IN VITRO INVESTIGATIONS OF ANTIBIOTIC INFLUENCES ON NERVE CELL NETWORK RESPONSES TO PHARMACOLOGICAL AGENTS

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Neuronal networks, derived from mouse embryonic frontal cortex (FC) tissue grown on microelectrode arrays, were used to investigate effects of gentamicin pretreatment on pharmacological response to the L-type calcium channel blocker, verapamil. Gentamicin is a broad spectrum antibiotic used to control bacterial contamination in cell culture. The addition of gentamicin directly to medium affects the pharmacological and morphological properties of the cells in culture. A reproducible dose response curve to verapamil from untreated cultures was established and the mean EC50 was calculated to be 1.5 ± 0.5 μM (n=10). 40 μM bicuculline was added to some cell cultures to stabilize activity and verapamil dose response curves were performed in presence of bicuculline, EC50 1.4 ± 0.1 μM (n=9). Statistical analysis showed no significant difference in verapamil EC50s values obtained in presence of bicuculline and hence the data was combined and a standard verapamil EC50 was calculated as 1.4 ± 0.13 μM (n=19). This EC50 was then used to compare verapamil EC50s obtained from neuronal cell cultures with chronic and acute exposures to gentamicin. FC cultures (21- 38 days old) were found to be stable in presence of 2300 μM gentamicin. The recommended concentration of gentamicin for contamination control is 5uL /1 ml medium (108 μM). At this concentration, the verapamil EC50 shifted from 1.4 ± 0.13 μM to 0.9 ± 0.2 μM. Given the limited data points and only two complete CRCs, statistical comparison was not feasible. However, there is a definite trend that shows sensitization of cells to verapamil in presence of gentamicin. The cultures

exposed to 108 μM gentamicin for 5 days after seeding showed loss of adhesion and no data could be collected for pharmacological analysis. To conclude, acute gentamicin exposure of neuronal cell cultures causes increased sensitivity to verapamil and chronic or long term exposure to gentamicin may cause loss of adhesion of the cell culture by affecting the glial growth. The effect of chronic exposure to gentamicin on pharmacological responses to verapamil remains inconclusive.

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ABBREVIATIONS

AC: Auditory cortex

ANOVA: Analysis of variance CAA: Calcium channel antagonist

CRC: Concentration response curve DIV: Days in vitro

DMEM: Dulbecco's Modified Minimal Essential Medium

FC: Frontal cortex

IC50: Concentration at which 50% of the spike activity is inhibited MEA: Microelectrode array

MMEP: Multi-microelectrode plate PTZ: Pentylenetetrazole

SD: Standard deviation Bic: Bicuculline

MC: Medium change ITO: Indium tin oxide

CNS: Central nervous system GABA: Gaba amino butyric acid NA: Native activity

RA: Reference activity

Pen-Strep: Penicillin Streptomycin W: Wash/medium change

CHAPTER 1

INTRODUCTION

No cell culture problem is as universal as that of culture loss due to contamination. Most bacterial contamination control protocols for cell culture recommend the use of gentamicin, a broad spectrum antibiotic. The detrimental effects of gentamicin exposure on growing cell culture has been studied in 1975 by Fischer et al., who showed that exposure to gentamicin, during various stages of growth, caused morphological damage in mammalian cell cultures at a concentrations >125µg/ml (270µM[1]). In rats, gentamicin causes ototoxicity by inducing damage and eventual death of the cochlear hair cells (Huth et al., 2011). These observations have led to the hypothesis that neuronal cell cultures may also be affected by gentamicin and may shift pharmacological profiles.

The hypothesis was supported by previous research with penicillin-streptomycin (pen-strep) conducted by Sabnam Oli-Rijal, 2006. The data showed that the addition of pen-strep sensitized the cells in culture and caused notable shifts to the left in the EC_{50}s of muscimol titration, indicative of increased sensitivity. Cultures at 5 days *in vitro* were treated with antibiotics (penicillin-streptomycin, 170 µM), washed after 48 hrs and left in the incubator with regular maintenance until used for experiments after approximately 4 –5 weeks. The total exposure time to this antibiotic was only 48 hrs and yet the EC50s were sensitized 4 weeks later (Fig 1). These observations were not the primary focus of the Oli-Rijal thesis and were not pursued further.

[1] Concentration calculated assuming the gentamicin solution used by Fischer et al was the same as that used in this study (gentamicin, Sigma, G1272).

In light of these preliminary data, it becomes important to continue the investigation of chronic and acute effects of antibiotics on cells in culture and their pharmacological responses. For this study, gentamicin was used as the stressor and verapamil as the primary test substance.

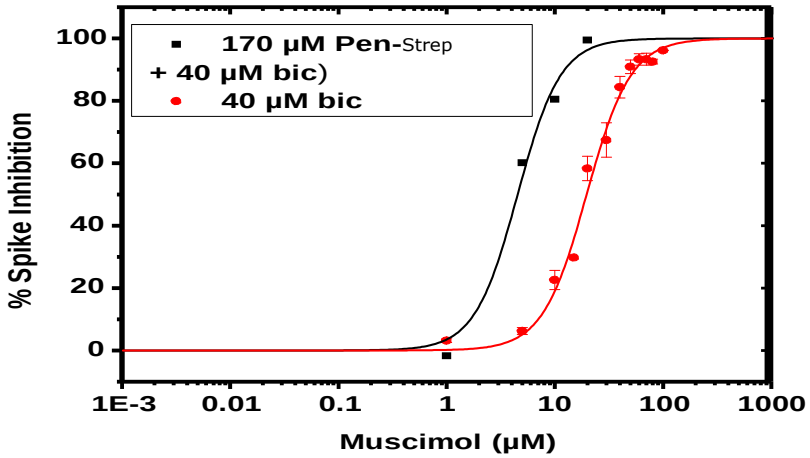

Figure 1 Sensitization of pen-strep treated cell culture to muscimol titrations. Concentration-response curve of pen-strep treated culture shifts to the left without affecting maximum response, indicating greater sensitivity. Culture pre-treated with 170 µM pen-strep for 48 hrs on day 5, culture age *27* days *in vitro* (recording 22 days after pen-strep exposure). From Oli-Rijal, MS Thesis, UNT 2006

1.1 Overview of Gentamicin

Gentamicin is a potent, heat-stable aminoglycoside antibiotic synthesized by bacteria of genus *Micromonospora* and is used to treat bacterial infections caused by Gram-negative organisms. Unlike pen-strep, gentamicin was stable at pH 2 to 10 for 15 days at 37^0 C in tissue culture medium, and its activity was unaffected by the presence of serum (Schafer et al., 1972). Gentamicin may be uniquely useful for shipment of clinical specimens and long-term tissue culture. Gentamicin remains effective even after

autoclaving (Moulds et al., 2010). However, gentamicin toxicity is a major problem in clinical applications as it has shown to have ototoxic and nephrotoxic effects (Moulds et al., 2010). Gentamicin binds to the 30S subunit of the bacterial ribosome, interrupting protein synthesis. The recommended concentration of gentamicin for cell culture use is approximately 100 µM in the medium.

1.2 Overview of L-type Ca^{2+} Channels and Verapamil

L-type calcium channels (LTCC) are voltage-dependent calcium channels. "L" stands for long lasting referring to the length of activation. Investigators have relied on their unique pharmacological sensitivity to dihydropyridine to establish the contribution of L-type calcium channels to various signaling cascades. However, dihydropyridines are vascular selective. A schematic overview of the drug interaction sites of the calcium channel antagonist (CAA) (dihydropyridines, diltiazem, verapamil, fantofarone, mibefradil) with the L-type calcium channel is shown in Figure 2. The currently available CCA interact predominantly or exclusively with the L-type calcium channel (Sandmann et al., 1999).

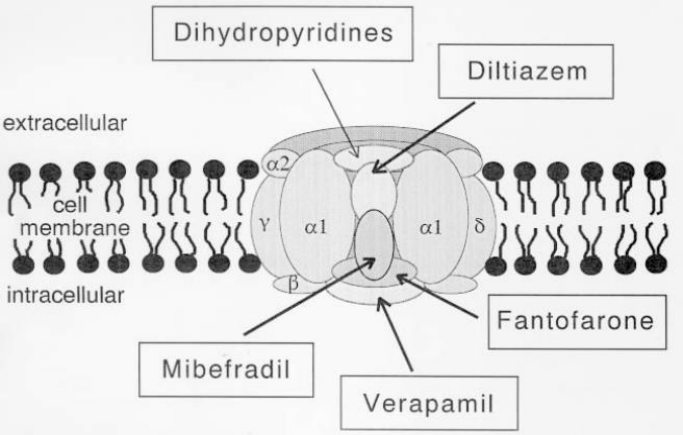

Figure 2. Schematic overview of the drug interaction sites of the CCA (dihydropyridines, diltiazem, verapamil, fantofarone, mibefradil) with the L-type calcium channel (Sandmann et al., 1999)

LTCCs are found predominantly in neural and muscle tissue, but exist in many other cell types. Their cell physiological functions in neurons range from nuclear pCREB signaling and activity dependent gene expression (Zhang et al., 2006; Helton et al., 2005), to synaptic efficacy (Lipscombe et al., 2004), and general signaling cascades (*ibid*). Neuronal L-type calcium channels open with fast kinetics and carry substantial calcium currents in response to individual action potential waveforms. Although the traditional view of dihydropyridine-sensitive L-type calcium channels is that they are high-voltage-activating and have slow activation kinetics, the activation and their presumed lack of contribution to single action potentials is a reflection of the state-dependent nature of the antagonists used to study them (Helton et al., 2005). For example, LTCC Ca_v 3.1 in thalamocortical neurons has been shown to cause low threshold Ca^{2+} spikes that mediate burst firing (Helton et al., 2005).

Electrophysiological data of functional influences of verapamil on spontaneous activity are difficult to find. Verapamil is a prototypical phenylalkylamine, and it was the first calcium channel blocker to be used clinically. Verapamil has been routinely prescribed to treat hypertension, angina and cardiac arrhythmia. A recent use of verapamil has been to cure cluster headaches and migraine (L. Wing, 1997). It tonically blocks L-type calcium channels with micro-molar affinity, and its affinity increases at depolarized membrane potentials (Bergson et al., 2011). Low concentrations of verapamil (0.5–30 μM) have shown to block uptake of Ca^{2+} into incubated cerebrocortical synaptosomes whereas at higher concentrations (30–200 μM) verapamil acts additionally at sodium (Na^{-}) channels, reducing or preventing both depolarization-induced K+ efflux and neurotransmitter release (Norris et al., 1985). It is very interesting

that both verapamil and diltiazem decrease spontaneous network activity in a concentration -dependent manner and were found reversible after complete cessation of activity (Jason Brauner, aborted MS Thesis - UNT, 2004). Given the broad effects of L-type Ca^{2+}channels on cell physiological functions in many tissues, it was decided to use verapamil as the pharmacological reference compound and gentamycin as the stressor. Any major shift in verapamil dose responses would have implications also in areas other than neurobiology.

1.3 Overview of Bicuculline

Bicuculline is a $GABA_A$ antagonist that causes epileptiform activity in neurons by reversibly blocking *γ*-aminobutyric $acid_A$ ($GABA_A$) receptors (Uneo, 1997; Birnir, 2000) with an EC_{50} value of 1.7 μM (Feigenspan, 2004). Bicuculline acts as a competitive antagonist at $GABA_A$ receptors in that it competitively inhibits GABA binding to these receptors and, in turn, GABA competitively inhibits bicuculline binding (Andrews and Johnston, 1979). This evidence is supported by functional studies. Single channel studies have shown that bicuculline reduces GABA activated conductance by reducing both channel open times and opening frequency (Macdonald et al., 1989). Studies indicate that bicuculline binds at the orthosteric site resulting in stabilization of the receptor in a closed state. In this sense bicuculline has also been described as an allosteric inhibitor (Ueno et al., 1997). This epileptogenic effect elicited by bicuculline, often interpreted to be due to disinhibition, were shown to be abolished by application of the L-type calcium channel blocker, verapamil (Straub et al., 1990; 1994).

Bicuculline is used in many studies to eliminate any interference by GABA and to obtain comprehensible data on L- type Ca^{2+} channels. (Martella et al., 2005; Zhang et al., 2002; Bonci et al.,1998). Although there is some evidence that verapamil also affects $GABA_A$-receptors (Das et al., 2004), these modulations are related neither to calcium channel modulation nor to possible intracellular effects of L- type Ca^{2+} channels (Chesnoy-Marchais., 2001). In this study, bicuculline (40µM) was used to increase or stabilize activity of five neuronal networks to enhance the quantification of data for verapamil CRCs (dose response curve) as it would not interfere with the L-type Ca^{2+} channels.

1.4 Research Objectives

The research purpose was to investigate the effects of gentamicin exposure on pharmacological responses to verapamil and morphological properties of neuronal cell cultures. The approach was to quantify the shift of concentration-response curves of the test substance, verapamil, in the presence of stressor, gentamicin.

Specific Aims:

1. Establish intra-culture and inter-culture repeatability of verapamil EC_{50} values.

2. Re-examine that bicuculline has minimal effect on verapamil EC_{50} in frontal cortex networks to allow the use of network responses obtained under bicuculline.

3. Investigate the effects of gentamycin exposure on verapamil dose response profiles (acute exposures).

4. Investigate the effects of chronic exposures to gentamycin on verapamil dose response profiles.

CHAPTER 2

MATERIALS AND METHODS

2.1 Microelectrode Array Fabrication and Cell Culture

Microelectrode arrays (MEA) were fabricated in house according to methods defined formerly (Gross 1979; Gross, 1994; Gross et al, 1985). Briefly, photo-etched indium–tin oxide (ITO)-sputtered glass plates were spin-insulated with methyltrimethoxysilane, cured, de-insulated at the electrode tips with laser shots, and electrolytically gold-plated to adjust the interface impedance to 1 MΩ at 1 kHz (Gross et al., 1985). The MEA insulation material is hydrophobic, and butane flaming through masks was used to activate the surface and generate a hydrophilic adhesion island (3 mm in diameter) centered on the MEA (Lucas et al., 1986).

Frontal cortex tissue was dissociated from 15- to 16-day-old BALB/c/ICR mouse embryos and cultured according to the methods of Ransom et al., (1977) with minor modification that included the use of DNAse during tissue dissociation. 50 µL of cell pool containing approximately 2.5 x 10^5 cells was placed directly over the electrode grid in each well with subsequent addition of 2 ml of medium (after 2 hrs adhesion period) confined to a 4-cm^2 area by a silicone gasket. The care and use of, as well as all procedures involving, animals in the study were approved by the institutional animal care and use committee of the University of North Texas and are in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996).

The cells seeded on the electrode grid were incubated in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10 ml/L B27 (GIBCO, pH 7.4), 1.25 ml/L L- glutamine, 5% horse serum, in a 90% air and 10% CO^2 atmosphere.

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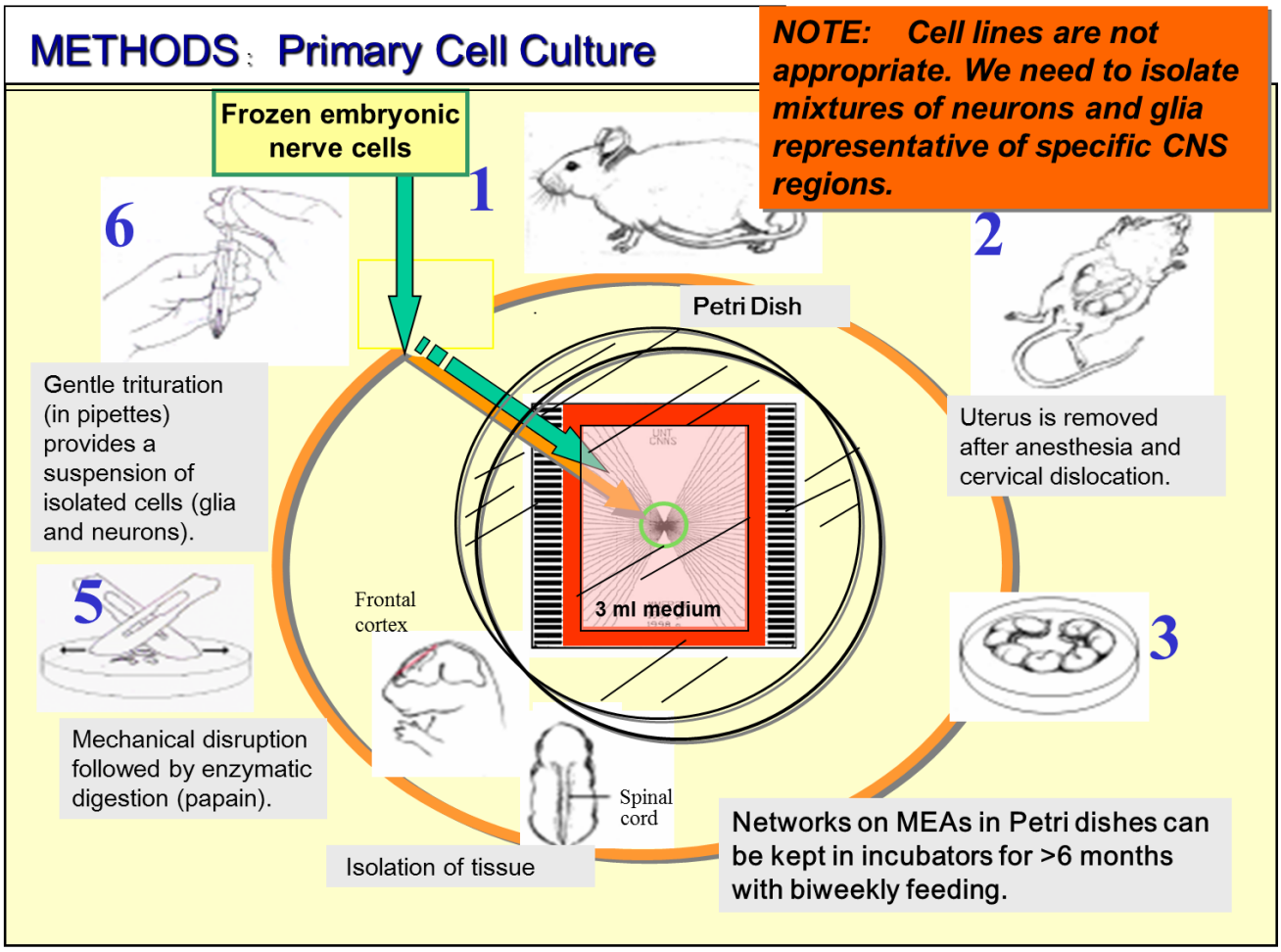


