CONCENTRATION-DEPENDENT EFFECTS OF D-METHYLPHENIDATE ON FRONTAL CORTEX AND SPINAL CORD NETWORKS IN VITRO

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Spontaneously active frontal cortex and spinal cord networks grown on microelectrode arrays were used to study effects of D-methylphenidate. These central nervous system tissues have relatively low concentrations of dopaminergic and noradrenergic neurons compared to the richly populated loci, yet exhibit similar neurophysiological responses to methylphenidate. The spontaneous spike activity of both tissues was inhibited in a concentration-dependent manner by serial additions of 1-500 µM methylphenidate. Methylphenidate is non-toxic as spike inhibition was recovered following washes. The average concentrations for 50% spike rate inhibition (IC_{50} ± SD) were 118 ± 52 (n= 6) and 57 ± 43 (n = 11) for frontal cortex and spinal cord networks, respectively. A 3 hour exposure of a network to 1 mM methylphenidate was nontoxic. The effective concentrations described in this study are within the therapeutic dosage range. Therefore, the platform may be used for further investigations of drug mechanisms.

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LIST OF ABBREVIATIONS

A	Adrenaline
ADHD	Attention-deficit hyperactivity disorder
ANOVA	Analysis of variance
CA	Catecholaminergic
CNS	Central nervous system
CRC	Concentration-response curve
DA	Dopamine
DAB	Diaminobenzidine
DAT	Dopamine transporter
DIV	Days in vitro
DMEM	Dulbecco’s modified minimal essential medium
MPH	Methylphenidate
D-MPH	D-methylphenidate
DOPA	3,4-Dihydroxyphenylalanine
DSP	Digital signal processor
EPSP	Excitatory postsynaptic current
FC	Frontal cortex
IC_{50}	Concentration at which 50% of the spike activity is inhibited
i.v.	Intravenous
LC	Locus coeruleus
MEA	Microelectrode array
MEM	Minimal essential medium

MMEP	Multimicroelectrode plate
MPH	D,L-methylphenidate
NE	Norepinephrine
NET	Norepinephrine transporter
PFC	Prefrontal cortex
SC	Spinal cord
SD	Standard deviation
SE	Standard error
TH	Tyrosine hydroxylase
VTA	Ventral tegmental area

1. INTRODUCTION

1.1 Overview of Cultured Neuronal Networks

It has been demonstrated that spontaneously active neuronal networks, grown on microelectrode arrays (MEAs) *in vitro*, are uniquely suited to study the pharmacological responses of different tissues to increasing concentrations of neuroactive compounds, therefore providing an effective platform to study neurotoxicology and drug development (Gross et al.,1997). Based on previous pharmacological data, it is now apparent that receptors, synapses, and cellular mechanisms responsible for pattern generation in a specific central nervous system (CNS) tissue are retained and represented in neuronal primary cell culture (Gramowski et al., 2000; Morefield et al., 2000; Keefer et al., 2001 a,b,c). Compound-specific neuroactivity and/or toxicity can be investigated quantitatively through analyses of the temporal changes in spike, burst, and waveshape production of such networks. These changes are considered “cell culture correlate responses” to what occurs *in vivo*, as networks *in vitro* are capable of responding to compounds in approximately the same concentration range that alter the neurophysiology of living animals.

The observed high degree of inter-culture repeatability and sensitivity of tissue-specific and histiotypic network responses support the validation of this platform, and suggest application to more complex and dynamic problems (Xia et al. 2003, a,b). Thus, it is realistic to take further steps in the expansion of platform capabilities by investigating the electrophysiological responses to drugs that produce complex psychoactive reactions in animals. Methylphenidate is the most common psychoactive medication prescribed to children with attention-deficit hyperactivity disorder (ADHD) in

the United States. However, its therapeutic mechanisms of action are not well understood (Solanto 1998; Volkow et al., 2001, 2002). Thus it is a prime candidate for investigation.

1.2 Attention-Deficit Hyperactivity Disorder

Attention-deficit hyperactivity disorder is estimated to affect 3-10% of the children in the United States and around the world (Sandoval et al., 2002; Volkow, 2003; Roman et al., 2004; Ding et al., 2004). ADHD symptoms are manifested early in childhood and may extend into adulthood (Schachter et al., 2001). The most inherent physiological characteristics of ADHD patients are motor over-activity (hyperkinesis), inattentiveness, and impulsivity (Solanto M.V., 1998, 2002; Overtoom et al., 2003). Dysfunctions in both the dopaminergic and noradrenergic (catecholaminergic) systems are thought to be crucial in the manifestation of ADHD etiology (Biederman et al., 1999; Barr et al., 2001, 2002; Kuczenski et al., 1997, 2002; Overtoom et al., 2003; Roman et al., 2003, 2004). The dopaminergic system is known to be involved in mediating motor output and the response to reinforcement, while the noradrenergic system regulates the orienting response, selective attention, and vigilance (Solanto, 1998). The pathophysiology of the dopaminergic and noradrenergic dysregulations include the inability to control and modulate higher cortical functions such as arousal, cognition, executive function, and motor control, all characteristic of ADHD patients (Biederman et al., 1999; Overtoom et al., 2003).

It has been shown that subjects with ADHD exhibit a greater concentration than normal of dopamine transporters (DAT) in the brain (Dougherty et al., 1999; Krause et al., 2000). In addition, studies have indicated several polymorphisms of the DAT1 gene

prevalent in ADHD patients (Barr et al., 2001; Kirley et al., 2002; Roman et al., 2004). The DAT is the primary mechanism for dopamine (DA) removal from the extracellular space, and thus ADHD patients show decreased levels of DA at the dopaminergic synapse. It is thought the decreased levels of DA in the brain may contribute to the stereotypic physiological characteristics expressed in ADHD patients (Solanto, 1998, 2002; Volkow et al., 2001, 2002). In contrast to the significant evidence on the presence of abnormalities of the DAT in ADHD patients, the norepinephrine transporter (NET) and the α-2 adrenergic autoreceptor have not been shown to be major genetic susceptibility factors in ADHD, although a plethora of studies suggest them to be an integral part of the disorder (Xu et al., 2001; Barr et al., 2001, 2002; Roman et al.; 2003). Methylphenidate has been the most widely accepted treatment for ADHD and is pharmacologically targeted to a combination of dopaminergic and noradrenergic mechanisms (Overtoom et al., 2003).

1.3 D-Methylphenidate and Mechanisms Underlying Its Effects

D,L-thereo-Methylphenidate (MPH) is an amphetamine-like psychostimulant classified as a DEA Schedule II controlled substance, is the most common psychotropic drug administered to treat ADHD in adolescent children, (Solanto et al., 1998, 2002; Volkow et al., 2001, 2002). It is estimated that 60-70% of ADHD subjects have favorable therapeutic responses to the drug (Volkow et al., 2001). Recently, there has been an increased recognition and diagnosis of ADHD patients which has made prescriptions of MPH much more prevalent (Kuczenski et al., 2002). The increase in therapeutic administration of MPH has introduced a heightened awareness of possible MPH abuse, overdose, and medical errors, which can include side effects such as

tachycardia, headache, insomnia, and nervousness (Klein-Schwartz et al., 2002; Sandoval et al., 2002; Volkow et al., 2003). The physiology, mechanisms of action, and toxicity of MPH have not been sufficiently studied, even though it has been used as a therapeutic agent for over fifty years (Solanto 1998; Volkow et al., 2001; Kuczenski et al., 2002; Teo et al., 2002 a, b; Prieto-Gomez et al., 2004).

Methylphenidate is known to alleviate the stereotypical symptoms in people diagnosed with ADHD (Mehta et al., 2000). There are three mechanisms of action of MPH, each leading to a subsequent increase in either DA or norepinephrine (NE) in the synapse. Converging evidence has led recent studies to focus on the DAT and NET as the primary therapeutic mechanisms of action of MPH in ADHD patients (Kuczenski et al., 1997; Volkow et al., 2001, 2002). It has been shown that MPH binds both the DAT and NET, thus blocking the presynaptic reuptake of DA and NE at the synapse, respectively (Overtoom et al., 2003). The presynaptic blockade of DA and NE via the DAT and NET causes a substantial increase in DA and NE concentration in the synapse. It is thought the buildup in DA and NE at the synapse facilitates an amplification of spontaneously released DA and NE from reserpine-sensitive storage vesicle pools, further increasing their concentrations. To a much lesser extent, methylphenidate is thought to inhibit the catabolic activity of monoamine oxidase (MAO), an enzyme that breaks down free DA and NE (Solanto, 1998). Due to the law of mass action, DA and NE binding to postsynaptic receptors increases dramatically. Many studies have shown that MPH induces a dose-dependent reduction of spontaneous neuronal activity in the dopaminergic and noradrenergic pathways of various tissues, thus increasing the signal-to-noise in the target neuron (Rebec et al.,

1978; Lacroix et al., 1988; Solanto, 1998; Ruskin et al., 2001; Volkow et al., 2001; Prieto-Gomez et al., 2004). This mechanism may be caused by the fact that MPH enhances the amplitude and duration of the inhibitory postsynaptic potential (Kuwahata, 2002). The methylphenidate-induced amplification of presumably weak DA and NE signals in ADHD patients enhances task-specific signaling, thus improving attention and decreasing distractibility (Volkow et al., 2001). Likewise, the synaptic increase of DA is thought to provoke stimulation of the motivation and reward pathways, which enhances interest in task-specific goals and thus improves the performance on such salient tasks (Volkow et al., 2001).

Psychophysiological, clinical, and basic neuroscience research has helped to elucidate the mechanisms, therapeutic effects, and other basic properties of MPH. However, there has been little research on the electrophysiological effects of MPH on individual neurons, nor on the global response on neuronal networks. Most research has also focused on the effects of MPH on specific dopaminergic and noradrenergic loci in the brain such as the nigrostriatal, mesocorticolimbic systems, and efferent projections from the LC to the cortex, rather than in sparsely populated areas such as the frontal cortex and spinal cord. This study reports the effect of D-methylphenidate (D-MPH) on the spontaneous firing, bursting, and waveshape properties of individual neurons, connected in networks and grown on microelectrode arrays *in vitro*, and derived from frontal cortex (FC) and spinal cord (SC) tissue. Methylphenidate prescriptions contain a 50:50 racemic mixture of the D-threo-MPH and L-threo-MPH enantiomers; however, studies have shown that D-MPH is the pharmacologically active enantiomer for the treatment of ADHD, and thus was used in this study (Teo et al.,

2003a, b; Ding et al., 2004). Furthermore, immunocytochemistry was performed to illuminate the dopaminergic and noradrenergic content of the frontal cortex and spinal cord cultures.

1.4 Further Research Validation

The pharmaceutical industry is facing serious time-consuming problems in the establishment of the pharmacological efficacy and toxicity of novel drug candidates. Given that networks growing in culture on microelectrode arrays show histiotypic responses to many compounds, it is reasonable to suggest that such systems can be used as effective screening platforms for drug development (Keefer et al., 2000; Xia et al., 2003a, b). Quantitative data on efficacy ranges, potential toxicity, secondary-binding effects, spike and burst properties, and other intrinsic neuronal properties are all reflected in the dynamic responses of spontaneously active networks to pharmacological exposures. However, before this new system is accepted by industry, an extensive validation process is required. Such validation has already started with compounds such as ethanol, quinine, trymethylolpropane, and fluoxetine (Keefer et al., 2001, a, b; Xia et al., 2003, a, b; Gopal et al., 2004). Clearly, the most important validations are established with compounds that have complex psychophysiological effects rather than with toxins. Methylphenidate, a compound that has been extensively used as a human therapeutic, is considered an important substance to study.

1.5 Objectives of Study

This research effort explored the effects of D-methylphenidate on cultured frontal cortex and spinal cord neuronal networks *in vitro*. The drug-induced electrophysiological responses of the networks are expressed as pharmacological

response profiles of D-MPH on each type of network. Immunocytochemistry was used to elucidate the presence of a subset of pertinent receptors present in both FC and SC cultures.

Specific Aims:

1. Determine the global and unit-specific concentration-dependent responses of frontal cortex and spinal cord networks to D-methylphenidate.
2. Quantify tissue-specific effects, inter- and intra-culture repeatability, and response uniformity of single units by constructing concentration-response curves (CRCs) and associated IC_{50} values.
3. Identify if D-MPH-induced electrophysiological responses are reversible following maximum concentration exposure to determine possible toxic concentrations.
4. Quantify the effect of D-MPH on spike waveshapes to determine whether voltage-gated sodium and potassium conductance are involved.
5. Determine the presence of catecholaminergic neurons in FC and SC cultures.

2. MATERIALS AND METHODS

2.1 MMEP Fabrication

Multimicroelectrode plates (MMEPs) are an essential part of neuronal network research. The electrical activity of neurons is monitored with 64 electrodes located in the center of the MMEPs, which are fabricated at the Center for Network Neuroscience. Detailed procedures of in-house MMEP fabrication have been previously documented (Gross et al., 1985; Gross and Kowalski, 1991; Gross, 1994). Briefly, commercially available glass, sputtered with 1000A layer of indium tin oxide (Applied Films Corp., Boulder, CO) is used to make a 5 cm^2 and 1 mm thick MMEP with 32 amplifier contact strips on either side. The conductors are etched out of the indium tin oxide layer using photolithography, and the resulting 64 recording measures 0.8 mm X 0.8 mm. The thin-film electrodes are 8 – 10 μm wide and are arranged in rows and columns were they are separated by 40 and 200 μm, respectively, for MMEP 3 and by 150 μm for MMEP 4. The MMEP is then spin insulated with polysiloxane (PS233; United Chemical Technologies). The tips of the electrodes are de-insulated via a laser shot and electrolytically gold-plated to lower impedances at 1 KHz to 1-2 MΩ. The electrode matrix is treated with poly-D-lysine and laminin for better cell/surface adhesion.

2.2 Neuronal Cell Culture

Cell culture technicians obtain CNS tissue from timed pregnancy mice at 14-15 days gestational age for spinal cord cultures and 16-17 days for frontal cortex. The tissue is mechanically minced, enzymatically dissociated, triturated, and combined with medium. Medium used for culturing frontal cortex (FC) tissue is Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine

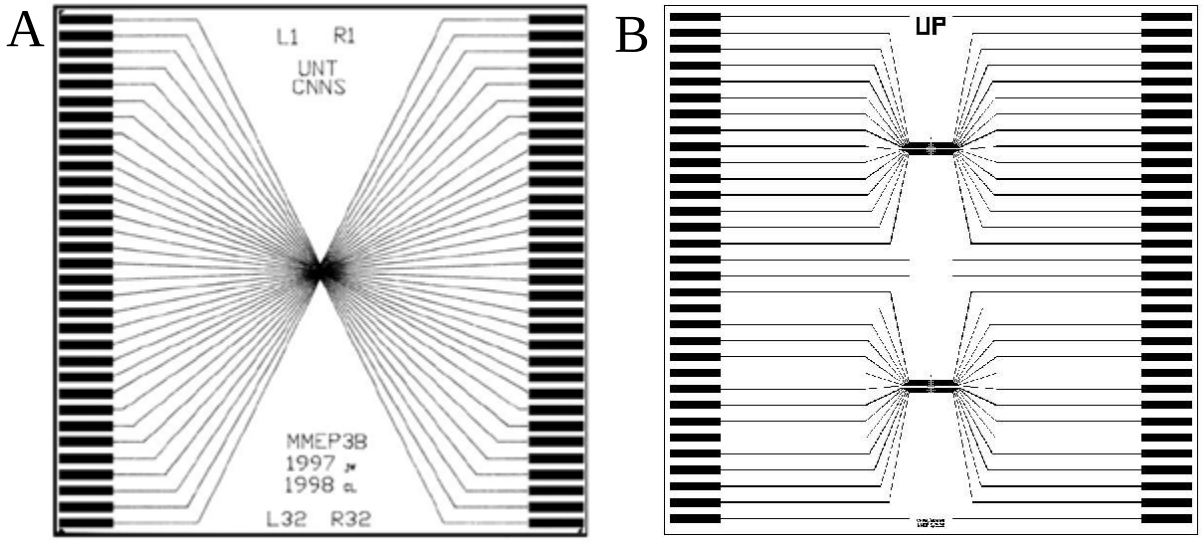

Figure 1. MMEP designs. (A) Single network photoetched micro-electrode plate with 64 indium-tin oxide microelectrodes. (B) Two network array with 32 microelectrodes per recording matrix (Center for Network Neuroscience archives. Center for Network Neuroscience, University of North Texas, http://www.cnns.org).

serum and 10% horse serum. Maintenance medium was DMEM containing 10% horse serum. Medium used for seeding and maintenance of spinal cord (SC) tissue is minimal essential medium 10 (MEM). Before seeding, the microelectrode array (MEA) hydrophobic polysiloxane surfaces are activated by flaming with a butane flame to enhance cell adhesion. The adhesion area is restricted to a 2-5 mm^2 area over the matrix by flaming through a stainless steel mask. Polylysine and laminin, which adhere only to the flamed hydrophilic regions, are used to enhance cell attachment and growth. A tissue pool aliquot of 50 μl is seeded at 500k/ml onto a centrally located recording island (typically 2-6 mm in diameter) on the MMEP. The cultures are incubated at 37°C in a 10% $C0_2$ atmosphere and ½ medium changes are performed two times a week until they are used for experiments, generally three weeks to three months after seeding.

