple sites of the network using multielectrode arrays. Such systems including total number of channels as high as a few hundreds requires using VLSI technologies and integration of at least a few dozens of independent stimulation circuits in one chip.

Based on our experience with electrical stimulation using MEA [1] and works of others we have specified the following requirements for the Stimchip:
- ability to generate bipolar stimulation signal in each channel independently of other channels,
- temporal resolution of 50 μs,
- selectable current mode or voltage mode,
- selectable ranges of current amplitude,
- control of signal levels with a precision of ~1%,
- output voltage range -1.5V to +1.5V,
- stimulus artefacts suppressor in each channel.

1.2 Design overview

A simplified block diagram of the Stimchip is shown in Fig. 1. The chip consists of 64 channels and a logic block. Each channel is equipped with a 7-bit digital-to-analogue converter, which controls in real time the output signal waveform. The data for the DACs, as well as the control signals for the artefact suppression circuits are sent through a 4-bit bus clocked with a nominal frequency of 5 MHz. The signal range and the mode (current or voltage) can be defined for each channel independently. Operating configuration of each channel is controlled by commands sent to the logic block through single serial command line. Each chip has 5-bit identification address so that up to 32 chips in a system can be controlled by one common command line.

1.3 Channel functionality

A simplified functional schematic of a single channel is shown in Fig. 2. The typical stimulation protocol is as follows:
- disconnect the recording system from the electrode,
- apply a stimulation waveform,
- discharge the electrode to the previously sampled DC electrode potential (optional),
- connect again the recording system to the electrode for recording neuron response.

Fig. 1 A block diagram of the Stimchip ASIC

Fig. 2 Functional diagram of single channel. Real time control signals are underlined.
The stimulation waveform can be applied either in a current or in a voltage mode. In the current mode the output current is controlled by 7-bit DAC within each one of eight selectable ranges: 60 nA, 250 nA, 1 µA, 4 µA, 16 µA, 64 µA, 250 µA and 1mA. The output current is sent directly to electrode in the current mode, or to a current-to-voltage converter with a transresistance of 24 kΩ, producing a voltage output signal. In the voltage mode electrode DC voltage can be sampled before stimulation and added to the generated waveform so that the levels of stimulation signals are corrected for the electrode offset.

Given that the Stimchips are foreseen to be mounted in the immediate vicinity of microelectrode arrays, power dissipation is a critical parameter. Depending on the output current range the chip dissipates between 3 mW and 5 mW per channel.

1.4 Layout

The chip has been designed and fabricated in a 0.35 µm CMOS technology. The pitch of the input and output pads is 100 µm. Single channel occupies area 0.18 mm² and the total die area is 6.4×2.8 mm². The mask layout is shown in Fig. 3.

2 Test results

Initial evaluation testing of the chip has been performed using a dedicated test set-up. Two naked Stimchips and an array with 5 µm diameter platinized microelectrodes [1] were mounted on a printed circuit board (PCB). The input and output pads were wire bonded directly to traces on the PCB and the connection to the MEA were made using a zebra connector.

Figure 4 presents the exemplary stimulation waveforms recorded at the stimulation outputs connected to the MEA. Although our minimum requirements for temporal resolution was 50 µs, the prototype works well for the real-time data transfer up to 20 MHz. Thus, 64 stimulation waveforms can be controlled independently with a time resolution as good as 10 µs.

Accordine to design requirements the voltage across the load impedance should not exceed +/-1.5V. Within this range the maximum deviation of the DAC response curve from the linear fit is below 1.5LSB for any current or voltage amplitude range and any load impedance. Figure 5 shows a family of voltage response curves, superimposed on an external DC offset changing from -0.5V to +0.5V. Measurements performed for 32 channels show very good uniformity of DAC slope, with standard deviation below 1% for any current or voltage amplitude range.

3 Outlook

The results of initial tests performed on the Stimchip ASIC prove that the prototype meets all requirements. In a future the chips will be used for extension of the existing 512-channel recording system [2], adding independent stimulation circuit for each channel. The system is foreseen to be used for research on acute brain slices and continuation of the work on high resolution retinal prostheses [1].

Acknowledgement

Work supported by the Polish Ministry of Education and Science - Project no. 3 T11E 011 27.

References

Magnetic Stimulation and Depression of Non-homogenous Networks in Primary Neuronal Cell Cultures

Jochen F. Meyer1*, Guenter W. Gross2

1 Department of Medical Electronics, Technical University Munich, Munich, Germany
2 Department of Biological Sciences, University of North Texas, Denton, TX, USA
* Corresponding author. E-mail address: meyer@tum.de

Transcranial magnetic stimulation has been used for brain mapping, diagnostics, and clinical treatments of disease for about twenty years. However, the coupling of induced magnetic and electric fields with neurons in gray matter is not well understood, and there is little information on optimal stimulation parameters. For this purpose, magnetic stimulation of neuronal networks in culture provides an efficient test environment, allowing use of smaller coils and less restricted electronic circuits. The coils were fed stimulation pulses by a HiFi mono amplifier with input signals from a PC sound-card, so that stimulation pulses of any frequency and shape could be used. Pulses were rectangular and 500 µsec long. Both single pulse and bursting stimulation patterns were used, with the latter being the more effective. Both spinal cord and frontal cortex cultures were used which showed no obvious response differences. For evaluation of response categories, spike activities of all channels were averaged in one-minute bins and plotted as a function of time. Activity depression generally resulted either from decreased spike production or a temporary loss of active units. Although averaged network responses were repeatable, individual units showed excitatory and inhibitory unit-specific responses. Disinhibition of cell cultures with bicuculline or strychnine had a great influence on the outcome of stimulations.

1 Introduction

Although much speculation has been raised about the possible mechanisms underlying the effects of transcranial and peripheral magnetic nerve stimulation, there is still considerable uncertainty about what really happens to the cells when exposed to alternating electromagnetic fields. Furthermore, there is not enough quantitative information on effects of different stimulation patterns and the associated mechanisms. This study represents an attempt to directly quantify the effects of alternating magnetic fields in the field strength range of commercially available TMS-stimulators and -coils with several different stimulation patterns of intensities of 0.5 - 100 pulses/sec. Commercially available TMS-stimulators and coils cannot repeat their stimulation pulses at frequencies exceeding 30-40 Hz. Furthermore, there is a restriction in the maximum total application time of these high intensity stimulations. In this study, these restrictions were overcome and possible mechanisms are discussed.

2 Materials and Methods

2.1 Cell Cultures

Frontal cortex (FC) or spinal cord (SC) neurons were collected from dissected embryos of 15-16 days old, timed pregnancy, mice. Cells were mechanically and enzymatically dissociated and then seeded on poly-d-lysine coated glass microelectrode array plates (MEAs). SC cultures used Minimum Essential Medium (MEM) with 10 % horse serum (HS) and FC cultures used Dulbecco’s Modified Eagle Medium (DMEM) with 5% HS Cultures were incubated at 37 °C under a 10% CO2 atmosphere for at least 4 weeks until mature networks had developed. 50% medium changes were performed twice a week.

During experiments, cell cultures were kept alive by means of a closed chamber setup with constant flow of heated, pH- and osmolarity-controlled media, at flow rates of ~30 µL/min. The cell chamber consisted of an aluminum base plate with attached heating resistors and a stainless steel chamber with a 100 µm thick, 2 cm wide circular glass window glued to the bottom of the chamber with biocompatible silicone sealer. The cell chamber setup was placed on a heated inverted phase contrast microscope stage for optical control.
2.2 Stimulation Environment

The source of the stimulation signal was a Velleman 200 W mono amplifier fed by a standard PC soundcard output. The stimulation files were in the .wav format and designed by the author with the sound editing program Goldwave. They were replayed by free winamp software.

The design of the stimulation coil was based on the intention of having a maximum magnetic flux density (and thus a high electric field strength). Several different coil geometries were simulated by means of finite element electromagnetic CAD software (EM Studio, CST GmbH, Darmstadt, Germany). According to the simulation results, a design was chosen that allowed the cell culture to be placed between the poles of the coil. Thus, a uniform high field exposure of cells located above the recording matrix was achieved. Figure 1 shows the custom made stimulation coil. The wiring was applied in such a way that the current direction was the same for both the upper and the lower pole of the coil, ensuring that the alternating fields of both poles did not annihilate each other. The picture to the right details the magnetic field simulation at the site of the cell culture.

Equal numbers of the same stimulation pattern were applied to cultures in both native and disinhibited (with bicuculline and/or strychnine) cultures. These patterns were either made up of single pulses repeated at 0.5-5 Hz, or pulse trains (“bursts”) which consisted of 5, 10 or 20 pulses at repetition rates of 30-300 Hz within one burst. Bursts were, again, repeated at 0.5-5 Hz. The single pulses were generally 500 µs long, rectangular and biphasic. Rise and fall times were always around 1-2 µs.

2.3 Elimination of Artifacts

In the experimental setup discussed here, there were a large number of potential artifacts that could have obscured the accuracy of the results. To prevent these, stimuli were plotted in the online Rasputin window as unsorted waveforms but not discriminated and recorded, except for one channel for later control of start and end points of stimulation episodes. At pulse frequencies above 300 Hz, occasional dropouts were observed in the sorted waveforms occurring at the same time as stimulation bursts. Hence, only pulse frequencies up to 300 Hz were used. In a temperature test temperature elevations to 41 °C were only measured for stimulation doses >30000 pulses and at burst frequencies of 5 Hz. In a calculation of the currents induced by the magnetic fields in the ITO, it was found that the resulting voltage between two conductors could not reach values high enough to stimulate the neurons electrically. The calculated voltages were compared with values from earlier publications [1]. For long-term stability of the reference activity, a closed chamber setup with a slow, constant laminar flow was selected. A flow rate of 25 µL/min was chosen to allow for continuous removal of potentially toxic metabolites with relatively low shear forces. In this manner, the cells were kept active over a period of 3-4 days, so that long recovery times between stimulations could be used.

3 Results

The effect of the magnetic field did not elicit immediate spiking, as is characteristic of electrical stimulation. Rather, it resulted in a slow cumulative activity suppression or excitation with onset times varying between 10 seconds and several minutes. Consequently the effect is subthreshold and may be elicited by enhanced or suppressed synaptic activity. Stimulation effects were generally dose-dependent. Figure 2 shows three consecutive episodes with the same intensity (5 Hz burst frequency, 200 Hz pulse frequency) but increasing numbers of applied pulses (2,500, 10,000, 15,000). Clearly, the maximum effect (suppression of activity) increased nearly linearly with dose.

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Additionally, a rebound excitation effect was usually seen, shortly after the end of the stimulation and increased in amplitude and duration with increasing pulse number. Effects from all three stimulation protocols were 100% reversible. The number of bursts per minute (blue data points) varied similarly indicat-
ing that the reduction of spikes is linked to the reduction in burst rate. Green bars show start and end of the stimulations.

Stimulations with single pulse patterns were generally much less effective than bursting stimulations. The observed effects, as depicted in Figure 3, are a good example for a strong temporary depression caused by a bursting stimulation episode (1 Hz burst repetition frequency, 100 Hz pulse frequency, 10,000 applied pulses total) followed by two single-pulse stimulations (3 Hz and 5 Hz pulse frequency, respectively; 2500 total dose each).

![Fig. 3 Bursting stimulation (1) followed by two single pulse episodes (2, 3) in the same cell culture. Spike rate/min: red; burst rate/min: blue; stimulation start/end: green bars.](image)

Another interesting result of the present study was that the responses were different in normal and disinhibited cultures. Under disinhibited conditions (blockage of inhibitory circuitry with bicuculline or strychnine), the networks' reactions were 59% excitation, 39% depression and 10% no effect, as opposed to 14% excitation, 76% depression and 10% no effect under native conditions with the same stimulation numbers and patterns. This observation suggests that the applied magnetic fields generally affect both the inhibitory and excitatory circuits, but that inhibition normally dominates. Whenever they are blocked, the enhancement of activity in excitatory circuits dominates.

To quantify the observed effects, the data were divided into categories and then analyzed with respect to which parameters were significant for maximum response, onset time and recovery time. All the episodes resulting in a temporary increase in activity were grouped in category one, all the temporary depressions in category two. The variable stimulation parameters were number of bursts per second, pulse frequency within the bursts, number of pulses per burst and total number of applied pulses. For the experiments performed, we used not the total number of pulses applied, but the number of pulses required to reach maximum effect. The bursts/sec and the number of pulses to maximum effect were identified as the most significant parameters for a dose-response-display of the obtained data (Figure 4), since the number of pulses per burst and the number of bursts per second are redundant if the total dose is plotted in the same diagram. Also, varying the burst frequency between 1 and 5 Hz usually resulted in larger variations of activity change than varying pulse frequency.

![Fig. 4 Maximum stimulation (inhibition and excitation) effects in percent of reference activity observed in a particular culture.](image)

4 Discussion

The use of TMS as a diagnostic and therapeutic tool for clinical applications is primarily based on trial and error and empirical data. Unlike the peripheral magnetic stimulation, where nerve bundles are stimulated and action potentials are elicited directly, there is no satisfying theory on the mode of action of low-frequency electromagnetic fields on heterogeneous neural networks as found in mammalian gray matter. The effects observed here are clearly sub-threshold and, un-like electrical stimulation of neural cell cultures, do not consist of direct network responses or long-term entrainment of activity patterns. The effects were relatively slow (onset times between 10 seconds and several minutes) and generally exhibited dose dependence. Out of the stimulation variables (total applied dose, number of pulses per burst, burst frequency and pulse frequency), the pulse frequency was the least significant one, although effects of its variation need further examination. We observed strong intra-culture repeatability, even after a sequence of several stimulations with different parameters.

