

ACUTE EFFECTS OF THE ANTIBIOTIC STREPTOMYCIN ON NEURAL NETWORK
ACTIVITY AND PHARMACOLOGICAL RESPONSES

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The purpose of this study is to find out that if antibiotic streptomycin decreases neuronal network activity or affects the pharmacological responses. The experiments in this study were conducted via MEA (multi-electrode array) technology which records neuronal activity from devices that have multiple small electrodes, serve as neural interfaces connecting neurons to electronic circuitry. The result of this study shows that streptomycin lowered the spike production of neuronal network, and also, sensitization was seen when neuronal network pre-exposed to streptomycin.

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CHAPTER 1

INTRODUCTION

The validation of in vitro platforms such as nerve cell networks on microelectrode arrays (MEAs), for use in toxicology, pharmacology, and drug development requires test result consistency across all laboratories. In vitro platforms can mimic the electrical signaling and pharmacological sensitivities of the parent tissue. In vitro platforms are essential to supplement animal experiments, especially in the field of pharmacology and toxicology. In the field of drug development, rapid pre-screening of compounds is necessary for detection of potential toxicity at the early stage. The current method of toxicity determination, which is the direct application on animals, not only fail to keep pace with the high rate of new chemical compound production but also gives pain and distress to animals (Russell & Burch, 1959). According to the report of the U.S. Environmental Protection Agency, from 3000 high production volume organic chemicals, produced more than a million pounds, only 7% received all the toxicity tests agreed internationally and 43% of them do not provide toxicity data to the public (EPA, 1998). There is no doubt that in vitro methods will start to be utilized widely. One in vitro method measures the electrical activity from many neurons in a spontaneously active neural network. The electrical activity is highly responsive to the change of pharmacological environment and the activity change is quantified. The Center for Network Neuroscience (CNNS) is the pioneer of MEAs technology, which allows us to record the change of electrical responses from many neurons through as many as 64 electrodes. CNNS was also the first laboratory that used in vitro MEA technology for studies of toxicology and pharmacology.

Neural networks for pharmacological investigations are grown on a MEA for 3 to 4 weeks. Although grown in a sterile environment, contaminations still occur in cell culture during

maturation. Because neural networks do not contain cells of the immune system such as phagocytic cells or natural killer cells, a few bacteria or spores of fungus in the network will grow without restriction and eventually stress cell culture leading irreversible damage to the network. Most laboratories solve this problem by applying antibiotics to the culture medium. However, according to previous data which will be discussed in the next section, antibiotics may alter neuronal networks. It is essential to determine whether the biochemical environment biases network responses, and one of these biochemical variables is the use of antibiotics.

Streptomycin is an antibiotic that is produced by the soil actinomycete *Streptomyces griseus*. It is widely used to get control over bacteria, fungi and algae growth. Streptomycin is a member of the aminoglycoside family. By binding to the 16S rRNA of the bacterial ribosome, it will interfere with the binding between formyl-methionyl-tRNA and the 30S subunit, and which prevents initiation of protein synthesis in bacteria. As a result, streptomycin is widely applied for treatment of serious infectious diseases, such as tuberculosis and brucellosis (Zhu et al., 2001; Singh and Mitchison, 1954). In 1945, soon after streptomycin was introduced into clinical practice, it was found that long-term usage of the calcium and sulphate salts of streptomycin may lead to deafness due to the intoxication of the cochlear system (Hinshaw & Feldman, 1945). But scientists soon concluded that streptomycin was well tolerated as long as the period of treatment is within two weeks and the daily dose is less than 3 grams (Walsh, 1947). Nevertheless, streptomycin is important in many areas, such as sperm preparation, crop protection, and laboratory research. In the field of sperm preparation, streptomycin is used as a pre-treatment of donor semen and is added to semen storage solution to extend sperm shelf life (Dissanayake, 2014). Streptomycin is also used as a pesticide for crop protection (Vidaver, 2002). In laboratory

settings, combining with penicillin (collectively: pen-strep), it is routinely supplemented to culture media to prevent bacterial growth (Schantz & Ng, 2004).

The general and ubiquitous use of pen-strep in cell culture raises the question of how these antibiotics affect the spontaneous activity of nerve cell networks and, especially, their pharmacological responses. Recent studies have demonstrated that mammalian networks grown on microelectrode array plates in vitro are histiotypic in that they mimic the pharmacological responses of the parent tissue (Yun & Gross, 2003). However, preliminary data exists that some antibiotics change quantitative pharmacological responses. It was published as a side note in an MS thesis (Rijal-Oli & Gross, 2008), and has not been investigated since that time. The pertinent data are shown in Figure 1. Cortical networks, derived from mouse embryos were exposed to 170 μ M pen-strep on day 5 with washout on day 7 (48 hours exposure). When these networks were used for experiments on day 27, a substantial sensitization to muscimol was noticed with IC₅₀ values (50% network activity decrease) shifting from 20 μ M to 5 μ M. Also, in Table 1, it's shown that different concentration of pen-strep has distinct effect on a cell culture. If these observations can be verified with more experiments, they would lead to an important experimental result that would place limits on the use of antibiotics and lead to re-examinations of earlier pharmacological data obtained from networks under antibiotics. It's shown that short exposure to pen-strep will sensitize the response to muscimol. That interested us since many papers have used the cell cultures that were treated with pen-strep, and if it will sensitize the pharmacological effect of other chemical, those data may not be trustworthy.