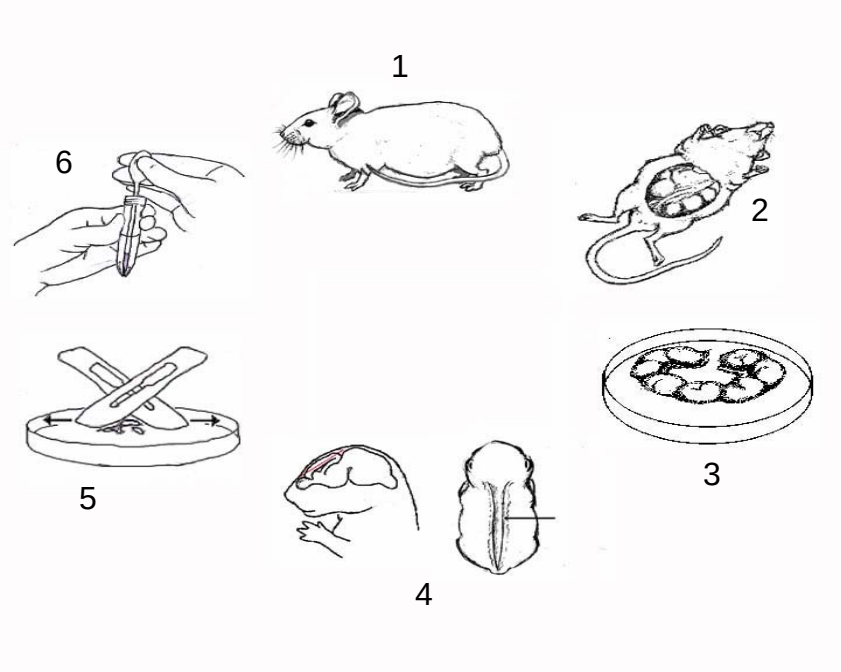

Figure 2. Mouse dissection and cell preparation.
1. Balb-C/ICR mice are mated for 24 hours, 14 to 18 days before culturing. A single pregnant mouse is anesthetized for each batch of cultures, sacrificed by cervical dislocation, and dissected under sterile conditions to remove the uterus.
2-3. Ten to fourteen embryos are delivered from the uterus and placed in H1SG medium.
4. The frontal cortex and/or spinal cord is removed from each fetus after decapitation.
5. Tissue is mechanically minced with two sterile scalpel blades.
6. Minced cortex is triturated in 5 ml DMEM, 5% horse serum, 5% fetal bovine serum, 2% B27, 8 µg vitamin C/ml (DMEM5/5).
(Center for Network Neuroscience archives. Center for Network Neuroscience, University of North Texas, http://www.cnns.org).

Neuronal networks grown on the recording electrode matrix typically develop a confluent glial carpet within 3-5 days after seeding (Fig. 3, 4). A shallow three-dimensional neural network forms with neuronal somata generally found on top of the carpet. Axonal processes are found both below and at the surface of the glial layer. Such networks can remain viable, stable, spontaneously active, and pharmacologically responsive for more than six months (Gross, 1994).

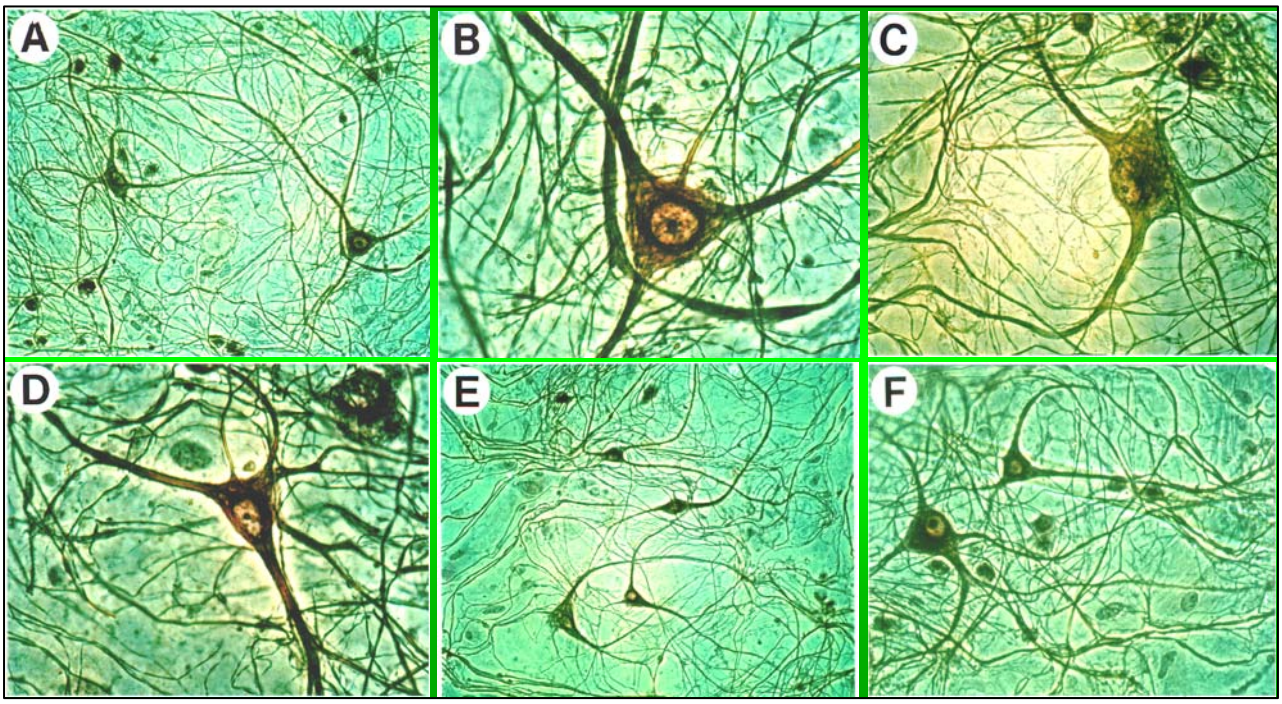

Figure 3. Example of a neuronal cell culture. Spinal cord culture 138 days (4.6 months) after seeding. Loots modified Bodian stain shows the distinct somata, axons, and dendritic arbors. Useful lifetime, > 6 months. (Center for Network Neuroscience archives. Center for Network Neuroscience, University of North Texas, http://www.cnns.org).

2.3 Assembly of Recording Apparatus

On the day of the experiment, a mature network growing on a MMEP is removed from the storage incubator and assembled into a recording apparatus, which includes a protective chamber, ITO cover cap to prevent condensation and that allows microscopy, and chamber base-plate connected to a DC power source that maintains a temperature of 37 ± 1 °C. The pH is stabilized at 7.4 by passing a stream of humidified 10% CO_2 in air through the chamber cap. A syringe pump maintains the osmolarity of the bath medium at approximately 320 mosmol/kg via 35 µl/hr ultra-pure water injections.

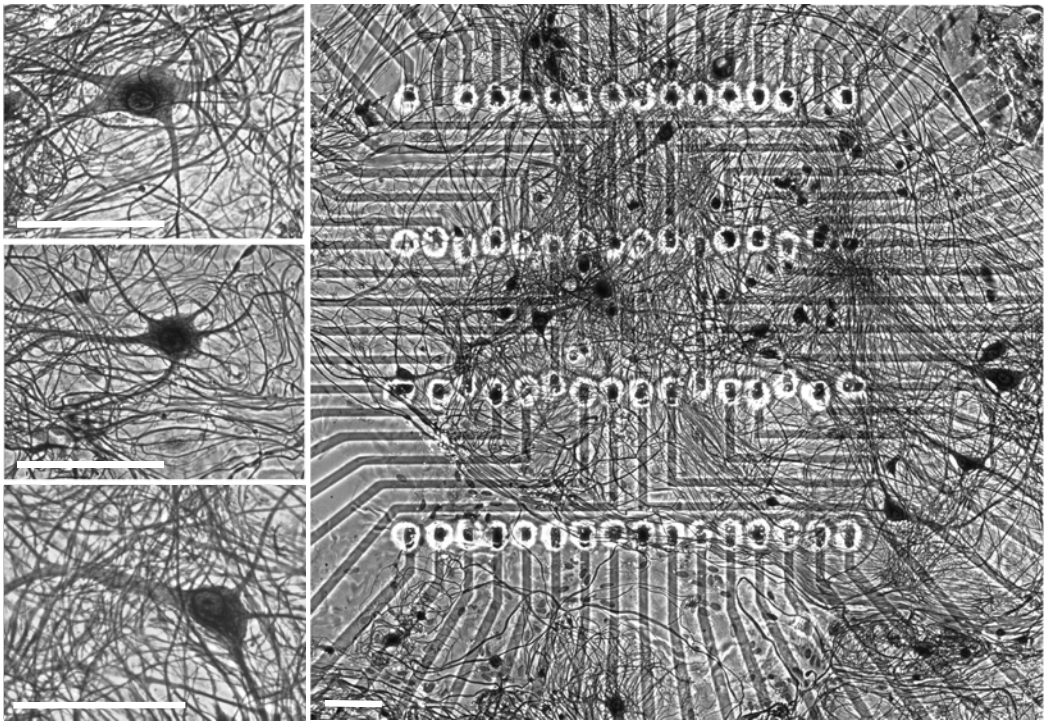

Figure 4. Neuronal network derived from murine spinal cord tissue (92 days *in vitro*), grown on the recording matrix of a 64-electrode array plate. Higher magnifications of neurons from this network are shown in the inserts. The transparent indium-tin oxide conductors are 8 μm wide and 1,200Å thick (Bars = 40μm) (Center for Network Neuroscience archives, Center for Network Neuroscience, University of North Texas, http://www.cnns.org)

2.4 Data Acquisition and Analyses

Extracellularly recorded action potentials are processed through 64 capacitively-coupled pre-amplifiers situated on an inverted microscope stage (32 channels per side, amplification factor: 100) to a box housing 64 main amplifiers and 32 digital signal processors (DSPs). Total system gain was 10k. The analog signal output from the box is sent to: (1) several oscilloscopes that allow monitoring of electrode activity (2) audio speakers that allow monitoring of spike and burst activity. A DSP is user-assigned to

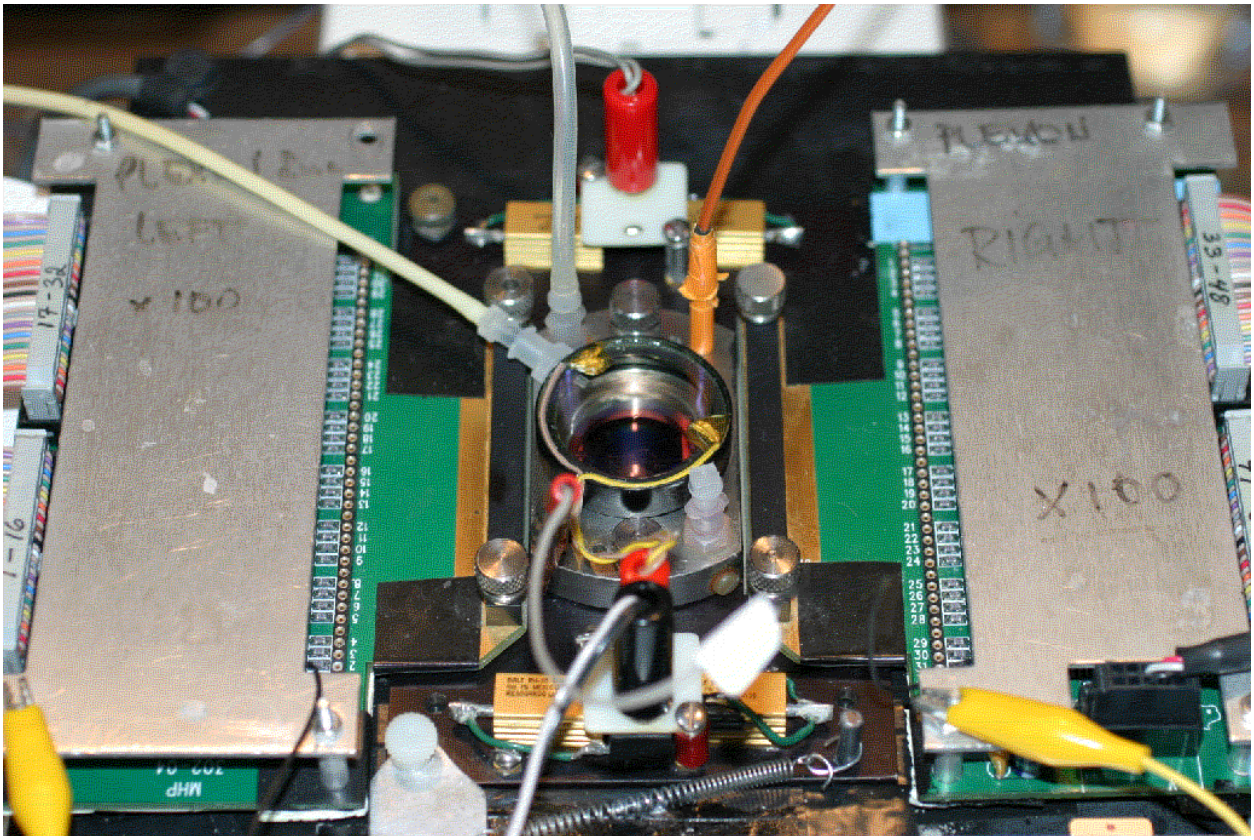

Figure 5. Recording apparatus on inverted microscope stage. A chamber containing the neuronal network growing on a MMEP with a constant medium bath of 2 ml. The chamber features fluidic syringe ports for access to bath medium for drug and water applications. The ITO chamber cap confines CO_2/air flow to maintain a constant 7.4 pH. The base plate with power resistor maintains a constant temperature of 37 ± 1 °C. A syringe pump injects 35 µl/hr water through the syringe port input to maintain a constant bath osmolarity of 320 mosmol/kg. Left and right pre-amplifier outputs (32 channels each side) feed data to a Plexon box for digital processing.

each electrode channel with a neuronal signal, and all subsequent processing is done on a digitized signal (40 kHz sampling rate per DSP). Only neuronal action potentials (units) with a signal to noise ratio of 2/1 or higher are selected. The signal from the electrode usually consists of action potentials from multiple cells. The software supplied with the amplifier/DSP hardware (Plexon, Inc., Dallas, TX) allows the discrimination of up to 4 signals (units) per DSP (Fig 6). The time stamps of spike events are sent to the host PC through a 16 bit parallel bus to a MXI interface board. The timestamp data is

recorded to hard disk in a proprietary Plexon file format with the option of also storing waveform information. An in-house program allows real-time monitoring of spike and burst rate. After the conclusion of an experiment, the recorded spike train data is viewed with software specialized for multiple neuron data sets (Neuroexplorer®, Nex Technologies, Littleton, MA), which incorporates several standard analysis routines (rate histograms, interspike intervals, waveshape data etc.). Additional data analysis (integrated burst analysis, concentration-response curves, tests for significance, waveform viewing, etc.) is performed with a variety of custom and commercial software packages.

2.5 Pharmacological Manipulations

Before drug application, native network activity was recorded for at least 30 minutes to reach a stable baseline in spiking and bursting activity. In most experiments subsequent to native activity and before drug addition, a complete medium change was performed to reach a stable reference activity. Stable reference activity is important because all subsequent changes were measured relative to this reference activity pattern. Medium changes were performed with either DMEM or MEM, for FC and SC, respectively, and were mixed with the appropriate fresh and conditioned medium to obtain a 50/50 solution. The 50/50 mixture is termed “wash medium”. Experiments were performed only on networks with an average reference activity of 200 spikes per minute or greater. In some networks the reference spike rate fell below 200 spikes/min. In such cases 40 μM bicuculline, a $GABA_A$ receptor antagonist, was used to disinhibit the network activity to allow an increase in spike rate activity. In addition, networks

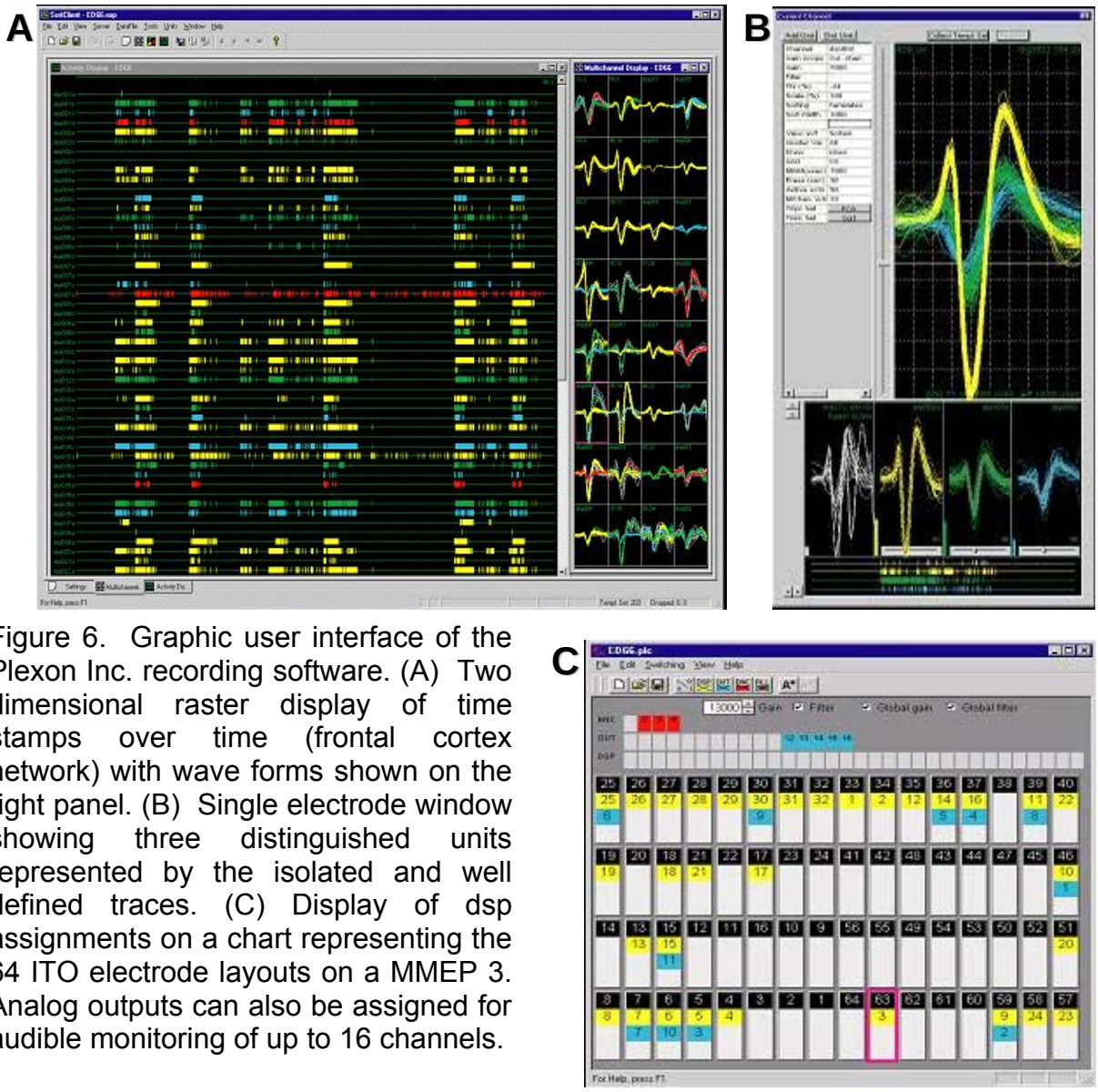

Figure 6. Graphic user interface of the Plexon Inc. recording software. (A) Two dimensional raster display of time stamps over time (frontal cortex network) with wave forms shown on the right panel. (B) Single electrode window showing three distinguished units represented by the isolated and well defined traces. (C) Display of dsp assignments on a chart representing the 64 ITO electrode layouts on a MMEP 3. Analog outputs can also be assigned for audible monitoring of up to 16 channels.

were not used experimentally if the native or reference activity fluctuated more than ± 10 %. Drug-induced activity changes were statistically compared to native, reference, or bicuculline-induced activity. For ease of terminology, the term reference will often be used to identify any of these three activity-states. D-threo-methylphenidate (Sigma, St. Louis, MO) was applied to the culture milieu in either of two ways: (1) pipetted directly into the bath medium by lifting up the chamber cap to allow access to the medium or (2) through the syringe ports of the chamber, which allow access to the bath medium

without removing the ITO cap. When the direct pipette method was used, the drug was dispensed at 12:00 and 6:00 positions and the pipette was rinsed by drawing up medium and subsequently dispensing it into the bath medium. Both methods have been used, and thus far the data do not suggest that one application is better than the other. After each drug addition, the network activity was not manipulated until a stable baseline was reached (in most experiments, this period was 30 min). In many of the experiments, a complete wash of the bath medium, after addition of the maximal drug concentration, was performed to assess drug-induced response reversibility. In several experiments, drug applications often followed the last wash, and the entire drug application procedure could be repeated to determine intra-culture reproducibility.