Figure 3. Summary of steps involved in the generation of primary cell cultures for growth on MEAs. All cultures were provided to the author by the CNNS culture staff. Picture: CNNS archives (Dian/Gross)

After the first week the cultures were fed biweekly with DMEM and 5% horse serum until the day of testing. On the day of testing, the MEAs were integrated onto a recording chamber (Figure 5) and a complete wash was done with DMEM that contained

no serum. The pH and osmolarity levels were maintained at 7.4 and 320 mOsmoles, respectively. Only mature (21 days or older) cultures were used for all pharmacological testing. Figure 4 shows a 13-week old culture on a 64-electrode MEA together with examples of phase contrast images of living neurons on such arrays (B-D)

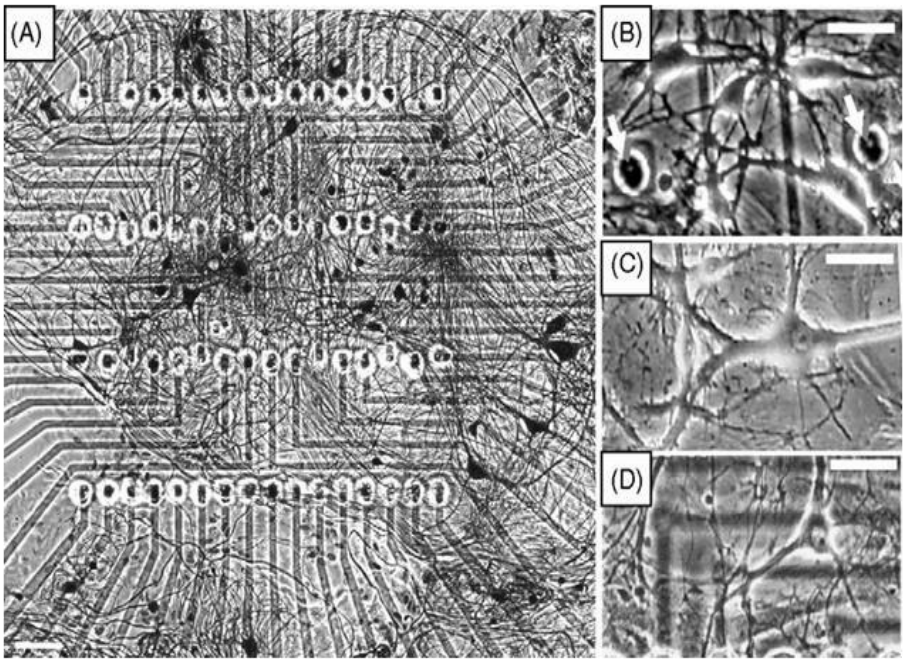


Figure 4. Example of neuronal circuits on microelectrode arrays. Transparent indiumtin oxide (ITO) conductors allow extensive optical access to the network morphology.(A) Neuronal network derived from murine spinal cord tissue (92 days *in vitro*), grown on the recording matrix of a 64-electrode array plate (Bodian stained). (B-D) Living neurons on MEAs. Recording sites (gold-plated, exposed ITO conductors are shown by arrows in (B). The ITO conductors are 8 µm wide and 1200 Å thick. bars = 50 µm. (CNNS Archives)

2.2 Recording Assembly and Data Analysis

The neuronal networks were maintained in a constant bath of recording medium using recording chambers. The assembly consists of an aluminum base plate that holds the MEA and a stainless steel chamber (Figure 5).

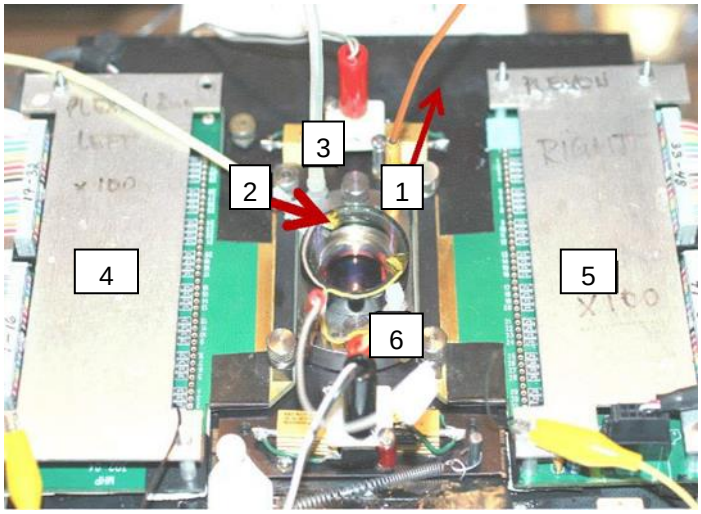


Figure 5. Recording apparatus on inverted microscope stage. Chamber containing the neuronal network on MMEP with a constant medium bath of 2 ml. (1) Heated base plate with thermocouple maintains a constant temperature at 37^0C, (2) ITO chamber cap with CO_2 air flow which maintains constant 7.4 pH, (3) A syringe pump injects 35 µL/hr water through the syringe port input for constant bath osmolarity of 320 mosmol/kg. (4-5) Left and right pre-amplifiers (32 channels on each side), Plexon Inc., Dallas. (6) Syringe port for drug application.

Preamplifiers were placed on the microscope stage to both sides of the recording chamber and connected to the MEA by means of zebra strips (Fujipoly America Corporation, Carteret, NJ). Total system gain was set to 10,000. The amplifier ground was connected to the stainless steel chamber confining the culture medium.

Single-unit activity was averaged across the network to yield mean spike rate. All analyses were done with binned data (bin size of 60 s). In order to avoid serum-binding of test substance and excessive network responses to medium changes required for the

washout of substances, the native medium was exchanged for the wash medium (fresh DMEM stock) at the beginning of the experiment, and the cultures were allowed to stabilize before any drugs were added (termed: reference activity/RA). The percent change in activity for each test substance at each drug concentration was always calculated relative to this 20- to 60-min reference spontaneous activity. This procedure provides an internal normalization and allows effective comparisons among networks with different initial activities. To follow the changes in network activity with time, total activity or spike rates averaged across all active units per minute, were plotted as a sequence of "1 minute binned values" in real time on computer screens (CNNS programs).

The percent change in spike activity for each episode of drug application was calculated for each titration and plotted in a semilog graph. The semilog data was fitted by a sigmoidal function to give individual concentration response curves or CRCs (see definition of CRC page 19; all CRCs were plotted on Origin, Microcal Software, Inc). Not all data sets have enough data points to generate reliable CRCs. However, even single additions with responses represent data. To include single, double and triple additions that do not have CRC data were pooled (Appendix 2). Such tables allow the calculation of means for certain concentrations of test substance and allow the generation of a pooled data CRC. The Ec_{50}s obtained from individual experiments were not significantly different with overlapping SDs. For statistical analysis pooled data CRC was used.

2.3 Pharmacological Manipulations and Life Support

Gentamicin, bicuculline and verapamil were obtained from Sigma Aldrich (Sigma Aldrich, inc., St. Louis, MO, www.sigmaaldrich.com). Verapamil HCl is soluble in water (below 83 mg/ml) and stable for one year when refrigerated in amber bottles (Sigma).

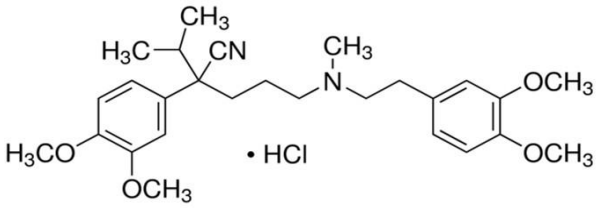


Figure 6. Chemical structure of verapamil hydrochloride. Photo courtesy: Sigma

Table 1 lists the compounds along with the concentration ranges used in this study, chemical class, CAS numbers, purity of the compounds, source form which the compound was obtained, and previous literature where these compounds were used or analyzed using MEAs. All compounds were diluted in water since all were water soluble.

Table1. List of compounds used

Chemical	Chemical class	CAS #	Conc. range used (μM)	Purity (%)	Source	MEA application references
Bicuculline	$GABA_A$ antagonist	40709-69-1	40	≥90	Sigma	Gross et al., 1997
Verapamil hydrochloride	L type Ca^{2+} channel blocker	152-11-4	0.1-104	≥ 99	SIgma	Novellino et al. 2011
Gentamicin	Broad spectrum antibiotic	1403-66-3	250-2300	≥98	Sigma	Baldrich et al., 2011
Muscimol	$GABA_A$ agonist	2763-96-4	0.1-0.7	≥98%	Sigma	Wu et al., 2014

CAS #: Chemical Abstract Service number.

In this study, micro-scale concentrations of verapamil, bicuculline and gentamicin were used. To ensure that the accurate amount of drug was transferred from the micro-pipette tip to the culture bath, about 200 – 300 μL of medium from the cell culture bath was aspirated which was just enough to fill the tip of a 3 ml syringe. The drug was then

pipetted into the syringe and mixed with the medium in the syringe by aspirating air bubbles. This medium was then reintroduced into the bath and mixed further by gentle syringe movement of the medium (normally about 50% per cycle) to ensure even distribution of the drug in the medium of the recording chamber.

To remove the test drug, syringes were used to extract the medium through the same Luer connections at the edge of the recording chamber. These connections lead through 0.8 mm conduits in the stainless steel to an orifice inside the 'O' ring situated approximately 0.2 mm above the surface of the MEA inside the 'O' ring (Gross, 1994).

Microelectrode arrays were placed into recording chambers (Gross, 1994, Gross and Schwalm, 1994) and sustained at 37°C on a microscope stage. The pH was maintained at 7.4 with a continuous stream of humidified 10% CO_2 and 90% air at 5–10 mL/min into a special cap fitted with a heated ITO window to prevent condensation. The syringe pump, Harvard Apparatus® Pump II (Harvard Apparatus, inc., Holliston, Massachusetts) compensated for water evaporation (30 to 60 µl/hr depending on set-up). During experiments, pH and osmolarity were tested at intervals of 2-3 hours. Despite the limited medium volume in the chamber (2 mL), the pH could be tested by extracting 100µL volumes with a pipette and measuring pH in the pipette tip. This was accomplished with an Accumet flexible pH microelectrode that has such a small sensor diameter as to allow entry into the pipette tip and a friction-fit stabilization of the pipette tip.

After pH measurements, 10 µL of the medium were extracted for osmolarity determination with a Wescore 5500 vapor pressure osmometer. This special protocol allowed frequent measurements without exhausting the medium volume in the chamber

and compromising sterility. Osmolarities were not allowed to fluctuate more than 10% from a reference of 320 mOsmoles. Osmolarities were regularly monitored and 100 µL sterilized water was added if the osmolarities were found to be over 340 mOsmoles. Between 290 and 340 mOsmoles, neurons osmoregulate without measurable activity variations if the changes are slow (10 mOsm per minute). Rapid medium replacements require osmolarity matching to within 10 mOsm. Water additions are tolerated generally up to 5% of the total volume.

2.4 Statistics

Data are presented in terms of mean ± standard deviation (SD), the number of cells (units), and/or the total number of experiments performed. The network spike rates were binned data (1 min bin size) and displayed as either total activity or average activity. The latter divides total activity by the number active units for 1 minute bin. Active units are recognized by the computer as discriminated waveforms that have at least 10 threshold crossings per minute (floating average). It should be noted that a physical channel can have up to four discriminated waveforms that can be separated in real time, yielding 4 logical channels.

As shown in Table 2, data from the experiments with only two (eg. In MS005) or three applications of verapamil cannot be used for the generation of verapamil dose response curves. However, such data points can be included in tables (Table 2) from which mean response data at certain concentrations can be obtained, which yields a pooled data concentration response curve (CRC) (figs 8, 10 and 15).

Table 2. Example of pooled data table of verapamil titrations.

Summary of spike rate change (inhibition) Verapamil DRC

Single runs

Tissue	Expt no.	Age (DIV)	Ref Activity	Active Units	Sp. Cond	EC50	Concentrations of Verapamil (µM) 0.5	1	1.5	2	2.5	3	3.5	4	5	6	7	8	9	10	11	12	13	14	15	18
							Percent spike rate																			
FC	MS005	35	235T	30	SERUM	3.8						47					66									
FC	MS007	21	145M	35		3.3		21		24		48		59	69		76	79	73	74	74	81	83	85	87	91
FC	MS008R1		3250T	22		2.6		23		38		54		74	78		85	89	79	83	83					
FC	MS030AR1		26500T	108		1.5	6	34	75	91	92	96	98							97	100					
FC	MS030BR1		12500T			1.4	20	39	62	78	87	92	96													
FC	MS042		7250T	77		2.3		27		41		59		76			93	100								
					Avg.	2.4	13	29	69	54	90	66	97	70	74		80	89	76	85	86					
					SD	1.1		7.6		29		22		11	6.4		9.5	7.1		12	13					

Table 2. MS005 has 2 data points at concentration 3µM and 7µM verapamil. A verapamil CRC is not possible with just 2 data points but they can be pooled with the data points for concentration 3 µM and 7 µM from all experiments (circles) to get a mean percent spike rate inhibition and standard deviation for individual concentrations of verapamil (squares). These mean percent inhibition in spike rates can then be used to give a verapamil CRC and a pooled or mean EC_{50} ± SD for verapamil (For complete data see Appendix 2).

The unpaired t-test (Graphpad Quickcals, GraphPad Software, Inc) was used to test the difference between the means EC_{50}s obtained from verapamil pooled data (no bicuculline and verapamil data in the presence of bicuculline. In this statistical test, $P < 0.05$ was defined as the probability that the compared means have statistically significant difference, data are presented as mean ± standard deviations and (n) number of experiments performed.

CHAPTER 3

RESULTS

3.1 Basic Network Responses to Verapamil

The typical network activity response to increasing concentrations of L-type calcium channel blocker, verapamil is shown in the spike rate plot of Fig 7. This example shows that, after a 60 min period of stable spike activity, the addition of increasing concentrations of verapamil to the bath medium resulted in a step-wise decrease in spike activity. The activity loss was instantaneously reversible, as demonstrated by the response to two complete medium replacements. Verapamil was applied in 2µL aliquots of a 1 mM stock solution to bath (no serum) to give final concentrations ranging from 1 - 11µM in a constant volume medium bath. The addition of 22µL (maximum) verapamil constitutes a 1.1% change in medium volume which does not generate osmolarity or pH changes.

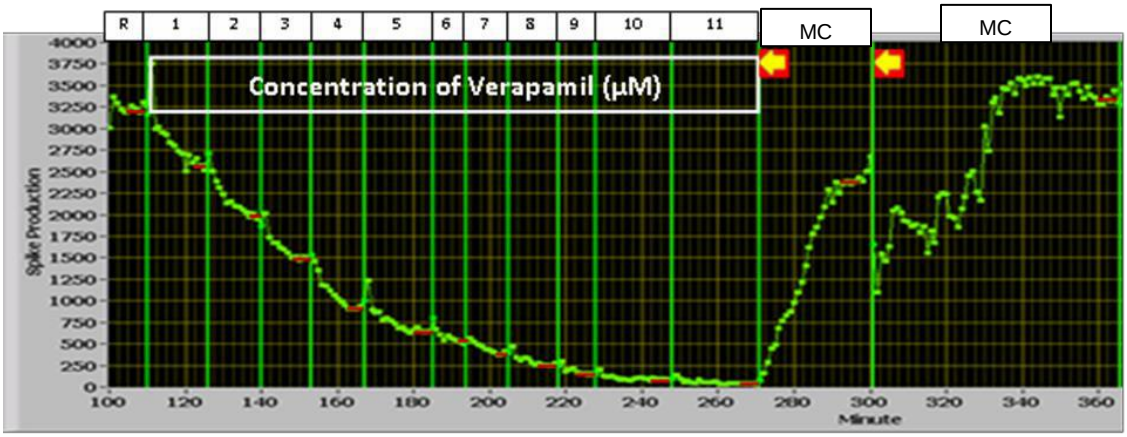


Figure 7 Verapamil titration using an untreated cell culture on a MMEP 4 MEA. Example of a well behaved titration with verapamil showing a concentration dependent decrease in the total activity followed by 100% recovery in activity after 2 medium changes or MC (⟸) . The activity stabilized after each addition of verapamil as indicated by the added plateau line (▬).

Table 3. List of complete verapamil CRCs and calculated EC_{50}s (n=7, frontal cortex cultures).

Expt No. N=7	Age*	Units	RA (total)	RA (mean)	EC50	Date
MS005	30	29	6815	235	(3.8)	3-Jan-10
MS008	31	22	3500	159	1.4	13-Jan-10
MS030AR1	31	108	26500	245	1.1	14-Apr-10
MS030AR2	31	108	31600	292	1	14-Apr-10
MS030BR1	31	108	12500	115	1.2	14-Apr-10
MS030BR2	32	108	14000	129	1.1	14-Apr-10
MS030BR3**	32	77	50820	660	(0.5)	14-Apr-10
MS042	33	79	7250	92	2.3	6-Jun-10
MS053	33	122	23000	188	1.1	26-Oct-10
			Group EC50±SD		1.30±0.45 µM	

Age : days *in vitro*. R1 and R2: First, second and third titrations of verapamil on a single culture, respectively. A and B refer to two networks on an MMEP-5 in separate wells and served by 32 electrodes. Refer to Appendix 1 for individual CRCs. Reference activity total and mean. **MS030BR3 was not included in the calculations because it was used by Calvin Wu for PTZ experiments between R2 and R3. MS005 was also barred because of poor application technique in early experiments. This represents the elimination of the highest and lowest value from the data set. RA: reference activity.

