IN VITRO CORTICAL NETWORKS FOR DISEASE MODELING
AND DRUG EVALUATION

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Dissertation Prepared for the Degree of

DOCTOR OF PHILOSOPHY

UNIVERSITY OF NORTH TEXAS

December 2013

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Wu, Calvin. *In Vitro Cortical Networks for Disease Modeling and Drug Evaluation.*
Doctor of Philosophy (Biological Sciences), December 2013, 67 pp., 20 figures, references, 150 titles.

In translational research, disease models in preclinical studies are used as media for discovery of drugs or novel therapeutics. Development of in vitro models for various neurological diseases that enable efficient pharmacological or toxicological screening has been ongoing but challenging. Recognizing the potential benefit of in vitro disease models, dysfunctions in the cortical neuronal networks were induced to mimic the functional pathology of neurological symptoms using microelectrode arrays. Two different disease states – tinnitus and excitotoxicity – were investigated and discussed. In this model, pentylenetetrazol-induced increase in spontaneous firing rate and synchrony in the auditory cortical networks was used as correlate of tinnitus. Potential tinnitus treatment drugs from several different classes – including the novel class of potassium channel openers – were screened and quantified. The potential therapeutic values of these drugs were also discussed as the basis for drug repurposing.

Functional excitotoxicity was induced by cisplatin (a cancer drug that causes neurological side-effects) and glutamate (the major excitatory neurotransmitter). As proof-of-principle that the model may contribute to expediting the development of therapeutics, cisplatin excitotoxicity was prevented by the antioxidant D-methionine, while glutamate excitotoxicity was prevented by ceftriaxone (a modulator of a glutamate reuptake transporter). In the latter part of the study, with results linking two of the screened drugs L-carnitine and D-methionine to GABA_\text{A} receptor activation, it was demonstrated that this model not only served as an efficient drug-screening platform, but can be utilized to functionally investigate the underlying mechanism of drugs. In
addition, several practical or conceptual directions for future studies to improve on this \textit{in vitro}
disease model are suggested.
ACKNOWLEDGMENTS

The present study was performed at the Center for Network Neuroscience (CNNS) in the Science Research Building, University of North Texas (UNT), Denton, TX. Funding for the study came in part from the Charles and Josephine Bowen memorial endowment to the CNNS, and a Faculty Research Grant (GA9289) from UNT, as well as a gift from the Once Upon a Time Foundation awarded to Dr. Ernest J. Moore. Among the many people who supported my studies in the past years, I especially thank the following:

Dr. Guenter W. Gross, my supervisor, “The Wizard of Hickory Street,” your enthusiasm in research has been an inspiration. Your knowledge in neurophysiology as well as wisdom of life in and out of the laboratory has been invaluable. Lessons learned under your supervision will remain with me throughout life.

Dr. Ernest J. Moore, Department of Speech and Hearing Sciences, my co-supervisor, a mentor who introduced me to scientific research, your guidance throughout my training has been instrumental and your insights continues to be a great asset.

Dr. Kamakshi Gopal, Department of Speech and Hearing Sciences, thank you for your valuable techniques and experience in clinical applications of MEA, as well as and your kindness and patience that accompany me throughout the past years.

Ahmet Ors, thank you for your expertise in MEA manufacturing, a constant supply of high quality MEA has been a cornerstone of the laboratory and indispensable to my study. Sarah Valliere (2009–2011), Nga Nguyen (2011–2012), Nicole Calderon (2012–2013), thank you for your efforts in the difficult task of maintaining primary neuronal cultures.
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<tr>
<td>ACN</td>
<td>Auditory cortical network</td>
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<tr>
<td>AC</td>
<td>Auditory cortex</td>
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<td>AED</td>
<td>Anti-epileptic drug</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>BK</td>
<td>Big conductance voltage-sensitive and calcium-activated K⁺ channel</td>
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<td>L-Car</td>
<td>L-Carnitine</td>
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<td>CN</td>
<td>Cortical network</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DIV</td>
<td>Days in vitro</td>
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<tr>
<td>DSP/dsp</td>
<td>Digital signal processor</td>
</tr>
<tr>
<td>EC₅₀/ECₓₓ</td>
<td>Half maximal (50%) or xx% effective concentration</td>
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<tr>
<td>K⁺</td>
<td>Potassium</td>
</tr>
<tr>
<td>Kᵥ</td>
<td>Voltage-sensitive potassium channels</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>Half maximal (50%) lethal dose</td>
</tr>
<tr>
<td>MEA</td>
<td>Microelectrode array</td>
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<tr>
<td>D-Met</td>
<td>D-Methionine</td>
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<tr>
<td>L-Met</td>
<td>L-Methionine</td>
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<tr>
<td>PTZ</td>
<td>Pentylenetetrazol</td>
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<td>SNEPCO</td>
<td>Selective neuronal potassium channel opener</td>
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A primary goal of scientific research is to benefit society. In medicine, the adaptation of basic medical discoveries for application in health care is termed “translational research.” The early stage of translational research, T1, is the “bench-to-bedside” process where observations in basic research are brought to use in the clinical settings (Woolf, 2008). The intermediate process between basic research and clinical studies requires disease models as media for drug discovery or development of novel therapeutics. The disease model may be an animal presenting induced symptoms or genetic components of a particular disease. For instance, mice may be induced with behavioral Parkinsonism using a toxin MPTP, or with transgenic mutations on leucine rich repeat of the serine/threonine kinase, both widely used Parkinson’s disease models (Blandini and Armentero, 2012). A disease model can also be based on cells grown in a dish: “in vitro,” especially for diseases that have well-defined cellular origin, such as microglial-mediated neuroinflammation, which is attained by using pro-inflammatory cytokines on neuron-microglial co-culture (Gresa-Arribas et al., 2012). The in vitro models often complement the animal (in vivo) models. Compared to the complexity of a whole organism, an in vitro model consists of only a single component (cell or tissue type), and is thus suitable for investigating a simplified and defined problem. With reduced cost and labor for animal care, in vitro models are also ideal for pharmacological or toxicological screening studies. However, for diseases in the central nervous system (CNS), in vitro models are less common. Unlike many other cell types in the
body, neurons do not divide, once differentiated. Thus, an in vitro model based on non-proliferating cells is more difficult to maintain. The pathological origins of many neurological diseases also remain largely unresolved. Despite these challenges, primary culture models based on hypothesized disease mechanisms, are being pursued and commercialized (see Charles River catalog of in vitro CNS models, Charles River Inc., Wilmington, MA).

**Neuronal Networks on Microelectrode Arrays (MEAs)**

A unique characteristic and primary purpose of nerve cells is that they conduct electrical signals via excitable membranes and intricate synaptic connections among cells. In electrophysiology, functional properties of neurons or other excitable cells are studied by recording the electrical activity (either intracellular or extracellular). This functional aspect of neurons is applied in modeling neurological diseases – diseases that involve aberrant electrical activity, such as an in vitro model for epilepsy (Wong, 2011), and interestingly, models for psychosis and schizophrenia (Greene, 2001). Within electrophysiological techniques, however, there are different levels of observations: at one end of the spectrum are trans-membrane current/voltage recordings from single cells, at the other end, are electric field potential recordings from multiple neurons (Boulton, 1990). Each approach provides a different perspective. Single cell recordings investigate the mechanism of specific ion channels or synaptic transmission, whereas, field potential recordings provide insight into the dynamics of collective actions of neuronal tissue. In between the two extremes lies the microelectrode array (MEA) technique based on simultaneous multi-unit recordings, capable of studying the collective actions of neurons while retaining single-unit information (Gross, 1979; Gross et al., 1997). Another advantage of the
multi-unit recording is fault tolerance, which avoids errors in single-unit observations (Gross and Kowalski, 1997). This platform is thus well-suited for studying the properties of spontaneously active neuronal networks.

A Cultured Cortical Network Model of Disease

Dysfunctions of neuronal networks in the cerebral cortex are exhibited as neurological symptoms. Although some diseases such as Alzheimer’s or Parkinson’s disease have initial progressions in other brain regions, the cortical neurons are targeted and responsible for the maladies in patients (Braak et al., 2006). Other diseases such as epilepsy or tinnitus have variety of hypothesized pathological origins but appear as hyperactivities in the cortical networks (Lossin et al., 2003; Roberts et al., 2010). Thus, an in vitro model of cortical networks (CN) – with a presumed inducible disease state – may contribute to the understanding of dysfunctional mechanism and more importantly, provide a platform for pharmacological screening. Currently, there is no in vitro model based on cultured CNs for functional observations. As a proof-of-principle that disease models can be studied using the MEA model, two different disease states – correlates of tinnitus and excitotoxicity – were investigated and discussed.