2.6 Concentration Response Curves (CRCs) and Subpopulation Studies

Concentration-Response Curves were generated using the above drug application technique and spike rates were used as the quantifiable variable. The concentration-response data (stable spike rate for each episode) were normalized to 0 and 100% from the raw % spike inhibition and plotted on a semi-log graph (Origin, Microcal Software, Inc.). CRCs were constructed using an automated logistical concentration-response sigmoidal curve fitting function, and IC_{50}s were interpolated.

Unit-specific subpopulation responses to D-MPH was explored by grouping individual units into subpopulations based on the single unit spike activity rate graphs, which may be viewed using Neuroexplorer, a neurophysiological data analysis package (Nex Technologies). Each unit was either classified as inhibitory, excitatory, or no change in response to D-MPH by using a 20% increase or decrease from reference activity as the parameter of definition.

2.7 Waveshape Analysis

The action potential waveshapes of SC and FC networks were recorded with a 40 kHz sampling rate within a 1,000 μsec window. Therefore, 40 points are sampled every 40 μsec and are used to construct the waveforms. The data points were plotted and absolute values of two amplitudes were calculated. Amplitude one and two are defined by using three peak assignments to each waveform (Fig. 7). Amplitude one is the absolute value of the amplitude (μV) between peak 1 and peak 2, and amplitude two is the amplitude (μV) between peak 2 and peak 3. The values of the amplitudes were obtained from an average of 25 or 10 consecutive waveforms taken at the appropriate activity state, 50, 200, and 400 μM D-MPH. The first 20 waveforms from 3 experiments and all 12 of another were used in the waveform analysis.

2.8 Immunocytochemistry

Frontal cortex and spinal cord cultures were seeded and maintained on glass coverslips for immunocytochemical procedures and analyses. After approximately three weeks of incubation, the networks were processed for tyrosine hydroxylase (TH) immunocytochemistry. Prior to fixation, multiple microscopic pictures were taken of each culture to ensure quality control in the fixation process. After fixation, the cultures were again examined, and if any apparent abnormalities existed, the cultures were not used for immmunocytochemstry. Briefly, the networks were fixed with 4% paraformaldehyde by adding serial drops into the culture dish until proper fixation was established. Following fixation, the cultures were permeabilized with 0.1% Triton X-100 in PBS and blocked in 0.1% Triton X-100 in PBS + normal horse serum (NHS).

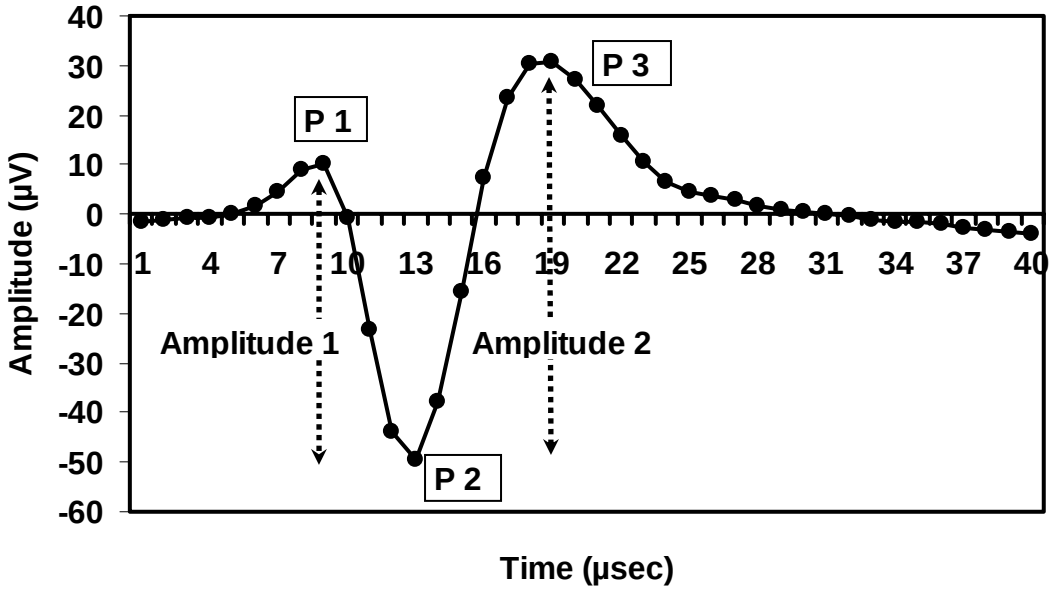

Figure 7. Action potential waveform recorded from one discriminated frontal cortex unit. P1, P2, and P3 represent peak 1, 2, and 3, respectively. Amplitude 1 is derived from the absolute value of the amplitude from the apex of P1 to the apex of P2. Amplitude 2 is determined by measuring the absolute value of the amplitude from the apex of P2 to the apex of P3. All waveforms were constructed from 40 digitized data points obtained at each µsec.

The networks were then incubated in a solution containing a rabbit primary antibody to TH (1:800; Chemicon Int.) for 24 or 48 hours at 4 °C. Several washes were performed to remove any excess antibody and the tissue was incubated in a solution containing a universal biotynylated secondary antibody diluted in 5% NHS (Vector Laboratories, Burlingame, CA) for 40 minutes at room temperature. The secondary antibody was made visible by the avidin-biotin complex method (ABC Elite kit; Vector Laboratories) using the chromogen, diaminobenzidine (DAB). The cover slips were then mounted to glass slides using Aqua-polymount (Polysciences Inc., Warrignton, PA). Phase contrast and bright field illuminations were used to qualitatively assess positive and negative staining.

2.9 Statistical Analyses

Where appropriate, the data were presented as mean ± standard deviation (SD) or standard error (SE) and the number of units or networks (n). The network spike and burst rates are binned data (bin size = 60 seconds) averaged across the network. Within single experiments, significance of changes induced by drug applications were tested with a paired 2-tailed Student's t-test. Within multiple experiments, significance of changes induced by drug applications were tested with an independent Student's t-test. Parametric (one way repeated measures ANOVA) and nonparametric (Friedman repeated measures ANOVA) analysis followed by the appropriate post-hoc comparison test were used to test significance during different episodes with the same drug treatment regimen. Finally, a Pearson product moment correlation was used to identify any significant correlations. In all statistical tests, $p < 0.05$ was considered significant.

3. RESULTS

3.1 Vehicle Control and Normal Spontaneous Activity of FC and SC Networks

Stock solutions of D-methylphenidate (D-MPH) were dissolved in sterile aqueous solutions containing 1 and 10 μM concentrations and stored at 4° C. Serial aliquots of D-MPH were added to 2,000 μl of the culture medium bath to produce an additive increase in drug concentration until network activity reached near complete inhibition. Before drug additions, a complete medium change was performed with the appropriate wash medium until a stable base line was achieved. A vehicle study was performed to ensure the drug vehicle and wash medium did not overtly change the spontaneous activity of the network. Figure 8 demonstrates the effects of increasing sequential additions of water and wash medium into 2,000 μl of culture medium on a frontal cortex culture 36 days *in vitro* (D.I.V.) with a count of 47 active units. The mean spikes/min and burst/min averaged across channels were plotted against time. Serial applications of 2, 10, 20, and 40 μl of both water and the wash medium were added, resulting in a total increase of 72 μl of each solution. The water additions mimicked the amount of water/drug solution applied during the sequential drug applications performed in the concentration-response experiments. The average percent change in spike and burst rate during the water treatment decreased 0.48 ± 1.4 % and increased 4.8 ± 5.4 % from the bicuculline-induced activity, respectively. The average percent change in spike and burst rate of each serial addition of wash medium (DMEM) increased 2.9 ± 2.4 % and 14.0 ± 3.8 %, respectively.

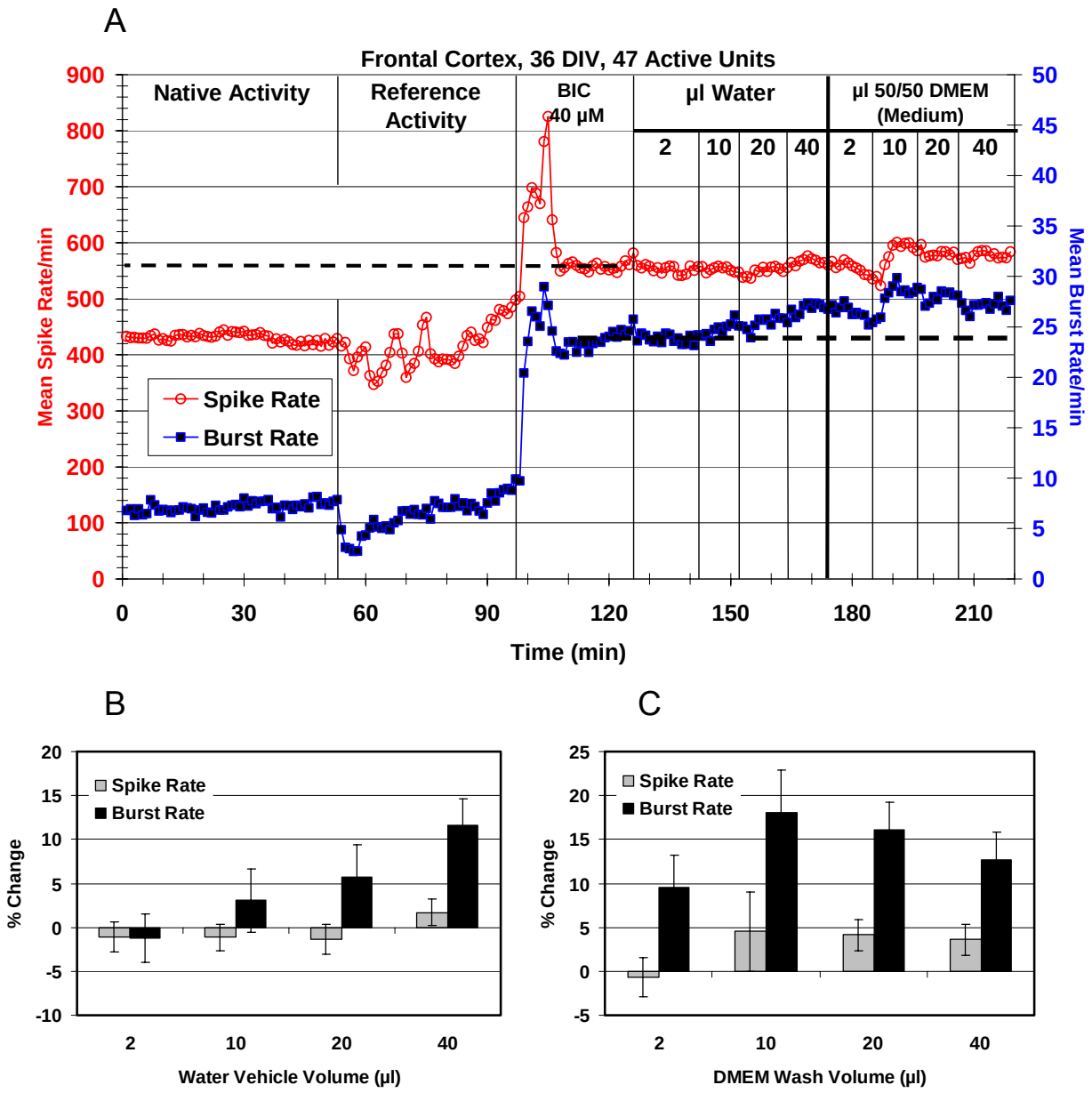

Fig. 8. Response of frontal cortex network to vehicle and wash medium. (A) Mean spike and burst rate/min plot shows typical response to water and DMEM serial additions as well as normal variations in spike and burst activity during Native, Reference, and Bicuculline-induced episodes. The horizontal bars indicate the bicuculline-induced activity used to calculate % inhibition. (B) The spontaneous spiking and bursting activity decreased 0.48 ± 1.4 % and increased 4.8 ± 5.4 %, respectively due to serial water additions. (C) The spontaneous spiking and bursting activity increased 2.9 ± 2.4 % and 14 ± 3.8 %, respectively, in response to DMEM additions. Bars indicate SD.

Figure 9 depicts the rate histograms of 47 discriminated units in the control experiment. A qualitative analysis suggests that spontaneous spiking activity did not change in 68%, increased in 21%, and decreased in 11%. This depicts the extensive variability of individual units and the relative stability of the neuronal ensemble, shown in Figure 8.

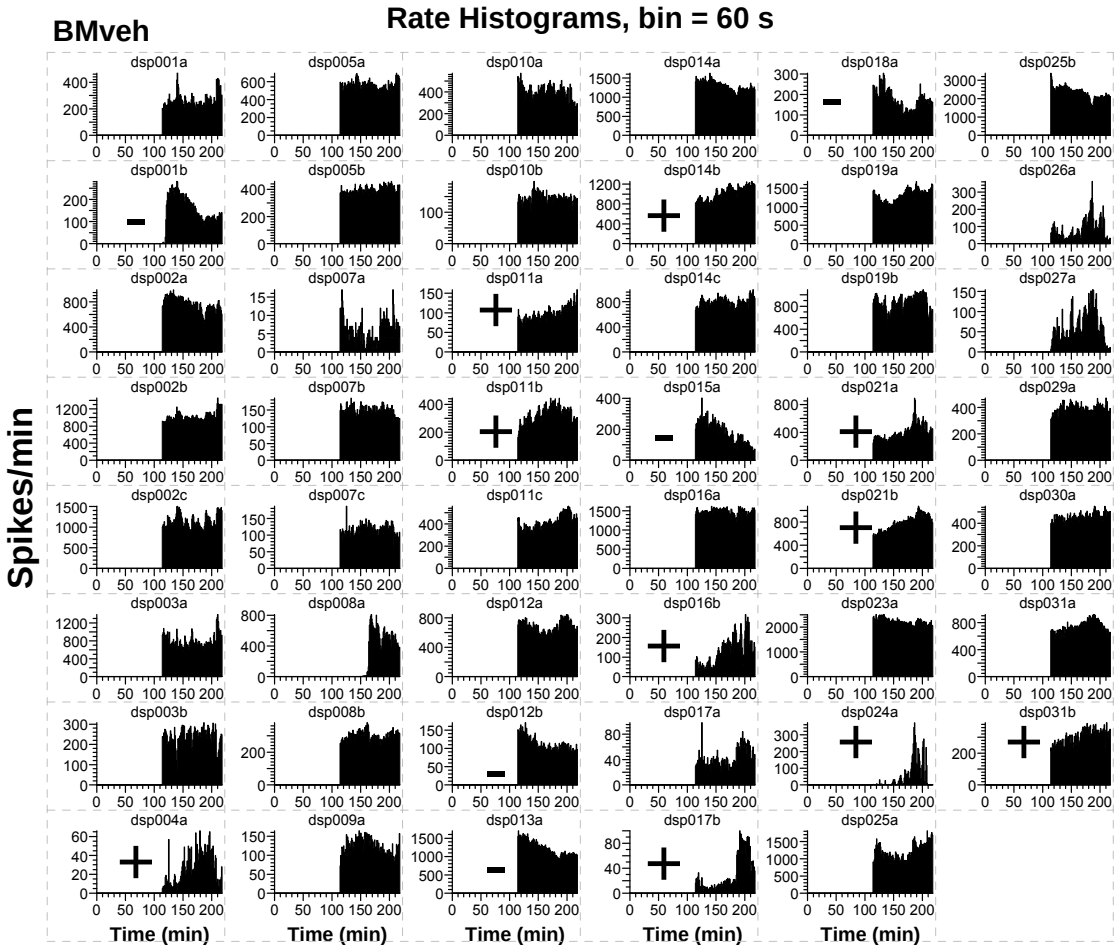

Figure 9. Individual spike activity response profiles of 47 discriminated units from the control experiment. The graph shows the evolution of spike rates (Y-axis) over time in minutes (X-axis) beginning at the bicuculline-induced activity period and ending at the final DMEM episode. + denotes an increase in spike activity, and – indicates a reduction in spike activity.

3.2 Global and Unit-Specific Responses of Frontal Cortex and Spinal Cord Networks to D-methylphenidate

Eight frontal cortex (FC) and 15 spinal cord (SC) cultures were used to study the effects of D-methylphenidate. Several of the neuronal networks were used multiple times to investigate intra-culture variability. The total neuron count for FC and SC networks was 456 and 589 with averages of 57 and 39 units per network, respectively. The ages of the cultures averaged 63 and 34 days *in vitro* for FC and SC cultures, respectively. The average experiment lasted 10 hours. Table 1 lists the entire set of D-MPH experiments.

Table 1. FC and SC cultures used in the D-MPH study.