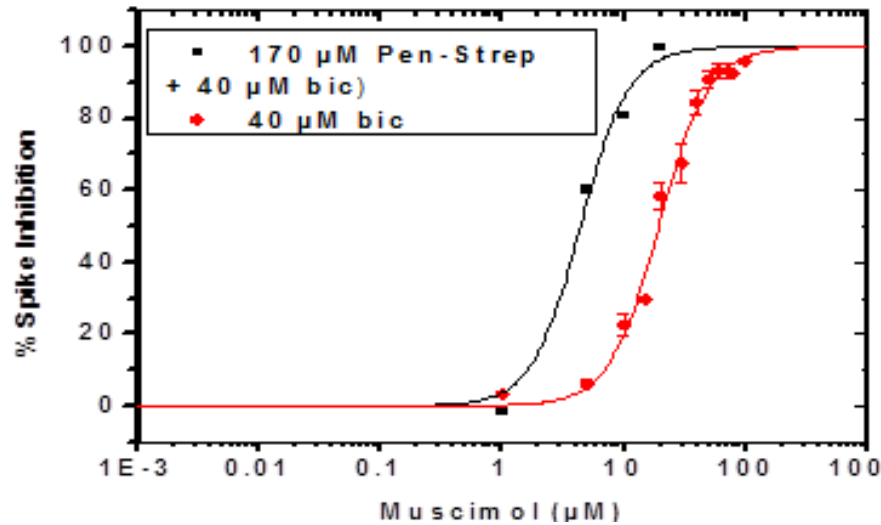


Figure 1. Concentration-response curve of penicillin-streptomycin treated culture shifts to the left without affecting maximum response, indicating greater sensitivity. Cell culture was pre-treated with 170 μM pen-strep for 48 hours on day 5, culture age 27 days in vitro (recording 22 days after pen-strep exposure). (From Rijal-Oli,. MS thesis, UNT 2008)

Table 1. Muscimol IC_{50} in Pen-Strep Pretreated Culture in the Presence of 40 μM Bicuculline (From Rijal-Oli,. MS thesis, UNT 2008)

Expt. No	EC_{50} (μM) in presence of 170 μM Pen-Strep (n = 5)	Expt. No	EC_{50} (μM) in presence of 57 μM Pen-Strep (n = 3)
SO068	4.4	SO054	13.2
SO068a	4.5	SO063	19.1
SO067	4.6	SO065	20.2
SO066a	7.9		
SO066b	4.2		
Mean \pm SD	5.1 \pm 1.6		17.5 \pm 3.8
Mean $\text{EC}_{50} \pm$ SD of non-treated culture is 19.25 \pm 3.54			

In a recently published paper, the neuronal effect of pen-strep was studied (Bahrami & Janahmadi, 2013). By using patch-clamp electrophysiology recording, the firing frequency of action potential was greatly reduced by the presence of pen-strep (100 $\mu\text{g}/\text{ml}$) at a seeding density of 1×10^6 cell/ml. In previous unpublished data from our lab (Figure 2), it's shown that even though penicillin and streptomycin both have effect on spike rate of primary neuronal cell culture, penicillin cause some excitability, while streptomycin is more influential and strongly inhibitory. Therefore, this study was focused on the study of the effect of streptomycin.