Aberrant Network Activity: Tinnitus

Neuroscience of Tinnitus

Tinnitus is a subjective phenomenon of hearing a phantom sound without a known physical stimulus present. The malady affects approximately 50 million people in the USA alone. It can be experienced by people with normal hearing (Thabet, 2009) as well as most noticeably
by individuals who have some type of auditory deficit (Littler, 1965). It is also exacerbated by the fact that no chemotherapeutic agents have been found to alleviate the problem over a long period of time (Baguley, 2002). The problem lies with the fact that the fundamental physiological and biochemical mechanisms that underlie tinnitus remain unclear (Kaltenbach, 2000, 2006; Roberts et al., 2011). Nevertheless, ongoing research during the past decades have established putative physiological correlates for this subjective pathological phenomenon, namely, an increase in neuronal firing rate and firing coordination, “neural synchrony,” in the auditory cortex (AC) (Eggermont, 2007; Eggermont, 2008; Kenmochi and Eggermont, 1997; Ochi and Eggermont, 1997; Roberts et al., 2010). As an emergent property of aberrant neuronal network activity, tinnitus cannot be observed at the single-cell level. Animal models using behavioral paradigms that infer the subjective experience have been the standard in tinnitus research (Bauer et al., 1999; Kaltenbach, 2011; Turner, 2007). In animal models, it was found that several mechanisms were implicated in the genesis of tinnitus in the AC network (Eggermont and Roberts, 2004):

- Deafferentation (loss of input) of thalamocortical connection to AC due to noise-trauma or drug-induced damage.
- Inactivation of feed-forward and feedback GABAergic interneuron connections because of reduced thalamocortical innervation.
- Adjacent neurons (which may be responding to different frequencies) increase excitatory inputs to the deafferentated region as they were released from inhibition (lateral disinhibition); adjacent frequency-specific regions expand into the deafferentated region (reorganization; Eggermont, 2008; Noreña et al., 2010).
Neurons in the AC, as well as in all subcortical relay structures of the auditory pathway, respond to specific stimulus frequencies. The connection between neurons at the nearby frequency and the deafferentated region strengthen and result in an over-represented frequency region.

The over-represented frequency correlates with the perceived pitch of tinnitus, which is usually of high frequency in nature. If the tinnitus has been induced by noise exposure, the frequency of the noise corresponds closely to the deafferentated region.

**In Vitro Model for Tinnitus**

Although animal models have been successful – with accepted and reliable behavioral paradigms – in constructing proposed mechanism and pointing to strategies to combat the disease, tinnitus in patients remains highly debilitating, and pharmacological treatments has not been forthcoming. It would be of great benefit to millions of tinnitus patients if effective pharmaceutical agents could be found to provide relief. Since pharmaceutical product development requires 10–15 years with costs estimated between $500 million and $2.0 billion dollars to bring a single drug to market, the concept of “repurposing” or “repositioning” of FDA approved drugs has been suggested (Boguski et al., 2009; Tobinick, 2009). However, there is currently no suitable screening assay for the necessary development or repurposing of drugs to treat tinnitus. In this light, using the *in vitro* CN (specifically the auditory CN) grown on MEAs, rapid neuronal firing was induced to mimic the aberrant network activity that may be similar to tinnitus, with the goal to establish a valid screening platform to assess and screen potential pharmacological agents for the treatment of tinnitus.
Pathophysiology of Excitotoxicity

Convergent Symptoms of Neurological Diseases

Excitotoxicity is a phenomenon observed in electrically excitable cells in which an ionic imbalance or the presence of toxic substances leads to pathological over-excitation, followed by degeneration (Doble, 1999). Although not a disease itself, excitotoxicity is implicated as a common symptom in various neurological diseases. In Parkinson disease, mitochondrial dysfunction of the substantia nigra neurons is the origin for increased vulnerability to excitotoxic damage (Beal, 1998); in Huntington disease, autosomal dominant mutation of the huntingtin gene produces huntingtin proteins with polyglutamine, which disrupts the intracellular homeostasis and leads to vulnerability to excitotoxicity (Raymond et al., 2011). Other neurological disorders – such as Alzheimer disease, which is associated with an accumulation of the beta-amyloid protein, or brain ischemia, associated with accumulation of excitatory neurotransmitters – directly causes excitotoxic damage (Hynd et al., 2004; Szydlowska and Tymianski, 2010).

Model and Mechanism of Excitotoxicity

The major excitatory neurotransmitter, glutamate, is the main contributor to excitotoxicity. Thus, in modeling excitotoxicity, increased glutamatergic responses of neurons are induced by glutamate, NMDA, AMPA, or kainate receptor agonists (Albensi, 2007; Bender et al., 2010). More recently, excitotoxicity can also be modeled by manipulating glutamate reuptake via the excitatory amino acid reuptake transporters of the presynaptic neurons and astrocytes (Kim et al., 2011). Other investigations regard excitotoxicity as non-neurotransmitter related and suggest that an intrinsic imbalance of ions would elicit excitation and subsequent degeneration,
modeled by using extracellularly-applied K\(^+\) ions to increase depolarization (Kucharz et al., 2011; Ramnath et al., 1992), or inhibiting Na\(^+\)/K\(^+\)–ATPase as well as other ion transporters to observe cell death (Veldhuis et al., 2003). However, regardless of agents used to induce excitotoxicity, cellular degeneration caused by over-excitation is ultimately a calcium-mediated process (Dong et al., 2009). In the glutamate model of excitotoxicity, sustained NMDA, AMPA, or kainate receptor activations cause excess influx of calcium ions (Ca\(^{2+}\)). In the model of disturbed membrane ionic balance, increased action potential firing exhausts the cation pump and cellular ATP supplies, which leads to the flow of Ca\(^{2+}\) down the electrochemical gradient into the cell. Once Ca\(^{2+}\) enters the cytosol, multiple signaling pathways are activated, including the mitochondria-mediated death signals. Ca\(^{2+}\) activates the mitochondrial permeability transition pore and signals the cytochrome and caspases to engage in apoptotic downstream processes (Nicotera et al., 1992). However, whether excitotoxicity always triggers apoptosis is unclear. The internal pathways may resist activations of apoptosis until necrosis. A simplistic view shown by López et al. (2003) is that lower Ca\(^{2+}\) influx over a longer period of time favors apoptosis as cells have time to engage in programmed response to external stimuli, whereas sudden influx of high concentration of Ca\(^{2+}\) causes catastrophic failures that injure cells and nearby tissues.

**Observing Excitotoxicity Using MEA**

Despite current knowledge and advances in modeling excitotoxicity, most investigations are only able to observe “toxicity” (the latter phase of excitotoxicity) in the form of cell viability, and few could observe “excitation” (the preceding phase). This is primarily due to the few experimental tools available to quantify excitation, aside from electrophysiology and calcium
imaging (Grienberger and Konnerth, 2012). Both are methodologically difficult and costly to perform. The other reason is that the excitatory phase that occurs before degeneration is often transient and perhaps regarded as less relevant pathologically to the end result of cell death. However, the neuronal over-excitation in patients with neurological disorders can translate into symptoms such as mood disorder, psychosis, and seizure (Tsapakis and Travis, 2002). Thus, it is imperative that the excitatory phase be taken into account in studying excitotoxicity, especially in screening for potential protective compounds that may attenuate excitotoxic damage. In this study, the CN model on the MEA was used to functionally observe both phases: excitation and toxicity. In screening and developing preventive or treatment strategies – “neuroprotectants” – against excitotoxicity, demonstrating the potential drug effect on modulating excitation is an indispensible aspect.
CHAPTER 2

AIMS

The overall aim of this study was to explore the use of in vitro models for diseases that are dominated by neuronal network dysfunctions, and establish drug-screening platforms for testing potential treatments or preventions of the modeled diseases. The specific aims were:

– To create an in vitro model for tinnitus or tinnitus-like behavior and screening of potential tinnitus treatment drugs.
– To investigate drug-induced excitotoxicity in cortical neuronal networks and explore the use of neuroprotectants.
– To explore mechanisms of drugs which were screened in the established disease models.
CHAPTER 3

MATERIALS AND METHODS

MEA Fabrication and Surface Preparation

Fabrication and surface preparation of MEA were performed by laboratory personnel. As described previously (Gross, 1979; Gross et al., 1985; Keefer et al., 2001), indium-tin oxide sputtered glass plates were photoetched, spin-insulated with methyltrimethoxysilane, cured, deinsulated at the electrode tips with laser shots and electrolytically gold-plated to reduce the interface impedance to 1 MΩ at 1.0 kHz. A hydrophilic adhesion island for cell growth in the center of the 64-electrode matrix was generated by butane flaming to approximately 3.0 mm in diameter. The surfaces were treated with poly-D-lysine (>300 kDa, 100 µg/ml) and laminin (20 µg/ml).