This intercultural variability may have several reasons. Cell cultures do have different numbers of intact and fully differentiated neurons, different cell densities, lengths of the axons, treatment with different concentrations of serum during maturation, etc. Also, we do not know yet, whether the age of a culture plays a role in the outcome of these experiments. For future investigations, there has to be greater emphasis on normed cell culture parameters and conditions. However, one obvious result of this study is that within one culture, the variation of doses and intensities, i. e. bursts per second, has clear relations to their
effects. This can be seen in Figure 1, where the same burst and pulse frequencies (thus the same stimulation intensities) caused the same type of a response, but the maximum effect achieved rose almost linearly with the number of applied pulses.

There exists a wide range of studies and publications on the biological effects of (electro-) magnetic fields. Essentially, what triggers any kind of cellular reaction must be a combination of the Lorentz force, which the magnetic component exerts on charged particles, and the electric field component that results in small ionic currents inside and between the cells. A large variety of possible mechanisms of electromagnetic field interaction with cellular substrutures has been proposed, among these conformation changes of (transmembrane) proteins [3], changes in calcium concentrations and oscillations through redistribution of calcium in intracellular stores [4], phosphorylation and activation of stress-activated protein kinase [5], alteration of gap junction function [6], induced membrane potential changes [7], cell surface receptor redistribution and cytoskeletal reorganization [8]. None of the proposed hypotheses could be sustained or disproved here, but we think that this approach will become a powerful tool to identify primary mechanisms.

The coil used induced a circular electrical field and thus circular currents in the cell medium and tissue. Since the neurons were distributed randomly on the glass chip surface, the electric field could not affect each cell in the same way. This correlates well with the observation of partly non-homogenous, unit-specific reactions. For future studies, we will have to simulate the induced electric field and its gradient in greater detail. That way, we will be able to correlate heterogeneities in the electric field with unit-specific reactions, which will help narrow down the list of possible mechanisms.

The results of this study show that the presented novel stimulation setup serves as an efficient tool to quantify changes in neural activity during and after exposure to alternating electromagnetic fields. With the experimental configuration, these fields can be of virtually any pulse shape, bursting configuration and dose up to a certain limit at which thermal effects obscure the electromagnetic field effects. This is possible because of the considerably lower energy needed to penetrate the thin cell culture as opposed to cortical tissue that is surrounded by bone and strong connective tissue. A coil cooling system can be implemented in the future.

References

Higher activity with increased connections in patterned hippocampal neurons

Gregory J. Brewer¹,², Michael D. Boehler¹, Bruce C. Wheeler³

¹ Medical Microbiology, Immunology and Cell Biology
² Neurology, Southern Illinois University School of Medicine, Springfield, IL, USA
³ Bioengineering Department and Beckman Institute, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Toward the long-term goal of growing defined live neuron circuits, we started with square patterns of poly-lysine aligned and stamped onto an MEA so that the nodal intersections were on top of electrodes. Increasing the number of patterned connections from 4 to 6 or 8 per node increased spontaneous activity above that for random cultures. This connection-dependent activity suggests that hippocampal neurons are designed to produce higher outputs with multiple inputs, an essential feature of associative learning and memory.

1 Rationale

We found square patterns of neurons produced low spontaneous spike rates compared to random cultures. Here we determined whether forced increases in the number of connections would further increase firing of spontaneous action potentials.

Fig. 1 Increase in forced connections by patterned growth of neurons increases spontaneous firing of action potentials. The number of forced neuronal connections on a square grid of electrodes was varied from A) four to B) 6 to C) 8 connections compared to D) an unpatterned control of neurons cultured from 19-23 days. In E) mean spike rate for each of 6 electrode arrays is significantly higher as a function of number of connecting paths allowed between electrodes.
2 Controlled patterns of neuron growth

Patterns of neuron growth were created by stamping adhesive poly-D-lysine as 10 um wide lines connecting the electrodes of a 60 element electrode array with 200 um spacing with either 4, 6 and 8 connections at each electrode (Fig. 1A-C). Stamps were fabricated from PDMS and loaded with poly-d-lysine according to our previous methods (Chang et al. 2003 Biomaterials 24: 2863). Stamps were aligned and poly-lysine was transferred to electrode arrays. Rat E18 hippocampal neurons were seeded onto these patterns or electrode arrays uniformly coated with poly-lysine (random cultures) at constant density. Neurons were cultured in 9% oxygen and 5% carbon dioxide for 19-23 days in Neurobasal/B27 serum-free medium to reduce deposition of stray adhesins in serum-containing cultures. Spontaneous activity was recorded using MultiChannel Systems amplifiers and software.

3 Effects of pattern connections on spontaneous activity

Fig. 2 shows a significant increase in activity as the number of connections increased from 4 to 8 connections per node (n=6/condition). Spontaneous activity for 8-connect patterns was higher than that for random arrays (ANOVA p=0.046).

4 Conclusion

Increasing the number of patterned connections from 4 to 8 per node increased spontaneous activity above that for random cultures. This connection-dependent activity suggests that hippocampal neurons are designed to produce higher outputs with multiple inputs, an essential feature of associative learning and memory.

Acknowledgement

Supported in part by NSF EIA 01328, NIH R01-EB00786 and RO1 NS052233
Progress on the Caged-Neuron MEA Project

Jon Erickson¹*, Gary Chow¹, Angela Tooker², Y-C. Tai², and Jerry Pine³

1 Dept. of Bioengineering
2 Dept. of Electrical Engineering
3 Dept. of Physics, California Institute of Technology, Pasadena, CA, USA
* Corresponding author. E-mail address: erickson@caltech.edu

In order to establish long-term, bi-directional communication with every constituent neuron of a cultured neural network, a caged-neuron MEA—a novel "neurochip"—is being developed. A laser tweezers system is also being developed to rapidly load neurons into neurocages. Here we report on key aspects of these projects.

1 Current Neurocage Design and Fabrication

Our initial device consists of an array of 16 “neurocages” (Figure 1) into which dissociated neurons are placed, one neuron per cage. The cages trap the neuron cell bodies near an extracellular electrode while allowing for normal axonal and dendritic outgrowth and network development over several weeks. Normal growth and development of cultured neural networks on the new neurochip has been regularly demonstrated (Figure 2). Cultured E18 Wistar rat hippocampal cells remain viable (>50% survival at 2 weeks in vitro) and trapped, with virtually no escape from the neurocages.

The parylene based micro-device is fabricated using conventional lithography processes on a silicon wafer. Full fabrication details have been published elsewhere [1]-[3]. Briefly, parylene cages are patterned on aluminum and photoresist sacrificial layers. The insulation layer is either parylene or silicon-nitride. A platinized gold extracellular electrode sits at the base of each cage.

Current pulses passing through the electrode have been demonstrated to reliably stimulate caged neurons. Using voltage-sensitive dye and a CCD recording system, 16 of 18 neurons had identified action potential responses, with a threshold current of 9 +/- 3 uA. Electrical recordings of action potentials have been made in an earlier version of neurochips with a typical SNR of about 50:1 [4].

2 Laser Tweezers for Moving Live Dissociated Neurons

A neurochip with 64 neurocages fabricated on a glass substrate is planned for the near future. Manually loading the neurons into cages is extremely cumbersome, so a better method is required. A laser tweezers system was constructed for transporting live, fully-dissociated neurons into neurocages, and the system was studied extensively [5].

The system consists of an inverted microscope, a 1064nm or 980nm laser module, a beam expander, a motorized mechanical stage, a CCD camera, and steering mirrors. The system uses a single, tightly focused, laser beam to trap a neuron. Once a neuron is

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Fig. 1 SEM of a neurocage. The scale bar is 10 um.

Fig. 2 Nomarski photograph of a neurochip culture, 8 days old.
trapped and lifted, the mechanical stage is moved to locate the neuron above its destination.

The survival of neurons at different laser intensities and exposure times has been studied. The results for 1064nm have shown that the survival of neurons depends strongly on laser intensity but less on exposure time. Preliminary results for 980nm have shown that survival is not dependent on laser intensity or exposure time. We also studied the maximum speed for moving a neuron through the medium at different laser intensities, which was 35 um/s at 17mW.

A preliminary version of the glass-substrate based neurochip has been fabricated. Neurons have been successfully placed into these neurocages using the laser tweezers system (Figure 3).

3 Summary

The development of a novel neurochip and neurocage loading system is nearly complete. This device will enable researchers to study cultured neural networks in new and more powerful ways.

Acknowledgement

We thank Sheri McKinney for expert technical assistance with cell culture; Pat Koen and Jean Edens for beautiful SEMs; Mike Roy and Steve Olson for precisely machined custom parts; NIH for funding this project (grant NS044134-02).

References

Ordered Neural Networks Grown on Patterned MEA

S. Gory-Faure¹, J. Brocard¹, P.O. Amblard², A. Depaulis³, P. Salin⁴, E. Dumas⁵, S. Roth¹,⁵ and C.Villard⁵*

¹ Organisation Fonctionnelle du Cytosquelette, U366 Inserm, CEA Grenoble, France
² Laboratoire Images et Signaux, UMR 5083, Institut National Polytechnique de Grenoble, France
³ Dynamique des Réseaux Neuronaux, U704 Inserm, Grenoble, France
⁴ Physio-pathologie des Réseaux Neuronaux Responsables du Cycle Veille-Sommeil, UMR 5167, Lyon I, France
⁵ Centre de Recherches sur les Très Basses Températures UPR 5001, Consortium de Recherches pour l’Emergence de Technologies Avancées UPS 2070, CNRS Grenoble, France
* Corresponding author. E-mail address: catherine.villard@grenoble.cnrs.fr

The use of photolithography and surface chemistry enables to grow constrained neural networks. Matching such living systems with a MicroElectrode Array will allow to circumvent the drawbacks of conventional cultures on MEA: partial recording of the network activity, partial use of the electrode array or, on the opposite, necessity of a spike sorting step for the electrodes that record more than one neuron. Such model systems are well adapted to the exploration of new dynamical or computational properties of neural networks in relation with their architectures. This is the basis of our project that develops the three axes of cell cultures on patterned substrates, MEA design, and signal recording using an home-made experiment.

1 Patterning and cell culture

1.1 Substrate patterning

Our protocol uses either silanized glass surfaces or polystyrene Petri dishes. Contrast of chemical properties is obtained using classical photo-lithographic steps, including spin-coating of the (silanized) substrate by a positive photoresist, UV exposure step through a mask, and development of the exposed parts of the photoresist.

Glial cells proliferation is stopped by AraC after 2 days (1 mg/ml, Sigma), while a medium coming from a separate culture of these dividing cells is added every week. Long term survival (4 weeks) is reached but patterns become less focused after 2 weeks. Beside a marginal process of somatic motion, neurites tends to increase their substrate colonisation by escaping from their original guiding lines (Fig. 3).

1.2 Cell culture

Pyramidal neurons from mouse hippocampus are deposited on these substrates and grow only on the permissive regions (Fig.1 & 2) coated by Polylysine.

Glial cells proliferation is stopped by AraC after 2 days (1 mg/ml, Sigma), while a medium coming from a separate culture of these dividing cells is added every week. Long term survival (4 weeks) is reached but patterns become less focused after 2 weeks. Beside a marginal process of somatic motion, neurites tends to increase their substrate colonisation by escaping from their original guiding lines (Fig. 3).
2 MEA design and Signal recording

A modular and versatile home-made acquisition/stimulation system has been designed. MEA are fabricated according our designs by Ayanda (Fig. 4) [2]. Their geometry are compatible with a 124 pins connector that is incorporated into a chamber regulated in temperature (flat flexible heaters). A 5% CO₂ concentration is delivered by a continuous flow of a 19% O₂, 76% N₂ and 5% CO₂ gas mixture while evaporation is prevented by closing the culture chamber with a thin film of fluorinated ethylene-propylene [3]. Acquisition and stimulation are performed by National Instrument DAQ cards driven by the Labview software (4x32 channels, 16 bits, 1Ms/sec) through an home-made electronic card (Fig.5).

3 Perspectives

We currently upgrade our elaboration protocol to a more complex one dedicated to axon/dendrites polarisation in addition to network topology control. For this purpose, a two steps photolithographic process allowing the successive deposition of two adhesive layers (Poly-L-Lysine and Laminin for example) will be used in a first step [4]. In parallel, we now develop patterned cultures on MEA that will lead to the first tests of our acquisition/stimulation system.

Acknowledgement

We thank L. Bourdieu for the very useful discussions we had at the start of this work.

This project is funded by the Contrat de Plan Etat-Région Rhône-Alpes NAPSV (2000-2006) and by the Institute of Condensed Matter (Grenoble).

References

Hydrogel-coated microelectrode array for neural interface

Sang Beom Jun¹,², Matthew R. Hynd³, Jae Kyoo Lee¹,², James Turner³,⁴, William Shain³,⁴, Sung June Kim¹,²*  

¹ Nano-Bioelectronics & Systems Research Center, Seoul, Korea  
² School of Electrical Engineering, Seoul National University, Seoul, Korea  
³ Wadsworth Center, New York State Department of Health, Albany NY, USA  
⁴ Department of Biomedical Science, School of Public Health, University at Albany, Albany NY, USA  
* Corresponding author. E-mail address: kimsj@snu.ac.kr

Recently, hydrogel has been extensively used in various biomedical applications including soft contact lenses, wound healing materials and as a means for controlled drug delivery and release. In this study, we propose to use hydrogel as a novel neural interface. Planar microelectrode arrays were coated with hydrogel in order to improve the quality of the interface and to aid in drug delivery to cells on microelectrode arrays (MEAs).