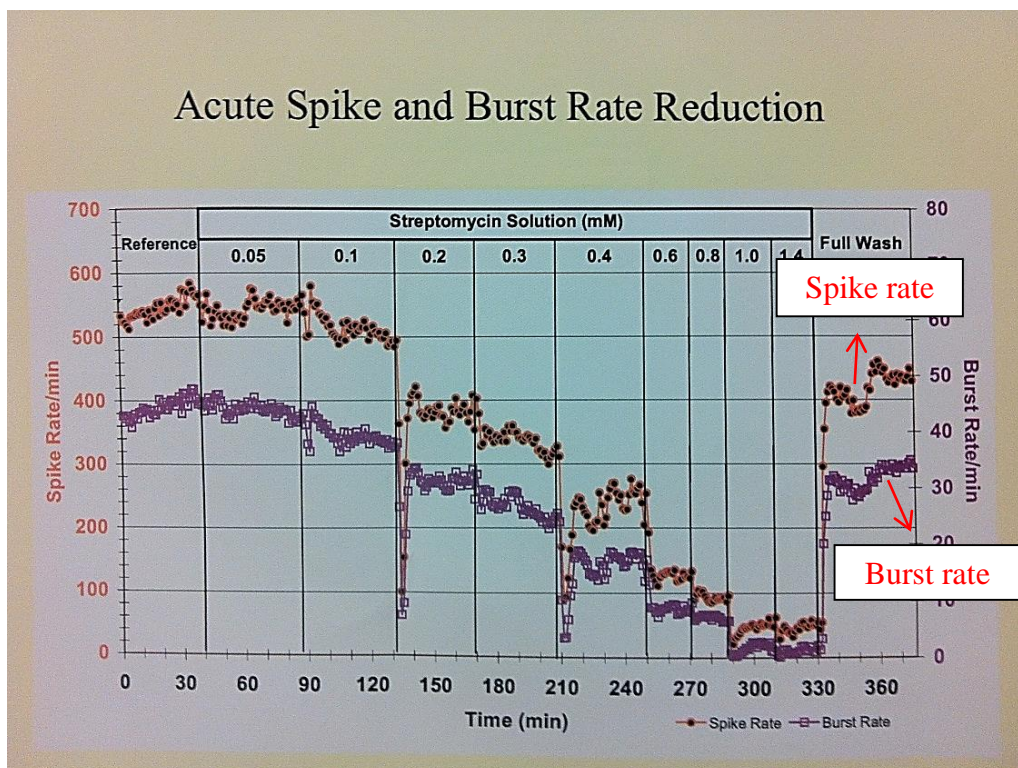
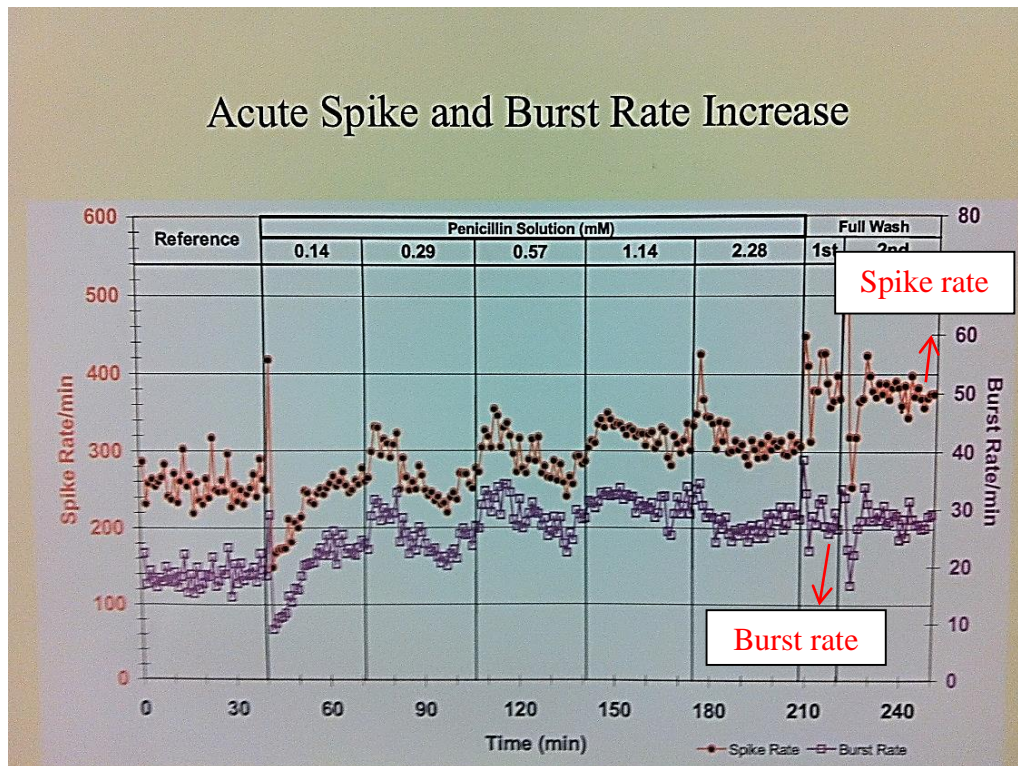


Figure 2. (a) Acute spike and burst rate increase in the presence of penicillin (From Hollmuller, Dayne, 2006, Biol 4900 project) unpublished results (b) Acute spike and burst rate decrease in the presence of streptomycin. For spike rate, IC_{50} of streptomycin is 0.33 mM. Spike rate didn't have recovery after a single medium change. (From Hollmuller, Dayne, 2006, Biol 4900 project) unpublished results

Table 2. Summary of Figure 2b

Molarity(mM)	REF	0.05	0.1	0.2	0.3	0.4	0.6	0.8	1.0	1.4	MC
Spike Production	560	550	500	380	320	250	120	100	50	50	0
Percent Decrease	N/A	1.8%	11%	32%	43%	55%	79%	82%	91%	91%	100%

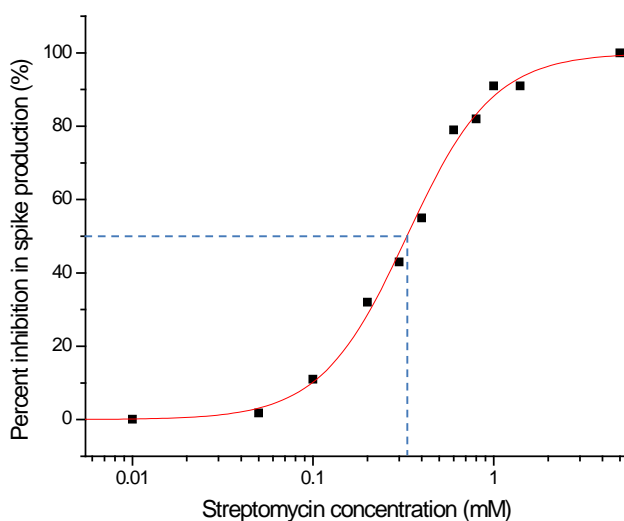


Figure 3. Concentration-response curve of Figure 2b. IC50 of streptomycin, indicated by the dash lines, is 0.33 mM. The graph was generated by using Origin Pro 7.0.