Exp.	Tissue	Neuron Count	Age (D.I.V.)	Culture Medium	BIC Used	Serial Additions of D-MPH [μM]
BM041	FC	50	54	DMEM	no	1, 10, 50, 100, 200, 300, 400
BM042	FC	52	54	DMEM	yes	1, 10, 50, 100, 200, 300, 400
BM044	FC	84	56	DMEM	no	1, 10, 50, 100, 200, 300, 400
BM044b	FC	50	56	DMEM	no	10, 100, 200, 300, 400
BM044c	FC	35	56	DMEM	no	1, 10, 50, 100, 200, 300, 400
BM056	FC	74	52	DMEM	no	1, 10, 50, 100, 200, 300, 400, 500
BM058	FC	54	88	DMEM	yes	1, 10, 50, 100, 20, 300
BM059	FC	57	88	DMEM	yes	1, 10, 50, 100, 200, 300
total	8	456				
average		56	63			
BM039	SC	71	32	MEM	no	100, 200, 300
BM039b	SC	68	32	MEM	no	100, 200, 300
BM040	SC	25	33	MEM	no	1, 10, 100, 300, 400
BM045	SC	15	28	MEM	yes	1,10, 50, 100, 200, 300, 400
BM046	SC	41	56	MEM	yes	1, 10, 50, 100, 200
BM047	SC	43	23	MEM	no	1, 10, 50, 100, 200, 300, 400
BM047a	SC	33	23	MEM	no	1, 10, 50, 100, 200, 300, 400, 500
BM048	SC	27	23	MEM	no	1, 10, 50, 100, 200, 300
BM048a	SC	17	23	MEM	yes	1, 10, 50, 100, 200, 300, 400
BM049	SC	22	37	MEM	yes	1, 10, 50, 100, 200
BM049a	SC	16	37	MEM	yes	1, 10, 50, 100, 200, 300
BM050	SC	68	47	MEM	yes	1, 10, 50, 100, 200
BM051	SC	45	47	MEM	yes	1, 10, 50, 100, 200
BM052	SC	52	34	MEM	yes	1, 10, 50, 100, 200, 300
BM053	SC	46	41	MEM	yes	1, 10, 50, 100, 200, 300
total	15	589				
average		39	34			

D.I.V. = days *in vitro*. Neuron count = number of units recorded in each experiment. BIC used = networks treated with 40 µM bicuculline before the addition of D-MPH. a, b, c = consecutive experiments with the same network.

It was observed that sequential additions of D-methylphenidate ranging from 1-500 µM produced a concentration-dependent inhibition of the spontaneous spike and burst activity in both FC and SC networks, n = 17. Figure 10 shows 30 seconds of representative spike patterns (spike time stamps) of 31 SC units in the reference state and in the presence of 100, 200, and 300 µM D-MPH. The raster plots indicate the reduction in spike production and the early loss of weak units following sequential additions of µM concentrations of D-MPH.

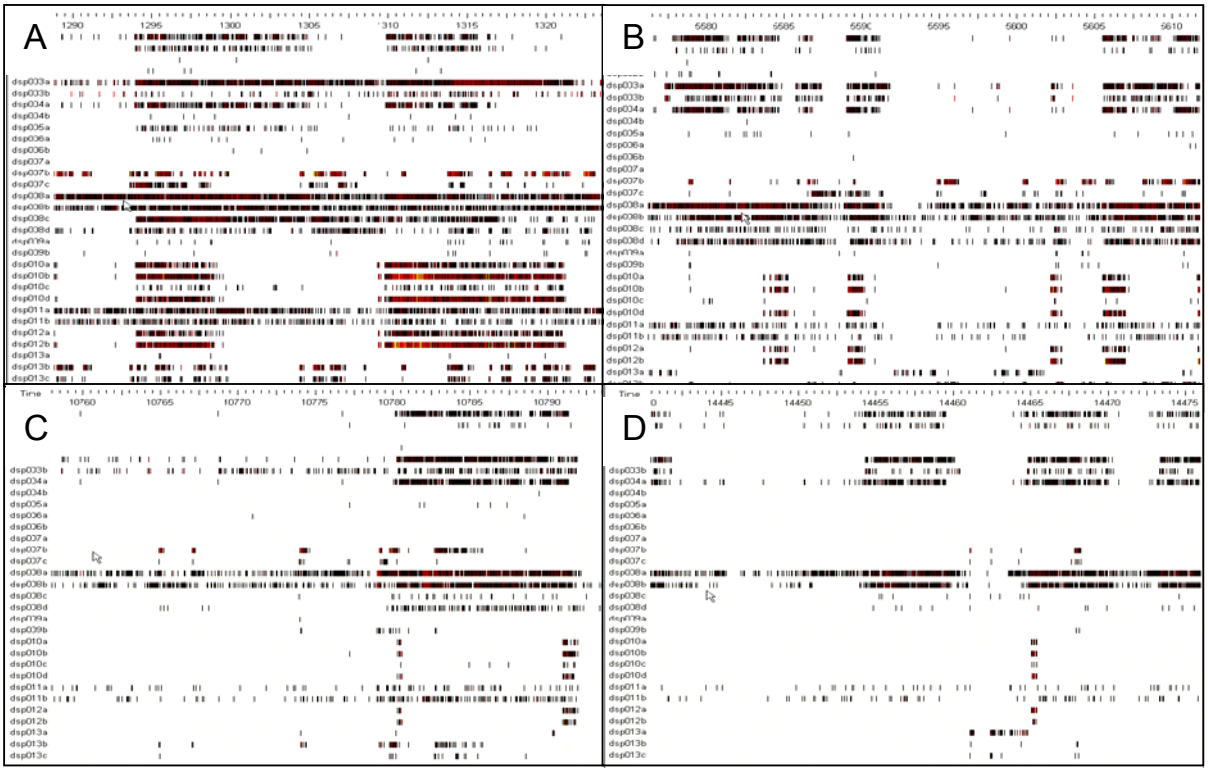

Figure 10. Raster displays obtained from Nex showing the concentration-dependent decrease in spike production and the disruption in the burst pattern in response to D-MPH on a SC network (BM039b). Shown are simultaneous 30 sec spike trains from a sample of 31 neurons. (A) Reference activity. (B) 100 µM D-MPH. (C) 200 µM D-MPH.

(D) 300 μM D-MPH. Notice the reduction in spike production at each concentration of D-MPH.

Figure 11 shows the typical concentration-dependent response of a FC network when exposed to a serial addition of 1-300 μM D-MPH (BM059). The mean spikes/minute and bursts/minute were plotted in one-minute bins over time. The electrophysiological recording lasted for 11 hours. Native activity was recorded for 50 min and a wash was performed with DMEM to obtain constant reference activity. The average reference activity was below 200 spikes/min therefore 40 μM bicuculline was added to disinhibit the activity. The bicuculline-induced spike rate and burst rates were 1,000/min and 35/min, respectively. At 1 μM, D-MPH inhibited the spike rate 16% compared to the bicuculline-induced activity-state. When concentrations reached 100 and 200 μM, D-MPH inhibited the spike rate by 46% and 68%, respectively. Near-total spike cessation (99%) occurred at 300 μM D-MPH. A subsequent wash performed without the addition of bicuculline, restored spike activity 100% to that of the reference activity.

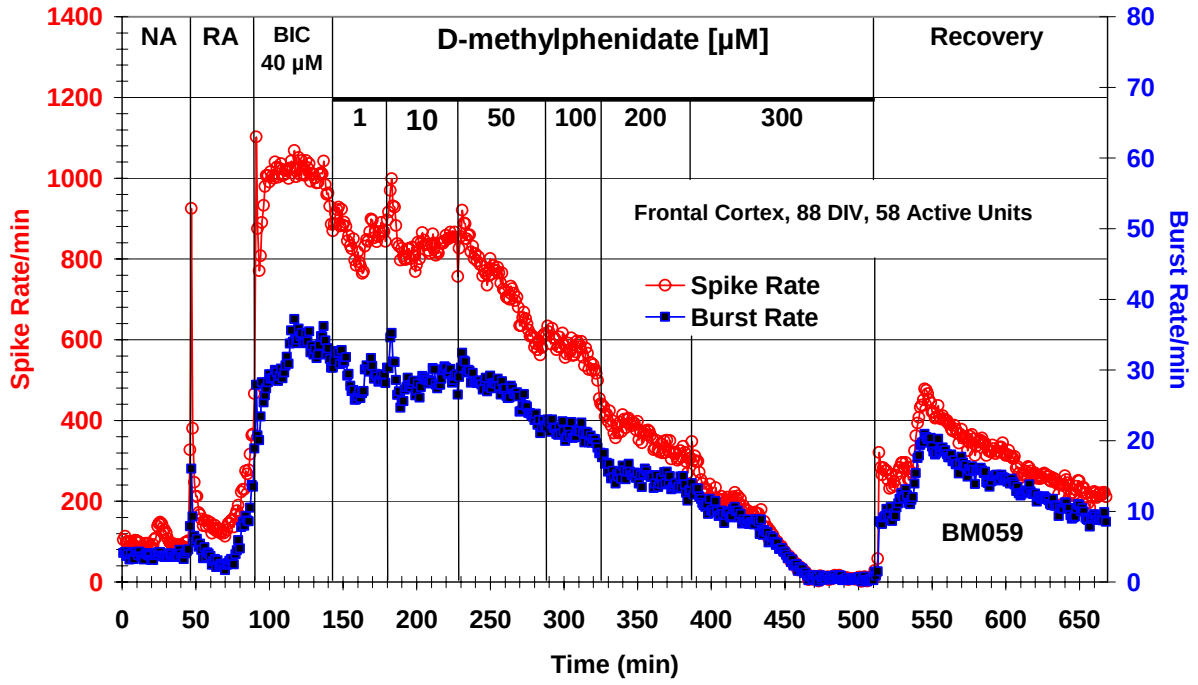

Figure 11. Global response of a frontal cortex network to increasing serial concentrations of D-MPH. The spike and burst activity decreased in a concentration-dependent manner. At 300 µM, D-MPH inhibited the spike rate by 99%. NA = native activity; RA = reference activity, BIC = bicuculline. Note: recovery medium did not contain BIC.

Figure 12 shows the network concentration-dependent response of a SC culture when exposed to a serial addition of 1-300 µM D-MPH (BM053). As before, the mean spikes/minute and bursts/minute were plotted over time. The electrophysiological recording lasted for 9 hours. Native activity was recorded for 50 min and a wash was performed with MEM to obtain constant reference activity. The average reference activity fell below 200 spikes/min, therefore 40 µM bicuculline was added. The bicuculline-induced spike rate and burst rate stabilized at 500/min and 14/min, respectively. At 1 µM, D-MPH inhibited the spike rate 15% compared to the bicuculline-induced activity-state. When concentrations reached 100 and 200 µM, D-MPH inhibited the spike rate by 82% and 94% from the bicuculline-induced activity, respectively. Similar to the FC network, near-total spike cessation (99%) occurred at 300 µM D-MPH.

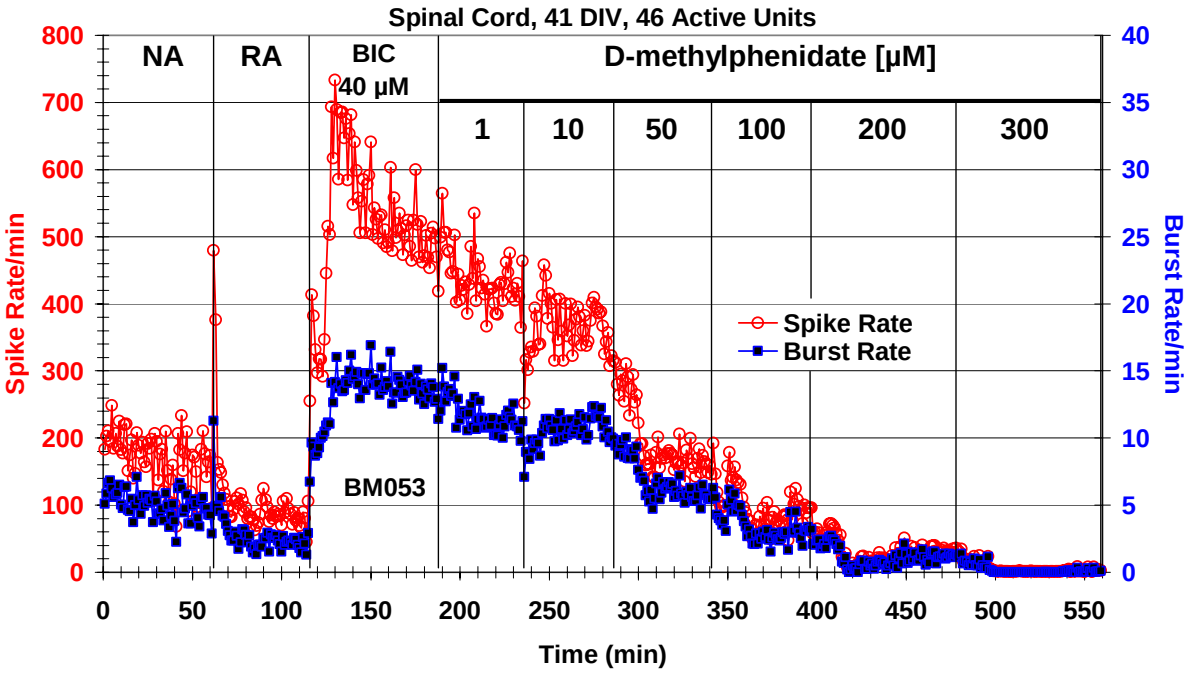

Figure 12. Global response of a spinal cord network to increasing serial concentrations of D-MPH. The spike and burst activity decreased in a concentration-dependent manner. At 300 µM, D-MPH inhibited the spike rate by 99%. NA = native activity; RA = reference activity, BIC = bicuculline.

Figure 13 shows a summary of the inhibition of spontaneous spike activity attributed to various µM concentrations of D-MPH for all FC and SC experiments (n =8 and 15, respectively). In the majority of experiments, complete cessation of spike activity was not obtained. At 400 µM, D-MPH inhibited the spike activity an average of 87 and 92% for FC and SC networks, respectively. A one way repeated measures ANOVA with post hoc Holm-Sidak pairwise multiple comparison test indicated that the inhibition in spike rate was significantly different among the 1, 10, 50, 100, 200, 300, and 400 µM concentrations of D-MPH in each FC and SC cultures ($p < 0.05$, n = 7 treatment groups). The 500 µM concentration of D-MPH was not represented in the statistical analysis because only one data point was collected for each tissue type.

A one-tailed independent t-test was used to determine if differences among the % inhibition values existed between the FC and SC networks. The test indicated that only the 10, 50, 100, and 200 μM concentrations were significantly different ($p < 0.05$).

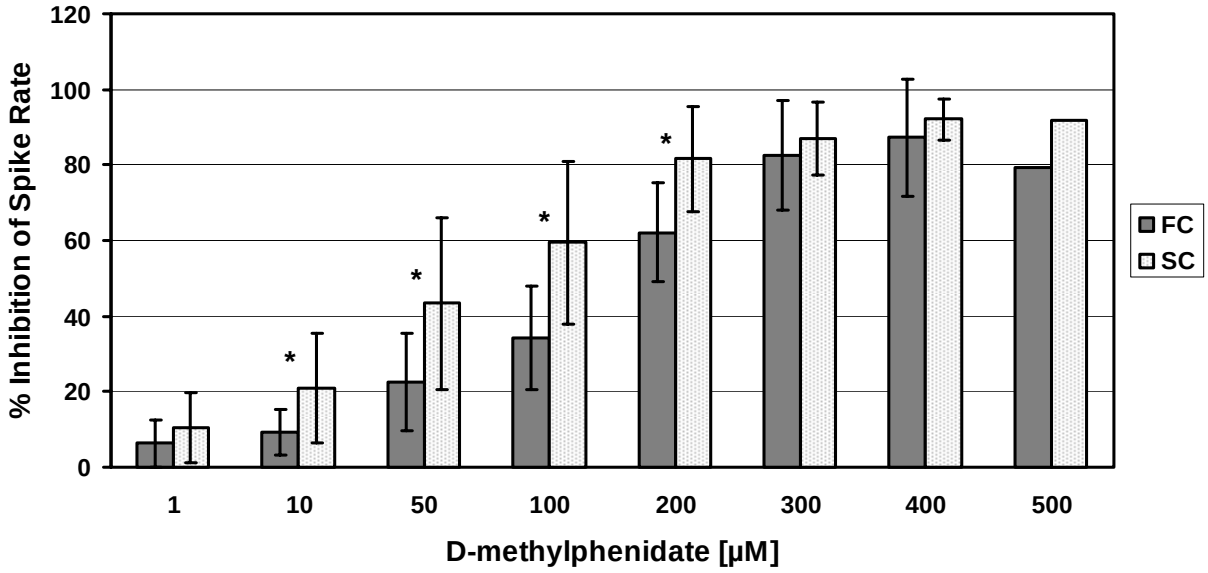

Figure 13. Summary of the average % inhibition of spike rate ± SD of 8 FC and 15 SC networks to serial additions of D-MPH. In each tissue type, the 1, 10, 50, 100, 200, 300, and 400 μM concentrations were all statistically different from one another (One Way Repeated Measures ANOVA with post hoc Holm-Sidak test, $p < 0.05$). A one-tailed independent t-test revealed that only the 10, 50, 100, and 200 μM were significantly different between the FC and SC networks ($p < 0.05$). * indicates $p < 0.05$.

To determine the sensitivity of the network to D-MPH, paired t-tests were conducted to identify the first concentration of D-MPH that significantly inhibited the spontaneous spike activity in each experiment. The test compared 10-30 min stable spiking from reference and 1, 10, or 50 μM D-MPH. It was found that 1 μM D-MPH significantly inhibited the spike activity in the majority of experiments (Table 2). Lower concentrations were not used in these experiments.

Table 2. Statistical summary of network sensitivity.