1 Hydrogel coating on MEAs

A polyacrylamide gel matrix consisting of acrylamide (19:1, 40% acrylamide:bis-acrylamide), in 30% glycerol, 0.83% methylene blue, 0.12% TEMED, and 100 mM Tris Cl (pH8.5) was prepared. Various compositions (12.5, 25, 50, 70%) of acrylamide were prepared to measure the difference of electrochemical impedances after polymerization over microelectrode sites. Nerve growth factor (0.1 mg/ml in PBS) was prepared and added to the hydrogel solution with the concentration of 10% (v/v). The mixture (10 μl) was applied onto MEAs pretreated with Bind-Silane (3-(trinethoxysilyl)-propyl acrylate, acetic acid, dH2O) for 1 hour. Quartz coverslips were used as a backing template during polymerization. Prior to use, coverslips were treated with PlusOne Repel-Silane ES for 30 min, then rinsed in dH2O and dried at room temperature. A treated glass coverslip was placed on top of the hydrogel solution dropped on MEAs. The acrylamide solution was photopolymerized by exposure to UV light for 15 min on a 312nm UV lamp. Following polymerization the backing coverslip was carefully detached from the MEAs and washed a stream phosphate buffered saline (pH7.4) for 10 min to remove any unpolymerized gel solution.

2 Electrochemical Characterization

The electrochemical impedances of microelectrode recording sites were measured through polymerized hydrogel in order to check if the neural signal can be detected through hydrogel. The impedances of electrode sites covered by various compositions of acrylamide were measured by potentiostat at 1 kHz in PBS. After hydrogel polymerization on MEAs, the impedances were increased by 1.8, 3.4, 10.8, and 57.1 times for 12.5, 25, 50, and 70% composition of hydrogel coating respectively.

3 Neuron culture and neural recording

Poly-L-lysine was patterned onto the surface of hydrogel using microcontact printing. Primary rat hippocampal neurons were applied to hydrogel surfaces and cultured on stamped patterns. Neural signals were successfully recorded from cultured neural networks through hydrogel (12.5% acrylamide) at 21 days in vitro. (Fig. 2)
4 Drug delivery from hydrogel

The release of NGF from hydrogel was verified by the differentiation of PC12 cell. PC12 cells were cultured on NGF-containing hydrogel. After 2 days in culture, PC12 cells successfully differentiated by released NGF as shown in Fig. 3.

5 Summary

We showed that hydrogel can be used as a neural interface material with a drug delivery function. Hydrogel coated on MEAs with a low concentration of acrylamide hardly affect the quality of neural recording. In near future, hydrogel could be employed in neural prosthetic devices using its noble properties such as responsiveness to pH, electrical fields, temperature, light, and organic compounds.

Acknowledgement

This work was supported by the International Collaboration Program, NBS-ERC (Nano Bioelectronics and Systems Engineering Research Center)/KOSEF (Korea Science and Engineering Foundation) and also supported in part by the Nanobiotechnology Center (NBTC), an STC Program of the National Science Foundation under Agreement No. ECS-9876771.

References

Bionic approach to defined networks on MEAs

Lautemann N.1(a)*, Künzel T.1(b), Ingebrandt S.2, Böcker-Meffert S.2, Offenhäuser A.2, Bräunig P. 1(a)

1 Institute for Biology II, (a) Department of Developmental Biology and Morphology of Animals / (b) Physiology, RWTH Aachen University, Aachen, Germany
2 Research Center Jülich, Institute for Thin Films and Interfaces (ISG-2), Jülich, Germany
* Corresponding author. E-mail address: n_lautemann@hotmail.com

In order to construct defined neuronal networks on MEAs for sensor or information-processing applications, and to study the development and plasticity of neuronal networks, we have combined different methods to develop a bionic solution. By now, we have an integrated, multifunctional and basically simple solution, which is inspired by different fundamental principles of in-formation of matter.

The integration is realized by the use of the already present electrodes of the MEA. By application of alternating fields we are able to position different cell types (i.e. neurons/glia cells) on different locations on the chip (electrodes/space in-between) being also a step towards biohybrid tissue engineering. This exceptionally potent method is called dielectrophoresis (DEP). In the case of travelling field DEP (TWD) it can be used for selection of defined cells out of a cell suspension (instead of marker-dependent FACS/MACS (Fluorescence and magnetic activated cell sorting)).

To ensure correct "wiring" of the previously positioned neurons an electrostatic field is applied via the electrodes of the chip. This method is termed galvanotropism and is of special interest in the migration of cells and axonal pathfinding processes within morphogenesis and regeneration.

1 Material and methods

1.1 Defined cell types

There are several reasons and aims which made us test different cell types.

Insect neurons are attractive because they are robust and relatively easy to handle in vitro. In vivo they are members of comparatively simple networks that are electrophysiologically well studied. In our case we use cells of the metathoracic ganglion of locusts. The neurons, having a diameter of 10 to about 100 µm, are well suited to the size of the MEA-electrodes (ranging from 8 to 30 µm), thus providing single-cell positioning/recordings with a very good signal-to-noise ratio.

The chick hindbrain contains a well-studied circuit for coincidence detection in auditory signals, a circuit that might be mimicked in vitro for further analysis.

By now only suspensions of different cell types (neurons/glia cells/neuronal subtypes) were used since the purification of cell suspensions by use of standard techniques (MACS, tracing, differential centrifugation) is complicated and still on progress. To improve the separation, we are going to try techniques which use combinations of fluid-flow and travelling wave dielectrophoresis (TWD). One important reason is that this technique is integrable providing an optional add-on for construction of µTAS (micro total analysis systems). The second one is the fact that these operations do not affect or alter the electrophysiological properties of the cells (which is not clear for the previously mentioned techniques).

Fig. 1 Positioned neurons (red/blue fluorescence) and glia cells (blue fluorescence) of metathoracic ganglion of Locusta mig. (double-stained with 4',6-Diamidino-2-phenylindole (DAPI, binding to DNA) and horseradish peroxidase (HRP, for staining of neurons)). Size of microelectrodes: 30x30 µm. Mainly neurons of size comparable to electrode size can be found on the electrodes or along the conducting paths. Since a comparably high frequency was used, the glia cells can be found on the electrodes too. V=8 Vpp, f=5 MHz, t=15 min.
1.2 Positioning/Patterning

The next step in re-/construction of neuronal networks on MEAs is the positioning of neurons onto the electrodes of the array.

This can be done by using an inhomogeneous alternating electrical field between the electrodes. This field induces a polarization of the cells in a frequency dependent manner. We are using frequencies in the radiofrequency range (10 kHz-10 MHz) and thus the adequate polarization mechanism is the Maxwell-Wagner-Polarization (interfacial polarization = dependence of membrane properties). The direction of polarization and the dielectrophoretic force depends on the Clausius-Mosotti-Factor (CM) which briefly reflects the relationship of the complex conductivities and permittivities of the cell/membrane and the surrounding medium. If the polarizability of the cells is higher, the cells will be accelerated towards the highest field intensities which can be found at the electrode edges (positive dielectrophoresis = pDEP). In the opposite case, they will be accelerated toward the lowest field intensities (= nDEP).

As mentioned above, the spectra of different cell types are different. Because of the mixed cell suspension we want to separate cells simultaneously which means in the simplest case: neurons get placed onto electrodes, glia cells in between. This is only possible if the crossover frequencies (nDEP → pDEP) of both cell types are somewhat different.

1.3 Axonal guidance

Since there is only very few work concerning arthropod galvanotropism, preliminary tests had to be done in order to find out the essential parameters respectively if those neurons react galvanotropistic at all.

Some experiments were based on classic works concerning galvanotropism [3] by using salt bridges to apply the electric field.

Subsequently we applied the static electric field via the MEA electrodes. Different voltages have been tested in combination with different substrates (Poly-D-Lysin, ConcanavalinA, Laminin, glass) and cell types.

2 Results and Discussion

We could show that a positioning of cells of various origins (arthropod ganglia and brain, chick hindbrain) is possible and can be done within a very short time (Fig. 1&2). Short in this case means in the minute range, depending upon the height of the cell suspension, density of the cells compared to density of the surrounding medium etc..

By now, the patterning experiments (positioning of neurons and glia cells at different locations on the chip) are still on progress because of the lack of the needed spectra of the different cell types.

Further, we found that previously positioned neurons of the embryonic chick hindbrain did grow out and that they seemed not to be negatively influenced by that procedure. This result will be evaluated and supplemented by electrophysiological data.

Our thoracic arthropod neurons grow out best in very low density cultures, meaning that the probability of right positioned neurons before DC field application, is reduced. In order to find a significant effect on the direction of outgrowth we will do further studies with respective to substrates, supplements and duration of field application. The galvanotropistic effect on rat neurons has already been shown by Shalini [1], for chick medullary neurons by Marsh and Beams [2] and for Xenopus neurons by McCaig et al. [3].

Acknowledgement

This project is funded by the Helmholtz-Society and part of the vIBHT (virtual network for biohybrid technology).

References


Photolithographical patterning of single cells and cell assemblies on commercial multielectrode arrays

Peter Molnar, Melissa Kuchma, Anupama Natarajan, Jung-Fong Kang, Neelima Bhargava, Mainak Das and James J. Hickman

Nanoscience Technology Center, University of Central Florida, Orlando, FL, USA

1 Engineered functional cellular assemblies

There are several properties of a biological system which are difficult to study using random cultures of cells because they result from system-level interactions among the composing elements such as synaptic communication in a neuronal system. The interest of our laboratory is the creation of simple functional biological systems from dissociated cells on silicon substrate and studying the process of their integration and their resulting cellular interactions. These engineered biological systems can find applications in functional pharmacological screening, toxin detection, as disease models or in robotics.

2 Laser ablation of self-assembled monolayers is a flexible method to pattern cells on multielectrode arrays

We were using photolithography to pattern self-assembled monolayers (SAMs) on glass or silicon substrates to guide cell attachment, axonal growth and differentiation of the cells. (Fig. 1). We have registered these surface patterns with substrate embedded microelectrodes in order to allow long-term recording of the activity of the cells or for their electrical stimulation. In certain experiments these SAMs were further modified with proteins. We have shown that commercial multielectrode arrays could be repetitively modified with SAMs and patterned at least 3 times without a decrease in the quality of the electrode recordings.

3 Neuronal cellular networks on MEAs with directed connectivity

A typical way to study synaptic transmission and plasticity is by extracellular stimulation of a presynaptic cell layer with concurrent recording of extracellular field potentials from a postsynaptic layer in a brain slice preparatum. In order to make a similar experiment possible with cultured cells we have created two layers of neurons on the top of substrate-embedded microelectrodes. (Fig. 2) The interconnectivity pattern between the two layers was asymmetrical, thus enhancing the probability of one-directional connections.
4 Two-cell neuronal networks

Another solution to study synaptic transmission in cultures is to create two-cell networks with determined interconnectivity patterns. We have optimized the patterning and culture conditions to obtain two-cell neuronal networks on microelectrodes as well (Fig. 3).

5 Patterned skeletal muscle myotubes

Based on photolithographic patterning of vitronectin as the growth substrate we have developed a method to create functional skeletal muscle myotubes on the top of a microelectrode array (Fig. 4).

Acknowledgement

Funded by NIH grant K01 EB003465-03
Microstamped electrodes with PDMS insulator: A design towards inexpensive, single-use MEAs

Katherine Musick1*, Yoonkey Nam1, Bruce Wheeler2

1 Electrical and Computer Engineering Department, University of Illinois at Urbana-Champaign, USA
2 Bioengineering Department, University of Illinois at Urbana-Champaign, USA
* Corresponding author. E-mail address: kmusick@uiuc.edu

The Microelectrode Array (MEA) is a versatile tool for monitoring the acute and chronic activity of electrogenic cells in vitro. However, many physiologists cannot benefit from MEAs because fabrication requires technical skill and extensive effort for non-experts. Fabrication generally includes specialized equipment, hazardous chemicals, and most require the use of a cleanroom. Due to the demands on equipment and personnel, commercially available MEAs are expensive. Many labs find the number of experiments that can be run simultaneously is primarily limited by number of MEAs in inventory. Therefore, a design for easy-to-prepare and inexpensive surface MEAs has been sought. With cheaper MEAs available, more labs will be able to use this tool, and labs presently strained by the price of MEAs will be able to do more experiments simultaneously.

1 Process Flow

1.1 Creating the stamp

The fabrication protocol has been designed to minimize demands on equipment and time, and exclude the use of a cleanroom. This process consists of five steps: fabrication of the stamp mold, fabrication of the stamp, deposition of the thin metallic films, creation of the desired electrode pattern, and fabrication of the insulation layer. The stamp mold can be fabricated out of standard SU-8 processing on a silicon wafer or can be outsourced. Once a stamp mold is fabricated, it can theoretically be used indefinitely until the mold breaks due to careless handling. The stamps themselves are fabricated by curing PDMS prepolymer poured onto the mold.

1.2 Microcontact Printing

Thin metallic films can be deposited with a dual-metal evaporator onto ordinary, cleaned glass slides. In the event that a lab does not have access to such equipment, gold-coated substrates are available commercially. The electrode pattern itself is achieved by using the fabricated stamps to transfer a layer of hexadecane thiol onto the gold-coated substrates (Fig. 1). An etching step removes all areas not protected by this hexadecane thiol layer.

1.3 Insulation

A patterned PDMS film is used to insulate the bare gold electrodes (Fig. 2). Holes in this PDMS film are aligned directly above the electrodes on the patterned gold substrate. Alignment can be done by hand under 5x magnification. An image of the fully-assembled device is shown in Fig. 3.