Neuronal Cell Culture

Animals

The care and use of animals in this study were approved by and performed in accordance with the guidelines of the institutional animal care and use committee of the University of North Texas. Female mice (Balb-C/ICR) carrying E17 embryos were obtained from Harlan Sprague Dawley (Indianapolis, IN). If all major regions of the CNS were to be used, it is estimated that tissue from one time pregnant mouse with 12 embryos can seed over 800 networks.
Dissection and Dissociation

Cell culture techniques were described previously for auditory cortical tissues (Gopal and Gross, 1996), and performed by laboratory personnel. Embryos were extracted from the dam after CO\(_2\) narcosis and cervical dislocation, followed by a double thoracotomy. Cortices located on the anterodorsomedial surface (frontal cortex) and the postero-lateral surface and depths of the left temporal cortex (auditory cortex) were dissected (~2 x 2 x 1 mm) from the embryos under sterile conditions. Tissues from the cortical regions were minced, digested with papain, and triturated in DMEM with 4% fetal bovine serum and 4% horse serum. The cell suspension was seeded on a microelectrode array at 60–90 K/100 µl, with all cell types present in the parent tissue at the time of isolation. Cultures were transitioned to 6% horse serum medium after 3–5 days and maintained biweekly by half medium changes (osmolarity: 300–320 mOsm/kg), under constant 10% CO\(_2\) and 90% air at 37°C.

Culture Age and Tissue Type

Cultures of minimum age of 18 days in vitro (DIV), with mean ± SD of 29 ± 10 DIV (n = 292), were used in this study. In the tinnitus studies, auditory cortical cultures were used. In other studies, attempts were not made to discriminate between auditory and frontal cortical cultures, and are identified only as cortical cultures. Both auditory and frontal cortical cultures exhibit statistically equal pharmacological response in all studies as well as undistinguishable patterns of spontaneous activity.
Drugs and Solutions

All drugs were obtained from Sigma–Aldrich (St. Louis, MO), unless otherwise specified. Pregabalin was obtained from Pfizer Pharmaceutical Company (Groton, CT). DMEM and B27 were obtained from GIBCO Products International, Inc. Pentylenetetrazol (PTZ), L-carnitine (L-Car), gabapentin, pregabalin, linopirdine, D-methionine (D-Met), L-methionine (L-Met), kainic acid, L-glutamate (L-Glu) and ceftriaxone were dissolved in deionized and filtered water (18.2 MΩ-cm resistivity at 25°C). Retigabine, flupirtine, NS1619 and isopimaric acid were dissolved in dimethyl sulfoxide (DMSO). Cisplatin was dissolved to 1.0 mg/ml in saline solution. All solutions were prepared to concentrations that minimized osmolarity changes in the 2.0 ml constant volume recording bath. Solutions were added to the bath using a 3-ml disposable syringe (Becton Dickinson) that enabled gentle mixing. After drug experiments were completed, washing was performed with two syringes (for application and extraction) to create a constant flow of fresh medium into the bath (total exchange volume: 6 ml, 2 x 3-ml syringes; flow rate: 0.1 ml/s).

Electrophysiological Recordings

As described previously (Gross and Schwalm, 1994), microelectrode arrays were assembled into sterile recording chambers consisting of stainless steel chamber blocks mounted on a heated base plate of an inverted microscope stage. Temperature was maintained at 37 ± 0.2 °C by a custom thermocouple controlled power supply providing DC current to power resistors on the base plate. All experiments were performed under DMEM stock medium without serum. The pH was maintained at 7.4 with a continuous 10 mL/min stream of filtered 10% CO₂ in air.
mixed by a gas flow controller (AFC 2600-PRO, Aalborg, Inc.), and confined by a chamber cap
featuring a heated window to prevent condensation. A syringe infusion pump (Harvard Apparatus
Pump 11, Holliston, MA) compensated for evaporation, due to the dry airflow, with sterile water
injection of approximately 50–70 µl/hr. Neuronal activity was recorded with a 64-channel
amplifier system (Plexon Inc., Dallas, TX), and digitized simultaneously at 40 kHz. Total system
gain was set to 1.1 x 10^4. Spike identification and separation was accomplished with a real time
template-matching algorithm (Plexon Inc., Dallas, TX) to provide single-unit spike data.
Electrodes/channels were assigned to 64 digital signal processors (DSP). Each processor could
discriminate up to four different units (cellular component that generated detectable action
potential waveforms) in real time.

Protein Extraction and Immuno-Detection of GLT1

Target protein of interest (GLT1) was isolated and the expression change quantified in
parallel with electrophysiology. CNs grown on 6-wells were rinsed with ice-cold PBS and lysed
with radioimmunoprecipitation assay (RIPA) buffer. Subsequent procedures were done off-site at
Texas Wesleyan University. Lysis was treated with 4X Laemmli buffer and separated by
electrophoresis in 10% SDS-PAGE gel at 150 V. Proteins were transferred from the gel to a
PVDF membrane by electrophoresis at 100 V. The membrane was blocked for 30 min, incubated
with GLT1 (1:1.000) and β-Actin (1:5.000) primary antibodies overnight at 4 °C before IgG
alkaline phosphatase secondary antibody (1:5.000) incubation for 2.0 hours. All antibodies were
obtained from Sigma-Aldrich (St. Louis, MO). Blot images were analyzed with densitometry
using the Image J software (NIH, Bethesda, MD).
Data Analysis

Spike and Burst

Spontaneous activity of the cortical neuronal networks was recorded as mean spike rate in one-minute bins. A DSP channel with firing rate higher than 10 spikes/min was considered an active unit. Networks with less than 20 active units were excluded. Average spike rate refers to spike production per minute averaged across all active units. Total spike production of an entire network was also used for quantification. Bursts were derived from spike integration with a time constant of 100 ms for each discriminated unit (Morefield et al., 2000). The integrated burst profiles were quantified using two-thresholds: the first threshold marked the starting point of the burst. This level is close to the noise line, resulting in frequent threshold crossing by noise line fluctuations. A second, higher threshold is necessary to confirm high frequency spike clusters and eliminate noise events, and determines whether the burst profile magnitudes were sufficient to be included in analysis. Threshold levels were determined off-line by comparing integrated burst identification with visual inspection of the raster plots that show the actual burst activity for the same time period. Parameters of burst quantified in this study were (1) average burst rate (bin: 1.0 min), (2) burst period (interval from the beginning of a burst to the beginning of the next burst), and (3) burst duration (timespan of spike occurrence within a burst).

Normalization and Identification of Activity Plateaus

A reference activity was established by the native activity of each respective network; valid reference activity was considered under equilibrium conditions presented as stable temporal network activity. Minute-mean activities were considered temporally stable if minute-
mean activities generated a 0-slope, or horizontal segment that could be visually identified. This stable activity plateau usually occurred after “transition states,” i.e. immediate response following drug applications, which may be influenced by mixing of the test substance with the bath medium and potentially also by the test substance itself. Thus, the length of the horizontal, 0-slope plateau was generally recorded at twice the length of the transition period. Consistent descending slopes would preclude further progression of experiments. Drug induced changes in minute-mean activity (averaged from the plateau period) were normalized to the reference activity as either percent change or relative fold change.

**Waveform Analysis**

Extracellular action potential waveforms from a single unit were sampled at 40 kHz and reconstructed from the average values of superimposed traces (n = 100). Changes in waveform were measured by peak values and action potential durations. In general, a sample of three units in each network from three networks was used for comparing drug-induced effects on waveforms.

**Computation of Concentration-Response Curves**

In experiments that attempted to characterize concentration-response relations of drugs, transition activity phases after compound addition was ignored, and only the horizontal "minute mean" activity plateaus were used for analyses (Gross, 2011). Each minute-mean data within the activity plateaus was averaged and the average value was normalized as percent decrease or relative activity. Sigmoidal regressions were conducted using the four-parameter sigmoidal
function. The minimum was always fixed as 0.0, while maximum was the asymptote of inhibition reaching by the highest concentration; EC\textsubscript{50} and slope were calculated after curve fitting. Curve fitting was conducted using Origin (OriginLab, Northampton, MA) and Prism softwares (Graphpad, La Jolla, CA).