Experiment #	D-MPH [μM]	Probability Different from Reference	n (# of minute means)
FC			
BM041	1	$p < 0.001$	30
BM042	10	$p < 0.001$	30
BM044	1	$p < 0.001$	30
BM056	1	$p < 0.001$	20
BM058	1	$p < 0.001$	20
BM059	1	$p < 0.001$	20
SC			
BM040	1	$p < 0.05$	20
BM045	10	$p < 0.001$	10
BM046	50	$p < 0.001$	20
BM047	10	$p < 0.001$	20
BM048	10	$p < 0.001$	20
BM049	1	$p < 0.001$	20
BM050	1	$p < 0.001$	20
BM051	1	$p < 0.001$	20
BM052	1	$p < 0.001$	20
BM053	1	$p < 0.001$	20

Single unit analysis indicated that the majority of the neurons recorded in FC and SC networks showed a concentration-dependent decrease in spontaneous spike activity following serial additions of D-MPH. In FC networks, a qualitative assessment showed that 97% of the neurons decreased, 0.2% increased, and 3% showed no change in spike activity in response to D-MPH applications (n = 456) (Fig. 14). In SC cultures 99% of units decreased, 0% increased, and 1% did not change in spike production (n = 589) (Fig. 15). Although the majority of neurons decreased, it is apparent that each unit has an intrinsically different spike activity profile and the times to decrease are different.

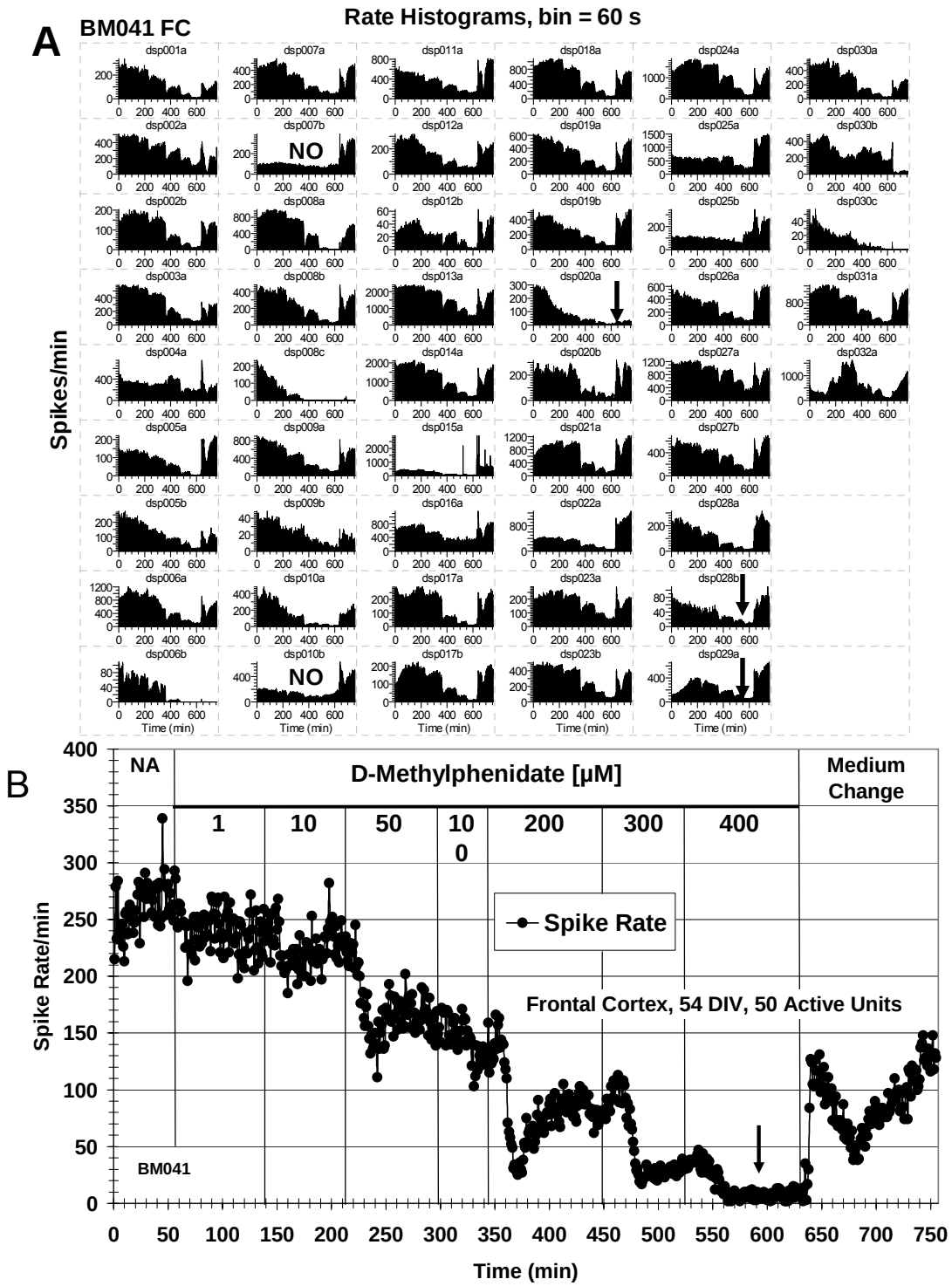

Figure 14. Unit-specific spike activity of a FC network in response to serial additions of D-MPH (BM041). (A) Individual spike rate histograms of 50 discriminated units depicting the variability across the units. 96% of the units increased, 4% did not change, and 0% increased in spike activity. NO = no change in activity. (B) Average spike activity plot of the 50 units. NA = native activity. Arrows serve as a reference point in time.

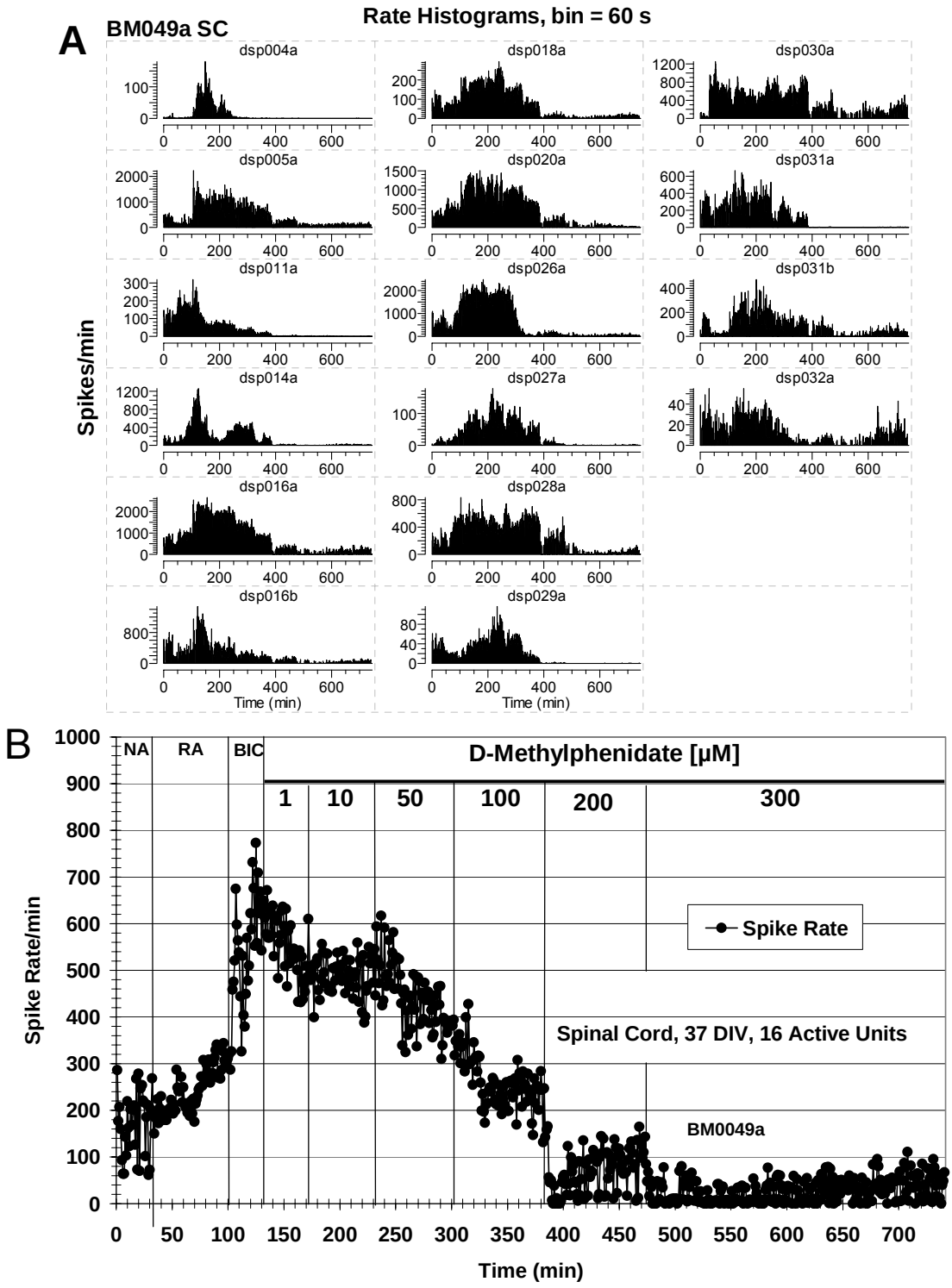

Figure 15. Unit-specific spike activity of a SC network in response to serial additions of D-MPH (BM049a). (A) Individual spike rate histograms of 16 discriminated units depicting different spike rate patterns across the units. All the units showed a concentration-dependent decrease in spike activity. (B) Average spike activity plot of the 16 units. NA = native activity, RA = reference activity, and BIC = 40 µM bicuculline.

3.3 Tissue Specific Responses, Inter-Culture Repeatability, and Uniformity of Units

To determine tissue specific responses, inter- and intra-culture variability, and uniformity of units to D-MPH on frontal cortex and spinal cord networks, concentration-response curves (CRCs) and associated IC_{50} values (concentration at which 50% of the spiking is inhibited) were calculated for each experiment in both culture types. The CRC data points are obtained from the normalized % inhibition of spike rates under each concentration of D-MPH (see Methods). The IC_{50} data were obtained from networks treated with and without bicuculline. A t-test indicated that there was no significant difference between the IC_{50} values of cultures treated with or without bicuculline in both FC and SC networks ($p > 0.05$). Therefore, the data were pooled.

Concentration-response summaries of 6 FC and 11 SC networks were constructed to explore tissue-specific and inter-culture responses to D-MPH (Fig. 16). Experiments that were subject to more than one concentration-response regimen were excluded from the tissue-specific IC_{50} summary analyses. The IC_{50} values ranged from 75-216 and 6-135 μM D-MPH and averaged 118 ± 52 and 57 ± 43 μM D-MPH for FC and SC networks, respectively (IC_{50} = mean ± SD). The average of the normalized % inhibition values for each D-MPH concentration were used to construct an average CRC for both FC and SC networks (Fig. 17). The corresponding IC_{50} values were 117 ± 13 and 38 ± 7 μM D-MPH for FC and SC cultures, respectively (IC_{50} = mean ± SE of the curve fit). The average CRC is another way to represent the average IC_{50}. The FC and SC IC_{50} values were shown to be significantly different (t-test, $p < 0.05$). Thus, DMPH induces a tissue-specific response which is primarily due to the difference in the drug potency of each tissue.

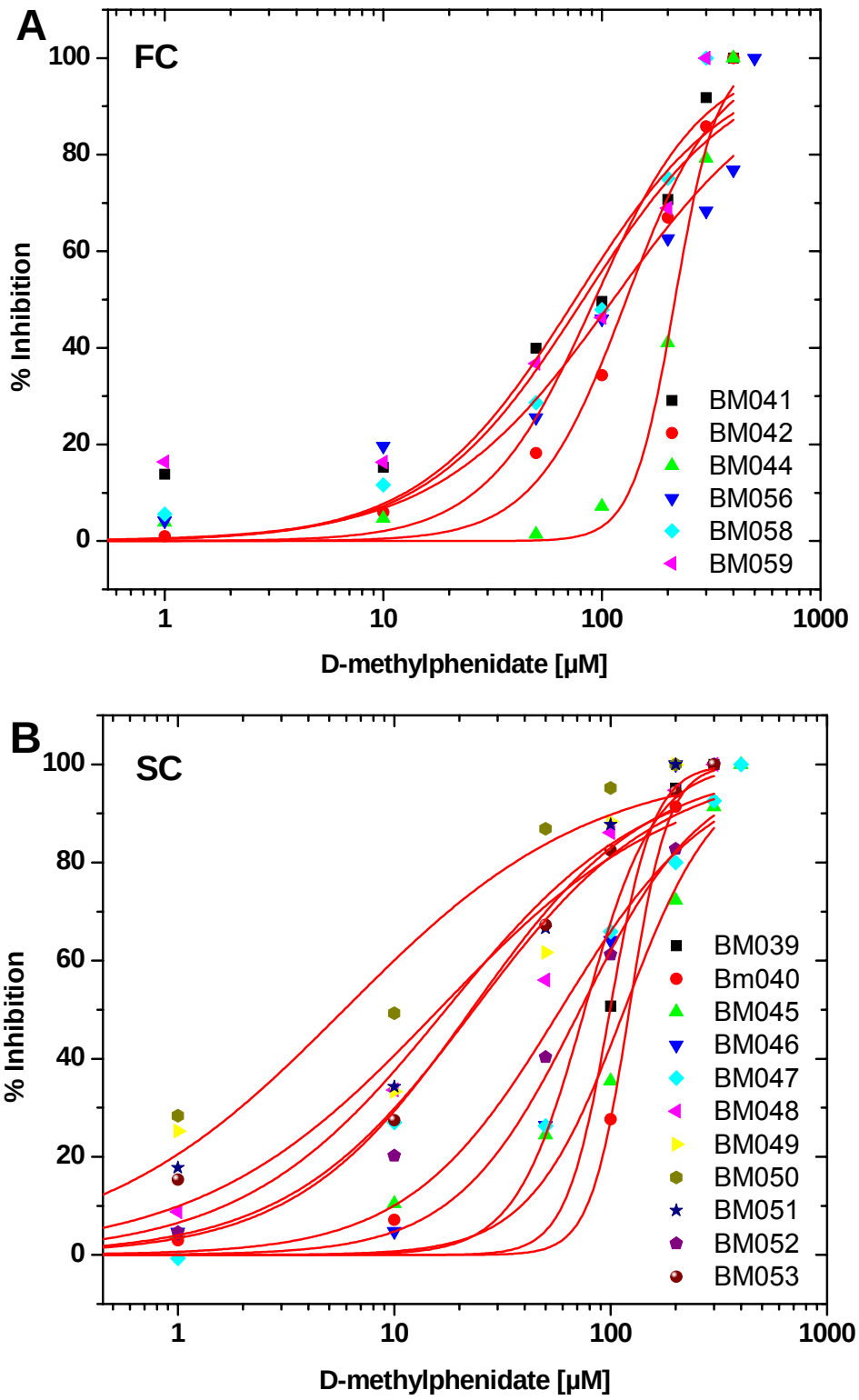

Figure 16. Individual IC_{50} curves for FC and SC experiments. (A) 6 FC IC_{50} curves ranged from 75-216 µM and averaged 118 ± 52 µM D-MPH. (B) 11 SC IC_{50} curves ranged from 6-135 µM and averaged 57 ± 43 µM D-MPH.

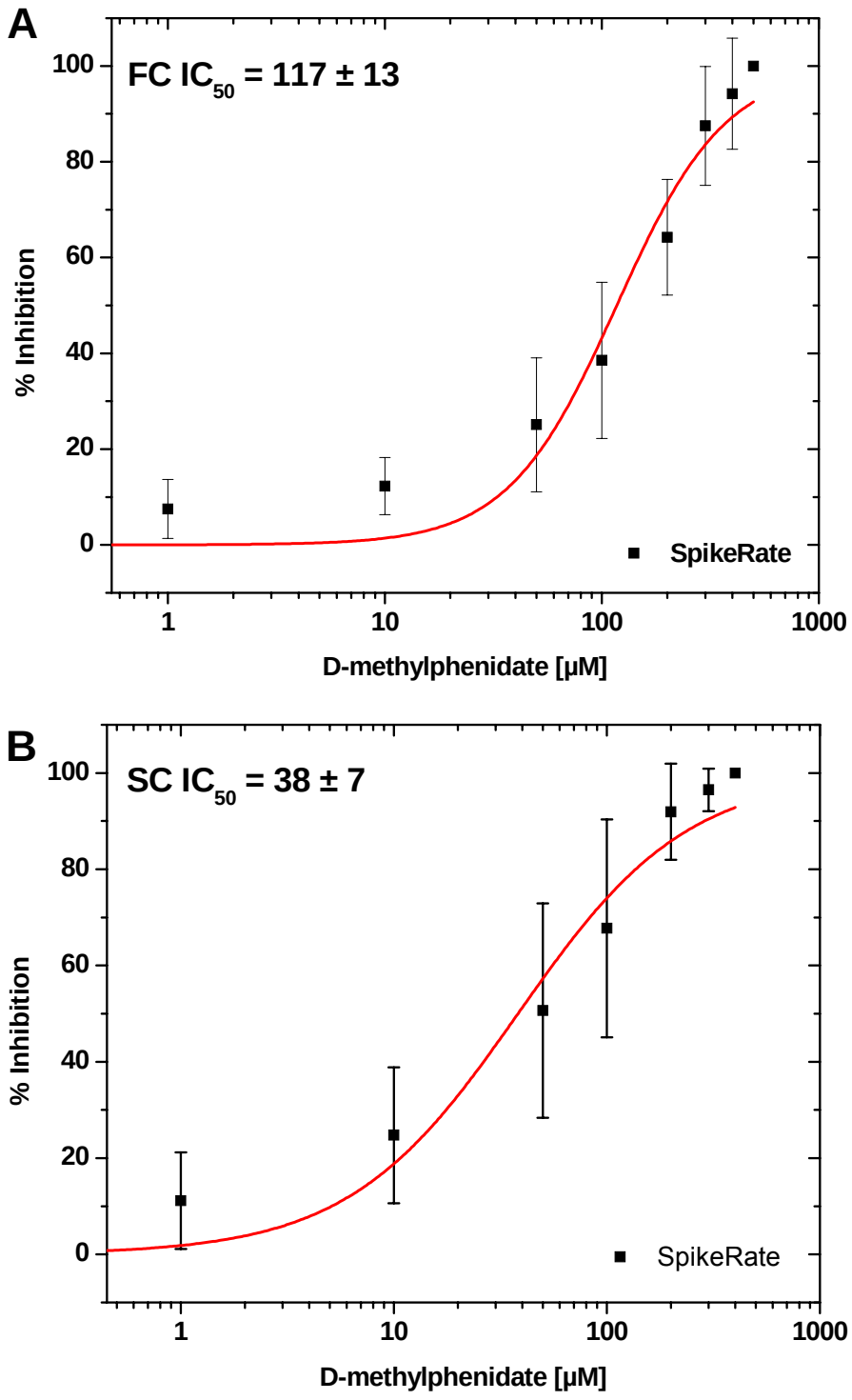

Figure 17. (A) Spike rate CRC summary of 6 FC networks in response to D-MPH. IC_{50} (mean ± SE of the curve fit) was 117 ± 13 µM D-MPH. (B) Spike rate CRC summary of 11 SC networks. The mean IC_{50} was 38 ± 7 µM. The FC and SC IC_{50} summaries were statistically different (t-test, $p < 0.05$). Bars indicate SD.