CHAPTER 2

OBJECTIVE AND SPECIFIC AIMS

2.1 Objective

Antibiotics are significant agents widely used to kill or inhibit the growth of bacteria. In vitro platforms, especially, rely on antibiotics to compensate for absence of an immune system. Nerve cell networks on microelectrode arrays (MEAs) have become popular for use in toxicology, pharmacology, and drug development. Antibiotics are used extensively by many laboratories using MEA technology. If antibiotics lead to the decrease of neuronal network activity, or affect the pharmacological responses, the application of antibiotics will need more caution. For this reason, I picked streptomycin, a major antibiotic that is often used in cell culture, as my object of investigation.

2.2 Specific Aims

1. Quantification of streptomycin effects on spontaneous activity of cortical networks
2. Effect of streptomycin on network pharmacological responses using muscimol as the primary test substance

CHAPTER 3

METHODS

3.1 MEA Preparation

Multi-electrode arrays (MEAs), also known as microelectrode arrays, are devices that have multiple small electrodes which serve as neural interfaces connecting neurons to electronic circuitry. There are two classes of MEAs: implantable MEAs that are used in vivo and non-implantable MEAs that are used in vitro. The latter are used by this laboratory.

MEA preparation and recording techniques have been described previously (Wu & Gross et al., 2014; Gopal & Gross et al., 2012; Gross, 1979). In short, the MEA staff etches the electrode pattern on glass plates that are sputtered with indium-tin oxide (ITO). This process generates a pattern of 8 μm wide conductors, terminating in 15 μm^2 terminal pads. The glass plates are spin-insulated with methyltrimethoxysilane (MTMS) followed by deinsulation of electrode tips with laser shots. Finally, the impedance is reduced to 1 $\text{M}\Omega$ at 1.0 kHz by electrolytical gold-plating. For the purpose of cell growth, a 3 mm diameter hydrophilic adhesion island in the center of the 64-electrode matrix is created by butane flaming and treated with poly-D-lysine and laminin.

3.2 Cell Culture

The care and use of animals that are involved in this study were approved by the guidelines of University of North Texas's institutional animal care and use committee. Mouse embryos were taken out on day E16 from mice under CO_2 narcosis followed by cervical dislocation. All culture procedure, were carried out by the CNNS culture staff.

The embryos' auditory cortices (AC) and frontal cortices (FC) were dissected and tissues were extracted. The culturing process was described previously (Gross, 1985). The AC and FC tissues are then mechanically dissociated and triturated, following with mixture with Dulbecco's modified minimal essential medium (DMEM) supplemented with 4% horse serum, 4% fetal bovine serum, and 2.0 ml/L B27 (obtained from GIBCO Products International; a cell culture supplement that contains vitamins, hormones, and other growth factors). The AC and FC cell suspension were seeded on the previously described adhesion island on MEAs at cellular concentration of 70K/100 μ l. To provide nutrition and remove waste, 50% of the medium of cell cultures were replaced twice weekly by fresh DMEM supplemented with 6% horse serum. After at least 21 days of growth in an incubator, the cell cultures were considered mature and were used in this study. Cultures were maintained at 37 degree Celsius, 300-320 mOsm, and pH of 7.3- 7.5. As an example, Figure 4 shows a 97 days old cortical neuronal network cultured on a 64-electrode MEA.

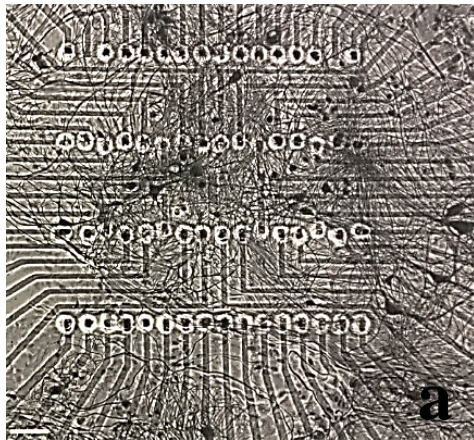


Figure 4. (a) Low density monolayer network consisting of approximately 90 neurons/ mm^2 growing over a 64 electrode-recording matrix 97 days after seeding. Transparent indium-tin-oxide conductors in center have 8 μm in diameter and are 100 nm thick. (b-d) Different regions of the matrix showing greater morphological details of the neurons in the cell culture Scale: Bar on left bottom corner is 80 μm (CNNS Archive)