Statistics

Average values were expressed as mean ± S.E.M, unless otherwise specified. Statistical significance was evaluated by unpaired, two-tailed t-test, one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test, or two-way ANOVA at \( \alpha = 0.05 \). Specific employments of significance tests are described in each section.
CHAPTER 4
RESULTS AND DISCUSSIONS

In Vitro Neuropharmacology

The dissociated cortical neurons grown in vitro form and retain many characteristics of a functional network in situ: spontaneous activity supported by various neuronal and non-neuronal cell types (Gross, 2011). To extrapolate results from this study for disease modeling, it is necessary to address if neurons – or certain components of neurons – from primary cultures were similar to those in vivo. Dichter (1978) had shown that neurons in dissociated cortical cultures were not only morphologically similar to neurons in the cortex, but retained abundant excitatory and inhibitory synapses with responses similar to that recorded in vivo. Later studies that demonstrated in vitro - in vivo correlations were summarized:

- The development of presynaptic GABAergic neurons in terms of enzyme-substrate specificity in vitro and in vivo were highly similar (Jong et al., 1986).
- Neuronal progenitor cells from embryonic mouse cerebral cortex – differentiated both in vitro and in vivo – showed similar electrophysiological properties as well as that expected of mature neurons in the responses of tetrodotoxin-sensitive Na⁺ channels and NMDA receptor channels (Wang et al., 2008).
- Serotonergic neurons served similar purposes in guiding the developmental process in vitro and in vivo, as well as similar interaction with glial components (Lauder et al., 1982).
- Dopaminergic neurons interact with glial cells during development with comparable neurite growth and protein expression in vitro and in vivo (Gates et al., 1993).
In terms of functional properties – the focus of this study – the spontaneous activities in dissociated cortical cultures retain similar characteristics as those in the cortex, such as recurrent spatiotemporal patterns (Ikegaya et al., 2004; Rolston et al., 2007) and population bursts (Chiu and Weliky, 2001; Pelt et al., 2005). In addition, the dissociated neuronal networks exhibited mechanisms of stimulus-specific memory – phenomena of recurrent activity patterns and synaptic facilitation – that are similar to those observed in vivo (Dranias et al., 2013). Relevant to this study, pharmacological manipulations of in vitro neuronal networks resulted in stereotypic excitation and inhibition (Gross et al., 1997). These responses were highly reproducible and consistent to each drug type (Xiang et al., 2007). We confirmed this feature by repeating previous experiments and comparing the result to published data that spanned over the past 10 years (Fig. 4.1). The consistency of the electrophysiological responses of cultured neuronal networks was well-established and has been applied as an effective mean for substance identification (Gramowski et al., 2004).
Fig. 4.1: Concentration-response curves of muscimol computed from experiments performed in various years. Upper left: 2004 (Rijal, 2005); raw data of which was used for burst analysis in Section 4.6); upper right: 2009 (Gonzalez, unpublished results); bottom: single experiment performed on July 2012 to confirm repeatability. EC$_{50}$ values (µM) showed excellent reproducibility.

The pharmacologic responsiveness of neural networks in culture at concentrations seen in animal experiments emerged early in the development of this methodology (Droge et al., 1986) but was not systematically investigated until parallel recording computer and data analysis systems became available in the late 1990s. Hereafter, histiotypic pharmacological network behavior was documented in numerous publications with increasingly quantitative data (Gramowski et al., 2000, Pancrazio et al., 2001; Keefer et al., 2001; O'Shaughnessy et al., 2003;
Xia and Gross, 2003; Xia et al., 2003). This development was summarized in two review papers (Gross and Gopal, 2006; Gross and Pancrazio, 2007). More recently, it was shown that these platforms allow a determination of dissociation constants in agreement with those found from other preparations (Rijal and Gross, 2008). Inter-laboratory reproducibility of pharmacological responses was highlighted in Johnstone et al. (2010) and Novellino et al. 2011. The research described in this study also contributes substantially to the now accepted histiotypic pharmacological performance of nerve cell networks in culture. Finally MEA investigations with botulinum toxin A also show close sensitivity overlaps with a variety of other investigative methods (Pancrazio et al., 2013).

Pentylenetetrazol (PTZ) as an Inducer of Tinnitus-Like Activity

Pentylenetetrazol [6,7,8,9-tetrahydro-5H-tetrazolo(1,5-a)azepine] (PTZ), is a respiratory and circulatory stimulant that in high doses causes convulsions. Its mechanism of action is GABA antagonism (Smiałowski, 1980). We used the pro-convulsant PTZ to induce tinnitus-like activity (or a cell culture correlate of tinnitus). The use of PTZ for mimicking tinnitus was a novel approach and unprecedented. Firstly, in animal models, noise-trauma and salicylate – the conventional inducers of tinnitus – as well as other ototoxic agents, exert their central effects through the postulated mechanisms of deafferentation that results in disinhibition of local GABAergic neurons (Stolzberg et al., 2011). Moreover, salicylate had been shown to directly target the fast-spiking GABAergic inhibitory interneuron of the auditory cortex (Su et al., 2009; 1)

Wang et al., 2006) to disrupt the balance between excitation and inhibition (Eggermont, 2008; Noreña et al., 2010). In addition, it should be noted that age-related tinnitus co-exhibited decreased GABAergic inhibitory neurotransmission along the subcortical auditory relay nuclei (Richardson et al., 2012), and that the age-related decrease in inhibition was also apparent in the AC (Caspary et al., 2013). It may be inferred perhaps that the GABAergic disinhibition in the AC is a correlate of age-related tinnitus. Thus, we used a simplified cortical disinhibition model with a defined GABA_A antagonist. PTZ was selected over other GABA_A antagonists because of its prevalent use in the in vivo and in vitro model of epilepsy (Piredda et al., 1985; Psarropoulou et al., 1994) – pathology of which, like tinnitus, also stemmed from maladaptive plasticity and hyperactivity (Scharfman, 2002). As there have been huge interests in repurposing anti-epileptic drugs (AED) for tinnitus (Hoekstra et al., 2011), the use of PTZ may facilitate the use of AED for tinnitus management, while confirming the validity of the MEA platform as an attractive in vitro disease model by comparing dosage data to the available epilepsy/AED literature.
In the auditory cortical network (ACN) cultures, sequential addition of PTZ was administered (Fig. 4.2A). A gradual increase was observed in spike rate and burst rate from concentrations of 0.1 to 0.9 mM PTZ, reaching a maximum plateau at 1.0 mM, while concentrations of PTZ higher than 1.0 mM did not cause a further increase in activity. PTZ of 1.0 mM was determined to maximally induce hyperactivity of neurons in the cultures – spike increase of $139.6 \pm 27\%$, burst rate increase of $129.7 \pm 28\%$ (Fig. 4.2B). The concentration of 1.0 mM was lower than that used in the guinea pig hippocampal slice culture of 5.0 mM (Hori and Katsuda, 1974) or 2–10 mM (Bingmann and Speckmann, 1986) for the induction of spontaneous
burst discharge of epilepsy. Hyperactivity was sustained in ACNs for more than 8.0 hours without morphological changes (Fig. 4.2C), which corresponds to the current understanding that the functional change in neural activity is the driving force of tinnitus in A1, not morphological change or cellular toxicity (Noreña et al., 2010). Another neural correlate of tinnitus – increased synchrony of neural activity in A1 (Eggermont, 2007) – was also observed in ACNs under PTZ (Fig. 4.3B).

Fig. 4.3. (A) Response of auditory cortical network to increasing concentrations of linopirdine (LP). Each data point represents spike count (left y-axis) and burst count (right y-axis) averaged across all units (n = 20) per minute. Horizontal bars identify regions of stabilized network activity used for concentration-response calculation. (B) Raster plots (spike sequence) display of spontaneous activity. (C) Cell morphology under phase-contrast microscopy (bar = 40 µm). (D) Action potential waveforms under reference, 1.0 mM PTZ, and 150 µM linopirdine.
Suppression of Hyperactivity

The purpose of a tinnitus-like state in ACNs was to establish a relevant drug screening platform and test for potential tinnitus treatment drugs. As proof-of-principle, drugs from different classes were tested and characterized by their efficacy and potency of suppressing the tinnitus-like hyperactivity. Among the experimental drugs were a K⁺ channel blocker linopirdine, an antioxidant L-carnitine, and selective voltage-gated Ca²⁺ channel antagonists pregabalin and gabapentin. Other channel antagonists were not tested since a myriad of off-labeled drugs such as the GABA agonists and NMDA channel blockers used in the investigation of tinnitus have proven ineffective (Elgoyhen and Langguth, 2009; Akula et al., 2009). The results of Paper I showed that the relative potential of these drugs of eliminating tinnitus was linopirdine > L-carnitine > pregabalin > gabapentin. It was not surprising that linopirdine showed the best effectiveness as that its supposed target, the voltage-gated K⁺ channels (Liang et al., 2005), has been implicated in the neurogenesis of tinnitus (Holt et al., 2007). However, the EC₅₀ of linopirdine (176 µM; Fig. 4.3, Fig. 4.4) was slightly above, but approaching the physiological concentration calculated from clinical studies (20 µM in brain per dose; Aiken et al., 1996).