A concentration response curve is defined by four parameters: the baseline response (Bottom), the maximum response (Top), the slope, and the drug concentration that provokes a response halfway between baseline and maximum (EC_{50}). EC_{50}s of individual verapamil titrations, are shown in Table 2 (complete CRCs, n=7). The mean EC_{50} ± SD for untreated networks is 1.3 ± 0.45 µM (pooled data, n=10, Appendix 2 - Table A). Based on the EC_{50} values, cultures showed low inter-culture variability. For some experiments, a single neuronal culture was used to perform two or more subsequent titrations (eg.MS030R1 and MS030R2) where R1 and R2 stand for verapamil run 1 and verapamil run 2. MS030A and MS030B are two verapamil titrations performed simultaneously on separate networks growing on a single MMEP 5 (2 matrices on one electrode plate). EC_{50} for each experiment was calculated from the

curve fit using statistical analysis package from Origin, Inc. The mean EC_{50} and standard deviation (SD) were calculated using Microsoft Excel (Table 3).

Second and third verapamil titrations were performed on the same network to examine the intra-culture variability (Appendix 2 Table A). Neuronal activity returned with removal of most verapamil from the medium bath via one or two medium changes. Thus, inhibition of activity by verapamil is reversible. The two displayed methods reveal different statistics. Table 3 provides a mean ± SD from separate CRCs. The mean percent inhibition in spike rate for pooled data (see Methods) is plotted in Fig. 8. Table 3 and Fig 8 represent the same data but different analysis strategies. The EC_{50}±SE calculated from the pooled data graph is 1.7±0.5 µM.

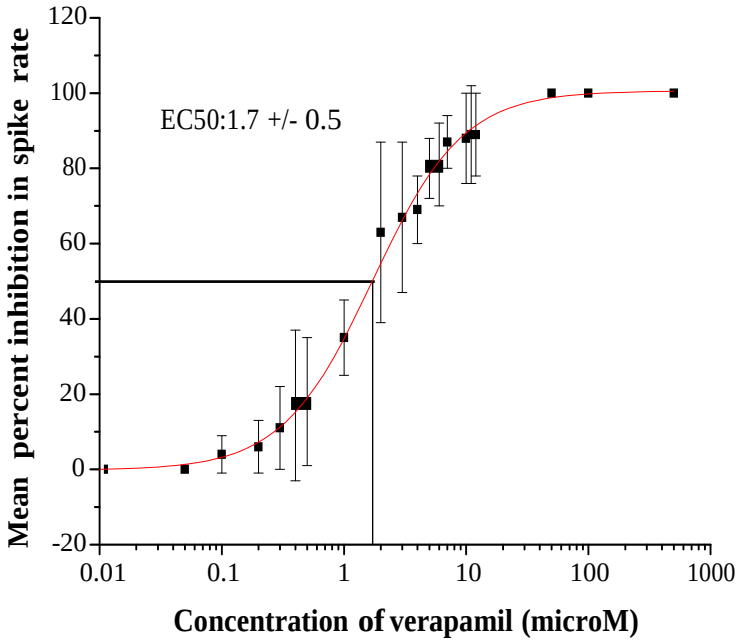


Figure 8. Concentration response curve for verapamil in untreated networks (data from 10 experiments (Appendix 2 Table 1). Graph of pooled percent inhibition in spike rate verses concentration of verapamil, EC50±SD calculated from the graph is 1.7±0.5 µM (n=10).

3.2 Network Responses to Verapamil in Presence of GABA Antagonist, Bicuculline

The GABA antagonist, bicuculline (40µM) was applied to networks with low or unstable activity to enhance the activity, and verapamil titrations were performed in the presence of bicuculline. It will be shown that the addition of bicuculline did not have significant influence on the verapamil EC_{50}, which suggests the action of verapamil is independent of GABA receptors.

Figure 9 shows a verapamil titration in presence of 40µM bicuculline. The native activity was recorded in presence of serum free medium (DMEM stock). Addition of 40µM bicuculline caused a 50% increase in total spike activity. The reference activity for the verapamil titration was taken under bicuculline (horizontal line). Verapamil was added to the culture in 1µM increments till all spike activity was inhibited at 8 µM verapamil. One medium change was done to wash out the verapamil and bicuculline. The activity returned almost instantaneously and stabilized between the native and bicuculline reference, presumably resulting from incomplete washout of bicuculline.

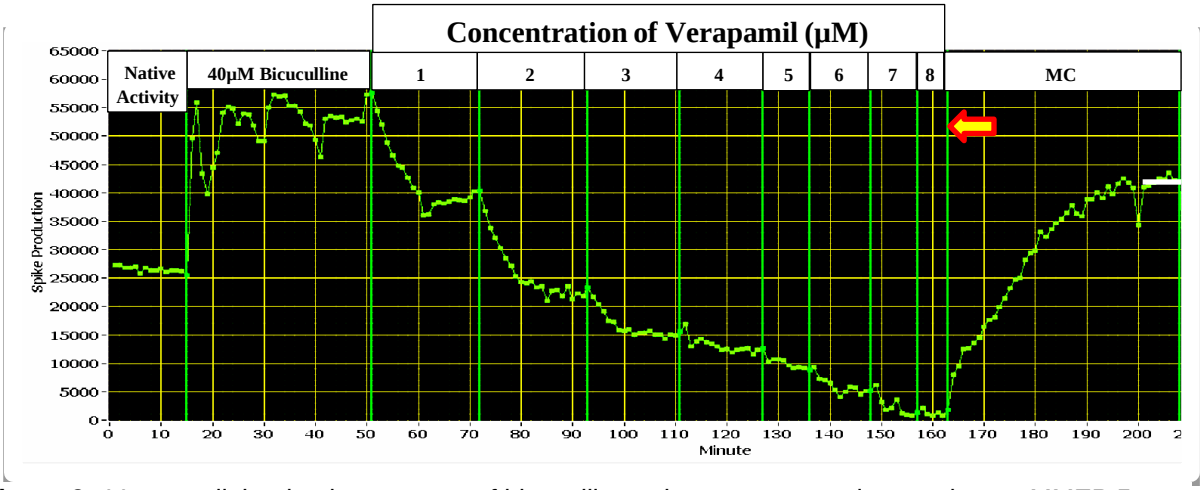


Figure 9. Verapamil titration in presence of bicuculline using one neuronal network on a MMEP 5, MS020B. The initial excitation caused by addition of 40µM bicuculline is followed by concentration dependent decrease to verapamil additions(——). The activity instantaneously returned after single medium change (⇦) but remained 60% over native activity. This can be attributed to the incomplete removal of bicuculline from the medium with a single wash.

Table 4 lists individual verapamil titrations in presence of 40 µM bicuculline (number of complete CRCs, n=5). The mean EC_{50} ± SD for verapamil in presence of bicuculline is 1.4 ± 0.2 µM. The total activity of the neuronal networks was used to calculate the reference activity in the presence of 40µM bicuculline and the percent inhibition by verapamil. Graph of percent inhibition of spike rate verses concentration of verapamil (µM) was generated and an EC_{50} was obtained for each verapamil titration using statistical software (Origin). Fig 10 shows the mean dose response curve for verapamil titration from pooled data. The mean EC_{50} ± SD from this curve is 1.4±0.1 µM.

Table 4. List of complete verapamil titrations in presence of 40 µM bicuculline

Expt no. n=5	Age*	Units	Reference activity (total)	EC50	Date
MS014	30	18	4000	1.6	2-Feb-10
MS015	33	76	17000	1.2	4-Feb-10
MS020B	35	95	52000	1.8	9-Mar-10
MS021R2	31	39	6000	1.3	15-Mar-10
MS021R3	33	39	12000	1.3	16-Mar-10
			Group EC_{50} ± SD	1.4 ± 0.2µM	

Reference activity was recorded in the presence of bicuculline (n=5, frontal cortex cultures). *Age: days *in vitro*. R2 and R3: Second and third titrations of verapamil on a single culture, respectively. Refer to Appendix 2 Table A for pooled data.

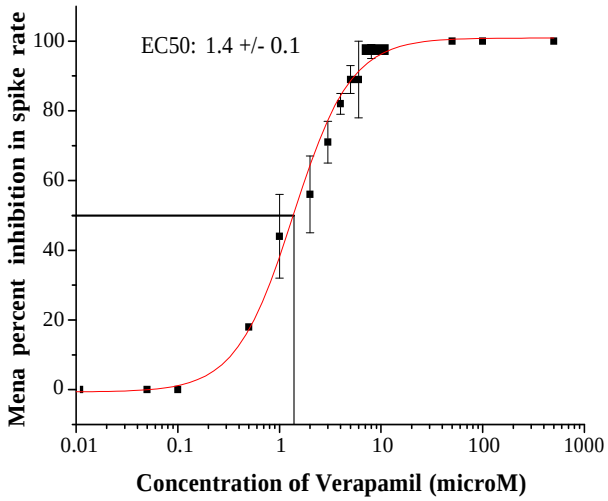


Figure 10. Pooled data concentration-response curve for verapamil in the presence of bicuculline 40 µM (n=9, frontal cortex). EC_{50} ± SD: 1.4±0.1µM. (See Appendix 2 Table B).

3.3 Significance Test: Effect of Bicuculline on Verapamil EC_{50}

Unpaired t- test was used to compare the mean EC_{50} from pooled verapamil (no bicuculline and verapamil titrations in presence of bicuculline. The Graphpad output for the t- test is shown in Table 5.

Table 5. Graphpad output for unpaired t- test

Group	Verapamil	Verapamil + Bicuculline
Mean	1.700	1.400
SD	1.800	0.900
SEM	0.680	0.402
N	10	9

P value and statistical significance:
The two-tailed P value equals 0.7407
By conventional criteria, this difference is considered to be **not statistically significant.**

Confidence interval:
The mean of Verapamil minus Verapamil + Bicuculline equals 0.300
95% confidence interval of this difference: From -1.665 to 2.265

Intermediate values used in calculations:
t = 0.3402
df = 10
standard error of difference = 0.882

Table 5. Unpaired t-test shows there is no statistically significant difference between the mean EC_{50} obtained from verapamil titration and verapamil + bicuculline titrations. Both data sets were combined to get the standard CRC for verapamil (Fig 11).

3.4 Pooled Data Analysis to Establish a Standard EC_{50} for Verapamil

Statistical analysis showed that the verapamil EC_{50}s obtained from untreated networks were not significantly different from those obtained in presence of bicuculline. The two data sets were thus pooled to give a standard verapamil dose-response curve (Fig. 11). The mean EC_{50}±SD for verapamil is 1.4 ± 0.13 µM (n=19). This EC_{50} was considered as a standard EC_{50} for verapamil and used to investigate the effect of gentamicin on pharmacological responses to verapamil as described in the following chapters.

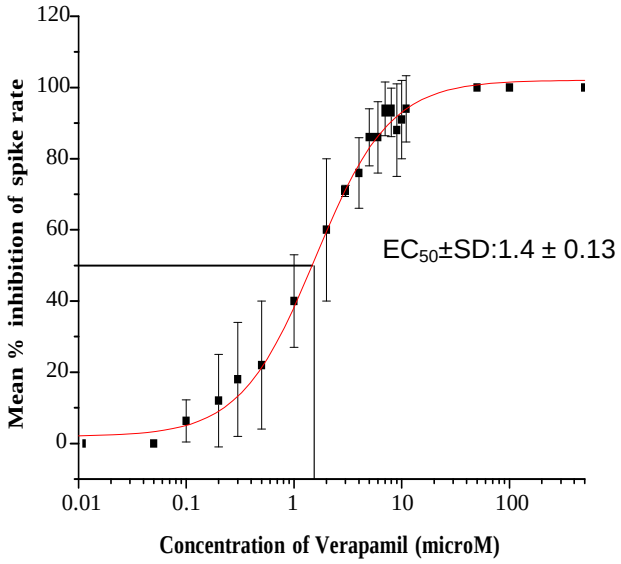


Figure 11 Standard concentration response curve for verapamil (n=19) obtained from combining data sets for verapamil titrations (no bicuculline and verapamil titrations in presence of 40 µM bicuculline, Verapamil EC_{50} ± SD:1.4 ± 0.13 µM.

3.5 Network Response to Increasing Concentrations of Gentamicin

Gentamicin is regularly used to control bacterial contamination in cell culture. The recommended concentration of gentamicin for cell culture use is 108 µM. As shown in Fig. 12, gentamicin titration was performed to test the effects of progressively increasing concentrations on neuronal network activity. The network activity was monitored overnight in the presence of 2300 µM gentamicin. This concentration is approximately 20 times the recommended concentration for cell culture use (108 µM, Sigma). The number of channels showed minimal fluctuations. However, activity decreased by 50% during the overnight hours when the assembly was not observed. This experiment shows that in normal experimental time of 7-8 hours at 100-200 µM gentamicin, no changes are expected (minimal effect at low concentrations).

Figure 12A shows the total activity of the network in presence of increasing concentrations of gentamicin. Figure 12 B shows the mean activity and number of active channels of the same network. At 7 hours (425 mins) the number of active channels increased after the addition of 2300 µM gentamicin. The sudden increase in active units can be attributed to the movement of the culture medium during mixing. Following the addition of 2.3 mM gentamicin, the assembly was left unsupervised overnight and a gradual decrease in activity was seen. This might be attributed to osmolatity changes and/or temperature fluctuations. The culture remained active for over 12 hours in the presence of 2.3 mM gentamicin.

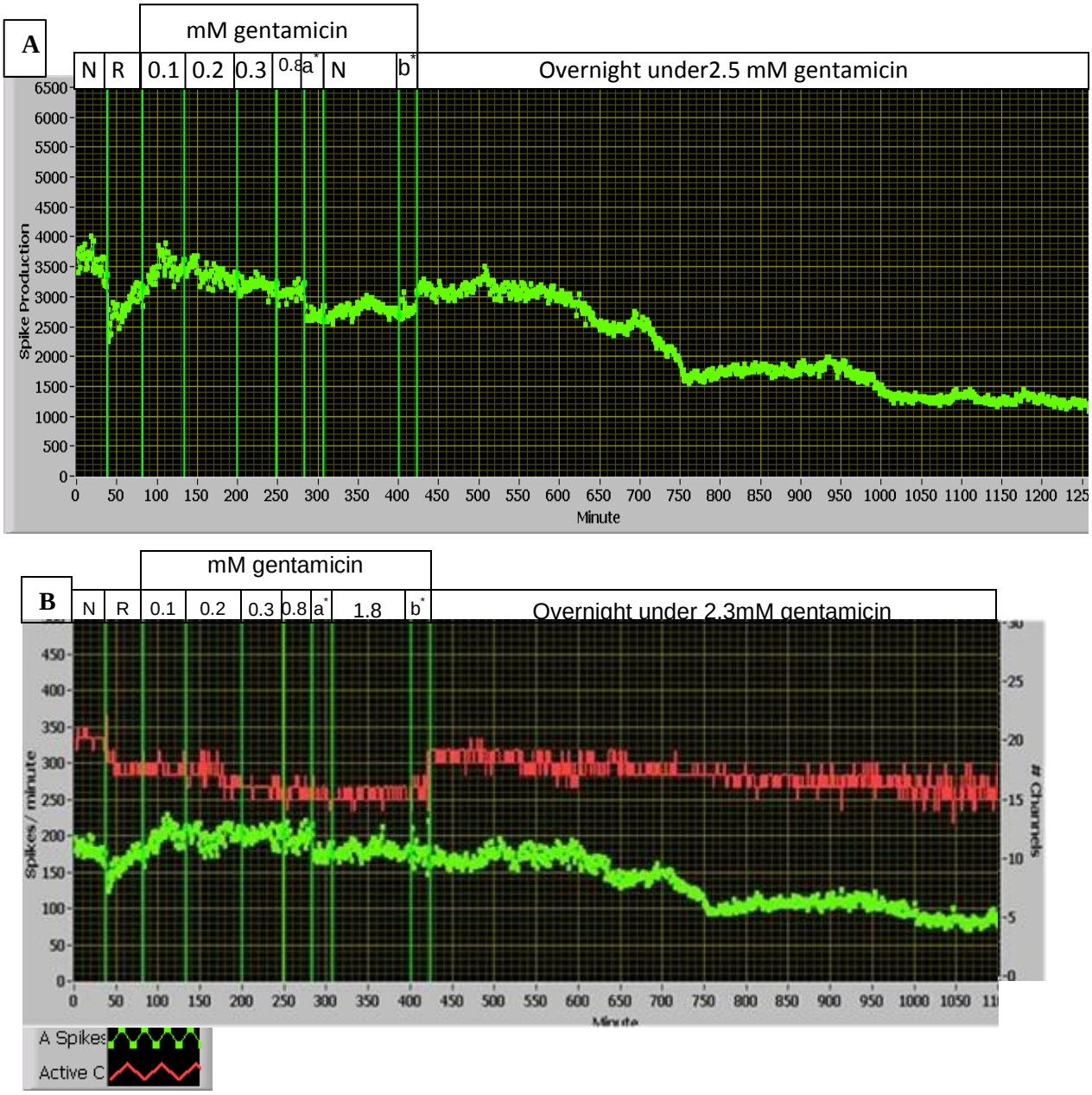


Figure 12. Neuronal network response to gentamicin. A. Total activity of the network in response to increasing concentrations of gentamicin. Native activity (N) was recorded in serum free medium and reference activity (R) was recorded in presence of 40µM bicuculline. The activity dropped by 50% over-night when the assembly was unsupervised. This might be attributed to changes in osmolarity and/or temperature fluctuations. B. The mean activity of the network and the number of active channels. At 7 hours the number of active channels increased after the addition of 2300 µM gentamicin. The sudden increase in active units can be attributed to the movement of the culture medium during mixing. *Concentrations a and b are 1.2 mM and 2.3 mM, respectively. The culture remained active for 12 hours under gentamicin.