3.3 MEA Recording

MEA recording was carried out under strict control of pH, temperature, osmolarity, and sterility (Gopal & Gross, 1996; Keefer & Gross, 2001). Cells that had been seeded on MEAs would be mature in an incubator after 21 days. MEAs were selected based on visible neurons on a carpet of glial cell and high number of interconnecting processes. The MEA was assembled into a custom recording chamber consisting of a heated base plate and a stainless steel chamber block (Figure 5). After 30 minutes of recording in original medium (DMEM), a medium change was performed to DMEM stock medium that didn't contain serum. This step was taken to avoid potential binding of test substance to serum. The pH of the medium was maintained between 7.3 and 7.5 by injecting 15 μ l air stream per minute with 10% CO². Sterility and clear microscopic observation were maintained by a cap on top of the chamber block featuring a heated indium-tin oxide window to prevent condensation. The temperature of the cell culture was maintained at 37 degree Celsius by connecting to a heater that had feedback from the thermocouple attached to the base plate. The osmolarity of the medium was maintained at about 320 mOsm by continuously pumping sterile water at the rate of 60 μ l/ hour to compensate for the loss of water from evaporation.

The activity of neuronal network was recorded by a 64-channel amplifier system from Plexon (Plexon, Dallas, TX). The system uses 64 digital signal processors (DSP), which digitize signals simultaneously at 40 kHz. The total system gain was set at around 11,000. To provide spike rate data from a single unit, spike identification and separation of spikes data acquired from a single electrode was processed by a real time template-matching algorithm (Plexon, Dallas, TX). Under optimal conditions (large signal-to-noise ratios), each DSP could collect data up to four different waveforms of action potential. After summing up multiple spike data per minute,

the total number of network spike production was divided by the number of active units detected each minute (floating average). Active units were defined as those with ten or more spikes per minute. Further analysis was performed using OriginPro (OriginLab, Northampton, MA).

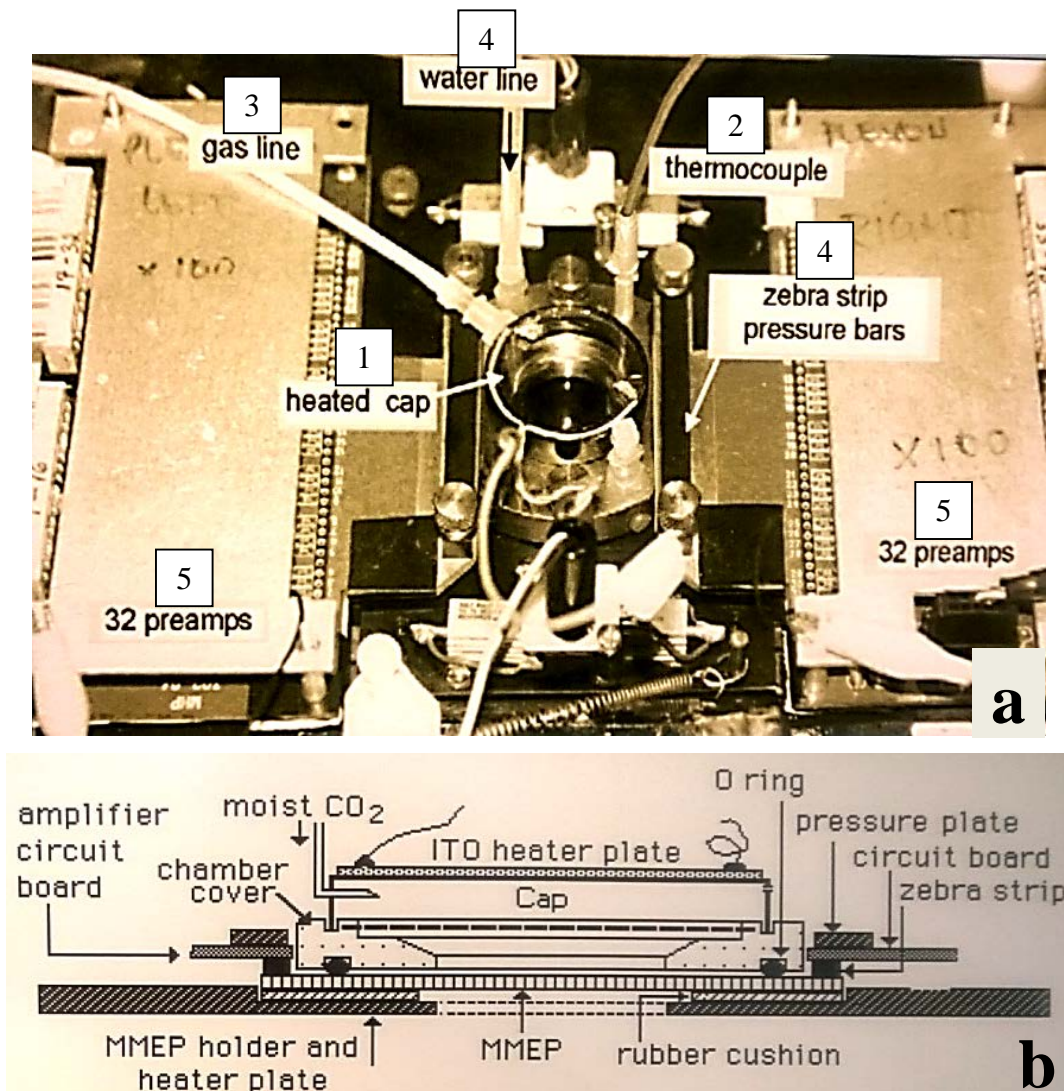
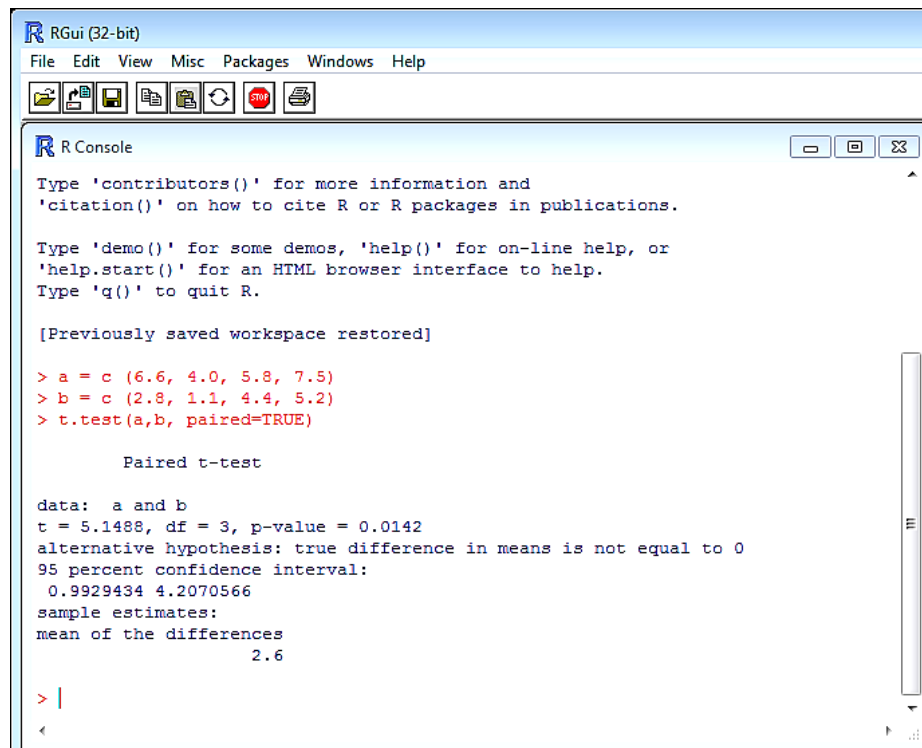