![Fig. 1 Process diagram for microcontact printing](image1)

![Fig. 2 Process diagram for insulation](image2)
2 Hippocampal Experiment
To ensure functionality and biocompatibility, hippocampal cells were grown on top of these single-use MEAs (Fig. 4). Cells were viable on the arrays in excess of 60 DIV. A recording of spontaneous activity on these arrays at 15 DIV showed noise bands with standard deviations of approximately 4 to 10 μV. The spike threshold was set at 5 times this value, and spikes were observed beyond this threshold—clearly distinct from the noise band (Fig. 5).

3 Summary
The MEA as outlined in this work is only marginally easier for a nonexpert to prepare. The real gains are that much of the process can be readily outsourced. For example, similar gold-coated slides can be obtained from a number of vendors (Platypus Technologies, LLC), which eliminates the need for a dual metal evaporator. The master mold containing the desired MEA and insulation patterns could be fabricated elsewhere, so the need for photolithographic equipment is eliminated as well. When these outsourcing measures are taken, the processing only requires PDMS, a spinner, an oven or hotplate, and a few chemicals for the microcontact printing and gold etching. With the addition of a spinner, even a novice should be able to successfully perform this process in any ordinary physiology lab with reasonable yield.

A possible next step for this work would be creating several arrays on one device. This is potentially nontrivial due to size limitations on contact pads, issues regarding routing the electrode lines, and the complexity of either multiplexing or providing additional amplifiers. The process as outlined here will allow labs to perform more experiments simultaneously, but further work must be done to achieve the orders of magnitude more that are desired.

Acknowledgement
This work was funded by the National Science Foundation under Grant EIA 0130828 and by the National Institutes of Health under Grant R01 EB000786.

Portions of this work have been previously presented:
Y. Nam, K. Musick, B.C. Wheeler, “Application of a PDMS microstencil as a replaceable insulator toward a single-use planar microelectrode array,” accepted in Biomedical Microdevices, April, 2006.
Polarized Axon Growth of Hippocampal Neurons Cultured on Tapered Lines of Laminin

Rudi F. Scharnweber1*, Gregory J. Brewer2, Bruce C. Wheeler1

1 Neuroscience Program, University of Illinois, Urbana, IL, USA
2 Southern Illinois University School of Medicine, Springfield, IL, USA
* Corresponding author. E-mail address: scharnwe@uiuc.edu

We generated tapered lines of laminin on glass substrates using microcontact printing. Hippocampal neurons cultured on these patterns primarily extended axons toward the wider end of the tapered laminin lines. Based on this finding, it is apparent that tapered lines provide a convenient alternative to traditional protein gradients for controlling the growth of individual neurons in culture. This report is an update of previously described results [1].

1 Introduction

Previous work has shown that cultured hippocampal neurons extend axons in the direction of an increasing substrate-bound laminin gradient [2]. However, most gradients are either difficult to manufacture or lack sufficient spatial resolution to guide axons to specific targets in a patterned neuronal culture. To circumvent these problems, we developed a tapered line pattern that approximates the properties of a laminin gradient with high spatial resolution and used it to direct the growth of individual axons in culture.

2 Methods

2.1 Microstamp fabrication

A transparency mask with the tapered line patterns (final dimensions: 2 mm long, 5 µm at wide end, 2 µm at narrow end, spaced 50 µm center-to-center; see Figure 1) was printed on a Linotype Herkules printer. Molds at the final dimensions were generated with a GCA DSW-6100 G-Line Series Wafer Stepper on 2-inch silicon wafers. Polydimethoxysiloxane (PDMS) was cured on top of the molds at 60°C for 12 hours to generate stamps. PDMS stamps were then coated with 10% SDS for 5 min, dried with nitrogen gas, and soaked in 100 µg/mL laminin (Invitrogen) for 2 hours at room temp. Protein-coated stamps were then dried with nitrogen gas and brought into contact with glass cover slips (VWR) for 2 min to transfer laminin. To facilitate cell body attachment, a final coating of 100 µg/mL poly-D-lysine was performed for 2 hours at room temp. Cover slips with protein patterns were then rinsed in DI water and sterilized in 75% EtOH prior to culturing.

2.2 Tissue culture

Embryonic day 18 rat hippocampal cells were purchased from BrainBits, LLC (Springfield, IL) and dissociated within 6 days of receipt. Cells were dissociated mechanically by trituration and plated on patterned substrates at 25 cells/mm² in Neurobasal/B27 serum-free medium (Gibco) with 0.5 mM glutamine, 25 µM glutamate, and 0.1% penicillin/streptomycin. Cultures were incubated at 37°C, 5% CO₂, and 9% O₂.

2.3 Microscopy and image analysis

After 2 days in culture, phase micrographs of active cultures were taken using an IX51 inverted microscope equipped with a QCOLOR cooled CCD and QCapturePro software package (all Olympus). A neurite was classified as an axon if it was at least 100 µm longer than the next-longest neurite. Only neurons that did not contact other cells or neurites were scored for the direction of axon growth.
3 Results and conclusions

We found that 72% of axons grew toward the wider end of the tapered line versus the 50% expected by chance (p < 0.02, n = 29; see Figure 2). Future experiments will determine the optimal degree of tapering for controlling the direction of axon outgrowth. With modest improvements, this technique may be useful for controlling the growth and synaptic connectivity of individual neurons in culture.

Acknowledgements

We thank Kathleen Motsegood and Yoonkey Nam for assistance in generating the molds for PDMS casting. Funded by NSF EIA 01-30828.

References


An Integrated System for Simultaneous Multichannel Stimulation and Recording

Edgar A. Brown1*, Richard A. Blum1, James D. Ross1, Yoonkey Nam2, Bruce C. Wheeler2, Stephen P. DeWeerth1,3

1 Georgia Institute of Technology, Atlanta, GA, USA
2 University of Illinois at Urbana-Champaign, Urbana, IL, USA
3 Emory University School of Medicine, Atlanta, GA, USA
* Corresponding author. E-mail address: ebrown@ece.gatech.edu

Experimental studies in neuronal development and plasticity require precision electronics capable of multielectrode stimulation and recording. To fulfill this need, we present a custom analog VLSI IC, fabricated in a 0.35 μm process, incorporating stimulation buffers and recording preamplifiers for 16 electrodes onto a single die. The architecture of the IC allows for arbitrary, independent configuration of electrodes for stimulation or recording, including stimulation artifact elimination circuitry that returns the stimulation electrode to its pre-stimulation voltage to minimize the interference with recording. The amplifier has an input–referred noise as low as 4.7 μVrms. We demonstrate that the artifact elimination reduces the duration of the stimulation artifact to 4 ms or less at the stimulation electrode.

1 IC design

1.1 Physical characteristics

We have designed and tested an analog VLSI system for extracellular stimulation and recording. The amplifier, artifact cancellation, and stimulation circuitry for a single channel occupy 33000 μm2 in the TSMC 0.35μm process. This implementation makes the design easily scalable to MEA systems consisting of thousands of electrodes. A novel feature of the system is the artifact elimination circuitry, which permits near–simultaneous stimulation and recording at the same electrode.

Fig. 1 Photomicrograph of the IC die showing 16 amplification, stimulation, and artifact cancellation channels with their control circuitry (this is Version II of the IC).

Two versions of this IC have been fabricated, with the second version overcoming the main limitation of the first, namely correcting stimulation circuitry problems. As testing for version II of the IC is still under way, for completeness, this document includes results from both versions. In both versions 16 channels have been implemented in a single IC, due to testing, control circuitry, and pin limitations. However the design is modular enough to be extended to 128 or more channels per IC in the near future.

1.2 Circuit operation

The main circuit consists of a low noise main amplifier (following [1]) with an active DC feedback amplifier in a Gm-C configuration that has been designed for very low current operation. The ratio of feedback capacitance defines the amplifier gain, while the feedback amplifier current defines the amplifier bandwidth, and noise characteristics.

An additional amplifier is added to this configuration to discharge the electrode after stimulation has taken place. The feedback loop, that includes the main amplifier and the discharge amplifier, work together to return the electrode to its pre-stimulation average voltage. This circuit has several improvements with respect to the one by Jimbo et al [2]. In addition to being simpler, integrated, and thus easier to extend to hundreds of channels, this circuit discharges to the average electrode voltage and not to a pre-stimulation sample of it. Additionally, the use of amplifier tail currents to turn the amplifiers on and off, mostly...
eliminates the introduction of the switching transients that would be generated by analog switches.

2 Testing

A board with a microcontroller, 12 biasing DACs, and 32 channels of additional amplification (+36) was built to interface the IC with a computer running MC Rack software and our custom stimulation interface. The ICs where interfaced to the MEA by connecting to an MCS pre-amplifier plate’s inputs.

2.1 Electronics testing

Results from experimental characterization with a commercial MEA validate the functionality of the electronics. The recording preamplifier provides an inverting gain of 50 (version I) or 100 (version II) with a noise level that is dependent on the setting of the highpass pole. Experimental characterization has measured input noise levels of 4.7μVrms, in the bandwidth relevant to neural action potentials for a cutoff frequency of 1Hz. After a biphasic stimulus of ±500mV on 10μm diameter TiN electrodes (Multi Channel Systems) the discharge of the stimulation electrode has been able to reduce its artifact duration to less than 4ms. This time is sufficient to observe responses to stimulation in experimental situations.

2.2 Biological testing

Initial biological testing was carried out with a dissociated hippocampal culture at 35 DIV. As shown in Fig. 5 we were able to elicit neural responses in the dish, and record on the stimulating channel 3–4 ms after stimulation.

2 Future work

Further efforts are underway on two fronts: additional biological validation and improved circuit development. The desired improvements to the system are to decrease discharge times and noise levels. Our desire to integrate the circuitry for 128 or more electrodes onto a single die demands limited per channel area and power requirements which would ultimately limit the achievable noise level. Our current design choices represent a balance among noise, stability, power, and area.

Acknowledgement

Funded by the National Institute of Health through a Bioengineering Research Partnership grant (1 RO1 EB00786-01)

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NEUROPLAT64 - low noise CMOS integrated circuit for neural recording applications

Pawel Grybos*, Władysław Dąbrowski, Paweł Hottowy, Tomasz Fiutowski, Bartosz Bielewicz

AGH University of Science and Technology, Faculty of Physics and Applied Computer Science, Cracow, Poland
* Corresponding author. E-mail address: grybos@ftj.agh.edu.pl

This paper describes the development of low noise multichannel integrated circuit for recording extracellular neuronal signals using microelectrode arrays. The IC called NEUROPLAT64 contains 64 readout channels and a single output analogue multiplexer. This chip has been fabricated in 0.5 μm CMOS technology and has been optimised for good noise and matching performance.

1 Integrated circuits for multichannel recording systems

Many neurobiological experiments need multichannel readout systems for simultaneous recording of extracellular signals from many neuronal cells. For simultaneous recording of neuronal signals from hundreds or thousands of neuronal cells one needs a high density multielectrode array, a multichannel readout electronic system and software for data acquisition and analysis. To increase the number of recording channels together with minimizing the volume and weight of electronic recording system, one can use the VLSI technology and develop an application specific integrated circuit (ASIC), which is suitable for these purposes [1]. Such an IC should fulfill the following requirements:

- low noise performance because of low amplitude of extracellular signals,
- AC coupling at the input of recording channel due to large input DC offsets from MEA,
- data output compression using analogue multiplexing,
- filtering of the input signals in low frequency range to improve signal-to-noise ratio and reduce aliasing problems,
- good uniformity of analogue parameters for all channels,
- low power dissipation and small area of silicon per single channel.

We present the design and test results of our 64-channel IC called NEUROPLAT64, which fulfils the requirements listed above.

2 Architecture and electrical parameters of NEUROPLAT64

The NEUROPLAT64 IC consists of 64 readout channels (Fig. 1), which can in parallel and independently process signals from 64 electrodes. The IC is designed in 0.5 μm CMOS technology and its total area is less than 20 mm².

The single readout channel is built of an AC coupling circuit at the input, a low noise preamplifier and a bandpass filter (Fig. 2). In order to reduce the number of output lines, 64 analogue signals from readout
channels are multiplexed to single output by an analog multiplexer. A single channel occupies 0.17 mm² of silicon area and dissipates 2 mW of power. The design has been optimized for low noise and good matching performance. For passband setting 10 Hz - 5 kHz an equivalent input noise of 5 μV rms has been achieved (with the inputs connected with 1 kΩ resistor to ground). However, the noise performance of the circuits strongly depends on the time constant of the AC coupling circuit at the input (see Fig.3).

![Fig.3](image)

**Fig.3** Power spectral density of output noise of NEUROPLAT64 - the noise strongly depends on time constant of the AC coupling input circuit ($\tau = C_0 R_0$ - see fig.2).

Thanks to internal digital to analogue converters implemented in NEUROPLAT64 IC, all the important chip parameters like gain, cut-off frequencies can be tuned in a wide range. For example, lower cut-off frequency can be controlled in the range from 10 Hz to 200 Hz, while the higher cut-off frequency from 200 Hz to 5 kHz. Internal calibration circuit allows one to test all analogue circuit parameters every time, even for the chip connected to MEA and assembled into multicore modules.

3 Measurements with MEA
The preliminary measurements with NEUROPLAT64 ICs were performed with 120 electrode array using the PCB with two NEUROPLAT64 ICs shown in Fig. 4. Data acquisition system was based on personal computer and NI-6110 card with 12-b ADC channels. We measured the gain and noise distributions for all 120 channels connected to MEA. The typical distributions of these parameters measured are shown in Fig. 5. The measured noise for bass band setting of 40 Hz - 4.5 kHz was about 18 μV (electrode impedance was of about 800 kΩ at 1 kHz). The spread of gain is 1.3%, while the spreads of the lower and the higher cut-off frequencies are about 1.8%.