3.6 Network Responses to Verapamil in Presence of Gentamicin (Acute Exposure)

Four frontal cortex cultures of ages ranging from 20 to 46 days in vitro were used to investigate the effect of gentamicin on the pharmacological response to verapamil (Table 6 and 7). Mean spike rates were averaged over the network using 1- minute bins. The individual experiment time ranged from 8 to 13 hours. One of the cultures was used twice to observe intra-culture repeatability (MS0315 and MS0316). Figure 13 A and B show a typical global response of the same frontal cortex network to two consecutive verapamil runs (0.1 – 16.5 µM) under 108 µM gentamicin. Figure 13 A represents the first verapamil run and is depicted as average activity per minute with active channels. Following the medium change to DMEM stock (DS), 108 µM of gentamicin was added. The culture showed low activity in DMEM stock but the activity increased with the addition of gentamicin. 40 µM bicuculline was added to stabilize the activity of the culture to obtain a stable reference activity (Bic). The activity ceased at 16.5 µM verapamil. The culture was washed twice with DMEM stock to wash out as much verapamil and bicuculline as possible before run 2 was performed. The two runs were performed with a 12 hours between experiments during which the culture was stable. No bicuculline was added before run 2 to minimize the use of bicuculline. Native activity (N) was recorded for 20 min and a wash was performed with DMEM stock (DS). The reference activity (G) of the culture, under 108 µM gentamicin, stabilized at a total spike rate of 8000 spikes / min. During run 2, all activity stopped at 8µM verapamil compared to the 16.5 µM required in run 1. This difference is partially an artifact of comparing mean and total data displays. To obtain mean values, the computer divides the total spike count by the number of active channels for each minute bin. The number of active channels is not

static but depends on at least 10 threshold crossings per minute. As channels drop out, the denominator decreases in magnitude and can artificially increase the mean value. Fig 13 A shows channel loss beginning at 5.5 µM. Beyond this point, the plateau levels are unreliable. Total activity for run 1 was not recorded. This sensitized response is typically seen in verapamil CRCs where a single culture was used to perform multiple titrations (Table 2 and Table 3).

Table 7, however, allows utilization of all data points and shows the summary of verapamil percent spike inhibition effect in the presence of 108 µM gentamicin. Cessation of spike activity (86- 99 %) occurred in the concentration range of 11.5-16.5 µM verapamil.

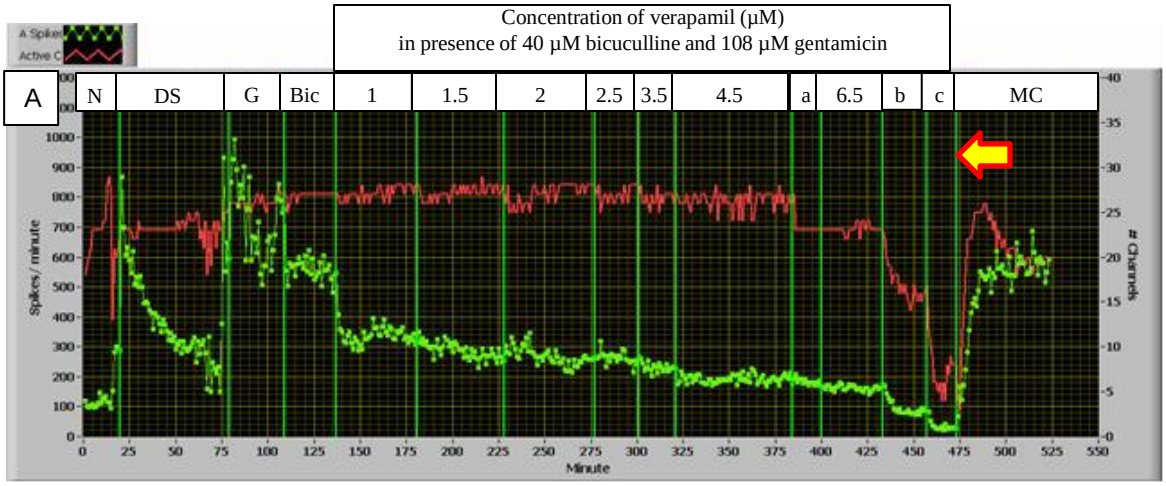


Figure 13 (A) Verapamil CRC in presence of gentamicin. (A) Run 1 of verapamil titration using neuronal network in presence of 108 µM gentamicin and 40 µM bicuculline (acute exposure). Bicuculline was added to stabilize the culture at 110 mins. The reference activity was recorded under bicuculline (bic). The mean activity shows predictable decreases with increasing doses of verapamil. The culture was washed with DMEM stock (⇦). The activity stabilized after each addition of verapamil as indicated by the plateau line (▬). The culture was washed again with DMEM stock (not shown) to remove as much bicuculline as possible and used for run 2 (Figure 13 B). a,b and c are 5.5, 11.5 and 16.5 µM verapamil, respectively.

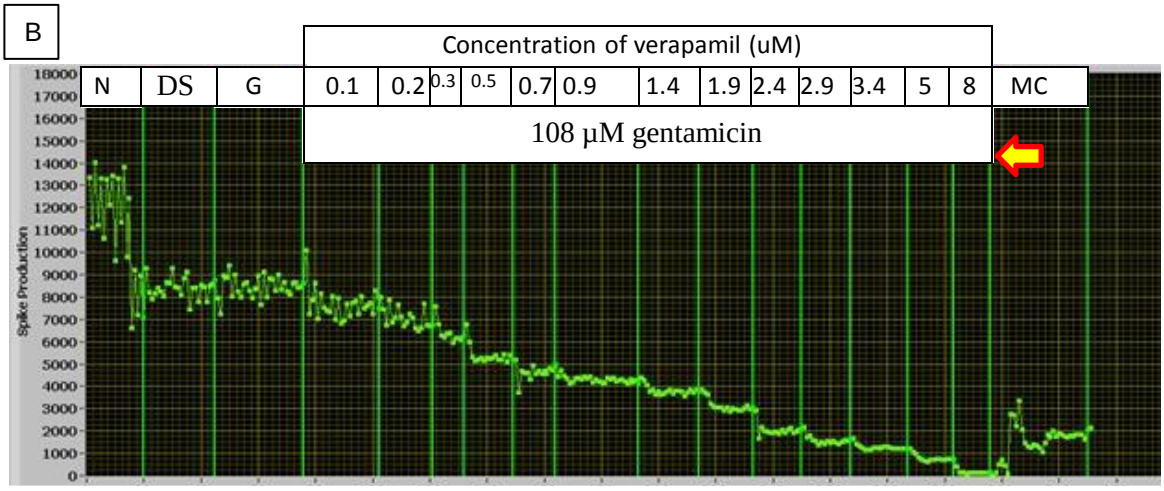


Figure 13 (B) Run 2 of verapamil titration using neuronal network in presence of 108 µM gentamicin (acute exposure). Mean activity of a well behaved titration with verapamil in presence of gentamicin showing a concentration decrease in total activity followed by a partial recovery in activity after a single medium change or MC with DMEM stock (⇦). The activity stabilized after each addition of verapamil as indicated by the plateau line (▬). The culture was treated with bicuculline, remained stable over night and was used for another experimenter (not shown).

Figure 14 shows the verapamil CRCs from figure 13. The average of 1.1 µM from these 2 experiments suggests a possible sensitization to verapamil in presence of gentamicin.

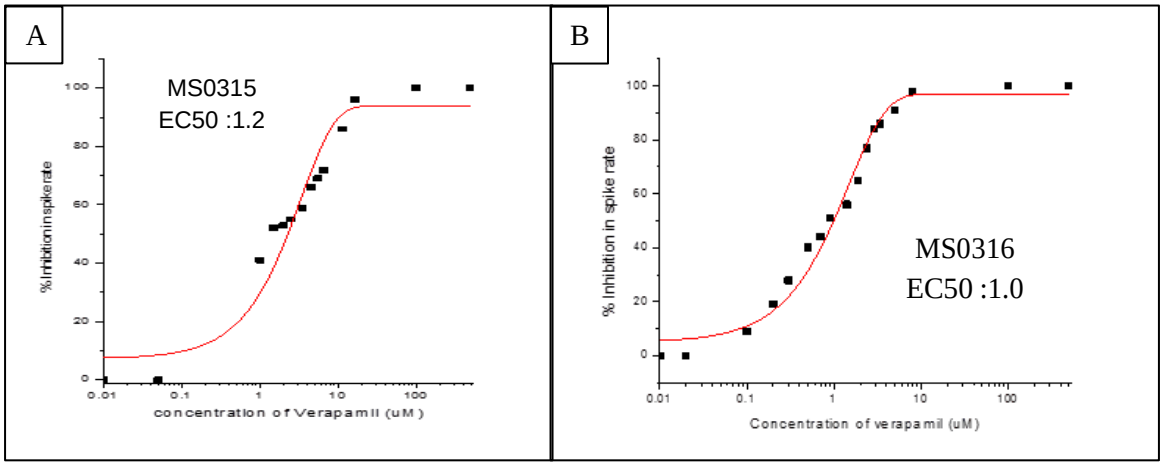


Figure 14. Verapamil CRC in presence of gentamicin. MS0315 and MS0316 are verapamil CRCs performed on the same culture in presence of 108 µM gentamicin. The two experiments were performed with a 12 hour gap between them. MS0315 (run 1) was performed in presence of 40 µM bicuculline to stabilize activity. The average EC_{50} of 1.1 µM from these two titrations suggests a possible sensitization verapamil in presence of gentamicin. Normal verapamil EC_{50}: 1.4 ± 0.13µM.

Table 6. Summary of verapamil titrations in presence of gentamicin.

Expt no. n=4	Age*	Units	Reference activity (total)	EC_{50} (µM)	ate
MS072	33	43	30000	1.09**	9-Jan-10
MS077	31	27	2000	0.86**	19-Feb-10
MS0315	29	21	16500	1.2	3-Jun-10
MS0316	29	19	8600	1	3-Aug-10
			Group EC50 ± SD	1.03 ± 0.14 µM	

*d.i.v: days *in vitro;* RA: reference activity. **Estimated due to low data points that prevented CRC generation. Units: number of active units.

Table 6 summarizes the verapamil CRCs in presence of gentamicin. The EC_{50} of MS072 is an estimated EC_{50} as this experiment had only 2 data points. Figure 15 represents the data from Table 7. Verapamil titrations performed in presence of 108 µM gentamicin (n=4) show a possible sensitization to verapamil by the shift in EC_{50} to 0.9 ± 0.2 µM, as compared to the standard EC_{50} (1.4 ± 0.13 µM, Fig. 11).

Table 7. Summary of verapamil percent spike inhibition in the presence of 108 µM gentamicin (acute application).

	VERAPAMIL (µM)																								
Expt no.	0.1	0.2	0.3	0.5	0.7	0.9	1	1.4	1.5	1.9	2	2.4	2.5	2.9	3	3.4	4	4.5	5	5.5	6.5	8	10	12	17
n=4																									
MS072																			67				100		
MS077							55				75				85										
MS0315R1							41		52		53		55				59	66		69	72			86	96
MS0315R2	9	19	28	40	44	51		56		65		77		84		86			91			98			
MEAN	9	19	28	40	44	51	48	56	52	65	64	77	55	84	85	86	59	66	79	69	72	98	100	86	96
SD							10				16								79						

n: number of experiments

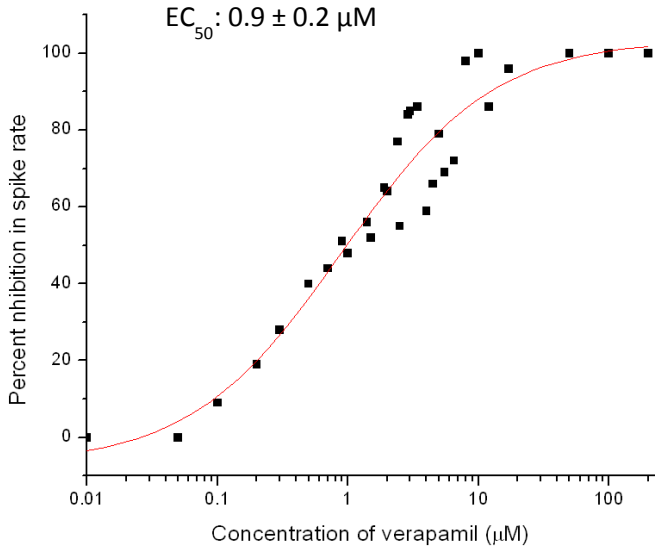


Figure 15 Mean verapamil CRC in presence of gentamicin (acute exposure, n=4). A. Verapamil CRC in presence of 108 µM gentamicin (EC_{50}: 0.9). Neuronal networks reveal a potential sensitization to verapamil under gentamicin as shown by the shift in verapamil EC_{50} from 1.4 ± 0.13 µM to 0.9 ± 0.2 µM.

3.7 Effect of Gentamicin Pre-Exposure on Network Responses to Verapamil (Chronic Exposure)

The exposure to gentamicin during the growth and adherence period helps understand the effect of antibiotics on development of neuronal cell culture. 5 µL gentamicin/ ml culture medium was added to six frontal cortex cultures on the fifth day after seeding onto the MEAs. On the tenth day after seeding gentamicin was washed out from the cells with a full medium change. Of the six matrices exposed to gentamicin, four lost adhesion and were displaced from above the recording matrix. Of the two cultures that adhered to the recording matrix, verapamil (n=1) and muscimol (n=1) titrations were performed using one matrix (i.e. network) (Figs. 16 and 17). The second gentamicin treated matrix lost adhesion during recording. Fig. 16 A and B show the percent decrease in activity of the neuronal culture pre-exposed to gentamicin in response to increasing doses of verapamil and muscimol, respectively. Note that the verapamil EC_{50} increased from 1.4 ± 0.13 µM (Fig 10) to 7 µM, a 5 fold increase whereas the muscimol EC_{50} increased from 0.13 ± 0.01 µM to 0.24 ± 0.01 µM. Only one titration each for verapamil and muscimol could be performed due to loss of adhesion of the cell culture from the MEA matrix which is not a normal occurrence.

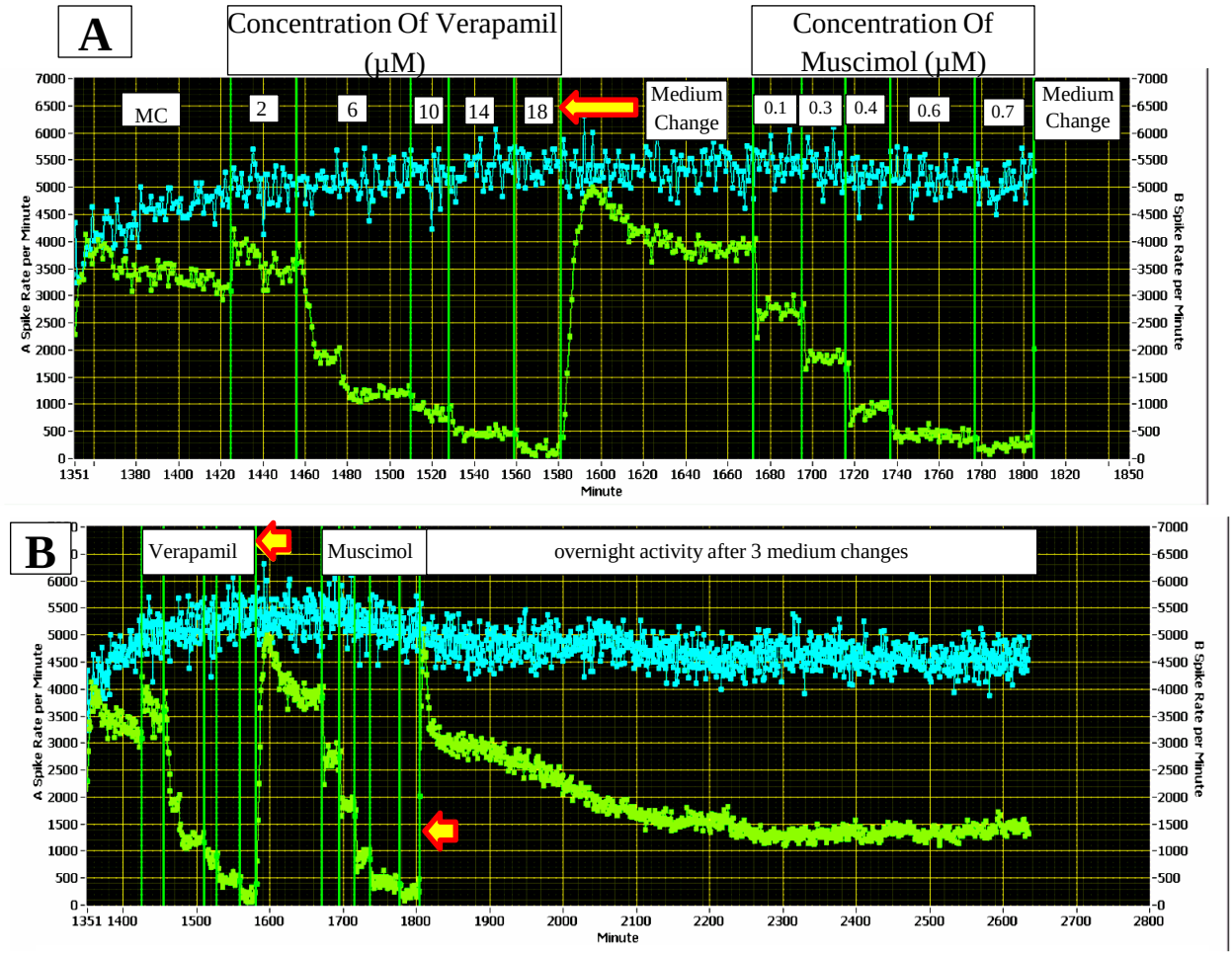


Figure 16 Direct comparison of verapamil and muscimol titration in the same network gentamicin 108 µM and in the presence of a control culture (MS073). A. Two sequential titrations with verapamil followed by muscimol using a MMEP 5 chamber with 2 networks. The first titration with verapamil showed an excitation followed by step wise decreases in activity. After 2 complete medium change (⇦) the activity recovered and showed step wise decreases to a muscimol titration. B. Shows the recovery from the second titration and survival overnight. Note that the control culture (top trace) shows minimal activity changes over the course of time. The control culture was not subjected to medium changes or any test substance.