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L-Car is an over-the-counter supplement that is classified as an antioxidant. Its mode of action is to act as a scavenger of reactive oxygen species – a possible cause of tinnitus in patients (Berni et al., 2008). Even though the lack of effect of most over-the-counter supplements in treating tinnitus patients (Enrico et al., 2007) may suggest that the mechanism of action of such agents is more complicated than previously considered, it is noted that over-the-counter drugs are touted as effective treatments for tinnitus in the lay literature, as well as in anecdotal claims. We found that L-Car was effective only second to linopirdine in suppressing tinnitus-like hyperactivity, with EC$_{50}$ of 1569 µM (Fig. 4.4). However, later studies on the mechanism of actions of L-carnitine shed more light on the use of L-Car as a potential tinnitus drug.

Fig. 4.4: Concentration-response curves for auditory cortical network pre-treated with 1.0 mM PTZ followed by application of linopirdine (LP; n = 7, black squares), L-carnitine (LC; n = 8, white circles), pregabalin (PG; n = 8, black upward triangles) and gabapentin (GP; n = 8, white downward triangles). The horizontal dashed line equals the 50% point of inhibition.
Pregabalin and gabapentin both bind to the voltage-gated Ca\textsuperscript{2+} channel subunit \(\alpha_2-\delta\) (Taylor et al., 2007). Pregabalin was more potent at a lower concentration than gabapentin in reducing the ongoing electrical activity induced by PTZ, albeit, at a rather high concentration (Fig. 4.4). Pregabalin and gabapentin are structurally similar, but pregabalin has greater efficacy at lower dosages in reducing certain types of pain and reducing the effects of electrical discharges in epilepsy patients (Gilron, 2007), which is consistent with the potency and efficacy comparison in this study. Gabapentin only exhibited 34% efficacy while pregabalin exhibited full efficacy and greater inhibitory response than gabapentin at lower concentrations (Fig. 4.4). The agreement with the clinical observations demonstrated the validity of our in vitro model. If epilepsy can serve as homolog of tinnitus, these drugs may prove to be effective in modulating tinnitus.

**K+ Channel Openers and Their Potential as Tinnitus Drugs\(^3\)**

As primary regulators of neuronal excitability, potassium (K\(^+\)) channels have been a major research focus in drug discovery and development (Shieh et al., 2000). A new anti-epileptic drug approved by the FDA in 2011 – retigabine – targets the KCNQ/K\(_V\)7 family of voltage-gated K\(^+\) channels by activating the K\(^+\) current, and thereby reducing membrane excitability to attenuate seizure hyperactivity (Stafstrom and Grippon, 2011; Gunthorpe et al., 2012; Large et al., 2012). Retigabine, with its structural congener flupirtine (Devulder, 2010) as well as other channel openers that activate other types of potassium channels – such as the

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\(^3\) Parts of this section have been previously published from Wu, C., Gopal., K.V., Lukas, T.J., Gross, G.W., Moore, E.J., 2014. Pharmacodynamics of potassium channel openers in cultured neuronal networks. Eur J Pharmacol. 732, 68-75. [doi: 10.1016/j.ejphar.2014.03.017]. Reproduced with permission from Elsevier.
calcium-activated large conductance K$^+$ channels, or BK/K$\text{Ca}_{1.1}$ (N’Gouemo, 2011) – constitute a novel drug class termed “selective neuronal potassium channel opener” or SNEPCO. SNEPCOs have been used to ameliorate various diseases: neuropathic pain, epilepsy, and cardiac arrhythmia (Chen et al., 2009; Jentsch, 2000; Liang et al., 2005; N’Gouemo, 2011; Wulff et al., 2009). A recent study showed that retigabine was successful in suppressing dorsal cochlear nucleus hyperacitivity (a correlate of tinnitus) and preventing the development of tinnitus (Li et al., 2013). Thus, four different SNEPCOs – retigabine, flupirtine, NS1619, and isopimaric acid – were tested and their therapeutic values assessed under the PTZ-induced tinnitus model.

Fig. 4.5. Concentration-response relations of K$^+$ channel openers. (A–D) Inhibition of spike activity normalized as percent decrease from reference activity of each network, and plotted as a function of concentration. Sigmoidal curve fitting allows computations of (E) EC$_{50}$ and (F) Hill coefficient ($n_H$). The EC$_{50}$ of RTG, FPT, NS1619, and IPA were 8.0, 4.0, 5.8, and 7.8 µM, respectively. (G) Inhibition of burst activity in percent (%) decrease was plotted against percent (%) decrease in spike activity (i.e., EC$_{50}$). The linear regression slope of 1.0 indicates equivalent response of spike rate and burst rate inhibition. Vertical dotted line denotes the EC$_{50}$ for % spike inhibition; the horizontal dotted line denotes the % burst inhibition at the respective EC$_{50}$ of spike for each drug.
ACN Response to SNEPCOs

ACN responses to SNEPCOs were first evaluated. The experimentally determined EC$_{50}$ of retigabine (8.0 µM; Fig. 4.5A, Fig. 4.5E) was comparable to the EC$_{50}$ for Kv7 subtype current activation of approximately 0.6 – 6.2 µM (Tatulian et al., 2001). The inhibitory effects on neuronal spike and burst properties were shown in the hippocampal slice preparation at similar concentrations (1.0 – 10 µM; Yue and Yaari, 2004). As a proof-of-principle, we showed that the response of ACN to retigabine was consistent with the existing literature at the reported concentrations. Flupirtine, a congener compound of retigabine, was developed before the Kv7 channels were recognized as therapeutic targets, and consequently categorized as a non-opioid and non-NSAID analgesic with an unknown mechanism of action (Devulder, 2010; Raffa and Pergolizzi, 2012). Recent discoveries of flupirtine as an activator of the Kv7 channels (EC$_{50}$: 2–6 µM; Kornhuber et al. 1999; Miceli et al., 2008) spurred the rethinking of its therapeutic potential. We found that flupirtine was twice as potent in its inhibitory effect on ACN as retigabine: 4.0 µM vs. 8.0 µM (Fig. 4.5B, 4.5E), and exerted significantly different effects on extracellular action potentials (Fig. 4.6). This is perhaps due to other mechanisms of flupirtine at comparable concentrations, such as the effect on the G-protein coupled inwardly rectifying K$^+$ current at the EC$_{50}$ of 0.6 µM (Jakob and Kriegstein, 1997), or the potentiation of GABA$_A$ current at <10 µM (Klinger et al., 2012). Expectedly, we observed significant GABAergic effects of flupirtine as the EC$_{50}$ increased 6-fold under the presence of the GABA$_A$ blocker PTZ, an effect not apparent for retigabine (Fig. 4.7G).
Fig. 4.6. Effects of K⁺ channel openers on extracellular action potential waveform. (A) Representative waveforms under untreated condition (Ref) and 20-μM retigabine (RTG). Drug-induced changes in the (B) negative peak amplitude (P_{Na}), (C) positive peak amplitude (P_K), and (D) AP duration (Δt) from multiple waveforms (see Methods) of each network were measured as percent change (%) of reference and compared for retigabine (RTG), flupirtine (FPT), NS1619, isopimaric acid (IPA) (20 μM for each), and the vehicle (0.2% v/v DMSO). * p < 0.05, ** p < 0.01, *** p < 0.001.

Fig. 4.7. Suppression of auditory cortical tinnitus-like activity by K⁺ channel openers. (A–D) Concentration-response curves of K⁺ channel openers characterized in networks pre-treated with 1.0 mM pentylenetetrazol (PTZ): bar graph in (A). Horizontal lines at the relative spike rate of 1.0 indicates the concentrations at which spike activity was suppressed to the native level –
defined as therapeutic concentration (TC). (E) TC for retigabine (RTG), flupirtine (FPT), NS1619, and isopimaric acid (IPA) were 7.4, 23.3, 15.2, and 30.1, respectively. (F) Increase in burst rate under PTZ was suppressed by K\(^+\) channel openers at their respective TC. (G) Shifts in the EC\(_{50}\) and Hill coefficient (n\(_H\)) values in the presence of 1.0 mM PTZ (PTZ (+)), from the no-treatment (PTZ (-)) condition shown in Fig. 4.5.