Table 3. Summary of IC_{50}s of each concentration-response experiment. The FC and SC IC_{50} values were significantly different (t-test, $p < 0.05$).

Experiment #	IC_{50} D-MPH [μM]
FC	
BM041	75
BM042	129
BM044	216
BM056	112
BM058	93
BM059	82
Mean ± SD	117 ± 52
SC	
BM039	99
BM040	122
BM045	116
BM046	76
BM047	72
BM048	23
BM049	16
BM050	6
BM051	17
BM052	59
BM053	23
Mean ± SD	57 ± 43

IC_{50} values were also used to investigate intraculture repeatability. Networks were exposed to multiple concentration-response iterations, and their respective IC_{50} values were calculated. It was shown that after sequential concentration-response experiments, there was either an increase or a decrease in the generated IC_{50} value from the respective network. One FC network endured 3 consecutive CRC regimens which produced three IC_{50} values of 216, 157, and 123 (BM044a, b, and c). Two sequential concentration-responses were produced in 4 SC cultures (Fig.18). It is

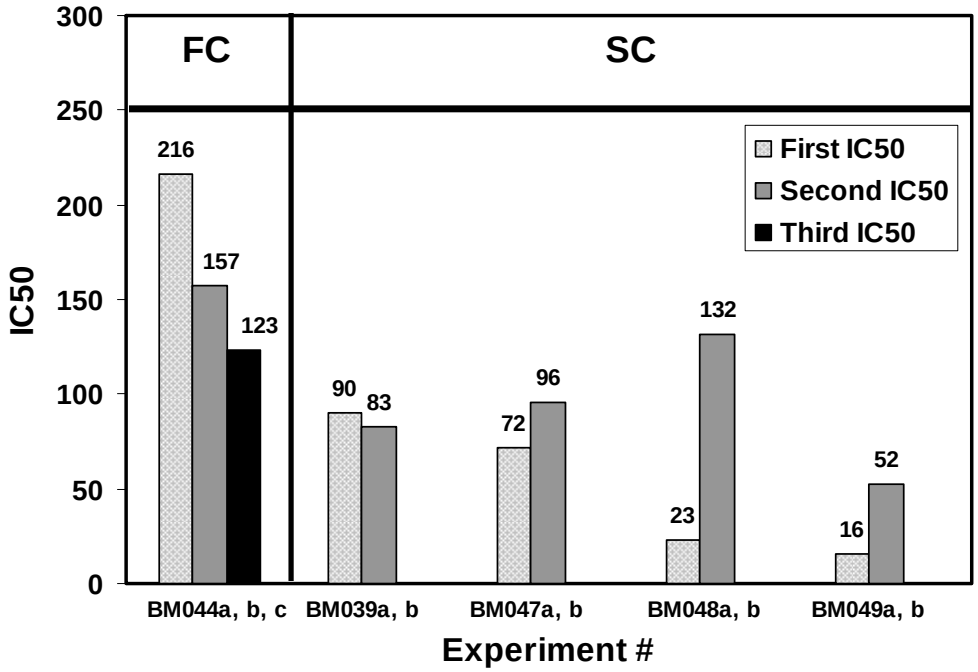

Figure 18. IC_{50} values showing the variability inherent in the concentration-responses of FC and SC networks to repeated exposures of D-MPH.

important to note, that the low number of experiments does not allow the establishment of a trend.

The uniformity of unit responses was analyzed by constructing CRCs for 31 individual units within the same SC network (BM047) (Fig. 19). It was shown that the single-unit IC_{50} values varied from one another, though they fell near the same range and average as the IC_{50}s of the respective tissue. The single-unit IC_{50}s ranged from 4-140 and averaged 63 ± 40. A t-test indicated that the SC single-unit IC_{50}s were not statistically different than the SC network IC_{50}s ($p > 0.05$). In contrast, the SC single-unit IC_{50}s were significantly different from the FC network IC_{50}s (t-test, $p< 0.01$). There was a dichotomous grouping of the IC_{50} values among the single units (see Discussion).

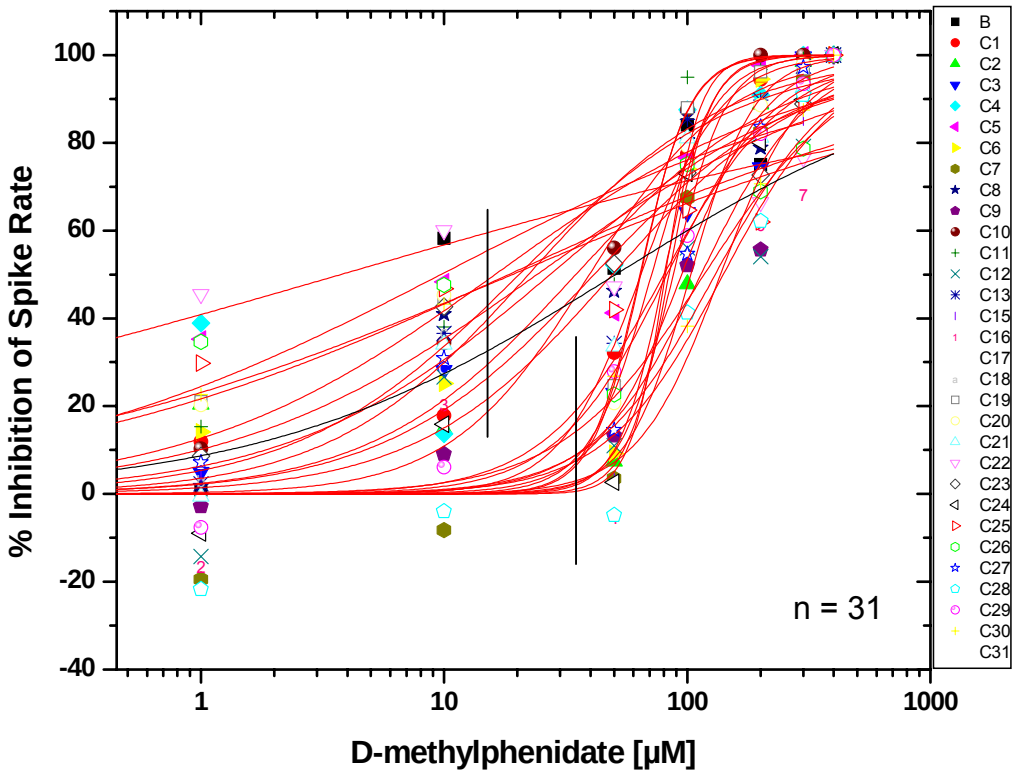

Figure 19. Spike rate CRC summary of 31 SC units from a single network (BM047) in response to D-MPH. This shows the variability of single units in the same network to D-MPH-induced changes. IC_{50} mean ± SD was 63 ± 40 µM D-MPH. The lines show the dichotomous grouping of the IC_{50}s.

Table 4. SC IC_{50} values from single-unit and network responses to D-MPH.

Spinal Cord IC_{50}s	n	Range	Mean	± SD
Single-Unit IC_{50}s (BM047)	31	4-140	63	40
Network IC_{50}s	11	6-135	57	43

3.4 Reversibility and Toxicity of D-MPH

Reversibility was quantified by performing a medium wash after the final serial addition of D-MPH, and the % recovery was measured. Figure 20 shows the responses of D-MPH followed by washes of FC and SC networks. In a FC culture, the native activity was followed by a wash to obtain reference activity, and a subsequent addition

of 40 μM bicuculline was added to increase the spike and burst rate. The % recovery was measured against the reference activity because no bicuculline was added to the wash. The spike and burst rates of the FC network recovered to 73 and 79% of the reference activity, respectively. The spike and burst rates of the SC network recovered 60 and 93% of the reference activity, respectively. Dual washes may be required in future experiments to bring the activity back to native levels. Among 2 FC and 7 SC networks, the recovery in spike rates after additions of D-MPH averaged 100% and 62 ± 21%, respectively. Both FC and SC networks showed spike recovery to serial additions of D-MPH.

To test if D-MPH had cytotoxic effects at high concentrations, a 1mM application of D-MPH was administered and followed by a wash. This concentration is close to 10X the average IC_{50} for FC networks. Three applications of 1 mM D-MPH each followed by a wash containing 40 μM bicuculline resulted in a 97.5% average spike inhibition and a 100% or higher average spike recovery, respectively (Fig. 21b). Inhibition and recovery were quantified from the original bicuculline-induced activity. Single unit analysis (n = 31) indicated that some of the neurons were not fully inhibited (Fig. 21a).

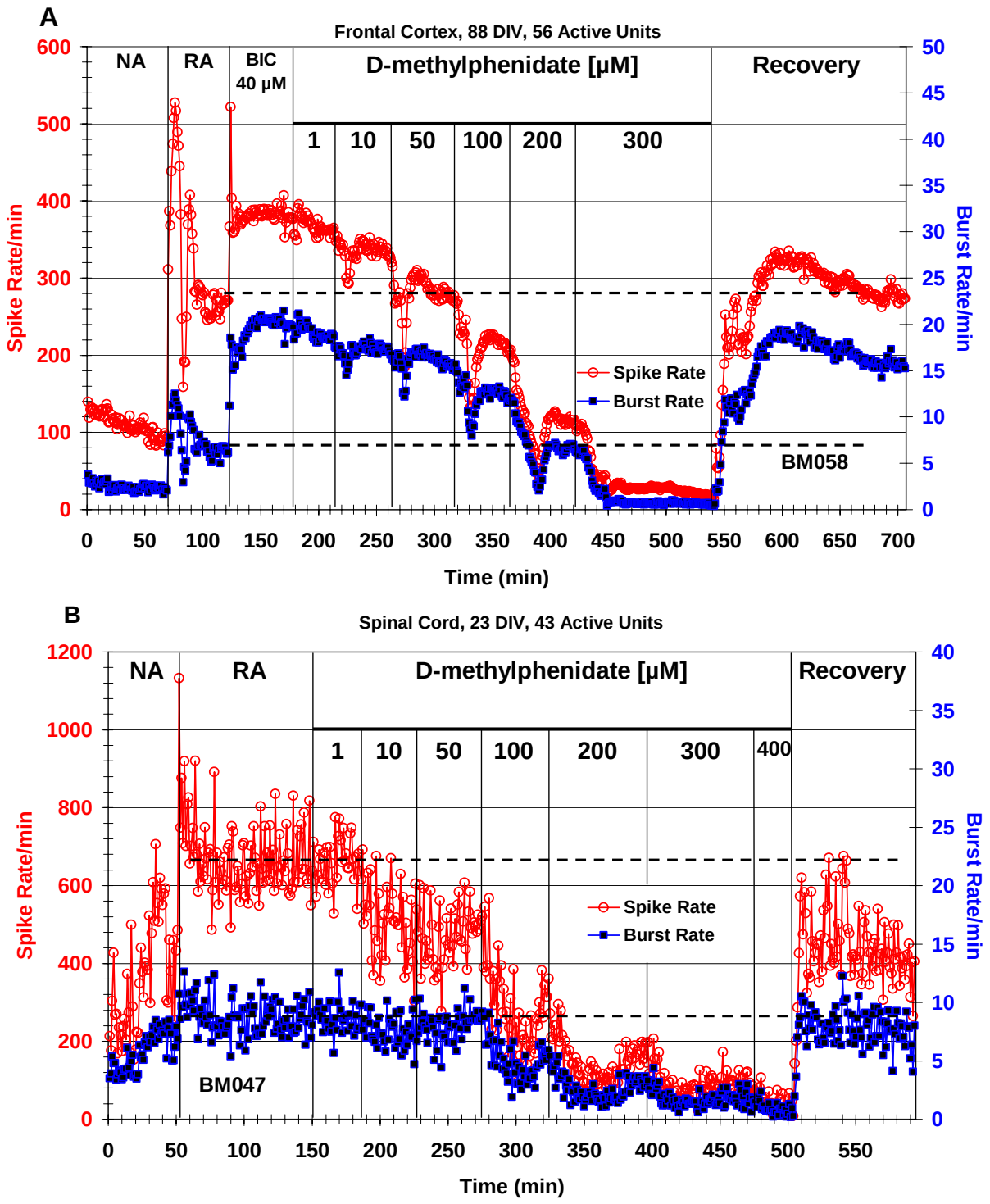

Figure 20. D-MPH-induced responses and subsequent recovery. (A) After a DMEM wash, a FC network (BM058, n = 56) recovered 100% for the spike and burst rates, after near-complete inhibition by 300 µM D-MPH. (B) SC network (BM047, n = 43). The spike and burst rates recovered 60 and 93%, respectively, from a complete wash with MEM after a 400 µM D-MPH-induced inhibition. NA = native activity; RA = reference activity. Percent recovery was measured using stable activity plateaus.

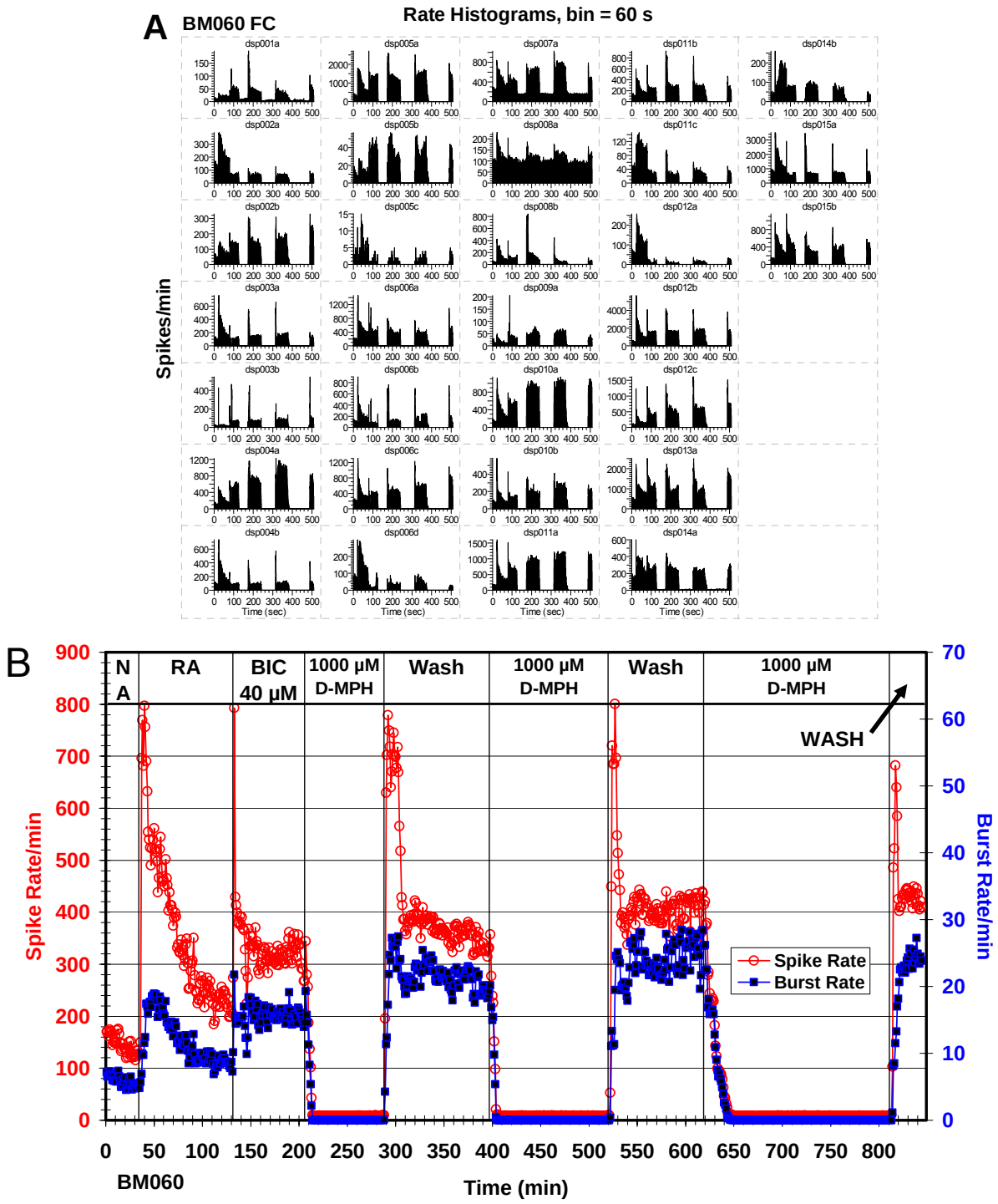

Figure 21. Spike and burst recovery of FC network after extended exposures to 1,000 µM D-MPH. (A) Spike activity of 31 individual units. Not all of the units were completely inhibited. (B) Network response showing complete recovery after washes. On average, the spike rate decreased 97.5 ± 0.02% in response to D-MPH, and recovered 100% after washes. The responses were calculated in relation to the first bicuculline-induced activity-state; therfore, the washes contained 40 µM bicuculline.

3.5 Effect of D-MPH on Waveforms

The waveform shapes of 40 FC and 32 SC units were analyzed to indicate if D-MPH had any influence on the voltage-gated Na^+ or K^+ channels involved in action potential generation. The waveshapes were analyzed by defining 3 peaks which consisted of 2 amplitudes. A change in either amplitude indicated a significant change in the waveform (see materials and method). Parametric and Nonparametric Repeated Measures ANOVA, followed by pairwise multiple comparisons found that no significant change in waveshapes occurred during treatments of 50, 200, and 400 μM D-MPH (Table 5, Fig. 22). Two ANOVA tests turned out to be slightly significant (*), however, the post hoc test indicated that there was no significant difference between the treatments. Since the multiple comparison tests showed no significant difference, an assumption will be made that the waveshapes did not change. The tests indicates that D-MPH has no significant effect on voltage-gated Na^+ and K^+ conductance. Figure 23 shows an example of 25 waveforms from a single unit separated by 30 minutes of native activity. This is representative of the inherent variability of consecutive single waveforms that are influenced by electronic noise in the multichannel amplification system.