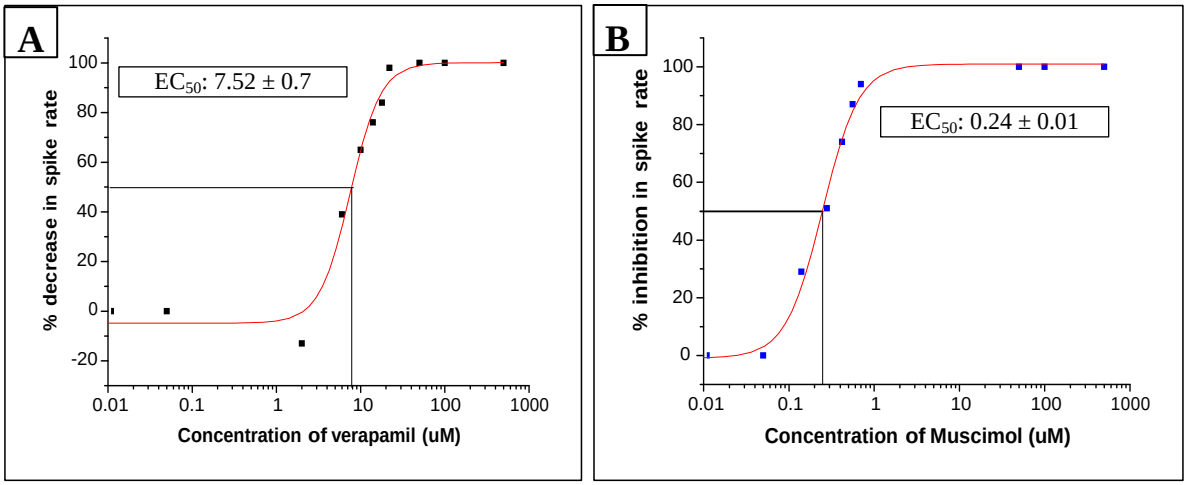


Figure 17 A and B. show the percent decrease in activity of the neuronal culture pre-exposed to 108 µM gentamicin in response to increasing doses of verapamil and muscimol, respectively. Note that the verapamil EC_{50} increased from 1.4 ± 0.13 µM to 7.52 ± 0.7 µM (a 7 fold increase) whereas the EC_{50} for muscimol increased from 0.13 ± 0.01 µM to 0.24 ± 0.01 µM. Standard EC_{50} for muscimol (0.13 µM) was taken from Sabnam Oli-Rijal MS thesis, 2006).

Morphology

A notable decrease in the glial confluence was seen in all cultures exposed to gentamicin during development. Loss of adhesion was observed 25 – 31 days after gentamicin exposure (n=4). Only two cultures pre-exposed to gentamicin that adhered to the electrode matrix could be used for further analysis.

One of above mentioned cultures (fig 18 A) showed unstable bursting patterns after medium change to DMEM stock – strong rapid bursts followed by long periods of no activity. 1 mM KCl was added to depolarize the neuronal culture and stabilize activity (total KCl = 6 mM; 5mM from DMEM stock +1mM added). Although the activity increased with the addition of KCl, the culture did not stabilize for 5 hours and lost adhesion. During the 12 hour experiment the osmolarity and pH of culture medium were controlled and

stable. 19 B and C show the electrode matrix on which this cell culture was seeded. 12 hours after assembly the culture detached from the ITO glass and drifted off of the electrodes and no activity could be recorded.

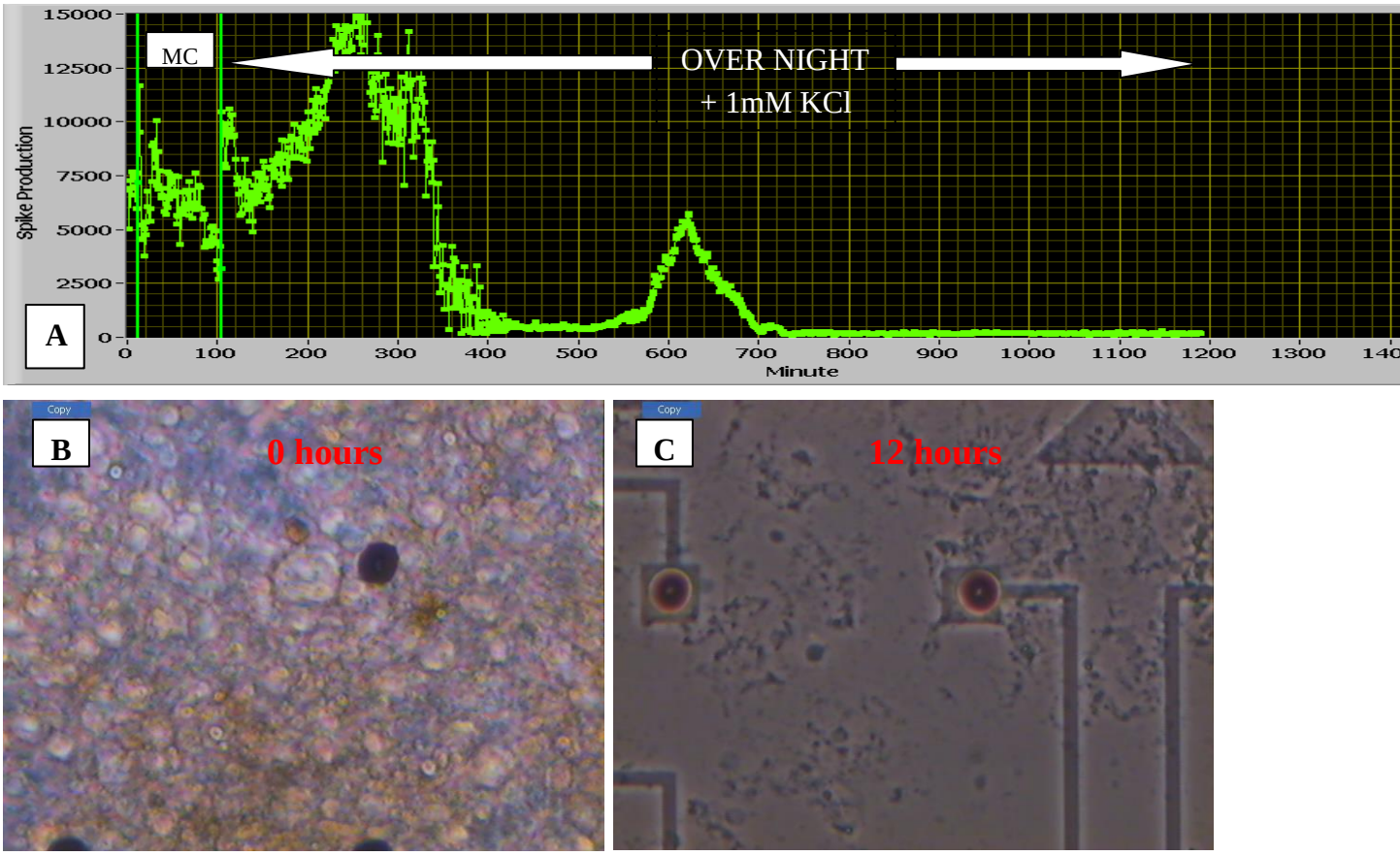


Figure 18. Loss of adhesion in gentamicin treated cell culture. A. Frontal cortex network adhered to the recording matrix 25 days after exposure to gentamicin and treated with KCl to stabilize activity. Loss of adhesion was seen within 5 hours of medium change indicative of weak adhesion. 18 B. and 18 C. Loss of adhesion of cells after 12 hours after medium change to DMEM stock.

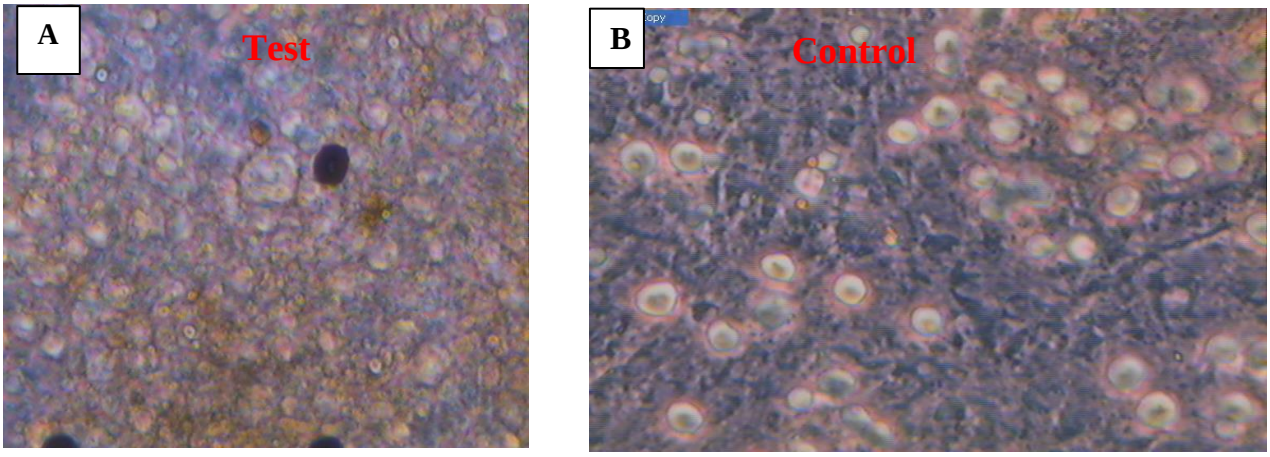


Figure 19 Comparison of morphology of neuronal cell cultures grown in normal culture medium and exposed to gentamicin during growth. A. Frontal cortex cells exposed to gentamicin for 5 days during development. B. Control frontal cortex cell culture from a batch seeded from the same mouse, seeded on the same day as culture A. No exposure to gentamicin. The pictures were taken at 21 days *in vitro.*

The neuronal cell cultures grown in normal growth medium showed marked difference in morphology and confluence than the cultures grown in presence of gentamicin. Fig 19 shows the comparison of a culture treated with gentamicin and a culture from the batch seeded at the same time that was grown with no gentamicin treatment. Retraction was also seen at the edges of gentamicin treated cultures.

CHAPTER 4

DISCUSSION

Contamination of cell cultures is easily the most common problem encountered in cell culture laboratories, sometimes with very serious consequences. In the last few decades, there have been numerous studies indicating the influence of antibiotics on biological organisms and cell membranes (Heyer et al., 1982; Marangoz et al., 2001). Antibiotics translocate across the target membrane (Ceccarelli et al., 2004), inhibit cell wall biosynthesis (Nestorovich et al, 2002) and change permeability of cell membrane (Berquand et al., 2005). Ion channels are also targets for the action of antibiotics (Wasko et al., 2012).

One of the important properties of antibiotics is their ability to disrupt the ion flow through cell membranes. This property becomes compounded when ionophore antibiotics are used. The stable concentration of ions necessary for normal function in the extracellular and intracellular media is imbalanced (Ronquist G, Waldenström A, 2003). The ion balance across the membrane can also be disrupted by application of ionophore antibiotics that form either ion channels or ion-ionophore complexes in biological membranes (Konnie, 2004). Gentamicin, an aminoglycoside, became the preferred antibiotic for cell culture use as it was heat and pH stable and was wide spectrum antibiotic in comparison to pen-strep.

This thesis investigated the effect of gentamicin on the pharmacological responses of neuronal cell culture using extracellular recordings from neuronal networks grown on microelectrode arrays. The research used spontaneously active neuronal FC networks to study the effect of verapamil on spiking activity in presence of high concentrations of gentamicin (108 µM).

The research also explored general verapamil effects, intra-culture repeatability and effect of gentamicin pre-treatment on verapamil dose response curves and morphology. A total of 19 cultures were used to study the effect of verapamil (n=10) and verapamil in presence of bicuculline (n=9). As shown in figures 7 and 9, verapamil inhibited the spike and burst rate in a concentration-dependent manner. The mean EC_{50} for verapamil in untreated cells was 1.7 ± 0.5 µM (Figure 8). This value agrees with the previous literature where Freedman et al. (1984) reported verapamil inhibited voltage-sensitive Ca^{2+} uptake at EC_{50} of 1.8 µM.

Bicuculline (40 µM) was added to stabilize activity in 9 cultures used for verapamil dose response curve. The verapamil EC_{50} in presence of bicuculline was 1.4 ± 0.1 µM (Figure 10). The EC_{50} verapamil values in presence of 40 µM bicuculline were not significantly different from the normal verapamil IC_{50} (unpaired t-test), indicating bicuculline does not affect the verapamil EC_{50}. Although other studies have shown that the L-type calcium channel antagonist verapamil blocked bicuculline-induced epileptiform activity (Straub et al., 1990), this is likely a calcium channel effect on strong bursting rather than interaction at the $GABA_A$ receptor channel complex. A statistically reliable and reproducible mean EC_{50} for verapamil (1.4 ± 0.13 µM) was established (Figure 11).

To investigate the effect of gentamicin on verapamil, verapamil dose response curves were performed in presence of 108 µM gentamicin (acute exposure). The mean EC_{50} for verapamil in presence of gentamicin from limited data was 0.9 ± 0.2 µM, n=4 (Fig 15). The change in verapamil EC_{50} from 1.4 µM (standard) to 0.9 µM (in presence of gentamicin) suggests possible sensitization. Given the limited data points and only two complete CRCs, no statistical comparison between these two data sets was feasible.

The concentration of gentamicin used in this research is much lower than a previous study showing that 5 mM gentamicin had no effect on the viability of the isolated cochlear outer hair cells for up to 6 hrs (Dulon, et al., 1989). Unlike vestibulotoxicity, involving reversible and dose-dependent inhibition of the L-type Ca^{2+} caused by gentamicin with an EC_{50} value of 36.3 ± 7.8 μM (Yu et al, 2014), 108 μM gentamicin did not affect the spontaneous, native activity of the neuronal networks. However, the pharmacological responses to verapamil in the presence of 108 μM gentamicin appear to be sensitized. The gentamicin dosage described to have caused toxicity in other cell cultures is stated in Table 8 (Schafer et al., 1972).

Table 8 Effect of gentamicin on cell cultures

Cell culture	Nontoxic conc. μM	Tolerated: suggested dose mM	Toxic conc. μM
Human amnion (AC amnion)	46(4)	20x	276 (2)
Chick fibroblast	46(5)	20x	138 (3)
Human foreskin (FS-1)	46(6)	20x	138 (2)
Human carcinoma of cervix (HeLa)	276 (3)	120x	
Mouse fibroblast (L-929)	46(3)	20x	276 (2)
Human amnion (U)	276 (3)	120x	
Monkey kidney (Vero)	184(4)	80x	230 (2)

a. Number of days observed noted in parentheses.
b. Tolerated dose was determined experimentally. The suggested dose was arbitrarily selected on the basis of in vitro antibacterial activity.
c. Numbers in parentheses indicate day at which gross morphological changes were observed

With the exception of the above stated vestibular toxicity, previous literature indicates that concentration at which gentamicin causes toxicity in cell culture is higher than 108 μM, the recommended concentration of gentamicin for cell culture.

Using canine renal brush border membrane vesicles, Sokol et al. (1989) showed that gentamicin and verapamil compete for a common transport mechanism. They postulated that high-affinity substrates protect against gentamicin-induced nephrotoxicity. Verapamil modifies some of morphological and functional kidney alterations induced by gentamicin (Stojiljković et al., 2005). Further, protective effect of verapamil against gentamicin nephrotoxicity is dose-dependent and the effect is enhanced at low doses (Boroushaki et al., 2005). These results indicate verapamil may have a protective effect against gentamicin toxicity in nephrons. However, in this study, the effect of gentamicin on the dose responses of frontal cortex neuronal networks to verapamil showed an increased sensitivity with short term or acute exposure (verapamil EC_{50} shifted from 1.4 µM to 0.9 µM).

In addition to nephrotoxicity, gentamicin can cause toxic side effects to the inner ear. While damage inflicted by gentamicin on the kidney is usually reversible (Hock et al. 1995, Toubeau et al., 1986), damage to the inner ear is permanent (Greenwood et al., 1959). Gentamicin is predominantly vestibulotoxic (Matz GJ, 1993). After systemic administration, gentamicin is detected in the cochlea within minutes. Fluorescently labeled gentamicin was detected in the stria vascularis 10 minutes after injection in mouse (Wang et al., 2009). Gentamicin enters the inner ear fluids from the strial capillaries through the strial marginal cells and hair cells demonstrate fluorescent gentamicin intra-cellularly after 3 hours (Wang et al., 2009). In outer hair cells (OHCs), gentamicin was detected after 30 minutes and peaked 6 hours after systemic injection (Imaimura et al., 2003). Fluorescently labeled gentamicin has been observed first in the tips of hair cell stereocilia before the fluorescent signal increases in the hair cell body

(Wang et al., 2009). Both endocytosis and transport through ion channels are proposed to mediate gentamicin uptake into sensory hair cells. While some publications describe endocytosis as the mechanism of entry into hair cells (Hashino et al., 1995; Richardson et al., 1997) others advocate for the mechanoelectrical transducer (MET) channel located at the top of hair cell stereocilia (Marcotti et al., 2005; Waguespack et al., 2005).

In this study, two verapamil titrations in presence of gentamicin were particularly interesting (Fig 20 and 21). In these experiments, the addition of 108μM gentamicin caused an increase in activity. Subsequent addition of verapamil doses caused further increase in activity which is not a normal response to verapamil. Figure 20 shows the activity increase with 108 μM gentamicin followed by a over 50% increase in the normalized spike activity with the addition of 1 μM verapamil. The increase in activity with the addition of 1 μM verapamil was seen even after the removal of gentamicin by two medium changes. To ensure this effect was not exclusive to verapamil, a muscimol titration was performed wherein the EC_{50} for muscimol was shifted to 0.28μM from the standard EC_{50} of 0.14 ± 0.05 μM. This abnormal response was persistent with a second muscimol titration performed 13 hours after the first muscimol titration.