4 Summary
We have shown an IC solution for low noise multichannel signals recording from multielectrode array. The IC has a good noise and matching performance, so it can be used in different neurobiological experiments (of both types in vivo and in vitro).

![Fig.4](image)

**Fig.4** PCB with two NEUROPLAT64 chips and MEA array.

![Fig.5](image)

**Fig. 5** Measured distribution of analogue parameters in two IC connected to MEA: a) gain, b) noise rms.

Acknowledgement
The work is supported by the Polish Ministry of Science and Education for financial support. Pawel Grybos thanks Raphael Holzer from EFFL BMI, Lausanne for multielectrode array.

Reference
Signal Processing Chain for CMOS Microelectrode Arrays Interfaced to Electrogenic Cells

S. Hafizovic1*, F. Heer, and A. Hierlemann

Physical Electronics Laboratory, ETH Zurich, Switzerland
* Corresponding author. E-mail address: sadik.hafizovic@ethz.ch

We report on the signal processing backend of a CMOS-chip-based system that is capable of bidirectionally communicating (stimulation and recording) with electrogenic cells and that is targeted at investigating electrical signal propagation within cellular networks in vitro. The system consists of three major subunits: The core component is a 6.5-by-6.5 mm² CMOS chip, on top of which the cells are cultured. It features 128 bidirectional electrodes, each sampled at 20 kHz and 8 bit resolution. Secondly, there is a field-programmable-gate-array-based device, which provides chip control, event detection, data buffering and a USB interface capable of processing the 2.56 million samples delivered by the chip per second. The third element includes software that is running on a standard PC performing data capturing, processing, and visualization.

1 Design

The chip is fabricated in industrial CMOS-technology [1] with post-processing steps described in [2]. Digital logic components on the MEA ASIC and on a field-programmable gate array (FPGA) connect the A/D-converters to the PC.

2 On-chip digital circuitry

The on-chip digital logic runs at 1.6 MHz and 5 V and serves two purposes. First, it performs control tasks like multiplexing, electrode selection for stimulation, reset of single electrodes, and it contains the successive-approximation registers of the A/D converters. Secondly, it provides the chip interface to the FPGA. In the architecture shown here, this digital interface relies on 16 lines for the readout data, 2 lines for stimulation and control data, and additionally, a clock, a reset and a frame sync line.

3 FPGA - Event Detection

To manage the data output of 3.2 MB/s and the input of 0.4 MB/s, an FPGA running at 48 MHz in conjunction with an USB 2.0 interface chip and 1 MB SRAM [3] has been utilized (Fig. 2). I/O buffering and digital signal processing like averaging and event detection are implemented on the FPGA to reduce the data volume transmitted to the PC. The event detection approach as presented in this paper is realized in a two-stage processing strategy.

Fig. 1 System overview and data flow. Left: Recorded data propagate from the left to the right (chip to PC) while stimulation data propagate from right to left (PC to chip). The left block labeled "Chip", includes many components in multiple realizations: numbers in brackets indicate the number of realizations implemented per chip, e.g. 128 electrodes or 16 A/D converters. For the sake of clarity, the analog signal processing chain is highly simplified. A more detailed FPGA schematic is given Fig. 2. b) Photo of the entire system setup. The PCB provides stabilized power supply and reference voltages for the CMOS chip.
1. During event detection, events are isolated from the continuous flow of data. In this context an event is a cutout of recorded data that contains potential events or spikes, e.g., a single spike, multiple (possibly overlapping) spikes, a spike train or barrage.

2. During spike discrimination, events are further processed to isolate spikes, perform a unit separation and resolve overlaps.

4 Results

An exemplary screen shot (Fig. 3) shows events that have been detected with the framework as described in this abstract. The combination of the CMOS chip with an FPGA-based device allows to perform online event detection and, on demand, full-scale transmission of all 128 20-kHz signals. The event detection is implemented on the FPGA and showed a maximum performance of $5 \times 10^3$ events per second, it is fully self-adjusting and requires no user inputs. The data volume transferred to the PC is effectively decimated by relocating the event detection from the PC to the FPGA. The system is capable of identifying an event and triggering a stimulation within less than 2 ms, so that it may be considered real-time with respect to the time scale of neuronal activity on the chip.

Acknowledgement

Funding has been generously provided by the European Information Technology (IST) Future and Emerging Technologies program, and the Swiss Bundesamt fuer Bildung und Wissenschaft (BBW), contract number IST-2000-26463.

References

[1] Standard 0.6-µm triple-metal, double-polysilicon CMOS process as provided by XFAB, Dresden, Germany.
CMOS Integrated Bidirectional 128-Electrode Array for Electrogenic Cells

Flavio Heer1*, Sadik Hafizovic1, Wendy Franks1, Urs Frey1, Frauke Greve1, Axel Blau2, Tanja Ugniwenko2, Christiane Ziegler2, Andreas Hierlemann 1

1 Physical Electronics Laboratory, ETH Zurich, Zurich, Switzerland
2 Department of Physics & Biophysics, Kaiserslautern, Germany
* Corresponding author. E-mail address: fheer@phys.ethz.ch

We report on a CMOS-based microelectrode array chip (6.5 by 6.5 mm²) for bidirectional communication (stimulation and recording) with electrogenic cells. The integration of on-chip circuitry, which includes analog signal amplification and filtering stages, analog-to-digital converters, digital-to-analog converter, stimulation buffers, temperature sensors, and a digital interface for data transmission, notably improves the overall system performance. Measurements with cardiomyocytes and neuronal cells were successfully carried out, and the circuitry characterization evidenced a total equivalent input noise of 5.9 μV_RMS (10 Hz - 100 kHz) at a gain of 1,000.

1 Fabrication
The chip is fabricated in industrial complementary metal oxide semiconductor (CMOS)-technology [1]. After the CMOS process, a 2-mask post-processing procedure is required to cover the Al electrodes with biocompatible platinum and to protect the Al from undesirable electrochemistry using a 1.6 μm thick passivation stack of alternating silicon nitride and silicon oxide [2]. The electrode (Fig. 1) diameter (10 to 40 μm) and location (pitch 50 to 500 μm) is defined during these post processing steps. Additionally the electrodes can be electroplated with porous platinum black to reduce the electrode impedance [2].

2 System Description
Each of the 128 electrode circuitry repeating units in the array includes a high-pass filter with 20 dB gain, a low-pass filter, a 30-dB amplifier, and a stimulation buffer (Fig. 2). Important advantages arise from the modular architecture with buffers and filters implemented at each electrode in comparison to other CMOS electrode arrays published so far [3, 4]: (i) The signal is amplified and filtered in close vicinity of the electrodes, which makes the design less sensitive to noise and distortion picked up along connection lines; (ii) a stimulation buffer makes the stimulation signal independent of the number of activated electrodes; (iii) the high-pass filter allows for immediate signal amplification; (vi) the low-pass filter limits the noise bandwidth and acts as an anti-aliasing filter. Additionally, the high-pass filter has a reset in order to ensure operability immediately after stimulation. Finally the signal is multiplexed for 20-kHz, 8-bit A/D-conversion. Total amplification is selectable, either 1,000 or 3,000.

Fig.1 Micrograph of the electrode array chip, close-up, and transducer schematic. Left: The chip features the 8-by-16 electrode array in the centre part and 16 A/D-converters and the digital block at the right-hand side. Centre: Close-up of the 128-fold repeated circuitry unit. Right: Schematic of the platinum-electrode processing.
Fig. 4 Left: Measurement of beating of primary cardiomyocytes from neonatal rats after 3 days in vitro. Raster plot of events vs. time and electrode. Clearly distinguishable is a synchronous beating, of the culture at 6 Hz. Further analysis revealed the existence of a natural pacemaker region in the cell culture. Right: Exemplary action-potential recorded from a spontaneously firing neural culture after 27 days in vitro. The amplitude of the signal is about 600 $\mu$V$_{pp}$, and the noise level is 17 $\mu$VRMS.

Fig. 2 Schematic of the chip architecture and the electronic components. The stacked frames indicate that these subunits are repeated for each electrode or each row. HPF denotes high-pass filter, LPF low-pass filter. The chip also includes a temperature sensor.

Fig. 3 Photo of the system setup except for the Faraday cage.

3 Results

Exemplary biological measurements include measurements from neuronal cell cultures with amplitudes of 600 $\mu$V and from cardiomyocytes with amplitudes of 1.3 mV (Fig. 4).

Acknowledgement

The authors are grateful to Evelyne Perriard for expertise on NRC culturing and Prof. Henry Baltes for sharing laboratory resources and for his ongoing stimulating interest in their work.

Funding has been generously provided by the European Information Society Technology (IST) Future and Emerging Technologies program, and the Swiss Bundesamt für Bildung und Wissenschaft (BBW), contract number IST-2000-26463.

Reference

Standard 0.6-$\mu$m triple-metal, double-polysilicon CMOS process @ XFab, Dresden, Germany. 
A Multichannel Recording Frontend for MEA

Ziv Yekutieli1, Yevgeny Perelman2, Ran Ginosar2*, Shimon Marom3

1 Intel Corporation, Haifa, Israel
2 Technion – Israel Institute of Technology, Faculty of Electrical Engineering, Haifa, Israel
3 Technion – Israel Institute of Technology, Physiology, Faculty of Medicine, Haifa, Israel

* Corresponding author. E-mail address: ran@ee.technion.ac.il

Glass-substrate Multi-electrode Arrays (MEAs) have become a valuable tool in neurophysiological research and drug screening. A typical setup for MEA recording is based on discrete-element preamplifiers located close to the MEA device and a multi-wire cable that conducts the pre-amplified analog signals to a data acquisition card. With the increase in the number of MEA sensing sites, this approach may turn impractical. We investigate the use of an integrated circuit as a mixed-signal analog/digital front-end for a MEA. This work presents a 0.35μm IC implementing a 12-channel mixed signal integrated front-end for neuronal recording successfully employed with a MEA system.

1 Conventional MEA Recording

Glass-substrate Multi-electrode Arrays (MEAs) have become a valuable tool in neurophysiological research and drug screening [1-4]. A typical setup for MEA recording (Fig. 1) is based on discrete-element preamplifiers located close to the MEA device and a multi-wire cable that conducts the pre-amplified analog signals to a data acquisition system.

![Laboratory MEA setup (left) and frontend chip micrograph](image)

Fig. 1

With the increase in the number of MEA sensing sites this approach may turn impractical, due to the large number of pre-amplifying channels, data acquisition channels, and, perhaps the most important, very thick wires.

A novel approach, based on integrated circuit for MEA recording front-end, is called for. The immediate advantage is the tight integration of the analog signal conditioning and data acquisition: A single IC can handle tens of MEA channels providing a digitized data stream in a suitable format. A less obvious advantage is associated with the integration of data processing on the same die with data acquisition: A single IC can handle tens of MEA channels providing a digitized data stream in a suitable format. A less obvious advantage is associated with the integration of data processing on the same die with data acquisition: A single IC can handle tens of MEA channels providing a digitized data stream in a suitable format. A less obvious advantage is associated with the integration of data processing on the same die with data acquisition: A single IC can handle tens of MEA channels providing a digitized data stream in a suitable format.

2 CMOS Multichannel Neuronal Recording Frontend

2.1 The Frontend IC

A mixed-signal CMOS front-end chip (Fig. 1) for 12-channel neuronal signal recording was fabricated with a standard 0.35μm mixed signal CMOS process. A detailed chip description and technical discussions can be found in [5].

Every recording channel (Fig. 1) included an ×50 pre-amplifier with digitally programmable input signal DC nulling and single-pole band splitter, breaking the signal at around 200Hz into high frequency spike activity and low frequency local field potential. The spike part was amplified another ten times and both parts then amplified by digitally programmable variable gain amplifiers with gain settings of ×2.5 / 5 / 7.5 / 10, providing for the total of ×5000 amplification at the spike chain and ×500 amplification at the LFP chain.

Digitally programmable offset calibration was provided by means of two calibration DACs at both spike and LFP chains. A sample and hold (SAH) circuit can sample either spike or LFP output at 50KSpS at 10-bit precision would generate a data stream of 50MSps that can be handled by a dedicated PC. A thousand MEA channels would already present a serious problem, especially if the system has to be responsive, i.e. generate stimuli as a reaction to the recorded signal. An integrated front-end with data processing capabilities can perform level detection and reduce the data rate by sending only the segments with suspected activity. Moreover, an advanced integrated front-end may even perform spike sorting and communicate only the times and the sources of the firing events, reducing the data-rate even further.

2.2 The Controller IC

A dedicated controller IC (Fig. 2) provides data acquisition timing and control functions. The controller is responsible for A/D timing, channel register access, recorded data serialization and host communication. The communica-
tion is bit-serial, carried over 5-wire serial bus (McBSP). The current system is capable of providing continuous sample rates of up to 40KSp on all twelve channels, while the communication bus is running at 12.5MHz.

An embedded FPGA board for host interface was also developed. The FPGA incorporates an Altera Nios II embedded processor running μC/II RTOS with custom developed low-level software. The interface communicates with the host over 100MBps Ethernet line, operating the UDP protocol.

2.2 Headstage and MEA Assembly

The front-end chip was mainly intended for in-vivo experiments; it was integrated on a miniature headstage (Fig. 4) and successfully used in in-vivo recording of rat cortical signals. To couple the headstage to an MEA, a commercial MEA holder (MultiChannelSystems, Inc.) was used with the internal pre-amplifiers removed. A special adaptor PCB was designed to provide the wiring (Fig. 5). Temperature control loop employed a commercial temperature controller with a temperature sensor and a heater within the MEA holder. Fig. 5 shows the complete assembled setup for MEA recording. The system was tested using a standard commercial MEA with a cortical neuronal tissue. A sample signal recorded with the integrated front-end and a close-up on a single spike are shown in shown in Fig. 6 (left). Signals and noise levels obtained with this system are very similar to those obtained through a standard commercial signal acquisition system.