Table 5. Experiments used to conduct the waveform analyses and appropriate tests.

Exp. #	Tissue	Amp.	ANOVA Test	p value	Multiple Comparison Test	n
BM041	FC	1	Nonparametric	* p = 0.03	Tukey, p > 0.05	20
		2	Nonparametric	p > 0.05	NO	20
BM044	FC	1	Nonparametric	p > 0.05	NO	20
		2	Nonparametric	p > 0.05	NO	20
BM045	SC	1	Nonparametric	* p = 0.03	Tukey, p > 0.05	12
		2	Parametric	p > 0.05	NO	12
BM047	SC	1	Nonparametric	p > 0.05	NO	20
		2	Nonparametric	p > 0.05	NO	20

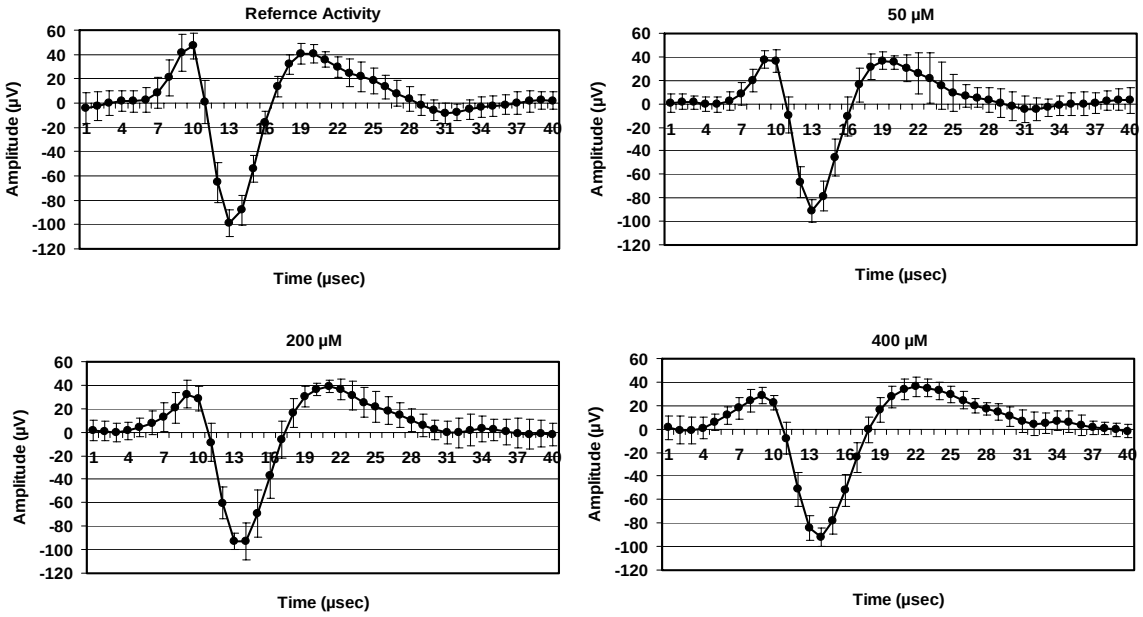

Figure 22. Discriminated waveform from a SC network (BM047, dsp 014b) showing the amplitude (µV) over time (µsec) during reference activity and 50, 200, and 400 µM D-MPH treatment periods. The waveform represents an average of 10 waveshapes during each episode ± SD. The experiment lasted for 10 hrs. The waveform showed little change in amplitude 1 and 2 during the different episodes. An analysis of 20 units in BM047 showed that amplitude 1 and 2 did not significantly change from reference (Friedman repeated measures ANOVA, $p > 0.05$, $n = 20$).

3.6 Immunocytochemical Determination of Catecholiminergic Neurons

A tyrosine hydroxylase (TH) immunocytochemical analysis was used to test for the presence of catecholaminergic (CA) neurons in FC and SC cultures. Tyrosine hydroxylase immunoreactivity was found in both tissue types (Fig. 26, 28). To ensure there were no false positives caused by non-specific chromogen (DAB) binding, the primary TH antibody was removed from the staining protocol (Fig. 25, 27). The stain gives a brownish/yellow color to TH positive cell bodies which then looks lighter in black and white pictures.

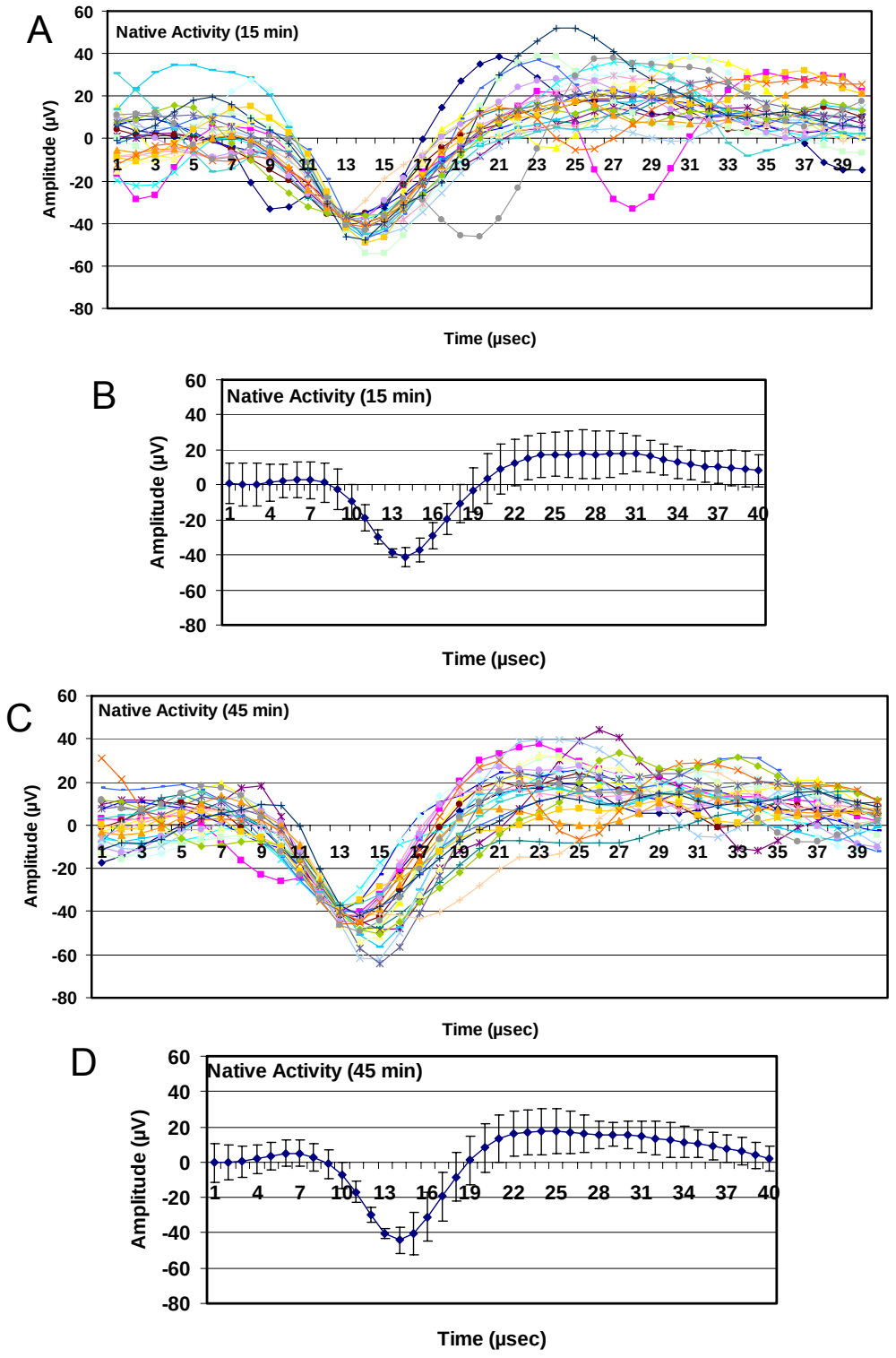

Figure 23. Discriminated waveform (BM041, dsp10b) showing the variability of individual waveforms from the same unit separated by 30 min of native activity. (A) 25 waveforms at min 15. (B) Average of the 25 waveforms at min 15. (C) 25 waveforms at min 45. (D) Average of 25 waveforms at min 45. There is inherent variability in the individual waveforms; however, the averages are very similar.

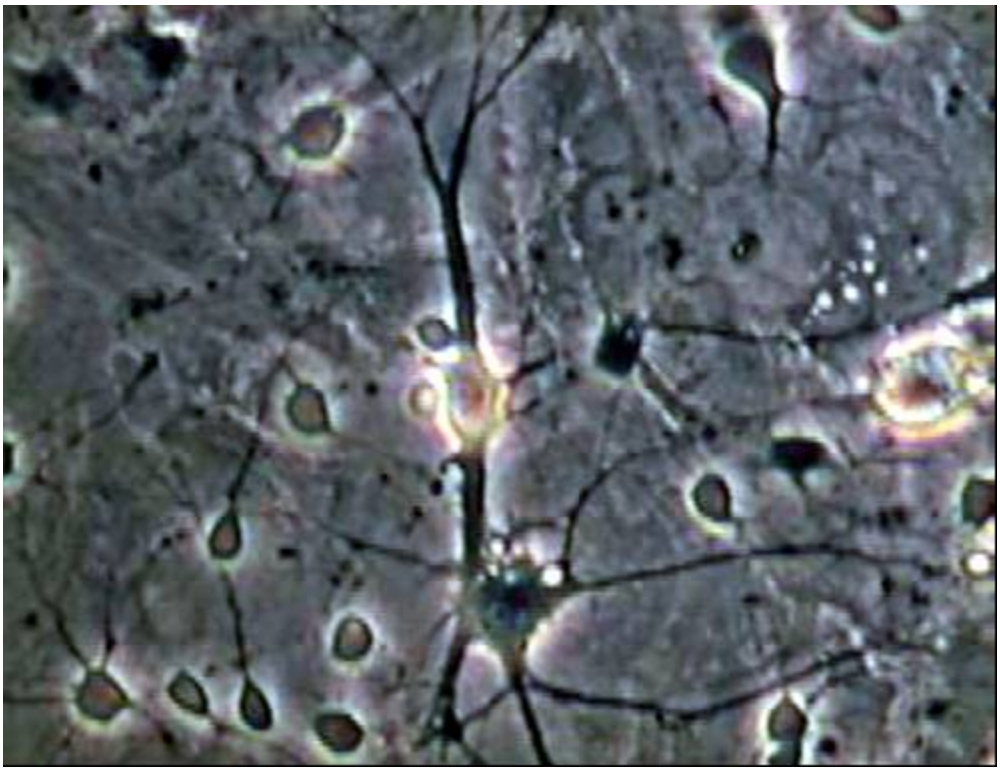

Figure 24. FC culture after fixation showing somata, neuritic projections, as well as the typical phase contrast image.

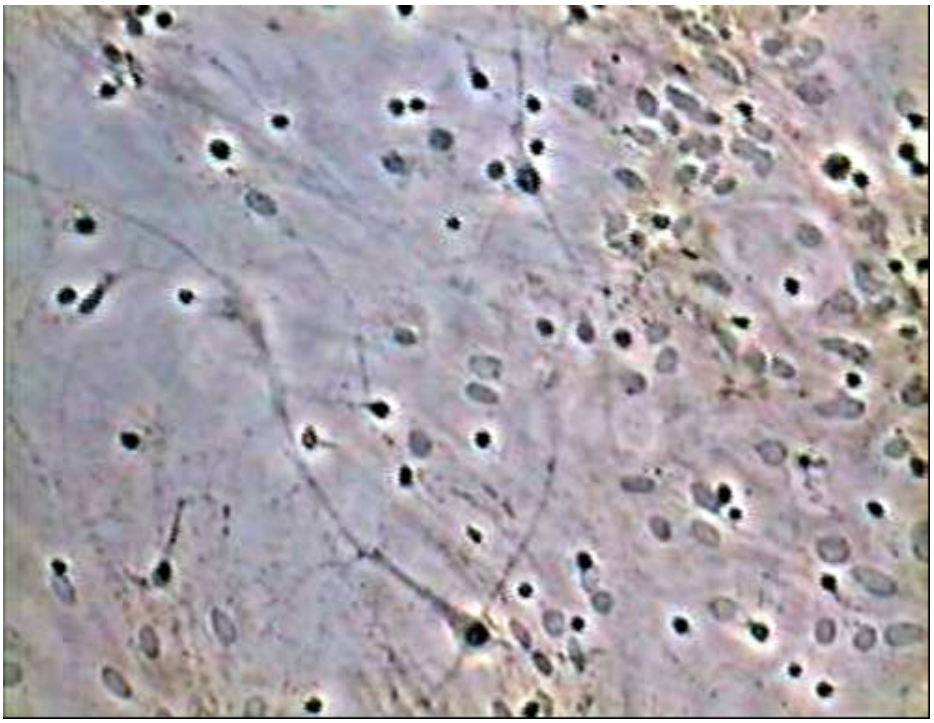

Figure 25. FC culture fixed and processed immunocytochemically without the TH primary antibody. DAB showed little nonspecific staining.

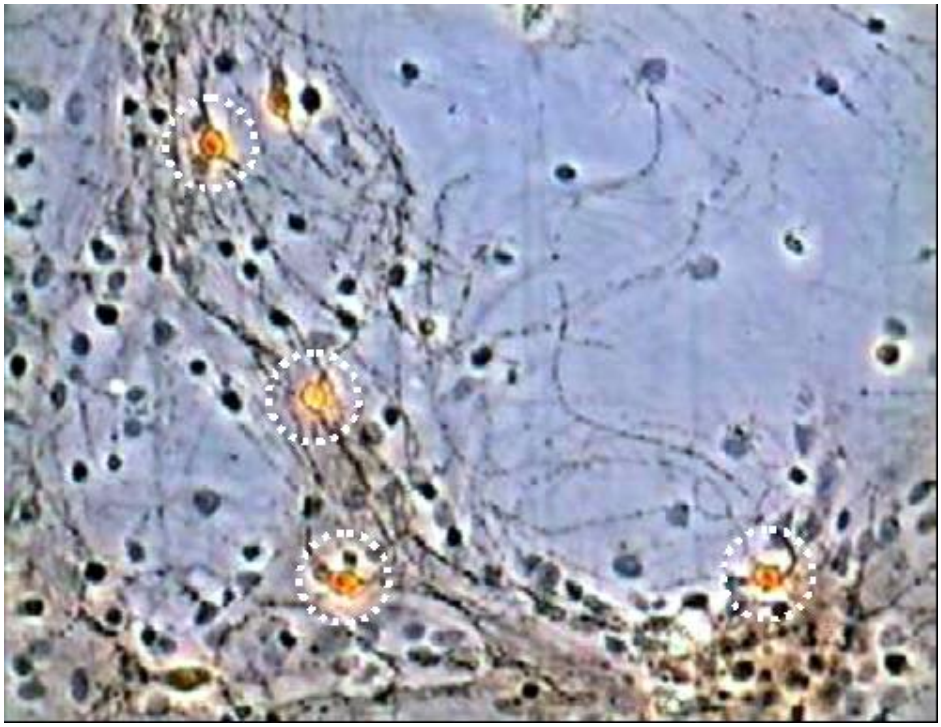

Figure 26. Shows the presence of TH positive neurons, as well as TH negative neurons. TH positive neurons are shown inside the broken circles.

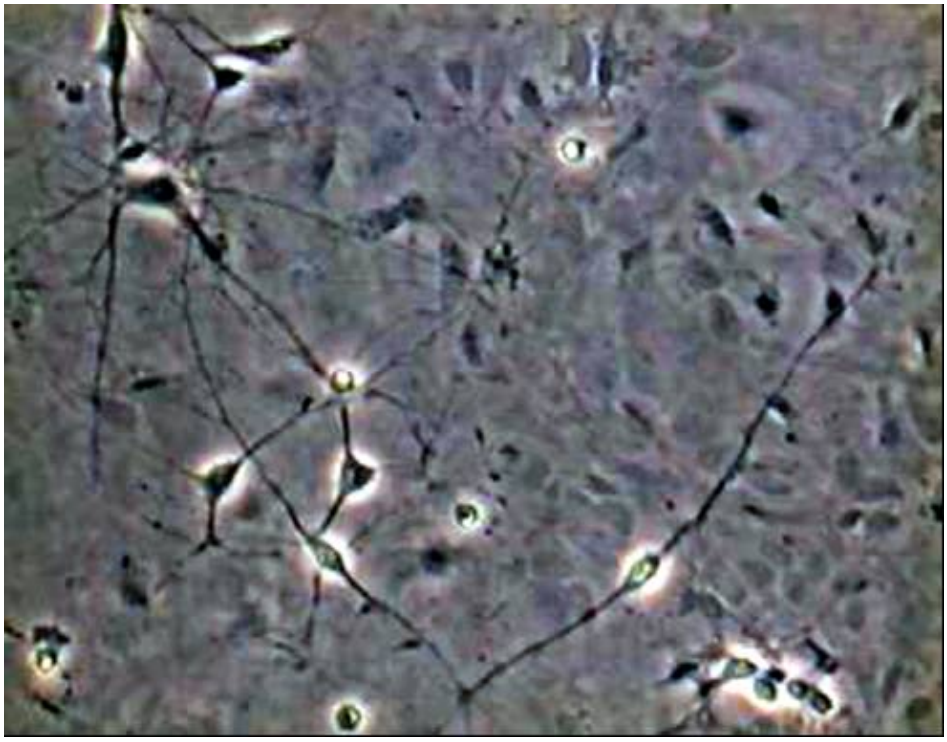

Figure 27. Representative SC culture processed without the TH antibody.