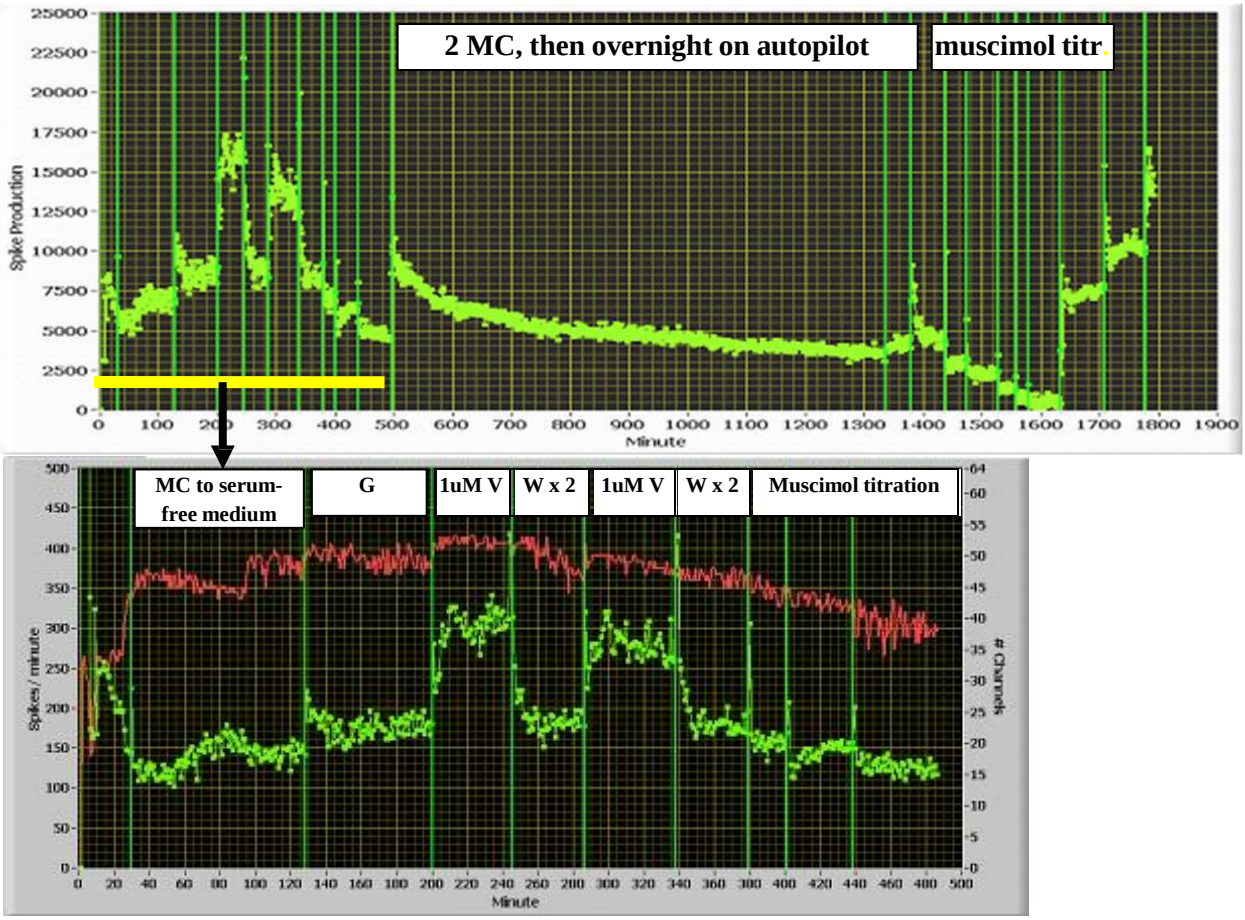


Figure 20 Culture sensitized by gentamicin acute exposure (80 mins), MS088. The addition of 108 µM gentamicin (G) caused an increase in activity. 1µM verapamil added in presence of gentamicin caused an approximately 50% increase in activity. The excitation to 1 µM verapamil is abnormal.To eliminate the possibility of an experimental error, the culture was washed two times with DMEM stock to remove gentamicin and verapamil. 1µM verapamil was added, this time without gentamicin. The culture mimicked the response from the first verapamil dose even when gentamicin was washed from the medium. Verapamil was removed with two medium changes. A muscimol titration was performed to test the response of the culture. Muscimol titration did not cause any increase in activity but the EC_{50} for muscimol shifted to approximately twice the normal EC_{50} (0.14 ± 0.05 µM). Following the muscimol titration the culture was given two more medium changes and left overnight on auto pilot. Another muscimol titration was performed after 12 hours and EC_{50} was still found to be around 0.28 µM.

Figure 21 shows another biphasic verapamil dose response in presence of 108 µM gentamicin. The activity increased with the addition of gentamicin followed by a 25% increase with the addition of 1 µM verapamil. The activity remained at +25% of the reference activity with 2µM verapamil and then continued to decrease with additional doses of verapamil. The EC_{50} of verapamil was shifted to 24µM which is 6 times the standard EC_{50} for verapamil. Activity was completely inhibited at 104 µM verapamil.

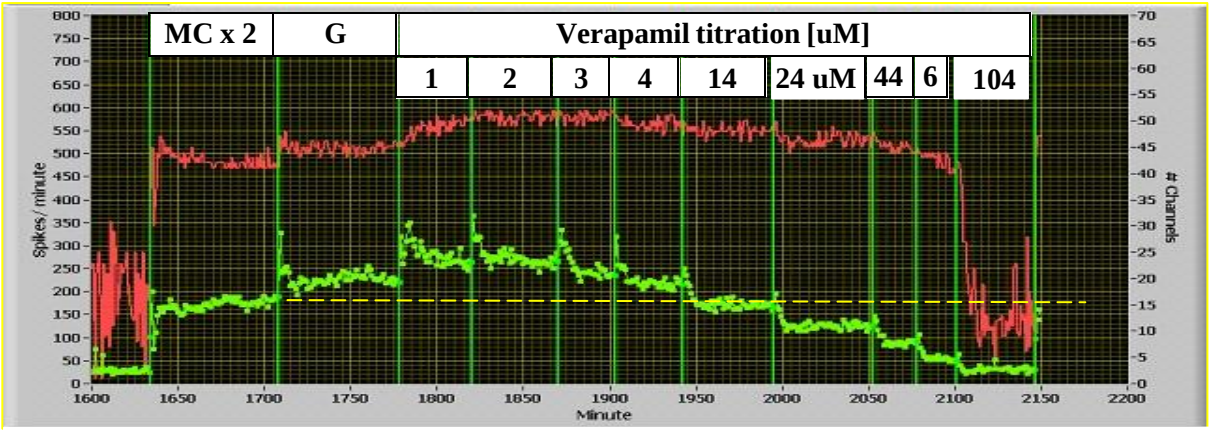


Figure 21 Biphasic verapamil dose response in the presence of gentamicin, MS087. The figure shows anomalous results with an initial excitation (normally not seen) and an EC_{50} of approximately 24 µM. The normal EC_{50} is 1.4 +/- 0.13 µM (n=12). This represents an enormous distortion of pharmacological responses. G: 108µM gentamicin, reference activity. MC: medium change.

Although these observations were intriguing, biphasic verapamil CRCs in presence of gentamicin could not be reproduced and hence were not included in the data. The verapamil EC_{50} in presence of 108 µM gentamicin was found to be 0.9 µM (n=4, Figure 15), which suggests the cultures were sensitized to verapamil (standard EC_{50}: 1.4 ± 0.13 µM).

Further, this research attempted to study the effect of gentamicin chronic pre-exposure on pharmacological responses of neuronal cell cultures to verapamil by exposing the cell cultures to gentamicin during growth phase (n=4). Three cell cultures

pre-exposed to gentamicin during growth showed lack of adhesion to the MEAs. Microscopic examination of these cells showed poor glial growth but good cell density (see Appendix 3). No spike activity could be obtained from these cultures as they were dislodged from center of the MEAs where the electrodes are located. The loss of adherence can be correlated to the low level of glial growth. The pattern of neuronal growth on these different mono-layers suggests that neurons are more adherent to glia than to other neurons but are more adherent to other neurons than to non-glia. Such an adherence hierarchy could explain the consistent finding of apposition of neurons to glial surfaces during neuronal migration and axon outgrowth (Noble et al.,1984). Previous studies on the effect of gentamicin on cell growth and metabolism, dose-response relationship showed that the antibiotic causes a depression of proliferation (Fischer, 1975). The observations of this research are consistent with the available literature.

A single gentamicin chronically pre-exposed culture which stayed adhered to the MMEP was used to perform one verapamil and one muscimol titrations each. The values of EC_{50} obtained for verapamil and muscimol titrations were 7.52 ± 0.7 µM and 0.24 ± 0.01µM, respectively. These values were higher than the normal EC_{50}s for verapamil (1.4 ± 0.13 µM) and muscimol (0.13 ± 0.01 µM), indicating major desensitization to verapamil. However, data collected from a single neuronal network is insufficient to determine if such pre-exposure to gentamicin causes a shift in the verapamil and muscimol EC_{50}s. This culture also lost adhesion after 12 hours of recording. Thus, due to lack of sufficient data, it remains inconclusive if long term pre-exposure to gentamicin changes the pharmacological responses to verapamil.

Even though cell culture researchers rely on the routine use of antibiotics in their cultures to avoid microbial contamination, leading cell culture experts strongly discourage the general use of antibiotics in culture (Coecke et al., 2005; Fogh et al., 1971; Wolf and Quimby, 1973; Freshney, 2000; Lincoln and Gabridge, 1998; McGarrity, 1976). Although the choice of antibiotics used to treat cell culture contaminations largely depends on the type of contamination, there is insufficient information about their effect on cell cultures. The present study was based on previous observations with chronic pen-strep done by Sabnam Oli-Rijal (2006) in which the cell cultures treated with pen-strep showed notable decreases in the EC_{50} of muscimol titration. In this study, verapamil titrations in presence of ten times the recommended concentration of gentamicin (acute studies) showed sensitization to verapamil. Neuronal cell cultures that were pre-exposed to gentamicin for 5 days after seeding on MEAs showed sparse glial growth and loss of adhesion of the neuronal networks to the MMEPs. Experiment with cells exposed to gentamicin during growth indicated extensive de-sensitization to verapamil.

Although the mode of action remains unclear, this research shows that acute exposure (20-40 mins before pharmacological testing) acute gentamicin exposure may cause sensitization to verapamil. Long term exposure to verapamil causes morphological changes that, like in this research, make it impossible for the cells to be used to measure pharmacological responses. The techniques of this research can be extended to other antibiotics and antibiotic-drug combinations that may influence pharmacological responses, morphology and even the genetic composition of cell cultures. Antibiotics and antimycotics are toxic and may affect the recovery and proliferation of cell lines

different than those of the neuronal frontal and auditory cortex. However, one may elect to introduce antibiotics for short periods to primary cultures as an absolute last resort.

APPENDIX A

ALL EXPIREMENTS SUMMARY

Name	Expt Date.	Units	Reason
MS001	12-Oct-09		Osmolarity maintenance
MS002	8-Oct-09		Osmolarity maintenance
MS003	23-Oct-09		Osmolarity maintenance
MS004	3-Nov-09		Verapamil
Expt. MS005	12-Nov-09		Verapamil
Expt. MS006	12-Jan-10		Verapamil
Expt. MS007	13-Jan-10	35	Verapamil
Expt. MS008R1		13-Jan-10	22
	Verapamil Expt. MS008R2	13-Jan-10	
	22	Verapamil Expt.	
MS009	19-Jan-10	0	Bad culture
MS0010	20-Jan-10	0	Verapamil
Expt. MS0011	25-Jan-10	0	Bad culture
MS0012R1	25-Jan-10		Bad culture
MS0012R2	25-Jan-10		Bad culture
MS0012R3	26-Jan-10		Bad culture
MS0013R1	26-Jan-10		Broken MEA
MS0013R2	26-Jan-10		Bad culture
MS0014	1-Feb-10	18	Verapamil
Expt. MS0015	1-Feb-10	76	Verapamil
Expt. MS0016A		2-Feb-10	89
	Verapamil Expt. MS0016B	4-Feb-10	
	89	Verapamil Expt.	
MS0017A	6-Feb-10		Bad culture
MS0017B	6-Feb-10		Bad culture
MS0017C	6-Feb-10		Bad culture
MS0018	1-Mar-10		Bad culture
MS0020	9-Mar-10	69	Bic-Verap
Expt MS0020B	9-Mar-10	95	Bic-Verap
Expt MS0021R1		15-Mar-10	39
	Bic-Verap Expt MS0021R2	15-Mar-10	
	39	Bic-Verap Expt	
MS0021R3	16-Mar-10	39	Bic-Verap
Expt MS0025	22-Mar-10	78	Bic-Verap
Expt			

Name	Expt Date.	Units	Reason
MS0026	29-Mar-10	40	No activity
MS0027	3-Apr-10		Temp Fluctuations
MS0028	5-Apr-10		Bad culture
MS0029	7-Apr-10		Bad culture
MS0029B	7-Apr-10		Bad culture
MS0030	14-Apr-10	108	Verapamil Expt.
MS0030R1	14-Apr-10	108	Verapamil Expt.
MS0030R2	3-Apr-10	77	Verapamil Expt.
MS0032	19-Apr-10		Verapamil Expt.
MS0033	25-Apr-10	10	Bad culture
MS0034	3-May-10		Bad culture
MS0035	3-May-10	2	Bad culture
MS0036	27-May-10	9	Bad culture
MS0037	31-May-10	27	Bad culture
MS0040	2-Jun-10	38	Bad culture
MS0041	6-Jun-10		Bad culture
MS0042	6-Jun-10	79	Verapamil Expt.
MS0043	20-Sep-10	10	Verapamil Expt.
MS0044	22-Sep-10	19	Adhesion lost
MS0046	27-Sep-10		No activity
MS0049	5-Oct-10	45	Verapamil Expt.
MS0053R1	26-Oct-10	122	Verapamil Expt.
MS0053R2	27-Oct-10	122	Expt terminated
MS0055	1-Nov-10	40	Verapamil Expt.
MS0056	3-Nov-10	115	Verapamil Expt.
MS0059	9-Nov-10	115	Verapamil Expt.
MS0060	12-Nov-10	178	Gentamicin expt
MS0061	20-Nov-10	123	Gentamicin expt
MS0062	20-Nov-10	44	Gentamicin expt
MS0063	24-Nov-10	55	Gentamicin expt
MS0065	29-Nov-10		Low density culture
MS0066	29-Nov-10	54	Gentamicin expt
MS0070	12-Dec-10		No activity
MS0071	18-Dec-10	55	Taken over for DARPA

Name	Expt Date.	Units	Reason
MS0026	29-Mar-10	40	No activity
MS0027	3-Apr-10		Temp Fluctuations
MS0028	5-Apr-10		Bad culture
MS0029	7-Apr-10		Bad culture
MS0029B	7-Apr-10		Bad culture
MS0030	14-Apr-10	108	Verapamil Expt.
MS0030R1	14-Apr-10	108	Verapamil Expt.
MS0030R2	3-Apr-10	77	Verapamil Expt. Verapamil Expt.
MS0032	19-Apr-10		
MS0033	25-Apr-10	10	Bad culture
MS0034	3-May-10		Bad culture
MS0035	3-May-10	2	Bad culture
MS0036	27-May-10	9	Bad culture
MS0037	31-May-10	27	Bad culture
MS0040	2-Jun-10	38	Bad culture
MS0041	6-Jun-10		Bad culture
MS0042	6-Jun-10	79	Verapamil Expt.
MS0043	20-Sep-10	10	Verapamil Expt.
MS0044	22-Sep-10	19	Adhesion lost
MS0046	27-Sep-10		No activity
MS0049	5-Oct-10	45	Verapamil Expt.
MS0053R1	26-Oct-10	122	Verapamil Expt.
MS0053R2	27-Oct-10	122	Expt terminated
MS0055	1-Nov-10	40	Verapamil Expt.
MS0056	3-Nov-10	115	Verapamil Expt.
MS0059	9-Nov-10	115	Verapamil Expt.
MS0060	12-Nov-10	178	Gentamicin expt
MS0061	20-Nov-10	123	Gentamicin expt
MS0062	20-Nov-10	44	Gentamicin expt
MS0063	24-Nov-10	55	Gentamicin expt
MS0065	29-Nov-10		Low density culture
MS0066	29-Nov-10	54	Gentamicin expt
MS0070	12-Dec-10		No activity
MS0071	18-Dec-10	55	Taken over for DARPA

Name	Expt Date.	Units	Reason
MS0071	18-Dec-10	55	Taken over for DARPA
MS0072	21/12/2010	0	Ver CRC + Chronic Genta
MS0073	18-Dec-10	43	Genta (Low activity)
MS0075	21-Dec-10	47	Small cells, No activity
MS0076	18-Dec-10	27	Ver/Mus + Chronic genta
MS0077	2-Jan-11	0	Vera CRC+ Gentamicin
MS078	21-Jan-11	50	Gentamicin CRC (2steps)
MS079	22-Jan-11	35	FDU treated (no activity)
MS080	1/29/2011	0	No activity
MS084	2/5/2011	44	Acute Gentamicin Expt
MS087	5/9/2011	50	Verapamil CRC
MS088A	5/10/2011	63	Acute Gentamicin Expt
MS089	5/12/2011	0	Chronic Gentamicin Expt (Adhesion lost)
MS090	5/24/2011	0	Chronic Gentamicin Expt (Adhesion lost)
MS091	5/25/2011		Verapamil CRC
MS092	6/7/2011		Chronic Gentamicin Expt (Adhesion lost)
MS093	6/7/2011		Verapamil CRC
MS0315	3/15/2014	21	Vera CRC+ Gentamicin
MS0316	3/15/2014	19	Vera CRC+ Gentamicin