Figure 5. (a) Recording apparatus on inverted microscope stage (vertical view). Chamber containing the neuronal network on MMEP 4 with a constant medium bath of 2 ml. (1) cap is heated to prevent condensation for clear microscopic observation, (2) thermocouple provides feedback to the heater, (3) gas line provides air contains 10% CO₂ to keep the pH at 7.4, (4, 5) preamplifiers were placed to both sides of the recording chamber and connected to the MEA by means of zebra strips.
(b) Recording apparatus (schematic view)

(CNNS Archive)

3.4 Statistics:

Statistics in this study utilized R programming software. R, as show in Figure 6, is a language for statistical computing and graphics. R programming software is available as free open-source software that can be downloaded on the website: <http://www.r-project.org/>. R was initially written by Robert Gentleman and Ross Ihaka at the Statistics Department of the University of Auckland and later on developed by a core group with write access to the R source. R programming software provides a wide variety of statistical techniques, such as classical statistical tests, time-series analysis, and linear and nonlinear modelling.



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File Edit View Misc Packages Windows Help

R Console
Type 'contributors()' for more information and
'citation()' on how to cite R or R packages in publications.

Type 'demo()' for some demos, 'help()' for on-line help, or
'help.start()' for an HTML browser interface to help.
Type 'q()' to quit R.

[Previously saved workspace restored]

> a = c (6.6, 4.0, 5.8, 7.5)
> b = c (2.8, 1.1, 4.4, 5.2)
> t.test(a,b, paired=TRUE)

      Paired t-test

data:  a and b
t = 5.1488, df = 3, p-value = 0.0142
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 0.9929434 4.2070566
sample estimates:
mean of the differences
                2.6