The BK channel openers NS1619 and isopimaric acid both exhibited concentration-dependent inhibitory effect with full efficacy. Isopimaric acid yielded an EC\(_{50}\) of 7.8 \(\mu\)M (Fig. 4.5D, E), near the concentrations at which BK channel activation was observed (10 \(\mu\)M; Imaizumi et al., 2002). However, the EC\(_{50}\) of 5.8 \(\mu\)M (Fig. 4.5C, E) for NS1619 was lower than the reported concentration for BK channel activation in cortical neurons at 30 \(\mu\)M (EC\(_{50}\); Lee et al., 1995), as well as that for spike activity modulation at 10 \(\mu\)M (Zhang et al., 2003). We speculated that the low EC\(_{50}\) of NS1619 in this study might perhaps be explained by its possible GABAergic mechanism. Although none of the studies have demonstrated a link between NS1619 and GABA neurotransmission, other derivatives of benzimidazole were developed as GABA\(_A\) ligands (Jain et al., 2010). We observed significant potency shifts of NS1619 and isopimaric acid under PTZ (Fig. 4.7G), and in a manner similar to flupirtine. However, unlike NS1619, isopimaric acid did not reduce the action potential duration (Fig. 4.6D), among which is a consequence of BK channel activation (Scholz et al., 1998). Isopimaric acid also completely abolished network burst structure at concentrations lower than the EC\(_{50}\) (Fig. 4.5G, 4.7F). Possible explanation of this phenomenon may be that the opening of the BK channel by isopimaric acid affects preferentially the calcium-activated process, and thereby, changes the calcium-dependent synaptic activity and burst characteristics (Kudela et al., 2009), while NS1619 modulates the voltage-gated properties of the BK channel, showing pharmacological
profiles more closely resembling the Kv7 openers. It must be noted that, at the concentrations of complete activity inhibition (50 µM), effects of retigabine and flupirtine were fully reversible (Fig. 4.8B–D), whereas that of NS1619 and isopimaric acid were partially reversible to different degrees (Fig. 4.8B, E–F). In Chinese hamster ovary cells, isopimaric acid extracted from natural compounds exhibited a LD$_{50}$ equivalent to 54 µM (Anaya et al., 2003); NS1619 was reported to be toxic to glial cells at 100 µM (Rundén-Pran et al., 2002). Both of these observations were corroborated by our data.

Fig. 4.8: Functional toxicity of K$^+$ channel openers. (A) Experimental protocol for quantifying activity reversibility as percent (%) recovery of spike rate. (B) Networks were exposed to each K$^+$ channel opener of 50 µM for 1.0 hour and the percent (%) recovery after wash (1.0 hour duration) were compared. * p < 0.05, *** p < 0.001. (C–F) Drug-induced changes in neuronal morphology under phase-contrast microscopy. Pictures were taken after 1.0 hour of exposure to 50 µM of each K$^+$ channel opener. Arrow marks the apparent cell deaths under NS1619 and IPA. Bar = 20 µm (C–F).
Clinical Relevance of SNEPCOs in Tinnitus Treatment

We estimated the therapeutic potential of SNEPCOs in suppressing tinnitus-like activity by quantifying the responses in ACN via calculations of concentrations at which hyperactivity was attenuated to the pre-PTZ level (this value was only estimated in Fig. 4.3). This therapeutic concentration was approximately the EC\textsubscript{42} of the sigmoidal curve – calculated by the inverse of the relative increase under PTZ (Fig. 4.7A). Retigabine had the highest therapeutic potential, with a therapeutic concentration of 7.4 µM, followed by NS1619 (15.2 µM), flupirtine (23.3 µM), and isopimaric acid (30.0 µM). These values were well within the range of their effective concentrations against epilepsy (3–100 µM; Kobayashi et al., 2008); thus, we surmise possible off-label treatment for tinnitus would be plausible and safe. Clinical studies had calculated the free brain concentration of retigabine taken at 1200 mg/day to be around 2.0 µM (Large et al., 2012) – a dose equivalent to that used in an animal model of PTZ-induced seizure (Rostock et al., 1996). The other three compounds have not undergone pharmacokinetic studies; however, as their EC\textsubscript{50} and therapeutic concentration values are almost comparable to retigabine, it is reasonable to assume that the potential effect on suppressing tinnitus-like activity may be observed at a similar dose. However, to be on the safe side, a controlled clinical trial of these other three drugs is warranted. As interest in this new class of drugs have gained traction, we surmise that the results of this study may serve as the basis for future research that might explore the pre-clinical and clinical effectiveness of SNEPCOs for the treatment of tinnitus.
Quantification of Functional Excitotoxicity and Neuroprotection

Cisplatin-Induced Neurotoxicity

The application of MEA methodology in studying excitotoxicity was demonstrated by using cisplatin. Cisplatin (cis-diamminedichloroplatinum(II)) is a platinum-based chemotherapeutic agent commonly used in treating various types of cancer (Kovarik et al., 1972; Stathopoulos, 2010). Cisplatin exerts its cytotoxic effect through formation of DNA adducts and disruption the mitochondria that trigger excitotoxic cell death by apoptosis (Eastman, 1990; Jamieson and Lippard, 1999). Previously not well characterized, we observed the neurotoxic effect of cisplatin on cultured cortical networks (CN), through which the functional aspect of excitotoxicity was then studied. Cisplatin concentrations ranging from 0.1 – 0.25 mM (100 – 250 µM) increased the mean spike activity in a concentration-dependent manner (Fig. 4.9A). This excitation lasted throughout the 7.0 hours of recording, but was not associated with any overt morphological neuronal and glial damage (Fig. 4.9B). Higher concentrations of cisplatin (0.5 and 0.75 mM) showed initial excitation followed by a marked irreversible decrease in activity (Fig. 4.9A), associated with massive cell death (Fig. 4.9B). These electrophysiological and morphological observations were in agreement with cell viability data published by Jiang et al. (2008), in which dorsal root ganglia were exposed to cisplatin for 24 hrs in culture. They observed that a cisplatin dosage of 25 µM had no significant effect on cell viability, however, at 400 µM, only about 4.0 % of the cells survived. It must be noted that with extrapolation from the maximum clinical dosage, the cisplatin concentrations used in this study included the upper limit

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of clinically relevant doses, as well as doses outside a physiological range, and that excitation observed in our results may account for the cognitive symptoms that occur in a majority of patients (Whitney et al., 2008) undergoing cisplatin treatment.

Fig. 4.9: (A): Concentration-response to cisplatin for 7.0 hours at concentrations (mM) of 0 (saline control, n=3), 0.05 (n = 3), 0.1 (n = 3), 0.25 (n = 3), 0.5 (n = 5), and 0.75 (n = 2). (B): Two sets of neurons under control (Ref) and 7.0 hours of 0.25 mM or 0.5 mM cisplatin. Bar: 20 µm. Arrows point to live neurons. Cisplatin concentration of 0.25 mM did not show obvious morphological changes of neurons (arrows), but 0.5 mM cisplatin induced cell death (no arrows).

*Neuroprotective Effect of D-Methionine (D-Met)*

The severe adverse effects from cisplatin exposure have led to the search for preventative treatments (Campbell et al., 1996, 1999; Rybak et al., 2007). The search for neuroprotectants for cisplatin has been successful in the peripheral nervous system, especially the inner ear. Sulfur containing compounds such as sodium thiosulfate and diethyl-dithiocarbamate were reported to provide protections against cisplatin-induced inner ear damage (Otto et al., 1988). In recent years, D-methionine (D-Met), also a sulfur-containing nucleophilic antioxidant, has shown
excellent otoprotection against cisplatin-induced hearing loss, and subsequent outer hair cell loss or stria vascularis damage (Campbell et al., 1996, 1999). Consequently, it is regarded as a potential otoprotective agent, and tested in clinical trials for prevention of noise induced hearing loss. Our study showed that the protective effects of D-Met, observed in the peripheral nervous system, and was also apparent in CNs. One hour pretreatment of D-Met at 1.0 mM (EC$_{50}$ assessed by concentration-response calculation (Fig. 4.10) significantly reduced excitation under 0.10 mM and 0.25 mM cisplatin (Fig. 4.11A, C). Pretreatment with D-Met showed even more remarkable protection at 0.5 mM cisplatin exposures. Without D-Met pretreatment, the CNs exhibited total loss of activity at 420 min with extensive cell death. In the presence of D-Met, however, the CN remained active and showed no overt morphological damage of the neurons (Fig. 4.11B). Pretreatment time was found to be a crucial factor in the protection of CN against cisplatin-induced neurotoxicity. D-Met applied to cultures 1.0 – 2.0 hrs prior to cisplatin exposure was found to exhibit protective effects, as opposed to simultaneous application of D-Met and cisplatin, wherein no protective effects were observed (Fig. 4.12). This result indicated that the downstream effect of D-Met may contribute to the protective effect – such as production of endogenous antioxidants (Campbell et al., 2003) – rather than directly acting as binding agents to cisplatin (Ekborn et al., 2002). In addition, we showed that the optical isomer L-Met was substantially less protective, as shown by the significant difference in activity and reversibility (pretreatment of D-Met vs. L-Met) after cisplatin exposure of 0.5 mM (Fig. 4.13). This result ruled out that endogenous transformation of D-Met into L-Met (Hasegawa et al., 2005) were responsible for neuroprotection. Although the detailed mechanism of D-Met requires further
studies, our study demonstrated that CNs grown on MEAs is an ideal platform for quantifying excitotoxicity and testing for potential neuroprotective compounds.