3 Summary

An integrated front-end was demonstrated with a standard MEA, replacing all pre-amplifiers and data acquisition circuits, thus reducing the physical size and complexity while maintaining the same signal quality. Such chips are useful for very large MEAs, enabling acquisition of thousands of channels and avoiding explosion of size, space, and power.

Acknowledgement

We thank the staff of Neurophysiology lab, Faculty of Medicine, Technion for helping us with MEA experiments.

References


Design of floating-gate field-effect transistor arrays for the bi-directional coupling with electrogenic cells

Mathias Schindler, Sven Meyburg, Günter Wrobel, Sven Ingebrandt, Andreas Offenhäusser*

Institute of Bio and Nano Systems, IBN 2: Bioelectronics, and CNI – Center of Nanoelectronic Systems for Information Technology, Forschungszentrum Jülich GmbH, Germany
* Corresponding author. E-mail address: a.offenhaeusser@fz-juelich.de

N- and p-type floating gate field-effect transistor (FG-FET) arrays for the detection of extracellular signals from electrogenic cells were fabricated in a complementary metal oxide semiconductor (CMOS) process. Additional passivation layers protected the transistor gates from the electrolyte solution. We present recordings acquired simultaneously with an n- and p-type FG-FET from one single HEK293 cell and show how the floating gate can be used to capacitively stimulate a cell positioned on the active device. Finally we give an outlook for a high density FG-FET array designed in a commercial 0.5μm CMOS process.

1 Introduction

For extracellular signal recordings from electrically active cells in culture, two main concepts have been developed in the past: multi-electrode arrays (MEAs) with metallised contacts on silicon or glass substrates [1,2] and arrays of field-effect transistors (FETs) [3,4]. With these non-invasive methods, the electrical activity of single cells and networks of neurons can be observed over an extended period of time.

We present here a floating-gate field-effect transistor (FG-FET) for the bi-directional coupling to electrogenic cells. The so-called floating gate architecture [5,6] combines a metal oxide semiconductor transistor with an independent sensing area as shown in Fig.1.

Fig. 1A Schematics of the floating gate sensor B: Schematics of the compound floating gate; Both drawings are not to scale

The sensors consisting of both n-type and p-type transistors were fabricated in a standard CMOS process followed by a post-process comprising interconnects and passivation layers. The concept of a floating gate does not only allow the transistor and sensing area to be optimised separately but also allows for a voltage pulse to be applied to the floating gate and thereby capacitively stimulating a cell positioned on the sensing area.

2 Results

Both, n-channel and p-channel devices were fabricated in close proximity and we were able to record signals from a single HEK293 cell with both devices, simultaneously (Fig. 2) [7].

Fig. 2 Stimulation pulse (A) and the respective current traces (B) of a HEK293 cell on a combined sensor area of n- and p-channel FETs measured by a patch-clamp pipette in voltage-clamp mode. The respective extracellular recordings of the two independent FET structures are shown in (C,D) (traces averaged over 42 sweeps).

These signals exhibited comparable amplitudes and identical time courses. The experiments indicate that both types of FETs express similar sensitivities. In a different experimental configuration, the FG-FETs were used to stimulate HEK293 cells. In this design, a capacitor on chip is connected to the floating gate structure and via this capacitor the stimulation pulses are applied. A successful stimulation experiment can be seen in Fig. 3 where a train of voltage pulses was applied to the floating gate. The resulting change in the intracellular potential of a HEK293 cell positioned on the sensing area was monitored using a patch-clamp pipette. We further integrated 32x32 FG-FETs into an addressable sensor array as depicted in Fig. 4.
The sensor array is flanked by a column and a row decoder, respectively.

Currently we are focusing the design of a high density FG-FET array in a commercial 0.5 µm technology. This new chip generation will include on-chip calibration and amplification as well as utilise the capacitive stimulation technique presented above.

![Figure 3](image3.png)

Fig. 3 Capacitive stimulation of a HEK293 cell in current clamp mode by a burst of 10 voltage pulses of 3V applied via a large capacitor to the floating-gate. A: Stimulation pulses. B: Depolarisation and relaxation of the membrane potential (average over 5 sweeps).

By increasing the number and density of sensor spots firstly we want to increase the redundancy and thereby the lifetime of our devices, and secondly we also hope that with this concept positioning of cells on the sensor array [8] becomes unnecessary in future experiments.

![Figure 4](image4.png)

Fig. 4 Photograph of a chip with an addressable 32 × 32 sensor array. Source line and drain line are marked with ‘S’ and ‘D’. The inset shows a DIC micrograph of the column decoder.

3 Summary

We showed the simultaneous recording of a cell signal with an n- and p-type FG-FET positioned in close proximity to each other. We further demonstrated how the floating gate can be used for stimulation and how it is possible to integrate the FG-FET structures into large arrays.

References

Replica-molded poly(dimethylsiloxane) cell culturing lids contribute to cell culture longevity

Axel Blau*, Tanja Ugniwenko, and Christiane Ziegler

University of Kaiserslautern, Dept. of Physics & Biophysics, Kaiserslautern, Germany
* Corresponding author. E-mail address: blau@physik.uni-kl.de

We present a new lid design to seal MEA glass rings against medium evaporation. It is based on replica-molded poly(dimethylsiloxane) and helps increasing cell culture longevity by means of stabilizing osmolarity at defined gas exchange. With such lids, osmolarity can be stabilized on average to increases of less than 5 mosmol/kg/month in humidified incubators, and to less than 10 mosmol/kg/month in non-humidified incubators when ¼ to ½ of the medium is exchanged on a 3-5 day schedule. However, if medium is not exchanged, osmolarity can increase by a factor of about 10 with respect to the aforementioned values. Extreme testing in a drying cabinet at 74°C yielded evaporation stability to 8 µL/h compared to 10 µL/h for PTFE-foil lids and 200 µL/h for regular Petri dishes. Incorporating pigments or objects to the pre-mixed resin furthermore allows adding other functionality to the lids. The lids were extensively tested on neural cultures of embryonic chicken.

1 Introduction
Osmolarity affects neuroregulatory mechanisms [1]. In a follow-up study to work by Steve Potter [2] we present a new lid design for increasing cell culture longevity by means of stabilizing osmolarity and gas exchange. Quite often, even in humidified incubators the longevity of a cell culture is limited by a slow increase in osmolarity over time due to the evaporation of water. Using a simple replica-molding strategy based on poly(dimethylsiloxane) (PDMS), self-sealing lids for small sized culturing vessels can easily be fabricated. PDMS is a very flexible, autoclavable, biocompatible, and almost indestructible material of excellent transparency. Its refractive index is n = 1.43.

2 Results
Two types of highly gas-permeable cell culturing lids made from PDMS are presented (figure 1). In one type, the optical properties were modified by pigments (e.g. for dark studies). In general, gas permeability can be tuned by embedding polymer windows (e.g. polyester). Once a template is available, unlimited numbers of lids can be fabricated.

Fig. 1 Transparent and pigmented PDMS lid for standard Ø 24 mm MEA glass rings.

When ¼ to ½ of the culturing medium is exchanged on a regular basis (every 3-5 days), the presented lids allow stabilization of osmolarity in humidified incubators (hi) to increases of less than 5 mosmol/kg/month, and to less than 10 mosmol/kg/month in non-humidified incubators ( nhi). In contrast, osmolarity rises by approx. 30 mosmol/kg/month (hi) and by 80 mosmol/kg/month ( nhi), respectively, when no medium is exchanged. Under the same conditions, osmolarity in regular Petri dishes rises by approx. 210 mosmol/kg/month (hi). A summary of the worst-case scenario is depicted in figure 2.
Comparison of changes in osmolarity over time

![Graph showing comparison of osmolarity over time for different lid types and culture containers.](image)

Fig. 2 Comparison of the increase in osmolarity for PDMS-lids, PTFE-foil lids as described by Potter et al., 24-well cell culturing containers, and standard polystyrene Petri dishes.

Extreme testing in a drying cabinet at 74°C yielded evaporation stability to 8 µL/h for PDMS lids compared to 10 µL/h for PTFE-foil lids and 200 µL/h for regular Petri dishes.

The new lids were extensively tested on neural cultures of embryonic chicken, which survived for more than 9 months; in all cases the cause of death was contamination.

3 Methods

A simple template can easily be fabricated from a combination of two stacked plastic rods in a hollow cylinder (not shown). An adjustable template design is depicted in figure 3. It gives a choice of vertical lid geometries and membrane thickness. Lids were fabricated using poly(dimethylsiloxane) (PDMS, Sylgard 184, Dow Corning) with high gas permeability but limited vapor permeability and negligible permeability for liquids. The two-component resin requires curing for 1 hour at 60°C or 0.5 hours at 85°C.

Extreme testing in a drying cabinet at 74°C yielded evaporation stability to 8 µL/h for PDMS lids compared to 10 µL/h for PTFE-foil lids and 200 µL/h for regular Petri dishes.

The new lids were extensively tested on neural cultures of embryonic chicken, which survived for more than 9 months; in all cases the cause of death was contamination.

4 Summary

While PDMS lids and PTFE-foil lids cannot prevent evaporation of water, they significantly help in keeping osmolarity constant when compared to regular cell culturing containers. Gas exchange with the environment is not compromised. Easy fabrication, robustness, and self-sealing properties make PDMS lids a suitable alternative to their PTFE-foil counterparts.

Acknowledgements

Many thanks to Simone Riedel, Jan Aigner and Tobias Budenz for excellent lab support. We would also like to thank the EU for partial funding of these studies (IST-2000-26463 FP5 IST FET open).

References


Multi-well microelectrode arrays for cell culture and tissue slices

Marc Olivier Heuschkel¹*, Valérie Perrin², Ruth Luthi-Carter², Solomzi Makohliso¹

1 Ayanda Biosystems SA, PSE Parc Scientifique, Lausanne, Switzerland
2 Functional Neurogenomics Laboratory, EPFL, Lausanne, Switzerland
* Corresponding author E-mail address: marc.heuschkel@ayanda-biosys.com

In order to address the need to conduct parallel experiments on a single microelectrode array (MEA) biochip, new multi-well MEA biochips been developed and tested using dissociated hippocampus neuron cultures. First results show that these new multi-well MEA biochips are well suited for dissociated cell culture experimentation.

1 Introduction
Microelectrode array (MEA) biochips are now a well-established tool for basic electrophysiological research and are gradually finding utility in applied and industrial research applications, such as pharmaceutical drug screening. The relative ease and rapidity to derive functional biological information from MEA experiments makes them ideal candidate tools for developing novel cell-based assays for ion channel screening. One of the key factors that may contribute or accelerate the adoption of MEA device in industry would be the capacity to conduct parallel experiments on a single MEA platform, e.g. to test various concentrations or types of drug candidates.

2 New multi-well MEA biochips
As a first step towards this objective, we currently present a 4-well MEA device, which has been adapted to the data acquisition system from Multi Channel Systems MCS (Fig. 1). Each well typically comprises 15 microelectrodes arranged in a 4x4 matrix allowing recording and/or stimulation of the cell culture/tissue slices. In addition, one large embedded ground counter electrode has been provided, as well as two supplementary stimulation electrodes (100 µm x 600 µm) on both sides of the central recording area in order to permit global electrical stimulation of all the cells/tissue located in between (Fig. 2).

3 Preliminary results
Preliminary experiments with dissociated hippocampal neuronal cells were carried out over a period of more than five weeks, and the spontaneous electrical activity of the cells was periodically monitored. Robust electrical activity could be recorded from the cultures as shown in Fig. 3, indicating that a reduced number of 15 electrodes (or less) per well may be sufficient for routine MEA experiments with neuronal cultures.

Fig. 1 Multi-well MEA biochip consisting of 4 wells.

Fig. 2 Top view of electrode geometry of one single well, with 15 planar platinum recording electrodes (ø 40 µm, spacing 200 µm) located at the centre of the well. Two large stimulation (100 µm x 600 µm) and GND electrodes are located around the central workspace of the MEA biochip.
Fig. 3  Top: typical recordings of spontaneous electrical activity from dissociated hippocampal neurons (from P1 rat, 28 days in vitro) on a 4-Well MEA Biochip, where each quadrant represents data from each well. Bottom: Electrical recording from one electrode located in the right-bottom quadrant of the data displayed in the left picture.

4 Perspectives
Future efforts will focus on developing high-density multi-well MEA platforms that would be suitable for medium-to-high throughput cell-based drug screening activities.
Recording chamber for glass sensor chips with MEA and integrated oxygen-, pH- and temperature sensors

Florian Ilchmann*, Johann Ressler, Jochen Meyer, Helmut Grothe, Bernhard Wolf

Technische Universität München, Heinz Nixdorf - Lehrstuhl für Medizinische Elektronik, Munich, Germany
* Corresponding author. E-mail address: ilchmann@tum.de

A multi-purpose recording chamber for long-time cell monitoring of bioelectronic signals has been developed. The chamber is designed for multiparametric sensor chips without external sensors, facilitating process sterility outside laminar flow benches. It contains a glass sensor chip integrating different sensors for extracellular cell recordings, i.e. a microelectrode array (MEA), an amperometric sensor to measure changes in dissolved oxygen, a pH sensor and a temperature sensor. The chamber includes the circuits for all these sensors except for the microelectrode array and provides temperature control for both the sensor chip and the culture medium.