> |
```

Figure 6. R programming software. R is a powerful program to use in statistical computing. It's an open-resource program that is available on <http://www.r-project.org/>. In this figure, a paired t-test is performed.

A paired t-test was utilized in Figure 20 when comparing IC₅₀ of muscimol before and after short exposure to 0.1 mM streptomycin. The null hypothesis is that the mean difference between paired observations is zero. Notice that unlike two-sample t-test, paired t-test focuses on the difference before and after treatment within individual experiments. Each experiment forms a pair (before and after treatment) and the difference within each pair are then compared by paired t-test. In this study, the paired t-test was picked rather than the two-sample t-test because the standard deviations within each group were large: the mean IC₅₀ of muscimol before exposure to streptomycin was $6 \pm 1.5 \mu\text{M}$, and the mean IC₅₀ after exposure was $3.4 \pm 1.8 \mu\text{M}$. Therefore, big sample sizes are needed to show the significant difference between two groups if the method was a two-sample t-test.

3.5 Chemicals

Muscimol is the major psychoactive alkaloid present in many mushrooms of the *Amanita* genus. Muscimol is a potent, selective agonist for the GABA_A receptors and displays sedative-hypnotic and dissociative psychoactive effects. According to the literature and previous data in this lab, muscimol will not easily break down and it has a rather stable IC₅₀ and great reversibility, which means that one to two medium changes can wash it out of the system. Thereby, dose-response curve of muscimol titration were used to test whether streptomycin has an effect on pharmacological responses. Muscimol was obtained from Sigma-Aldrich (St. Louis, MO) in powder form.

Bicuculline is a competitive antagonist of GABA_A receptors. It was found in plant alkaloid extracts and it is often used to mimic epilepsy since it blocks the inhibitory action of GABA receptors. In this study, bicuculline was used to stimulate cell cultures that had low spike

rate (<100 spikes/minute) or to regulate highly irregular native activity of neuronal network.

Bicuculline was obtained from Sigma-Aldrich (St. Louis, MO) in powder form.

Streptomycin was obtained from Sigma-Aldrich (St. Louis, MO) in powder form.

CHAPTER 4

RESULTS

4.1 Streptomycin Pharmacology and Toxicity

Streptomycin was added stepwise (0.1mM in each step except for the last 3 steps) to see its inhibiting effect on neuronal network. Figure 7 (Experiment WT025) is one of the experiments. In the beginning the spike rate per minute was around 15000, and after adding 0.2 mM and 0.3 mM of streptomycin, the spike rate per minute dropped to 8400 and 6000 relatively; hence we can tell that the IC₅₀ value of streptomycin lies between 0.2 and 0.3 mM. At the concentration of 1.0 mM of streptomycin, we can see that the spike rate per minute almost dropped to 200; comparing with the reference of 15000 spikes per minute, we can see that it lost 99% of activity. At the end of the experiment, there are 2 medium changes to show the recoverability. After acquiring data for each point, we created a titration table: Table 3. And by plotting two rows of molarity and percent decrease in OriginPro, we created a semi-logarithmic dose-response curve: Figure 8. OriginPro also showed the data of this experiment in Table 4 that contains chi square/degree of freedom, IC₅₀, and power. This research laid the focus on IC₅₀.