APPENDIX B

EXPLANATION OF ALL VERAPAMIL DOSE RESPONSE DATA AS GRAPHED IN FIGURE 8

. Summary of spike rate change (inhibition) **Verapamil DRC**

Single runs

Tissue	Expt no.	Age (DIV)	Ref Activity	Active Units	Sp. Cond	EC50	Concentrations of Verapamil (µM)																					
							0.5	1	1.5	2	2.5	3	3.5	4	5	6	7	8	9	10	11	12	13	14	15	18
							Percent spike rate																			
FC	MS005	35	235T	30	SERUM	3.8						47				66									
FC	MS007	21	145M	35		3.3		21		24		48		59	69	76	79	73	74	74	81	83	85	87	91
FC	MS008R1		3250T	22		2.6		23		38		54		74	78	85	89	79	83	83					
FC	MS030AR1		26500T	108		1.5	6	34	75	91	92	96	98						97	100					
FC	MS030BR1		12500T			1.4	20	39	62	78	87	92	96												
FC	MS042		7250T	77		2.3		27		41		59		76		93	100								
					Avg.	2.4	13	29	69	54	90	66	97	70	74	80	89	76	85	86					
					SD	1.1		7.6		29		22		11	6.4	9.5	7.1		12	13					

Low concentration verapamil 1st runs

					0.1	0.2	0.3	0.4	0.5	1	2	3	4	5	6	12
FC	MS053	31	23000T	1.1	9	13	22	39	43	46	52	61	63	83	87	96

Intraculture verapamil DRC (second runs)

						Concentrations of Verapamil (µM)																											
						0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1	1.1	1.2	1.3	1.4	1.5	2	2.5	3	3.5	4	5	5.5	6	7	8	9	10	11
FC	MS008R2	21	3500T	22	1									40						61		71		78	88		93	94	96	97	98	100
FC	MS030AR2		31600T		1.2	3	5	10	13	21				47					60	88	98											
FC	MS030BR2				1.2	0	0	0	0	0				29					57	71	93											
				Avg	1.3	5.3	12	16	18	23	55	57	59	62	45	67	71	74	76	65	73	96	71	88	78	88	94	93	94	96	97	98	100
				SD	y of	2.1	3.5	7.1	9.2	15				9.1					2.1	14	3.5											

*DIV: days *in vitro*

APPENDIX C

EXPLANATION OF ALL VERAPAMIL DOSE RESPONSE IN PRESENCE OF BICUCULLINE DATA AS GRAPHED IN FIGURE 10

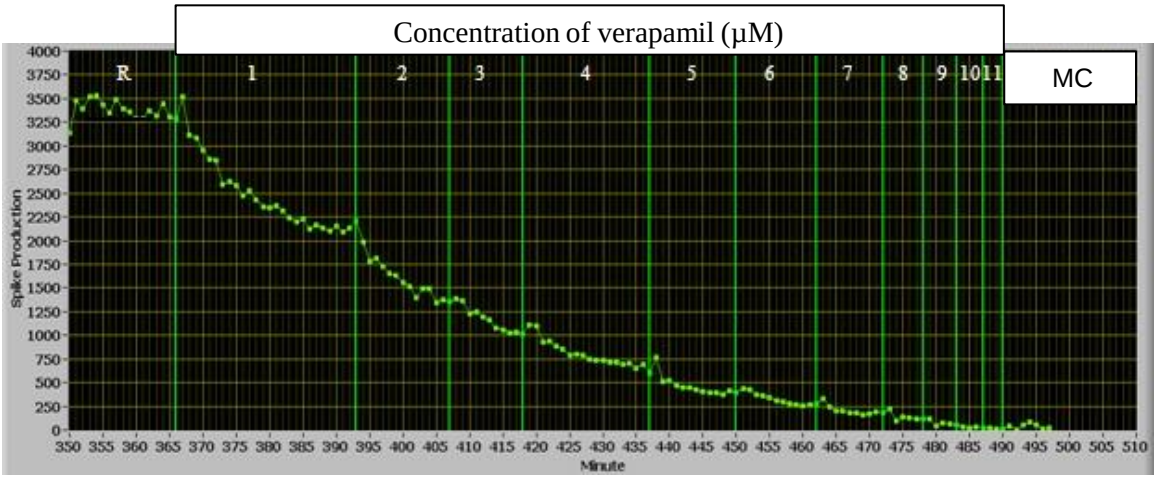
Summary of spike rate change (inhibition) **Verapamil DRC in presence of bicuculline**

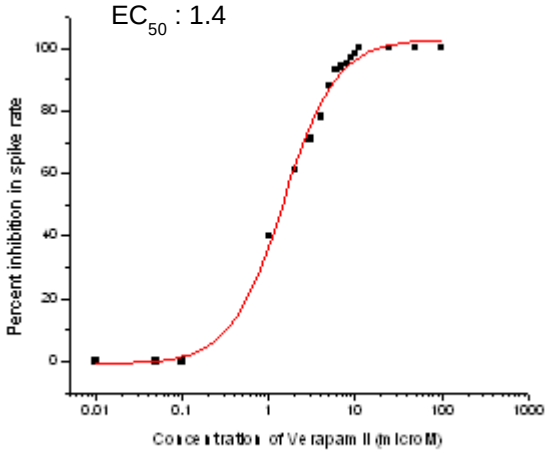
T	Expt no.	Age (DIV)	RA	Units	EC50	Concentrations of Verapamil (µM)																
						0.5	1	1.5	2	2.5	3	3.5	4	5	5.5	6	6.5	7	7.5	8	11	14	17
						Percent spike rate																	
FC	MS009	31	1750	24	5.7			9	12	15	15	23	39	43	46	55	58	63	66	69	86	98	100
FC	MS014	24	4000	18	1.6		38		50		75		83	84						95	98		
FC	MS015	35	17000	76	1.2	18	53		56		76		88	94		97		98					
FC	MS016A	23	21500	89	3.6	9	16	19	23		35		63	65		77		95					
FC	MS020A	27	35000	87	2.2		28		43		60		80	91		94		97		98			
FC	MS020B	22	52000	95	1.8		25		58		71		77	81		90		98		98			
FC	MS021R1	32	7500	39	1.1		57		70		76		80	90		93		97					
FC	MS021R2	33	6000	39	1.3		43		63		70		80	83		87		98					
FC	MS021R3	23	12000	39	1.2	8	29	70	83	96	100												

APPENDIX D

VERAPAMIL DOSE RESPONSE CURVES (UNTREATED)

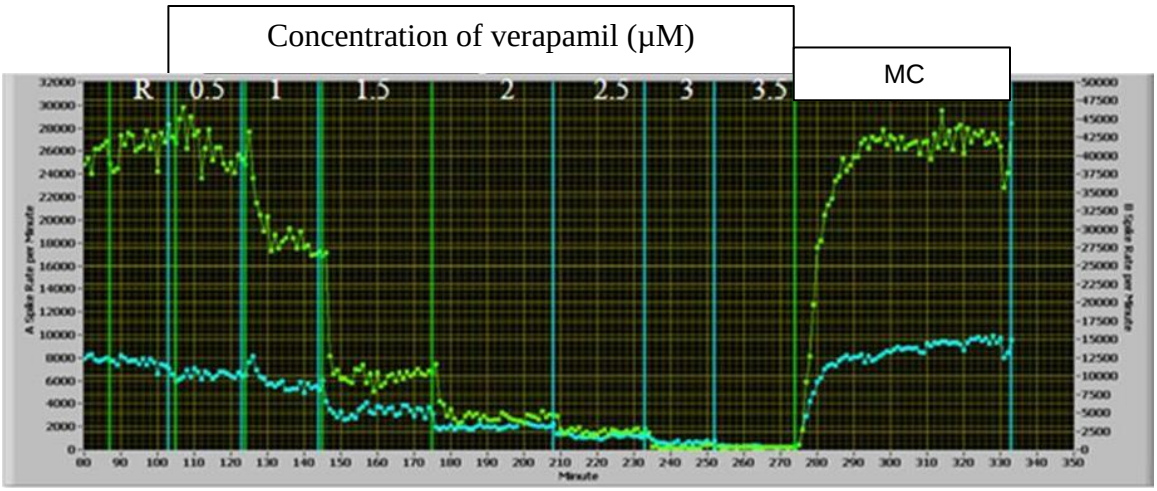
MS008R2

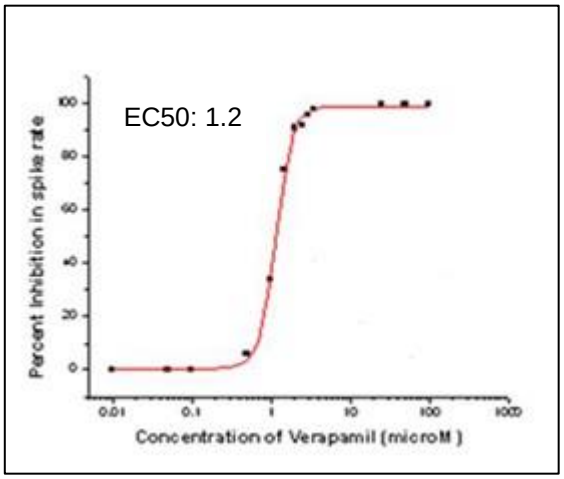




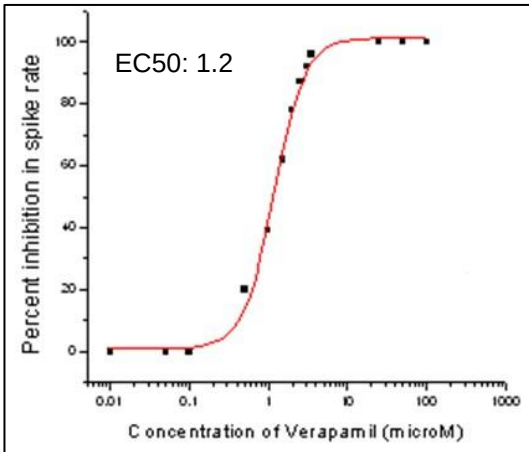
Conc.	Activity	%Inhib in spike rate
0.01	F	0
0.05	F	0
0.1	F	0
1	2100	40
2	1350	61
3	1000	71
4	750	78
5	400	88
6	250	93
7	200	94
8	150	95
9	100	97
10	50	98
11	0	100
25	F	100
50	F	100
100	F	100

MS030AR1 and MS030BR1



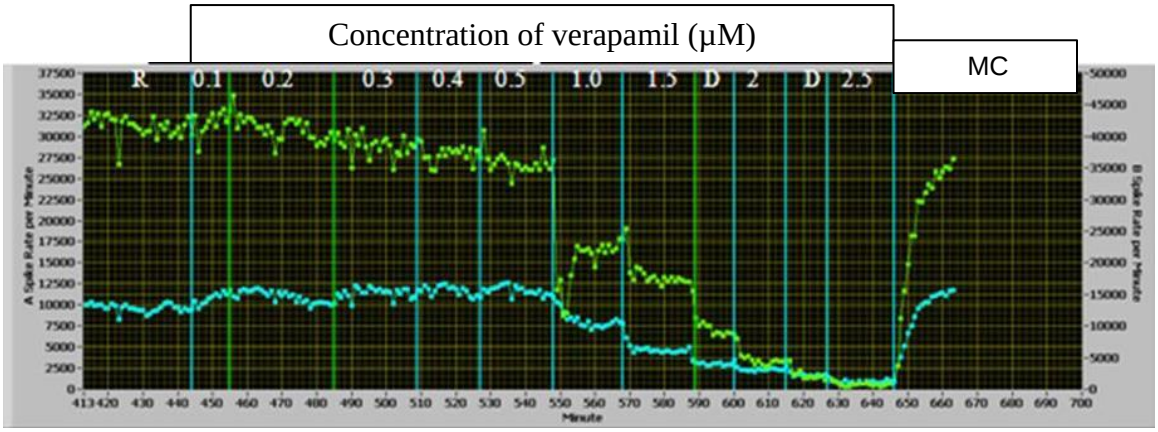


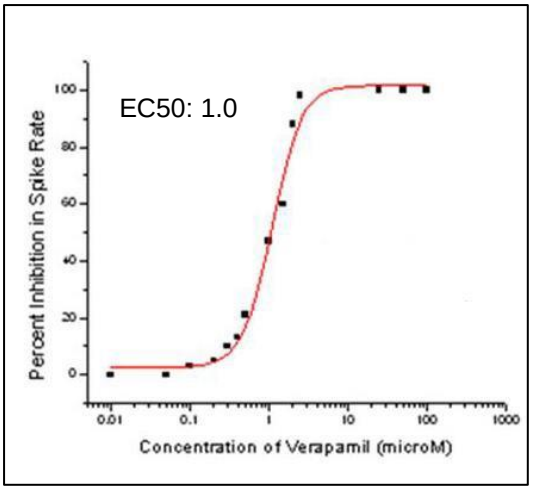
Ref Activity:	26500	TOT
Conc.	Activity	%Inhib in spike rate
0.01	F	0
0.05	F	0
0.1	F	0
0.5	25000	6
1	17500	34
1.5	6500	75
2	2500	91
2.5	2000	92
3	1000	96
3.5	500	98
25	F	100
50	F	100
100	F	100



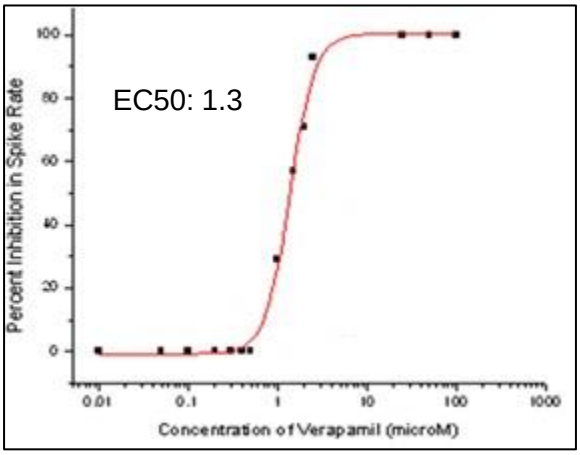
Conc	Activity	% Inhib in spike rate
0.01	F	0
0.05	F	0
0.1	F	0
0.5	10000	20
1	7600	39
1.5	4800	62
2	2700	78
2.5	1600	87
3	1000	92
3.5	500	96
25	F	100
50	F	100
100	F	100

MS030AR2 and MS030BR2



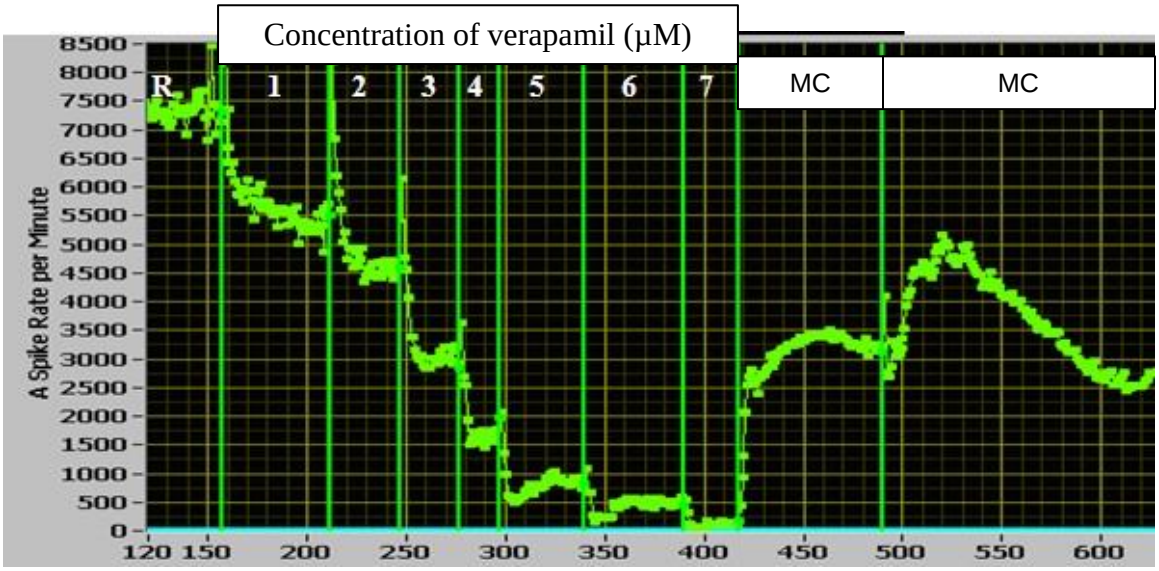


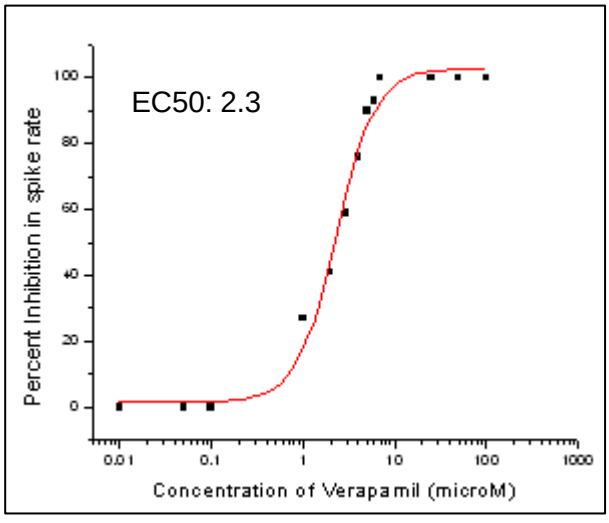
Conc.	Activity	% Inhib in spike rate
0.01	F	0
0.05	F	0
0.1	30800	3
0.2	30000	5
0.3	28300	10
0.4	27500	13
0.5	25000	21
1	16700	47
1.5	12500	60
2	3750	88
2.5	500	98
25	F	100
50	F	100
100	F	100



Conc.	Activity	% Inhib in spike rate
0.01	F	0
0.05	F	0
0.1	14000	0
0.2	14000	0
0.3	15000	0
0.4	15000	0
0.5	15000	0
1	10000	29
1.5	6000	57
2	4000	71
2.5	1000	93
25	F	100
50	F	100
100	F	100