Fig. 4.10: Concentration-response curve of D-Met (n = 4) and L-Met (n = 3). The IC$_{50}$ ± SEM values were 1.03 ± 0.21 mM (Hill slope: 0.94), and 0.64 ± 0.05 mM (Hill slope: 1.54), respectively. Note: L-Met has maximal efficacy of only 33% at a maximal concentration of 10 mM.
Fig. 4.11: (A): Concentration-response to cisplatin following 1.0 hour pre-exposure to 1.0 mM D-Met. Cisplatin concentrations in mM were: 0 (water control, n=3), 0.1 (n = 5), 0.25 (n = 3), 0.5 (n = 8), and 0.75 (n = 2). (B): Two sets of neurons under control (Ref), 1.0 hour in 1.0 mM D-Met, and subsequently 7.0 hours in 0.5 mM or 0.75 mM cisplatin. Bar: 20 mm. Arrows point to live neurons. In the presence of D-Met, 0.5 mM cisplatin did not induce cell death. However, there was no neuronal protection from D-Met when CNs were exposed to a cisplatin concentration of 0.75 mM. (C) Percent activity change at 180 minutes of exposure to cisplatin at various concentrations, with or without 1.0 hr pretreatment of 1.0 mM D-Met. All data represent comparisons with saline control. Error bars represent SEM values. One-way ANOVA was used to compare each concentration of cisplatin with the control. Significance is denoted by asterisks above the SEM bar. Students’ T-tests were used for individual comparisons of D-Met vs. no D-Met at a given concentration (thin-line with significance asterisks below the symbol). * p < 0.05, ** p < 0.001.
Fig. 4.12: Effect of D-Met (1.0 mM) pre-exposure time on network activity to cisplatin. Cisplatin of 0.1 mM (A) and 0.5 mM (B) was administered at t = 0. D-Met was administered either simultaneously (“0 min”), 30 min, 60 min or 120 min prior to the addition of cisplatin.

Fig. 4.13: (A): Effects of 60 min pretreatment with D-Met or L-Met at a concentration of 1.0 mM on network activity under 0.1 and 0.5 mM cisplatin (cis). (B): Percent recovery in spike activity from wash after D-Met and L-Met (both at a concentration of 1.0 mM) pretreatment and exposure of 0.1 or 0.5 mM cisplatin for 7 hours. Horizontal dotted line at 100% indicates full recovery to reference level. * p < 0.05.
Ceftriaxone Protects Against Glutamatergic Excitotoxicity

Glutamatergic agonist induced excitotoxicity has been a widely used model for studying various neurological diseases (Albensi, 2007; Bender et al., 2010). Even though the application of CNs grown on MEAs to functionally study the glutamatergic excitotoxicity model has been published by Frega et al. (2012), in order to discuss clinical relevance of using this model, an attempt to demonstrate the capability of screening for neuroprotective compounds must be made. Thus, we used the results from Frega et al. (2012) as a basis to improve the CN model. We showed a similar biphasic response of glutamate – not unlike that induced by cisplatin, as well as by kainic acid (unpublished observation) – with an excitatory phase from 1–20 μM, and inhibitory at >100 μM (Fig. 4.14). The inhibitory phase corresponded to the sigmoidal decreases in active unit count, with the EC$_{50}$ of 112 μM. The glutamate responses in the native state of CN agreed with the results of Frega et al., and were used as controls for studying the effects of neuroprotectants.

Fig. 4.14: Effect of glutamate on CN activity. (A) Raster plot display of 11 units (dsp’s) for 10 sec spike-train under glutamate concentrations of 5, 50, and 200 μM. (B) Spike rate and active unit count averaged to 1-min bin for the entire network as functions of time. Increasing concentrations of glutamate was administered sequentially network activity ceased at 500 μM.
Glutamatergic mechanisms play an essential role in the disease progression in models of Huntington’s disease in vivo (Cepeda et al., 2001). Miller et al. (2008) showed that the β-lactam antibiotic ceftriaxone, when administered in the transgenic mouse model, attenuated the symptoms of Huntington’s disease. Ceftriaxone was shown to up-regulate a glutamate transporter GLT1 (also known as excitatory amino acid transporter – EAAT2) (Miller et al., 2011; Rothstein et al., 2005). Thus, using an our in vitro model, we tested the hypothesis that ceftriaxone may be protective against glutamate excitotoxicity. As expected, pretreatment with ceftriaxone at 10 µM for 4 DIV indeed upregulated the expression of the GLT1 protein in CN cultures (Fig. 4.15). The functional consequence of GLT1 upregulation was observed by comparing the glutamate response in CN treated with ceftriaxone to those of untreated CN. We found that higher concentrations (10–200 µM) of glutamate were needed to induce excitation, albeit resulting in higher increases of spike activity (Fig. 4.16A). The decrease in active unit count, a measure of functional toxicity, also exhibited a shift toward higher concentration of glutamate (112 µM ! 560 µM; IV: Fig. 4.16B). A two-way ANOVA (df: 1,7) showed significant difference between ceftriaxone treated CN and control CN in terms of both spike rate and unit count (P < 0.001). Taken together, these results indicated that ceftriaxone may be a potential neuroprotectant for combating excitotoxicity, apparently through modulation of glutamate reuptake. Further studies using this model will attempt to quantify network spike patterns to understand the effect of glutamate reuptake in the CN, as well as screen for similar neuroprotective compounds.
Fig. 4.15: Cetriaxone increases GLT1 expression. Western blot images were quantified with densitometry using the Image J software. Beta-actin was used for normalization (n = 5 each; * P < 0.05).

Fig. 4.16: Pretreatment of ceftriaxone for 4 DIV changes the CN responses to glutamate. Spike rate (A) and unit count (B) are normalized as relative activities to references (n = 3 each) for characterization of excitation (rel. activity >1) and inhibition (rel. activity <1). Relative unit count (B), a measurement for loss of spike activity, was fitted with 4-parameter logistic function that allows calculation of EC_{50}’s.
Platform for Drug Mechanism Identification

The EC$_{50}$ of L-Car under PTZ-induced hyperactivity was determined to be 1570 µM. We found, however, that in the native state of CN (no-PTZ), L-carnitine has a potency of 220 µM (Fig. 4.17A, C). This observation had led us to explore the mechanism of the EC$_{50}$ shift under PTZ. The shifts in potency of the GABA$_A$ agonist (muscimol) in the presence of competitive GABA$_A$ antagonists (bicuculline, gabazine, and TMPP) were investigated by Rijal and Gross (2008). They quantified the change in muscimol potency and applied the results to the calculation of dissociation constants of antagonists, which demonstrated the feasibility of using network responses to study drug interactions at the GABA$_A$ receptor. Thus, as L-Car exhibited a large potency shift under PTZ (a GABA$_A$ antagonist) in a behavior similar to the muscimol potency shift under several antagonists, we hypothesized that L-Car may have an additional mechanism on the GABA$_A$ receptors, which would be antagonized by bicuculline. Another rationale for this hypothesis was based on another study by Xia et al. (2003), which observed complete inhibition of network activity under fluoxetine that could not be accounted for by its putative serotonergic mechanism. Later studies confirmed this observation by demonstrating a novel modulatory effect of fluoxetine on the GABA$_A$ receptor (Robinson et al., 2003). The complete inhibition of network activity could also be achieved by L-Car (Fig. 4.4). This prompted our attempts to investigate the hypothesis of L-Car as a GABA$_A$ modulator.

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Fig. 4.17. Inhibition of neuronal activity by L-carnitine (L-Car; A) and D-methionine (D-Met, B) plotted as total network activity/ min. Active units (right ordinate), defined by 10 or more threshold crossings/min, are also plotted (open squares). In both cases, spike activity decreased before the loss of units. The stepwise activity decreases as a function of drug concentration are demonstrated for D-Met (B) where constant level plateau values were reached within 4 min. L-Carnitine has a longer transitional period to quasi-stable plateaus. Bars above the spike plot in B indicate time periods from which plateau values were determined. W: wash with fresh medium that returned the activity to reference levels in both cases. (C) Concentration-response of L-Car (■, n = 6) or D-Met (○, n = 6) normalized as % inhibition of spike activity and fitted by a sigmoidal algorithm. The EC$_{50}$ for L-Car and D-Met are 0.22 (± 0.01), and 1.06 (± 0.06) mM, respectively.