1 Combined recording of electrophysiological activity and metabolic parameters

The utilization of cultured neuronal networks grown on sensor chips with MEAs for toxicological studies is straightforward and has yielded extensive knowledge in neurological research [1]. However, stable culturing of neuronal networks over long culturing periods requires strict control of microenvironmental parameters. Therefore the quantitative analysis of microenvironmental conditions and cell metabolic rates is helpful to interpret the changing patterns of electrophysiological activity.

For this reason a recording chamber has been developed, offering the possibility to handle sensors for dissolved oxygen, pH and temperature and providing accurate temperature control of sensor chip and culture medium.

2 Recording setup

2.1 Sensor chips

Glass-based sensor chips have been designed, including MEA and sensors for oxygen, pH and temperature. In addition to the obvious benefit for microscope inspection from both sides, glass-based sensor chips with metal oxide pH sensors need no silicon MOS technology. Glass chips are therefore not sensitive to electrostatic discharge, resulting in easier handling precaution. In addition, manufacturing costs are much lower for small quantities of large area chips making them ideal suited for one-time use.

For the detection of electrophysiological activity, a microelectrode array is placed in the centre of the sensor chip. It consists of 64 electrodes arranged in an 8x8 array.

To monitor the microenvironmental parameters of the living cell culture, three sensors were placed in immediate vicinity of the 8x8 microelectrode array resulting in accurate measurement of the cellular parameters.

For measuring the pH-value, a ruthenium oxide (RuO2) spot is applied on the surface of the sensor chip. Together with a reference electrode integrated in the recording chamber, pH measurement of the culture medium is possible.

Changes in the oxygen concentration can be monitored with a planar amperometric oxygen sensor. It consists of three electrodes (working-, quasi-reference- and auxiliary-electrode) without membrane structures. The diameter of the working electrode is \(\approx 30 \, \mu\text{m}\), the working potential -600 mV.

The temperature of the cell culture medium is measured by a platinum resistor (PT100 or PT1000) integrated on the sensor chip surface [2].

All conducting paths are fabricated from a thin platinum layer. The sensor chip is coated with an insulation layer which is opened only on the active sensor surface and the contact strips (shown in red in Fig. 1).
Due to the transparent substrate, all kinds of microscope monitoring are possible, making these chips powerful tools for neuro-bioinformatics.

2.3 Recording chamber

For multiparametric measurements with the sensor chip, a recording chamber was developed (Fig. 2). The measured parameters are directly amplified and converted to RS232 standard for computer readout within the platform. Furthermore, an integrated regulated heating foil underneath the sensor chip ensures stable temperature control for cell cultivation and enables the user to operate the chamber outside of an incubator.

In combination with a Ag/AgCl reference electrode integrated in the fluidic system, the \((\text{RuO}_2)\) spot on the sensor chip allows potentiometric pH measurement of the culture medium.

The upper part is the electronic circuit-board itself, fixed on a thin mounting plate. Gold-plated spring contact pins connect the MEA as well as the sensors and the heating elements. Thereby, reliable and constant contact resistance is achieved without the risk of damaging the glass sensor chip.

2.4 Electronics

The electronic circuits can be divided into three groups. Sensor amplification is the most important step, decisive for a precise measurement with low noise level. After A/D conversion, a microcontroller sends the measured data via RS232 interface to an external computer. The third part of the electronic section is the heating regulation circuit which works with an accuracy of up to 0.01°C using a proportional-integral-derivative (PID) controller.

Several LEDs give feedback to the user showing measurement and power supply status. In addition, separate over- and undertemperature outputs indicate when the chamber temperature is out of range.

Integrated electromagnetic shielding keeps the sensor signals, including MEA, from external noise influence. Optional, the internal software interrupts the heating process to safeguard critic measurements.

The entire circuit board is coated to protect against moisture, making it especially suitable for laboratory work. As mentioned before, the complete platform can be used outside an incubator resulting in flexible use and better performance.

Acknowledgement

The authors express their personal appreciation of the valuable assistance given them in their research by many students at the Heinz Nixdorf - Lehrstuhl für Medizinische Elektronik. Without their co-operation the extensive work involved in compiling background information and preparing test models for research would not have been possible.

Financial support by the Heinz Nixdorf Foundation is kindly acknowledged.

References


CHIMIA Vol. 59: No. 5, 243-246, 2005
Tools for Automated Stimulation and Connectivity Analysis of Neuronal Networks Grown on Multielectrode Arrays

David B. Khatami1,2*, Yoonkey Nam2,3, Bruce C. Wheeler1,2,3
1 Department of Electrical and Computer Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA
2 Beckman Institute, University of Illinois at Urbana-Champaign, Urbana, IL. USA
3 Department of Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL. USA
* Corresponding author. E-mail address: khatami@uiuc.edu

Although neural networks grown on top of microelectrode arrays appear to provide a great and powerful tool for the study of neuronal plasticity and mechanisms for learning and memory, the progress in this area has not been as rapid and earth shattering as one would have hoped. Apart from the profound complexity and huge variability which is inherent in such network, the lack of proper technological capacities has proved to be a hindrance. One such shortcoming is a lack of automated and dynamic stimulation capability; another is lack of a visually appealing software interface for automatically processing the massive amounts of data generated thus providing quick feedback to the researcher. The aim of this paper is to propose a simple scheme which can be employed on top of existing commercially available devices in an effort to address both issues.

1 Introduction

Planar multielectrode arrays (MEAs) have been used extensively in neural engineering research. In particular, they are recognized as a good tool for in vitro analysis of the properties of biological neuronal networks. A popular theme in neural engineering research deals with the notion of investigating mechanisms of learning and memory in vitro by attempting to induce short- and long-term changes in the properties of such networks. A hindrance to the progress of these projects stems from the technological shortcomings of commercially available devices for stimulation and data analysis. One such shortcoming is a lack of automated and dynamic stimulation capability; another is lack of a visually appealing software interface for automatically processing the massive amounts of data generated thus providing quick feedback to the researcher. The aim of this report is to address both issues. It provides a simple scheme for enhancing the functionality of the commercially available MultiChannel System’s hardware so as to provide semi-automated stimulation capability. We also introduce a Matlab-based tool that can be used to quickly and easily assess functional connectivity.

2 Methodology

2.1 Semi-automated stimulation capability

In this context, semi-automated stimulation capability refers to a process in which the stimulus can be automatically redirected from one channel to another during the experiment without the need for the user to intervene. The sequence of channels or groups of channels to be stimulated at a given time is extracted by the computer from a file outlining the experiment. Although commercial devices provide good hardware and software interfaces for stimulation and recording, the current state of these systems does not support automatic switching of the stimulus from one channel to another or between groups of channels as would be desirable in many practical experimental setups. Here we present a simple scheme for achieving semi-automated stimulation utilizing MultiChannel Systems’ hardware and software. A schematic diagram of the overall enhanced system is provided in Figure 1.
2.1 An analysis tool for quick assessment of functional connectivity

A Matlab-based tool was developed primarily as a way to effectively manage the wealth of raw data generated while attempting to decipher functional connectivity on a large scale. The main focus behind the design of this tool has been on creating rich visual diagrams that would capture the features in the data most appealing to the researcher. In particular, our software has engaged the challenging task of providing the user with a visual sense of the global connectivity patterns that exist within the network and to provide quick feedback on the spatial and temporal properties of such connections. The main display panel of the analysis software as well as a display indicating the detailed stimulus-response relationship for a single connection are shown in Figures 2 and 3 respectively.

3 Summary

We hope that these tools would speed up the analysis process and take away the burden of subjectively visually analyzing and interpreting tens and sometimes hundreds of data streams from the experimenter. Consequently, it would empower the researcher to design more elaborate experiments utilizing the full potential of the electrode array as well as employing many more stimulation trials without the fear of data processing becoming overwhelming and exceedingly challenging.

Fig. 1 The schematic of hardware/software integration and communication. The MC Select code is modified to accept an input file containing the sequence of channels to be stimulated which is communicated to the MEA1060-BC head-stage via the serial port. The timing of stimulus generation and transition between channels is coordinated via the parallel port.

Fig. 2 Main panel of the Matlab-based “Stimulation Multichannel Analyzer.” In this randomly seeded primary E-18 hippocampal culture (52 days in vitro), most of the connectivity appears to converge to one of 3 or 4 specific locations within the network. Note: in the actual software the connections appear in color resulting in a much more effective visualization of the connectivity patterns.

Fig. 3 Events elicited on Ch. 63 by applying 50 current pulses of varying amplitudes to Ch. 27. It is an example of one of several displays that can be called on from the main panel in order to gain a better understanding of the detailed features of a particular stimulus response pair or to check its validity. Note: in the actual software the response from each stimulus intensity is color-coded resulting in perceptual enhancement of the data.

Acknowledgement

This project was funded by the National Science Foundation under Grant EIA 0130828 and by the National Institutes of Health under Grant R01 EB000786. We thank Multichannel Systems for providing access to computer code to assist in the development of the stimulus protocol.
Recording electrophysiological activity by means of high density MEAs: theoretical models, extracellular signal simulations and measurements

Paolo Massobrio1*, Sergio Martinoia1, Luca Berdondini2, Kilian Imfeld2, Milena Koudelka-Hep2, André Garenne3, Gwendal Le Masson3

1 Neuroengineering and Bio-nano Technologies Group, Department of Biophysical and Electronic Engineering (DIBE), University of Genova, Genova, Italy.
2 Institute of Microtechnology (IMT), Université de Neuchâtel, Neuchâtel, Switzerland.
3 INSERM EMI 358, Institut François Magendie - Université Bordeaux 2, Bordeaux Cedex, France.
* Corresponding author. E-mail address: paolo.massobrio@dibe.unige.it

The increase of the number of recording electrodes of one order of magnitude is one of the main technological challenges for MEA applications. Here we present preliminary results of a new technology devoted to the realization of a new generation of MEA. The main features of these new MEAs are the high number and the close spacing among electrodes (e.g., 64 x 64 active microelectrodes with diameter and electrode separation of 20 μm). A simple electrical model circuit, which describes the behavior of the new devices coupled to a neuronal network, was developed and the results obtained by our simulations, compared with the experimental data.

1 Introduction

The use of microelectrode arrays (MEAs) for extracellular recordings from excitable cells cultures is now a well-known and accepted technique in both fundamental neuroscience research and applied electrophysiology [1]. Nevertheless, the current technological limitations restrict the mapping of spatial patterns capabilities of MEAs: the main drawback is constituted by a large under-sampling factor (only 100 neurons are probed on a square active area of 1.2 x 1.2 mm on a neuronal culture with a density of about 10^4 cells/mm^2) and by the impossibility of selecting precisely which neuron we are recording from or stimulating at. In order to overcome these limitations, a high-resolution MEA [2] is currently being developed. To understand the potential recording capability of these high-density devices, MEAs encompassing local high-density microelectrode areas (HD-MEAs) have been fabricated and tested. A simple electrical model circuit, which describes the behaviour of the new devices coupled to a neuronal network, was developed and the results obtained by our simulations, compared with the experimental data coming from cortical neurons.

2 High-density MEA

2.2 The main features

The main features of the HD-MEA are the high number and the close spacing among electrodes: in particular the final version of the device will be constituted by 64 x 64 active microelectrodes with diameter and electrode separation of 20 μm. The preliminary tests were realized by using devices made of 4 sub-regions, each constituted by a small array of 4 x 4 electrodes with electrode pitches of 10 μm and 20 μm respectively (Fig.1).

![Fig. 1 Cluster of a HD-MEA, and its schematic representation.](image-url)
Several simulations were performed in order to explain the genesis of the recorded signal waveforms. We simulated the actual configuration of the fabricated HD-MEAs (Fig.1). Different coupling configurations among the HD region and neuronal networks (by varying the number of neurons, and the synaptic connectivity) were tested. The simulations were carried out also by varying the adhesion conditions, the number of neurons coupled to one electrode (1:1, 3:1 correspondence, etc.).

3 Results

3.1 Simulation results

We presented the simulation results concerning the effect of the short distance between adjacent microelectrodes. We proved some simulations, by sweeping the value of the shunt resistor $R_{sh}$ of Fig.2.

Fig.3 shows the simulated results obtained by the SPICE simulations of a neuronal network made of 16 neurons synaptically connected and coupled to the microelectrodes in a 1:1 correspondence.

It should be noted that when the value of $R_{sh}$ increases, electrophysiological signals coming from neurons coupled to surrounding electrodes are detected. The amplitude of these artefacts is proportional to the value of $R_{sh}$. Below the value of 50 kΩ, these cross-talk like effects disappear.

3.2 Experimental data

We recorded spontaneous activity from different neurobiological preparations (cardiomyocytes, spinal cord, cortical and hippocampal neurons of rat embryos), in order to characterize and validate the behaviour of these new devices. We analyzed the results in term of raster plot, cross-correlation, ISI, and IBI (not shown). Fig. 4 shows an example of raster plot coming from a culture of cortical neurons at 20 DIV.

4 Conclusions

These preliminary results pointed out the good properties of the HD-MEAs, in terms of signal transduced, biocompatibility, and spatial resolution. Moreover, the simple developed model matches well the experimental data and offers a useful tool to understand the actual recorded signals.

Acknowledgement

Work supported by IDEA Project (EU, Contract n.: 516432 NEST).