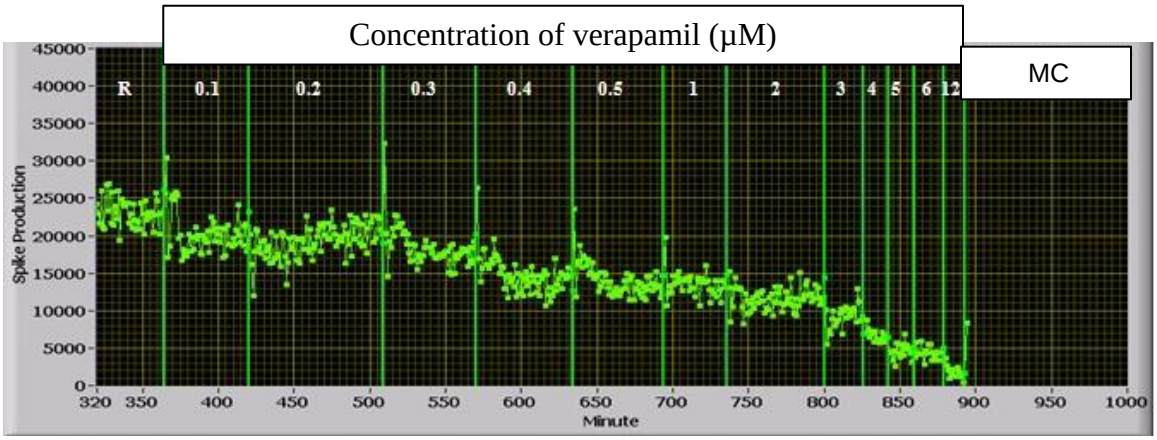
MS042

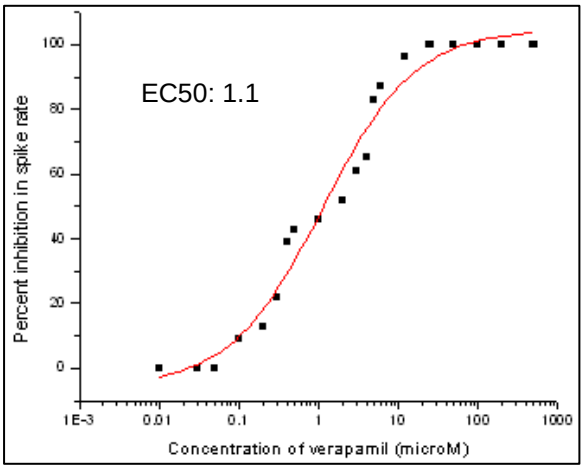




Conc.	Activity	% Inhib in spike rate
0.01	F	0
0.05	F	0
0.1	F	0
1	5250	27
2	4250	41
3	3000	59
4	1725	76
5	750	90
6	500	93
7	0	100
25	F	100
50	F	100
100	F	100

MS053



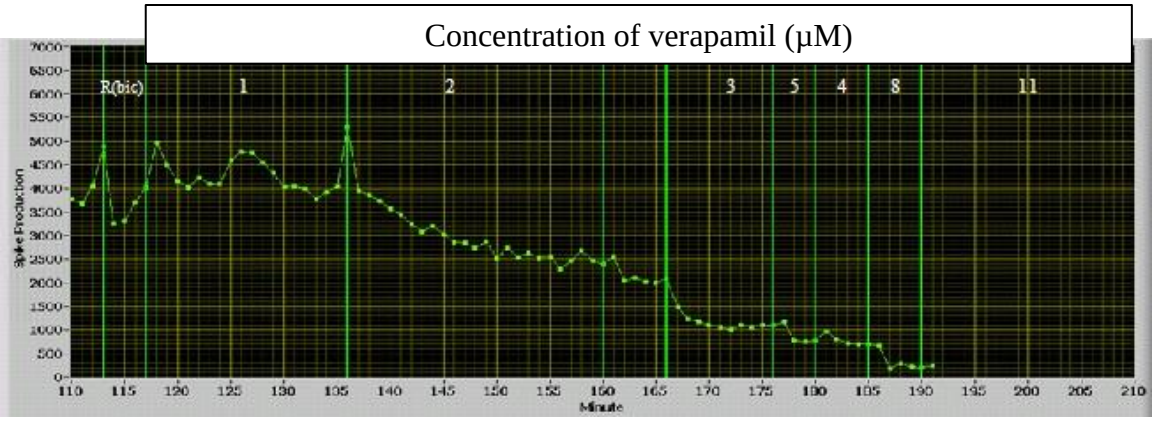


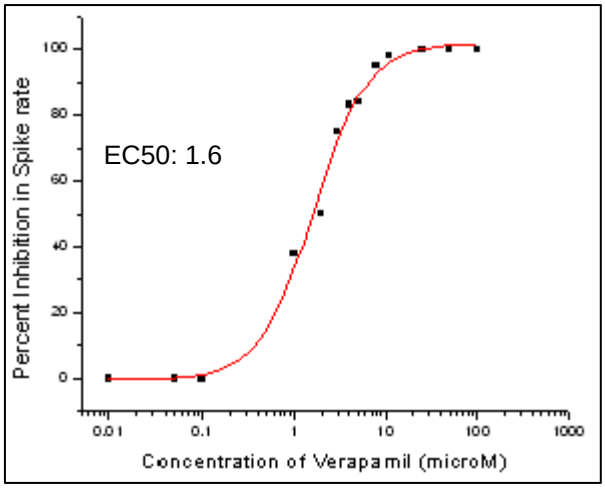
Conc.	Activity	% Inhib in spike rate
0.01	F	0
0.03	F	0
0.02	F	0
0.05	F	0
0.1	21000	9
0.2	20000	13
0.3	18000	22
0.4	14000	39
0.5	13000	43
1	12500	46
2	11000	52
3	9000	61
4	8000	65
5	4000	83
6	3000	87
12	1000	96
25	F	100
50	F	100
100	F	100
200	F	100
500	F	100

APPENDIX E

VERAPAMIL CRC IN PRESENCE OF BICUCULLINE

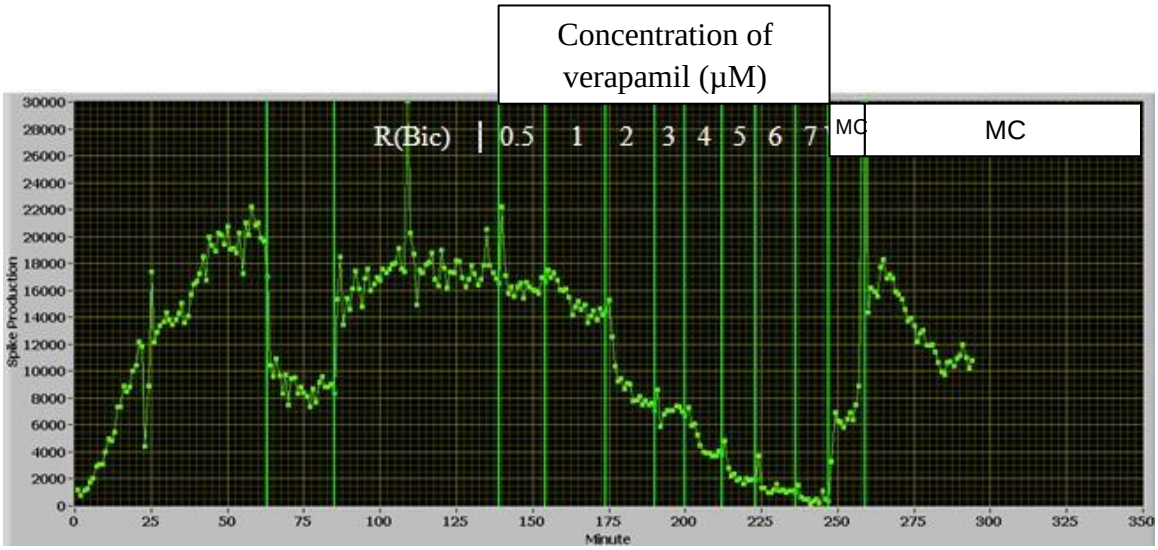
MS014

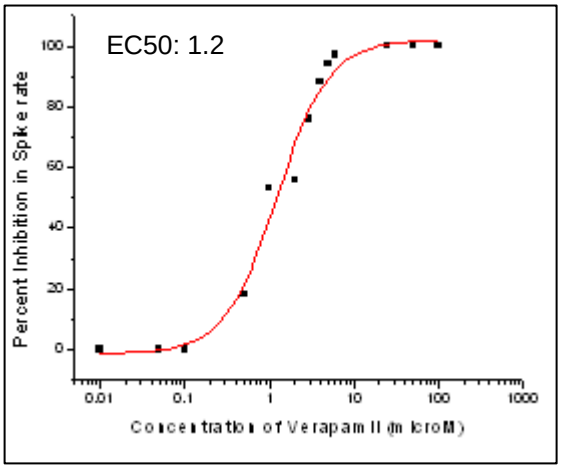




Conc.	Activity	% Inhib of Spike rate
0.01	F	0
0.05	F	0
0.1	F	0
1	2500	38
2	2000	50
3	1000	75
4	700	83
5	650	84
8	200	95
11	100	98
25	F	100
50	F	100
100	F	100

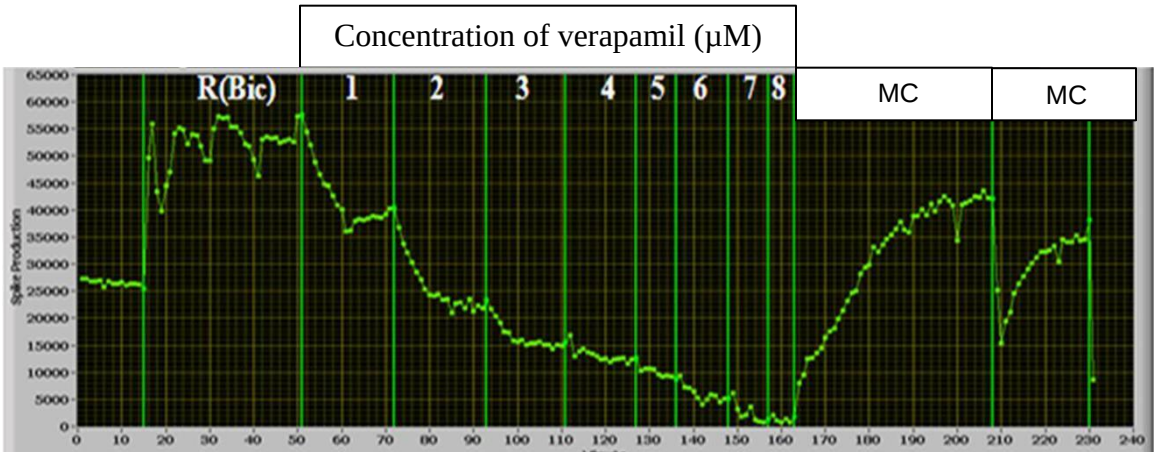
MS015

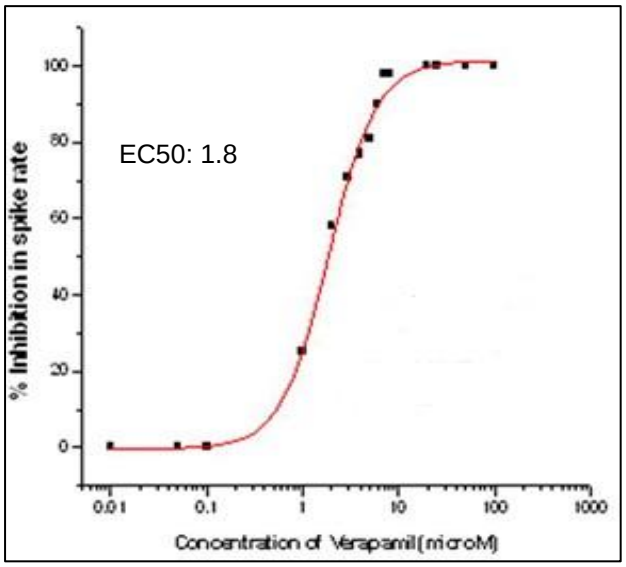




Conc.	Activity	% Inhib Of spike rate
0.01	F	0
0.05	F	0
0.1	F	0
0.5	14000	18
1	8000	53
2	7500	56
3	4000	76
4	2000	88
5	1000	94
6	500	97
7	100	98
25	F	100
50	F	100
100	F	100

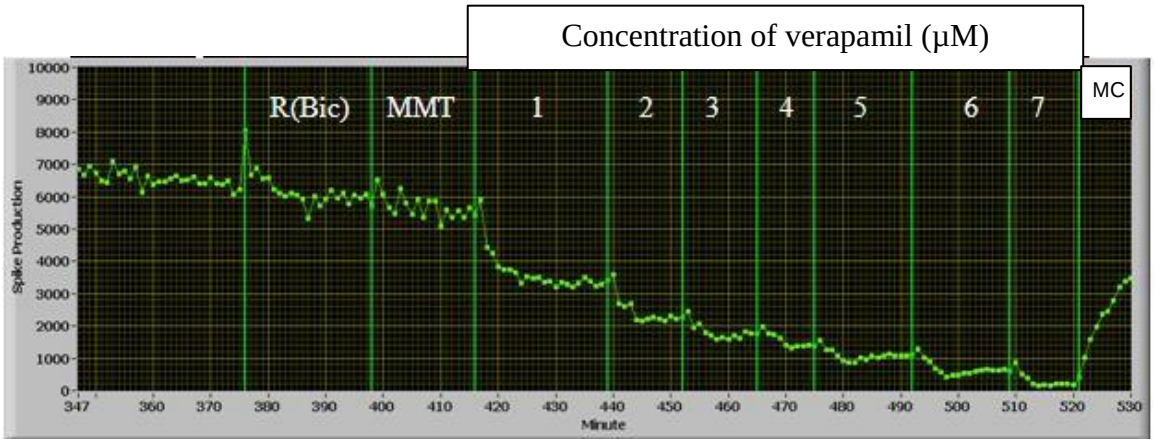
MS020B

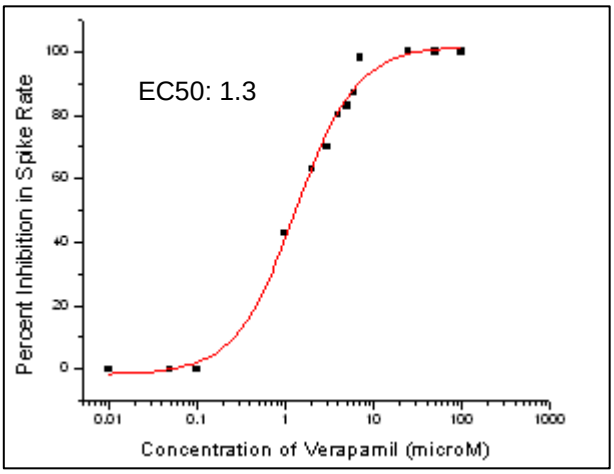




Conc.	Activity	%Inhib in spike rate
0.01	F	0
0.05	F	0
0.1	F	0
1	39000	25
2	22000	58
3	15000	71
4	12000	77
5	10000	81
6	5000	90
7	1000	98
8	900	98
20	F	100
25	F	100
50	F	100
100	F	100

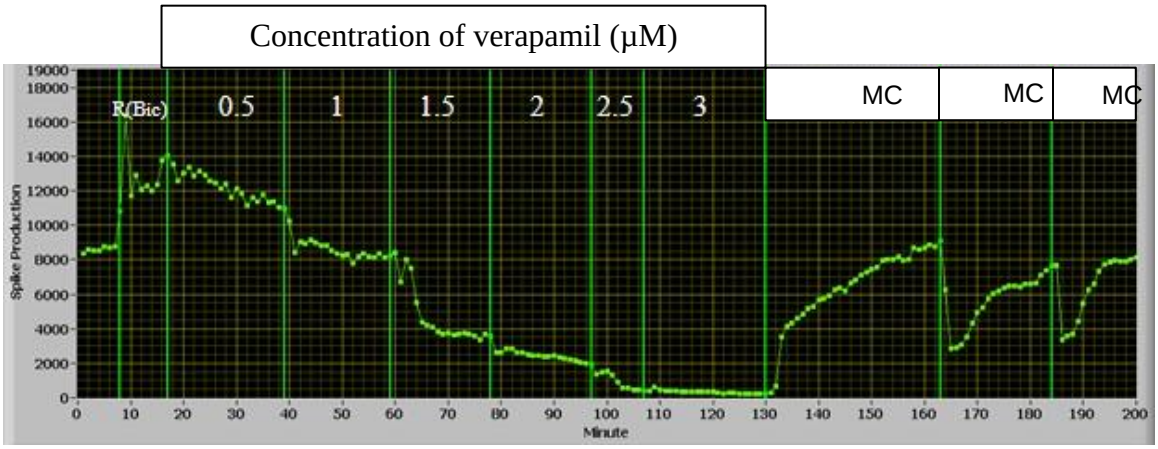
MS21R2

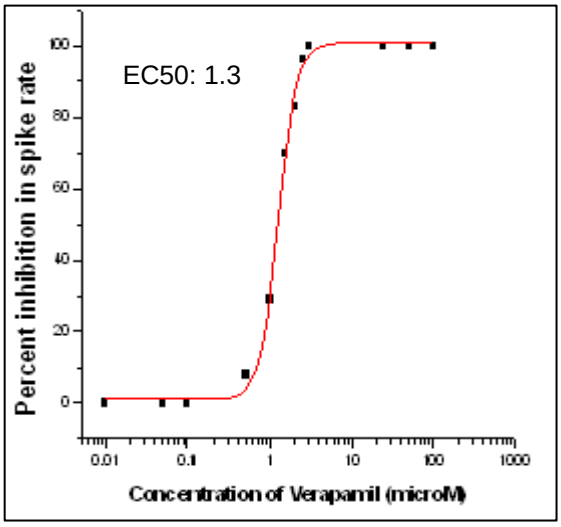




Conc.	Activity	% Inhib in spik rate
0.01	F	0
0.05	F	0
0.1	F	0
1	3400	43
2	2200	63
3	1800	70
4	1200	80
5	1000	83
6	800	87
7	100	98
25	F	100
50	F	100
100	F	100

MS21R3





Conc.	activity	% Inhib in spike rate
0.01	F	0
0.05	F	0
0.1	F	0
0.5	11000	8
1	8500	29
1.5	3500	70
2	2000	83
2.5	500	96
3	0	100
25	F	100
50	F	100
100	F	100

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