We found that like L-Car, D-Met also exhibited full efficacy, with potency around 1.0 mM (Fig. 4.17B, C). We investigated the inhibitory mechanism of L-Car and D-Met, and showed that L-Car and D-Met both resembled agonists of the GABA$_A$ receptor type. The hypothesis is corroborated by the clear shift of their concentration-response relations in the presence of bicuculline (Fig. 4.18A–B). The EC$_{50}$ of L-Car and D-Met exhibited stepwise shifts in increasing concentrations of bicuculline ranging from 0.5 to 40 µM. In the presence of 40 µM bicuculline, the EC$_{50}$ values of L-Car and D-Met both showed greater than 10-fold increases without affecting efficacy. The stepwise EC$_{50}$ shifts were analyzed using the Lineweaver-Burk

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plots to define antagonist behaviors (Fig. 4.18C–D). We found that both L-Car and D-Met plots showed bicuculline-dependent linear slope change but not for the y-intercept values – a characteristic of competitive antagonism. The EC$_{50}$ shift as a function of antagonist concentration can therefore be plotted to determine dissociation constants by the modified Gaddum equation (Gaddum, 1957; Lew and Angus, 1995). Using non-linear regression, the calculated dissociation constants of bicuculline were well within the established values in the literature – 5.7 and 5.2, using L-Car or D-Met titrations, respectively (Fig. 4.19; also see Rijal and Gross, 2008), which further validated the roles of L-Car and D-Met as GABA$_A$ agonists. As supporting evidence, the patterns of spike inhibition (for both compounds) resembled that elicited by the GABA$_A$ agonist muscimol (Fig. 4.20). Furthermore, high concentrations (50 mM, one-hour exposure) of the compounds did not cause morphological damage (data not shown) to the networks and the effects were completely reversible electrophysiologically (Fig. 4.17A, B). Even though only ligand-binding assays may offer direct evidence of physical interaction between the two antioxidant compounds and bicuculline at the GABA$_A$ site, our results presented the first functional evidences showing that GABA$_A$ receptor activation may be an important mechanism responsible for the modulatory actions of L-Car and D-Met.
Fig. 4.18: Concentration-response curves of L-carnitine (L-Car) (A) and D-methionine (D-Met) (B) in the presence of increasing concentrations of bicuculline (BCC; µM). (C–D) Same data plotted as reciprocal of L-Car (C) or D-Met (D) concentration (mM)$^{-1}$ vs. reciprocal of activity (% Inhibition)$^{-1}$ using the Lineweaver-Burk method.
Fig. 4.19. Extrapolated pEC\(_{50}\) values graphed as function of bicuculline concentrations. (A) Logarithmic regression curves are fitted with equation \(pEC_{50} = – \log ([B] + 10^{pA2}) – C\) for computation of pA2 values. (B) Clark plot display and linear relations of the pEC\(_{50}\) values graphed as function of log bicuculline concentration [B], adjusted with the calculated dissociation constants \((K_B = –\log(pA2))\).

Fig. 4.20. Network response pattern under L-carnitine (L-Car) and D-methionine (D-Met). (A–B) Raster plot displays (35 sec) of spontaneous spike and burst activities from samples of 7 units under native condition, and the EC\(_{50}\) condition for L-Car (A) and D-Met (B), bar = 3 sec. (C–D) Burst parameters quantified as average burst period (left, y-axis) and average burst duration (right, y-axis) as a function of time. Timeline of L-Car or D-Met applications corresponds to Fig. 1A & 1B. (E–F) Change in burst period (E) and burst duration (F) under muscimol (M), L-Car (L-C), D-Met (D-M) at EC\(_{50}\) (0.14 \(\mu\)M, 0.2 mM, 1.0 mM, respectively) and EC\(_{90}\) (0.5 \(\mu\)M, 4.0 mM, 12 mM, respectively). Significance indicates difference of drug response among M, L-C, and D-M; * \(P < 0.05\).
GABAergic mechanisms of L-Car and D-Met may be a key factor when discussing or explicating their roles in particular diseases. GABA\textsubscript{A} agonist actions, for instance, may be a direct cause of L-Car’s analgesic effect in neuropathy (Chiechio et al., 2007). Anesthetic-induced damage presented as potentiation of the GABA\textsubscript{A} receptor, perhaps, was rescued by L-Car due to its competition for receptor binding with isoflurane (Zou et al., 2008). The concept of excitotoxicity – such as that induced by excess glutamate in Huntington’s Disease symptoms or methamphetamine-activated dopamine surge (Virmani et al., 2003; Vamos et al., 2010) – may be more relevant in the discussion of the protective action of L-Car within the framework of the inhibitory neurotransmitter system. Likewise, we suspect that the protective mechanism of D-Met against platinum toxicity was perhaps a counteracting measure against excitotoxicity by GABAergic inhibition. This novel finding also reiterated the importance of the GABAergic system in neurotransmission and inhibitory plasticity (Vogels et al., 2011), as well as in neuroprotection. Moreover, as the search for and the development of neuroprotective compounds become an indispensible aspect of neurological research, we demonstrated that our \textit{in vitro} cortical network model may serve as an effective platform for screening and investigation of novel drug actions.
CHAPTER 5

CONCLUSIONS

Neuronal network dysfunctions are implicated in various neurological diseases. In vitro
CNs coupled with MEAs provides a powerful tool to functionally investigate the properties of
neuronal networks. By mimicking disease states in vitro, we created suitable models for drug
screening and probing of basic mechanisms underlying the disease states:

– We used PTZ, a proconvulsant agent that causes excitation of cortical neurons without
inducing cell necrosis or apoptosis, to model an in vitro state of tinnitus-like
hyperactivity. Drugs of the K⁺ channel blocker type, the Ca²⁺ channel antagonists, and
antioxidant, were used to attenuate the effects of PTZ. As proof-of-principle, testing the
effectiveness of FDA drugs for attenuating hyperactivity may be possible via this model.
As shown by the discussion of SNEPCO and their effects on CN hyperactivity, this model
may serve as a basis to repurpose drugs for the treatment of tinnitus.

– By quantifying the acute electrophysiological and cellular toxicity of cisplatin and
assessing the protective effects of D-Met against cisplatin-induced neurotoxicity, as well
as the CN response to glutamate and the protective effects of ceftriaxone, we
demonstrated that CNs on MEAs reveal the functional aspect of excitotoxicity, an area
lacking in the current literature, and that this model may serve as an efficient platform for
testing potential neuroprotective compounds against excitotoxicity.

– Out of the dozen or so compounds screened in this study, we investigated the mechanism
of action of L-Car and D-Met. We presented evidences that the antioxidants L-Car and D-
Met modulate neuronal activity through reversible activation of GABAₐ receptors,
causing systemic inhibition of spontaneous neuronal network activity. It served as a demonstration that this platform not only is a suitable model for drug screening and testing, but is also a valuable tool to investigate the mechanisms of potential therapeutic compounds and small molecules.

In future studies, using this model, we offer several insights into possible directions to pursue, whether conceptual or practical:

– Induced pluripotent stem cells (iPSC) have been developed as the *in vitro* model for Parkinson’s disease. The skin cells of patients suffering from neurological diseases can be extracted and transformed into neurons, through which components of patients’ neurological symptoms can be measured and studied *in vitro* (Martínez-Morales and Liste, 2012). The iPSC can theoretically be grown on MEAs, forming functional networks, and enable observation of their network properties and that of the diseases.

– Differentiated neurons derived from embryonic pluripotent stem cells can be used to treat deafness or hearing loss in children, especially through the use of cord blood or bone marrow stem cells (ref). Testing for functional viability and excitability during tissue generation may be feasible using MEA.

– Cell death assays using molecular markers may serve as an improvement to the observation of toxicity (we were only able to estimate toxicity by measuring functional reversibility), including discrimination between apoptosis or necrosis.

– Transgenic mice with mutant genotypes – such as Alzheimer’s or Huntington’s disease mice – may be used as sources of cortical tissue culture. The differences in the development of network activity, the pattern of spontaneous activity, or the responses to
pharmacological agents compared to that derived from wild-type mice can provide important insights into disease mechanisms.

- The viability of PNS or CNS substrates using animals other than mammals such as the juvenile zebrafish may be tested using the present in vitro electrophysiological model.


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