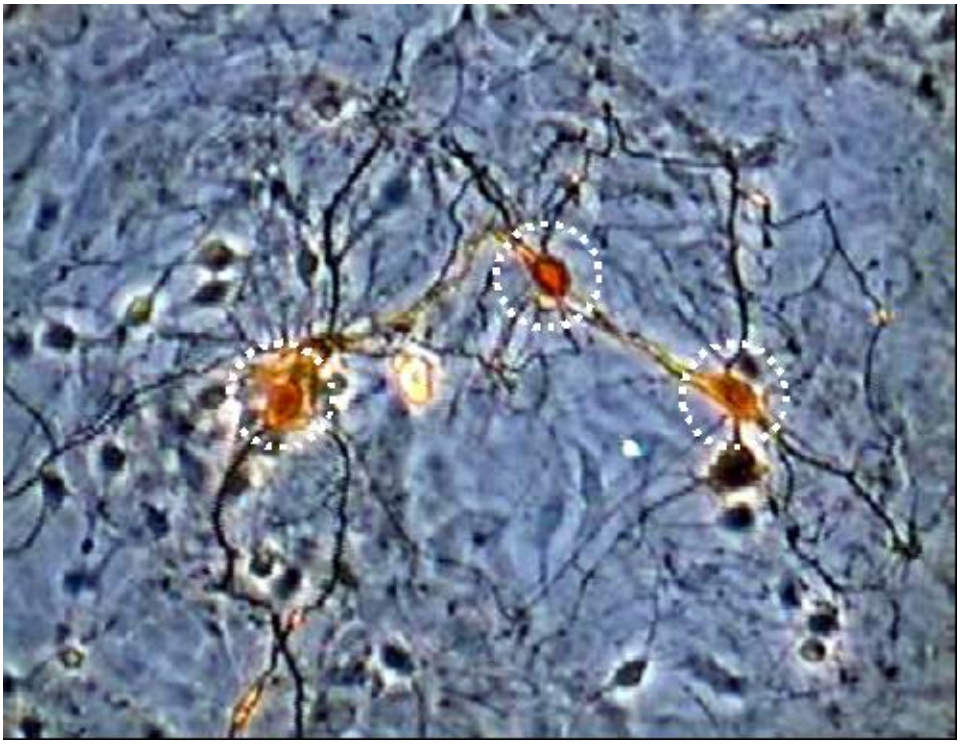

Figure 28. SC network showing both TH positive and negative neurons. The broken circles indicate TH positive.

4. DISCUSSION

Methylphenidate (MPH) is the most common psychoactive medication administered to children with attention-deficit hyperactivity disorder (ADHD); however, the therapeutic mechanisms of action are not well understood (Solanto 1998, 2002; Volkow et al., 2001, 2002). Recently, there has been widespread recognition and diagnoses of adolescents with ADHD in the United States, and thus MPH prescriptions have become much more prevalent (Kuczenski et al., 2002; Volkow et al., 2001, 2003). This has caused an increased awareness of its possible adverse side effects, such as abuse potential, neurological consequences of overdose, and toxicity. More importantly, this awareness provides a significant rationale for studying the general neurophysiological effects.

It is known that MPH preferentially binds both the dopamine transportger (DAT) and norepinephrine transporter (NET), thereby blocking the presynaptic reuptake of dopamine (DA) and norepenephrine (NE) (Kuczenski et al., 2002; Volkow et al., 2001, 2002; Overtoom et al., 2003). The increase in DA and/or NE concentration in the synapse is thought be the primary therapeutic mechanism of action. This mechanism, and its downstream neurophysiological manifestations have been studied to some extent in areas of the brain that contain high concentrations of dopaminergic and noradrenergic neurons. However, there has been little focus on these mechanisms in areas of the central nervous system (CNS) such as the frontal cortex (FC) and spinal cord (SC) where dopaminergic and noradrenergic neurons are sparse.

This research used spontaneously active primary neuronal FC and SC networks *in vitro* to study the effects of D-methylphenidate (D-MPH) on the spiking, bursting, and

waveshape properties of individual neurons connected in networks and grown on microelectrode arrays. It was found that the spontaneous spiking and busting pattern showed little change to serial water additions that paralleled the amount of water/drug solution used during the concentration-response experiments (Fig. 8a, b). The spontaneous spiking and bursting activity decreased 0.5 ± 1.4% and increased 4.8 ± 5.4%, respectively. It is not uncommon for the reference activity of such networks to fluctuate within in this range. Eight FC and 15 SC networks were used to study D-MPH. These networks contained a totaled 456 FC and 589 SC individual neurons with averages of 57 and 39 units per culture, respectively. Prior to D-MPH additions, the networks were screened for stable native activity. Bicuculline, a $GABA_A$ antagonist, was used to disinhibit the network if the stable activity fell below or near 200 spikes/min. The addition of bicuculline did not significantly affect the IC_{50} values within each tissue type, compared to those networks not treated with bicuculline (t-test, $p > 0.05$). Therefore, the data were pooled. The D-MPH-induced change in spiking and bursting were compared either to reference, or the bicuculline-induced activity-state.

Serial additions of D-MPH ranging from 1-500 μM produced concentration-dependent inhibitions of the spontaneous spike and burst activity in both FC and SC networks, n = 23 (Figs. 10, 11, 12). Likewise, single unit analysis showed that 97-99% of FC and SC neurons decreased spike activity in response to D-MPH (Fig. 14, 15). Each D-MPH concentration step used, caused a statistically significant reduction in spike production (one-way repeated-measures ANOVA with post hoc Holm-Sidak pairwise multiple comparison test, $p < 0.05$, n= 7 treatment groups). A one-tailed t-test

indicated that the 10, 50, and 100 μM concentrations were significantly different between the FC and SC networks ($P < 0.05$).

The concentration-dependent effects of D-MPH in this study were consistent with previous studies of both DA and NE mediated mechanisms. It has been shown that bath perfusion of 10 and 30 μM MPH suppressed the spontaneous neural activity of DA neurons in the ventral tegmental area (VTA) of rat brain slices (Prieto-Gomez et al., 2004). Prieto-Gomez et al. (2004) electrically stimulated afferents from the prefrontal cortex (PFC) to VTA dopaminergic neurons, thus causing an excitatory postsynaptic current (EPSC). Bath application of MPH induced a reduction of the PFC stimulation-induced inward EPSC resulting in a decrease in the evoked VTA dopaminergic activity. Russell et al. (1998) found that 0.1-10 μM MPH increased the amount of dopamine in the synapse from rat prefrontal cortex, nucleus accumbens, and caudate-putamen slices. It was also reported that extracellular single unit recordings of dopaminergic rat midbrain neurons show dose-dependent decreases in spike rate (Ruskin et al., 2001). They also showed that there was a maximum of 96% inhibition of spike activity at the highest cumulative MPH dose of 5.0 mg/kg. Neurons of rat brain slices of the locus coeruleus (LC), a noradrenergic locus, showed dose-dependent hyperpolarizations when exposed to bath applications of 1-300 μM MPH (Ishimatsu et al., 2002; Kuwahata et al., 2002). Noradrenergic neurons of the LC project recurrent collaterals that release NE on the LC neurons themselves. The release of NE on these neurons produces an inhibitory response, and is enhanced by MPH administration. Ishimatsu et al. and Kuwahata et al. showed that spontaneous firing action potentials of the LC were blocked by the NE mediated hyperpolarization induced by MPH. They also suggested

that the MPH-enhanced hyperpolarization is produced by an increase in potassium conductance via the α_2-adrenoceptor in LC neurons. Other *in vivo* and *in vitro* experiments have documented MPH-induced increases in both DA and NE at the synapse, as well as reduction in spontaneous activity in target neurons (Rebec et al., 1978; Lacroix et al., 1988; Volkow et al., 2001, 2002; Kuczenski et al., 2002).

The research presented in this thesis also explored tissue specific effects, inter- and intraculture repeatability, and the uniformity of response from units exposed to D-MPH by generating IC_{50} values from concentration-response data. The IC_{50}s of FC networks averaged 118 ± 52 μM and ranged from 75-216 μM, while the IC_{50}s of SC networks averaged 57 ± 43 μM and ranged from 6-135 μM D-MPH. The FC and SC IC_{50} values were significantly different (t-test, $p < 0.05$). The FC and SC IC_{50}s indicate that a tissue specific effect may be caused by different potencies of D-MPH in the respective tissue. This may be attributed to varying population densities of either dopaminergic or noradrenergic neurons in each tissue. However, there are no studies that demonstrate CA population differences in FC and SC tissue (Smeets et al., 2000). Future studies will help quantify the populations of each neuron type in FC and SC cultures. The ages of the cultures (D.I.V.), the number of active units, and drug application procedures were explored to insure the large IC_{50} ranges were not a product of these controllable variables. A Pearson product moment correlation indicated that the IC_{50} values were not correlated with either the number of active units recorded, nor the age at which the culture was used for experimentation ($p > 0.05$). There were no obvious differences in the IC_{50} values with respect to drug application procedures. There is also the possibility that because each network is its own entity, there could be

differences in neuronal development and network connectivity that give rise to varying response potencies. For instance, the single unit analysis showed that the degree of inhibition at specific D-MPH concentrations is not the same for all neurons. Each neuron has an intrinsically different response profile and this suggests why some networks were totally inhibited at either 200, 300, 400, or 500 μM D-MPH.

Intra-culture repeatability was used to investigate the response of individual networks to consecutive concentration-response experiments. The results obtained from this study were not extensive enough to make substantial claims about intra-culture repeatability. It is interesting to note that the two SC cultures that showed relatively good repeatability were not exposed to bicuculline (Table 4). The network that had the worst reproducibility was not exposed to bicuculline in the first concentration-response experiment, but was exposed to it before the second experiment (BM048a, b). Finally, the IC50 followed a trend that when the reference activity increased the IC_{50} increased, and when it decreased the IC_{50} decreased. Further studies should be performed before any conclusions can be made.

Table 6. Summary of the networks used in the intra-culture repeatability experiments.

Experiment #	Active Units a/b	Reference Activity Before/After (spikes/min)	Bicuculline a/b	IC_{50} Before/After
BM039a, b	71/68	600/550	NO/NO	90/83
BM047a, b	43/33	800/1,000	NO/NO	72/96
BM048a, b	23/17	300/500	NO/Yes	23/132
BM049a, b	22/16	400/600	Yes/Yes	16/52

Concentration-response curves were also constructed to analyze the uniformity of single unit responses to D-MPH within one SC network (BM047). It was found that the mean and range of the single-unit IC_{50} values were similar to that of all the SC networks (Table 2). The single-unit IC_{50}s were not statistically different from the SC network IC_{50}s ($p > 0.05$), but were different from the FC network IC_{50}s (t-test, $p < 0.01$). A qualitative assessment showed a dichotomous grouping within the single-unit IC_{50}s. The difference in IC_{50}s values may be caused by direct or indirect responses to D-MPH. Neurons with very low IC_{50}s may be inhibited by direct postsynaptic contact of dopaminergic or noradrenergic neurons, while neurons that exhibit high IC_{50}s may be secondarily influenced downstream from the direct mechanism of action.

The large range of the network IC_{50}s is still somewhat unclear. Major reasons for this variation are the neuronal culturing procedures, where neither the tissue harvesting nor the age of the tissue is exactly reproducible. It is difficult to precisely isolate and dissect only the FC from the embryonic mice. Therefore, it is possible to make conservative and non-conservative cuts to isolate the tissue. Non-conservative cuts in the FC could include portions of the midbrain. Because of the different populations of DA and NE neurons present in other CNS tissue, these cultures would indeed have comparatively different response profiles to D-MPH than would purely isolated FC networks. It is also very difficult to control the timed pregnancies of the mice and to determine the exact age of the tissue at dissection. Considering the rapidity of events during mammalian development, it is entirely possible that tissues differing in age by only 12 hours display different percentages of specific types of cells. The SC dissections are very conservative, which suggests that the major reason for the large

range in IC_{50} values in both FC and SC tissues is due to inadequate control over the precise age at which the embryos are dissected. Furthermore, there was a sudden use of antibiotics because of culture contamination in the Summer of 2004. Due to the lack of poor record keeping there is no way to tell which networks were exposed to varying concentrations of the antibiotics. Network exposure to antibiotics may have caused a shift in the IC_{50} curves, although there is no data to significantly support this hypothesis.

It is important to compare the IC_{50} results obtained in this thesis work with those obtained in brain slice and *in vivo* studies. It is known that terrestrial animals consist of 75% or more of water (Ekert et al., 1998). Using a conservative value to assume that the rat is approximately 70% water, and the water is evenly distributed throughout the body, calculations were made to convert MPH doses into μM concentrations for comparison. Lacroix et al. (1988), using extracellular unitary recordings from anesthetized rat noradrenergic LC neurons, found that intravenous (i.v.) administration of MPH produced an ID_{50} (dose at which 50% of the spike rate was inhibited) of 650 μg/kg, or 3 μM MPH. Ruskin et al. (2001) reported a MPH-induced ED_{50} (effective dose at which 50% of the neurons were inhibited) of 1.94 ± 0.25 mg/kg, or 10 μM, in midbrain dopaminergic neurons of anesthetized rats. It is somewhat established that 1-3 mg/kg doses (5-16 μM) of MPH in rats is equivalent to the therapeutic dose in humans, while 5-10 mg/kg doses (26-53 μM) typically exceed the therapeutic range (Kuczenski et al., 2000; Prieto-Gomez et al., 2003). However, there are still no universally recognized dosage guidelines and blood levels to achieve optimum therapeutic treatment with MPH (Santosh et al., 2000). The concentrations used this study are within the therapeutic range.

Clearly, many factors such as volume of drug distribution, drug metabolism, excretion rates, and species differences contribute to the difficulty in quantifying optimal doses of MPH (Kuczenski et al., 2002). The average network FC and SC IC_{50} values obtained in this thesis research, are somewhat higher than what has been previously reported. However, the previous studies used single-unit recordings of target neurons with direct dopaminergic and noradrenergic inputs. In both the FC and SC networks used in this study, the CA populations are rather sparse, and can be either directly or indirectly associated with target neurons.

This thesis research showed that, following washes, FC and SC networks recovered their spontaneous spike activity in response to serial additions of D-MPH. It was found that 2 FC networks recovered 100% and 7 SC networks recovered 62 ± 21% of their spike activity 3.9 ± 2.1 minutes after complete medium washes. Other studies have shown that MPH-induced inhibition of spike production is indeed reversible. Ishimatsu et al. (2001) reported that spike production from rat LC slices was blocked by the bath application of 30 μM MPH for 1-10 min. The spontaneous spike activity recovered within 15-20 min after MPH was removed from the superfusing solution. Kuwahata et al. (2002) showed that the reduction in spike activity caused by a 7 min bath application of 1 μM MPH in rat LC slices was reversed after 20-30 min following a complete wash. Compared to these published studies, the experiments in this thesis continued for very long durations (10 hrs). Many metabolic changes and the possibility of neuronal stress are likely to occur during this extended time. These conditions, as well as the relative difficulty in the wash procedure, due to medium biochemistry and

physical stress, may contribute to the inability to completely recover all spike activity in some networks.

To study possible toxic effects of D-MPH, three 1 mM aliquots were added to the bath medium, and each was followed by a wash. This concentration is close to 10 times greater than the average IC_{50} value for FC networks. The spike activity was inhibited an average of 97.5% and recovered an average of 100% of the original spike activity with all three applications and washes, n = 1 (Fig. 20). The toxic effects of high doses of D-MPH have not been well established in the literature. However, Teo et al. (2002) working with rats showed very high doses of D,L-MPH and D-MPH had reversible on all toxicological parameters.

There were no significant effects of D-MPH on the waveforms indicating that D-MPH has no effect no the voltage-gated Na^+ and K^+ channels (Table 3). It is not reported anywhere in the literature whether or not D-MPH has an effect on the voltage-gated Na^+ and K^+ channels. In the future, waveforms should be analyzed by defining amplitude 1 as the absolute value of the amplitude (μV) between baseline and peak 2, and amplitude two as the amplitude (μV) between baseline and peak 3. This will provide a more accurate representation of the Na^+ and K^+ conductance.

A tyrosine hydroxylase (TH) immunocytochemical analysis was used to test for the presence of CA neurons in FC and SC cultures. The TH antibody was used because it is the most widely used and accepted marker for CA neurons (Smeets et al., 2000). The TH enzyme converts L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA), a precursor for three catecholamines, DA, NE, and adrenaline (A). Positive immunoreactivity to TH indicates the presence of any of the three catecholamines.

Tyrosine hydroxylase (TH) immunoreactivity was found in both tissue types, suggesting the presence of either dopaminergic, noradrenergic, or adrenergic neurons in the network (Figs. 26 and 28). It is now established that catecholaminergic (CA) cell bodies are present in the telencephalon of vertebrates (Smeets et al., 2000; Solanto 2002). During FC dissection, much of the cortex is taken into the cell pool. Thus, TH immunoreactivity in the FC of our preparations is consistent with other studies. Although the literature is limited and conflicting, there is some evidence for CA perikarya in the vertebrate SC (Mouchet et al., 1986). Mouchet et al. (1986), using double labeling immunohistochemistry, reported that there were both dopaminergic and noradrenergic cells present in all levels of the spinal cord except the cervical location. These previous findings, as well as the TH positive results obtained in my research, suggest that the D-MPH-induced effects observed are indeed acting on either dopaminergic or noradrenergic mechanisms.

This research has contributed to the extensive validation process of the *in vitro* multi-microelectrode array recording platform. Previous research has shown that this platform provides remarkably repeatable results (Xia 2003, 2004; Gopal 2004). Thus far it is clear that the reproducibility of the IC_{50} values caused by the D-MPH-induced effects is somewhat variable. This is thought to be a direct reflection on the differences in the dopaminergic and noradrenergic populations in each network. These differences are largely attributed to several culturing techniques. First, the age at which the embryos are dissected and processed for seeding is variable within a 24 hour window. Second, it is difficult to precisely dissect the FC and SC tissue without cutting into and taking other CNS tissues. Finally, since each network is essentially its own entity, many

developmental processes may differ depending on the previously mentioned, as well as other culturing variables. It is likely that once a narrow range in IC_{50} values is achieved, it could be a direct reflection of the reproducibility of the culturing technique.

This study explored the effects of D-MPH on FC and SC networks *in vitro*. To the best of our knowledge, it is the only study that has explored the effects of D-MPH on SC tissue. Due to its complex psychoactive properties, it is important to characterize the effects of D-MPH in CNS tissue other than the major dopaminergic and noradrenergic loci. The D-MPH-induced effects on the FC and SC networks in this study are similar to those seen in other tissues. Although the dopaminergic and noradrenergic neurons of the FC and SC may not contribute to the primary therapeutic mechanisms of action in ADHD patients, they may provide subtle effects that together alleviate the stereotypical symptoms.

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