References


In vitro Assessments of Vascular Damage and Tissue Deformation Following the Insertion of Silicon Neural Probe

Seung Jae Oh\textsuperscript{1,2}, Jong Keun Song\textsuperscript{1,2}, CS Bjornsson\textsuperscript{3}, Y Al-Kohafi\textsuperscript{3}, Karen L. Smith\textsuperscript{3}, James Turner\textsuperscript{3,4}, William Shain\textsuperscript{3,4}, Sung June Kim\textsuperscript{1,2*}

\textsuperscript{1} Nano-Bioelectronics & Systems Research Center, Seoul, Korea
\textsuperscript{2} School of Electrical Engineering, Seoul National University, Seoul, Korea
\textsuperscript{3} Wadsworth Center, New York State Department of Health, Albany NY, USA
\textsuperscript{4} Department of Biomedical Science, School of Public Health, University at Albany, Albany NY, USA

* Corresponding author. E-mail address: kimsj@snu.ac.kr

Many kinds of micromachined silicon neural probes are developed and applied to brain research by using invasive method of surgical technique. While these micromachined silicon devices produce acceptable experimental results, the full potential of neuroprosthetic devices is presently limited by biological reactive responses that begin immediately upon their insertion into the body. The magnitude of these responses is affected by many insertion-related parameters, such as the insertion speed, device size, and tip design. A neuroprosthetic device inserted into a neural structure will disrupt the natural structure to some extent causing tearing, cutting, stretching, or compression. The goal of this paper is to improve our understanding of the relationship between insertion mechanics and vascular injury and tissue deformation in the brain.

1 In vitro experimental setup with living brain slices

Fig. 1 Scanning electron micrographs of the tips of fabricated silicon probes with different sharpness (A: 5degree-sharp probe, B: 90degree-medium probe and C: 150degree-blunt probe) and multishank array with various intershank spacing (D)

Fig. 1 shows scanning electron micrographs of the tips of fabricated silicon probes with different sharpness and multishank array with various intershank spacing. All devices used for this experiment had a 2-mm-long shaft, and the cross section dimensions of the shaft were 60μm(thickness) x 100μm(width). The schematic of the in vitro insertion and image capture setup are depicted in Fig. 2. Time-lapse images were collected during probe insertion and withdrawal into 500μm thick brain slices using a fluorescence microscope. The speed and moving distance of the device were automatically controlled with commands pro-
grammed into the microcontroller. To determine the mechanical behaviour during insertion and retraction of the probe into the dura meter of the brain surface, a high-sensitivity silicon strain gauge was used. And then immunohistochemistry for laminin was used as a measure of tissue trauma.

2 Brain damages according to the sharpness of the tips and speed of insertion

![Fig. 3](image) Time-lapse images of the surface deformation and haemorrhaging in the cortical area during insertion of a sharp probe with a tip angle of 5 degrees. These images were obtained during probe insertion at 2 mm/sec (left panels), 0.5 mm/sec (middle panels), and 0.125 mm/sec (right panels). Scale bars are 200 µm.

To study the effects of sharpness of the tips on tissue damage, three kinds of tips (5-, 90-, 150-degree) were inserted into the living brain slice with the speed of 2 mm/sec (high), 0.5 mm/sec (intermediate), 0.125 mm/sec (low) respectively. Time-lapse image of the surface deformation and haemorrhaging in the cortical area during insertion of a sharp probe are shown in Fig. 3. The results shows that sharp tips, faster insertion are better than blunt tips, slower insertion for penetrating the brain surface with less tissue compression, and although they can cause severe vascular rupture they cause less crush damage to neural cells. This may suppress the vascular response and inflammation in chronic applications.

3 Tissue-deformation forces during the insertion of neural probe array with multishanks

The insertion force required to fully advance the array was found to increase with decreasing insertion speed: when the high-density array with a shank spacing of 100 µm was inserted at 2, 0.5, and 0.125 mm/sec, the insertion forces were 88±39 (mean±SD), 270±97, and 962±171 mN, respectively (Fig. 4); and a similar dependence on probe velocity was observed for devices with shank spacings of 300 and 500 µm. The number of vascular elements presented by laminin expression was greatest for the tissue samples from the 100-µm-spacing insertion group, and least for the tissue sample from the 500-µm-spacing insertion group.

![Fig. 4](image) Raw data of distribution of insertion force in three-axes during epidural insertion using a type I probe (ten shanks, 100-µm spacing) into the brain at 2 mm/sec (A), 0.5 mm/sec (B), and 0.125 mm/sec (C). (D) Z-axis components of the insertion force at each speed. Z-axis is the direction of insertion.

4 Summary

In this paper, we successfully showed how insertion force and tissue trauma occur during epidural insertion of the multi-shank silicon arrays. The results presented here clearly demonstrate that the force required to insert an electrode array can be dramatically reduced by the use of high-velocity insertion. The experimental model developed and knowledge of geometry effects from the experimental data obtained can be used in the design of multishank arrays for large-scale neural interfaces.

Acknowledgement

This work was supported by the International Collaboration Program, NBS-ERC (Nano Bioelectronics and Systems Engineering Research Center)/KOSEF (Korea Science and Engineering Foundation) and also supported in part by the Nanobiotechnology Center (NBTC), an STC Program of the National Science Foundation under Agreement No. ECS-9876771.
Chronic Cellular Reactions to Silicon Neural Probe Implant

Seung Jae Oh$^{1,2}$, Jong Keun Song$^{1,2}$, CS Bjornsson$^3$, Y Al-Kohafi$^2$, Karen L. Smith$^3$, James Turner$^{3,4}$, William Shain$^{3,4}$, Sung June Kim$^{1,2*}$

1 Nano-Bioelectronics & Systems Research Center, Seoul, Korea
2 School of Electrical Engineering, Seoul National University, Seoul, Korea
3 Wadsworth Center, New York State Department of Health, Albany NY, USA
4 Department of Biomedical Science, School of Public Health, University at Albany, Albany NY, USA
* Corresponding author. E-mail address: kimsj@snu.ac.kr

Abstract
Chronic use of micromachined silicon neural probes is limited due to the formation of a complex sheath of cells and extracellular proteins that electrically isolates devices from adjacent neurons and neuronal damage. Understanding device-tissue interactions in the brain will provide a basis for developing successful interface strategies for biocompatible microdevices. While the reactive responses to single shank silicon devices inserted into neocortex are well characterized, responses in other regions have not been described. This study was designed to determine if the reactive responses observed in hippocampus and thalamus are similar to those observed in neocortex. This information is necessary to design and implement appropriate intervention strategies to control regional cell and tissue responses. Study of responses in hippocampus and thalamus has important scientific and clinical implications.

Fig. 1 Schematic diagrams showing probe placement in the brain. Silicon probes were implanted into the right hemisphere of adult Sprague-Dawley rats. (A) Sagittal section (B) Coronal section. Blocks were sectioned into tissue slices 100 μm thick from three different brain levels: neocortex, hippocampus, and thalamus.

1 In vivo implant surgery and in vitro experimental setup
A craniotomy was performed over the right hemisphere near the sensory motor cortex 3-mm distal and 1-mm lateral from Bregma(Fig.1). A hole, with a diameter larger than the tabs of the devices (700 x 700 μm2), was drilled through the skull, and the dura was reflected. Devices used for these experiments had a single 5-mm long shaft. The cross-section of the shaft was 50 μm (thickness) x 128 μm (width). Each silicon device was inserted using a custom made automated inserter at a speed of 2mm/sec. At 1 hr, 24 hr, 1 wk, or 6 wk following device insertion, animals were anesthetized with tribromoethanol and perfusion fixed with 4% paraformaldehyde in phosphate buffer, pH 7.4.

2 Chronic cellular reactions to silicon neural probe
We examined region-specific differences of reactive response in three different brain regions: neocortex, hippocampus, and thalamus. Silicon devices with shafts 5 mm long and 50 x 128 μm in cross-section were used. Immunohistochemistry was performed to identify reactive astrocytes (GFAP), vasculature (laminin), and activated microglia (CD11b) at 1 hr, 24 hrs, 1 wk, and 6 wk. Images were collected as three-dimensional data sets using laser scanning confocal
microscopy. Though the morphology, distribution, and numbers of reactive cells in the deeper brain regions differed from those observed in neocortex, the reactive responses in all three brain regions followed a similar pattern.

GFAP immunoreactivity was greatest in the hippocampus. CD11b was comparable in neocortex and hippocampus. Laminin expression in all regions extended considerable distances from insertion sites at 1 hr, and decreased at later times. Regional differences in glial cell activation may be caused by differences in microglia and astrocyte density or inflammation-related factors produced by these cells (Fig. 2).

3 Summary
In this paper, we successfully showed that regional differences of reactive responses may result from differences in vascular properties, differences in receptors or amounts of protein products responsible for the response, or sub-types of cells present in different brain regions. These data support our hypothesis that multi-shank devices produce overlapping reactive zones of influence. These results indicate that understanding cell and tissue reactive responses to micro-machined devices in deep brain regions is necessary for developing new generations of deep-brain prostheses.

Acknowledgement
This work was supported by the International Collaboration Program, NBS-ERC (Nano Bioelectronics and Systems Engineering Research Center)/KOSEF (Korea Science and Engineering Foundation) and also supported in part by the Nanobiotechnology Center (NBTC), an STC Program of the National Science Foundation under Agreement No. ECS-9876771.

References

Fig. 2 Triple-labeled images were obtained using a 40X objective lens. GFAP (green), laminin (blue), and CD11b (red) are markers for astrocytes, laminin, and microglia, respectively. Scale bars represent 100 µm. The integration of astroglial, microglial, and vascular responses provides a clearer representation of the reactive responses around inserted devices. The increase in CD11b signal and the delayed increase in GFAP signal clearly demonstrated that ultimately similar numbers of microglia and astrocytes are participating. In hippocampus there was a clear transient decrease followed by a large increase in GFAP signal (row B). The overall increase in CD11b signal/contribution appeared to be less than in neocortex at earlier times, but by six weeks the CD11b and GFAP signals appeared to be similar in both the numbers of cells described and their relative intensities. Clear differences in thalamus responses were observed (row C).
MEA recordings of the spontaneous behavior of Hen Embryonic Brain (HEB) Spheroids

Ivan S. Uroukov1,*, Mingwen Ma1, Larry Bull2 & Wendy M. Purcell3

1 Faculty of Applied Sciences, University of the West of England, Bristol, U.K.,
2 Faculty of Computing, Engineering, and Mathematical Sciences, University of the West of England, Bristol, U.K.,
3 Faculty of Health and Human Sciences, University of Hertfordshire, Hatfield, Herts, U.K.,
* Corresponding author: Ivan.Uroukov@uwe.ac.uk

It has long been known that aggregated neuronal cell cultures exhibit properties that are remarkably similar to their in vivo counterparts. Recently, a hen embryo brain aggregate model was presented as a suitable paradigm in which to perform in vitro toxicology studies. Termed spheroids, it builds upon previous studies with liver cell spheroid cultures in which it was shown that such artificial tissue constructs more closely mimic intact liver tissue than liver cell monolayer cultures. The majority of in vitro studies of the electrophysiological properties of neuronal networks exploit either tissue slices or monolayer cell cultures. The research into the electrophysiological properties of neuronal monolayers has shown complex spiking behavior in the later developmental stages, e.g., after 28 days in culture. However, because of the potential for more organotypic network formations in three-dimensional (3D) aggregated cell cultures, they may show significant differences in their electrophysiological behavior. Therefore hen embryo forebrain spheroids are being developed in order to study both the electrophysiological activity and structural properties of the cultures.

1 Study design and results

1.1 Materials and methods.

3D chick forebrain cell cultures were prepared under strict gyrotatory conditions to form spheroids. A structural study by means of SEM (ESEM) and CLSM was initiated to reveal the structural properties of the neuronal formation and gain knowledge about synaptic structuring and general organization of the spheroids. GFAP and NF stains were imaged to find the space distribution of glia and neurons in the spheroid. The levels of acetylcholine esterase as a synaptic activation was estimated in the time course of spheroid maturation. Multi Electrode Array MEA, (Multichannel Systems, Gmbh) dishes and DAQ PC (Multichannel Systems, Gmbh) were used to record the electrophysiological activity, across the in vitro lifetime of the spheroids, undertaken in 18 cultures in toto.

![SEM imaging of spheroids surface](image1)

![Confocal microscopy imaging of the spheroids surface](image2)
1.2 Results

Structural study

Brain spheroid surface structural information was obtained by means of ESEM, SEM (Figure 1), light microscopy (Figure 5) and confocal microscopy (Figure 2). In all cases, data showed an outer layer to the spheroid (termed glia limitans) consisting mostly of glial cells and glial fibres. GFAP and NF (Figure 3) imaging suggested an inner layer of neuronal distribution, approaching the centre of the spheroid. The concentration of micro vials structures were found in the peripheral regions of the spheroids. The limitan layer was built upon the ECM fabric network and majority of glia. The initial electrical measurements were designed with 2D electrode arrays. Due to the glia layer, the recorded activity was comparable to the noise levels. Using 3D electrode arrays, measurement of the inner network activity was realized due to penetration of the limitans.

Spiking activity

The typical spontaneous spike pattern (Figure 4) was observed during the lifetime course, gradually changing with the maturation, expressed in spike organization repertoire. The temporal spiking activity accorded with the activity biochemical assessment of neuronal activity. An independent estimation of acetylcholine esterase showed a maintained activity between DIV7 to DIV14. Ex-vivo evaluation of the recorded data showed increased spiking in the presence of acetylcholine $10^{-4}$ M over the spontaneous activity.

2 Conclusions

In comparison with the electrophysiological activity of monolayer cultures, the 3D spheroids exhibit similar behaviors in the early stages of development. In the mature stage, the spheroids tend to express organised spike-train behavior consistent of many spikes tightly packed in short trains. Thus it would appear that the stages of spontaneous spike activity in spheroids and monolayer do not show significant differences.

Acknowledgement

This work was supported under EPSRC grant no. GR/T11029/01.
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