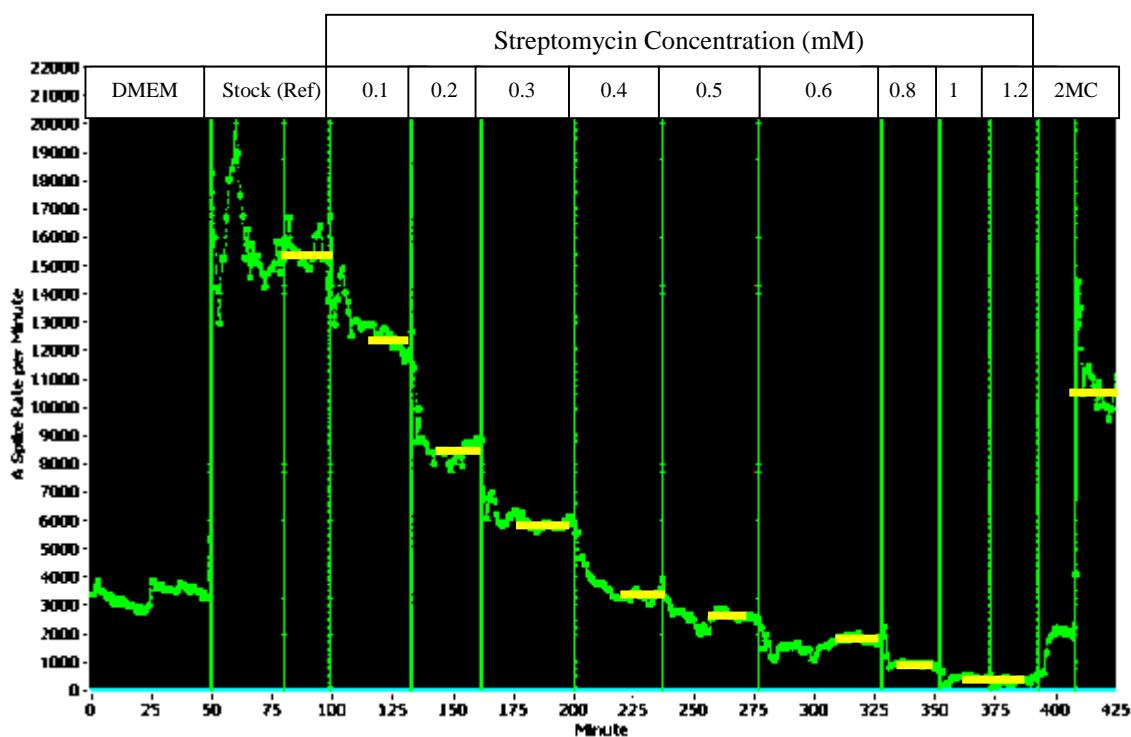


Figure 7. Streptomycin titration showing stepwise decreases in activity with increasing concentration of streptomycin (WT025). The unit of X axle is spike rate per minute while the unit of Y axle is minute. Each dot indicates the sum of spike production from all active units in a minute. Horizontal lines represent the time period of level plateau used for calculation. Partial recovery was shown after 2 medium changes at the end of experiment.

Table 3. Streptomycin Titration Table. This table was created according to data from Figure 7. In row 2, the horizontal lines in Figure 7 indicate the value of spike production for each concentration. In row 3, the value of percent decrease was created in relative to reference value (15000 as 100%).

Molarity (mM)	REF	0.1	0.2	0.3	0.4	0.5	0.6	0.8	1.0	1.2	2MC
Spike Production	15000	12500	9000	6000	3500	2500	1800	1000	500	400	10500
Percent Decrease	N/A	17%	40%	52%	77%	83%	88%	93%	97%	97%	30%

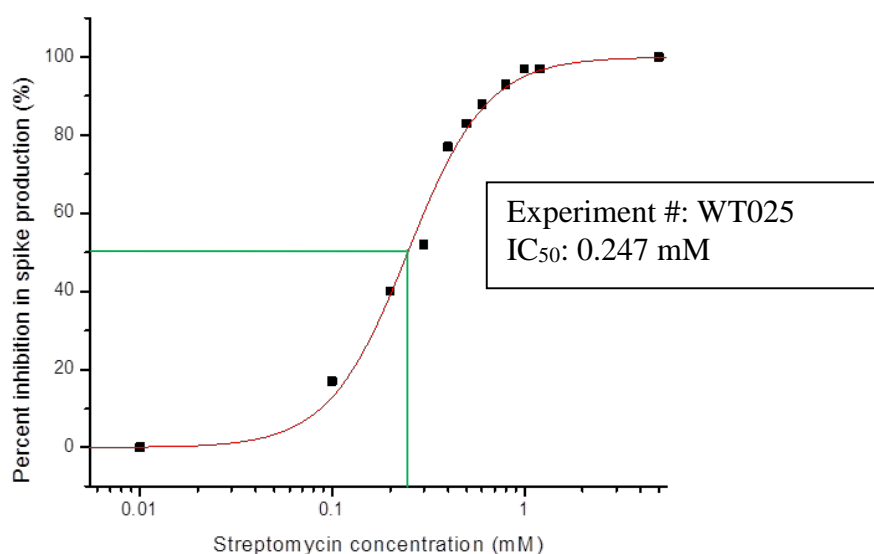


Figure 8. Dose-response curve of streptomycin titration. This figure was created from Table 3 by OriginPro. The two lines indicate the IC₅₀ value of streptomycin, which is at 0.247mM.

Table 4. Dose-Response Curve Data. This table was created according to data from Figure 7 by OriginPro. The value and error of chi square/degree of freedom, IC₅₀, and power are shown. The value of initial and final were fixed respectively at 0 and 100. IC₅₀ values will be the focus of this study.

Parameter	Value	Error

Chi ² / DoF	11.60243	
Initial (A1)	0	0
Final (A2)	100	0
IC50 (x0)	0.24725	0.00951
Power (p)	2.11103	0.15202

Table 5 and Figure 9 shows the inhibition effect of streptomycin from 4 experiments indicating high reproducibility of streptomycin inhibition. The mean IC₅₀ is 0.27 ± 0.07 mM (n=4), which is slightly higher than the recommended concentration for cell culture (0.17 mM) (<http://www.atcc.org/products/all/30-2300.aspx>). Noticeably, comparing to Figure 2b, which was conducted in 2007 in CNNS by Dayne Hollmuller, we can see that this experiment and his

experiment have very similar IC₅₀ of streptomycin (0.27 ± 0.07 and 0.33 mM respectively). At 0.17 mM of streptomycin, spike production is lower by 20%. Figure 10 shows that long-term exposure to high concentration of streptomycin (0.9 mM) may have permanent irreversible effect; only one experiment was done because the high concentration is not normally used in animal experiment. No further study was conducted.

Table 5. Data of Streptomycin Titration Experiments (n=4). All of the tissues are from frontal cortex. Stock medium were used as cell medium. Date of experiment, age of the cell culture after being seeded, number of units recorded, IC₅₀ of streptomycin, reversibility, and total shutoff time (at 95% shutoff compared to the reference) are shown. Notice that WT018 didn't recover after washes. The reason may be that the shutoff time is longer.

	Date	Age (days)	# of units	IC ₅₀ (mM)	Reversibility (after 2 medium changes)	Total shutoff time (minutes)
WT018	2/6/2013	49	18	0.36	None	1000
WT024-1*	4/4/2013	21	27	0.31	100%	80
WT024-2*	4/5/2013	22	19	0.19	100%	60
WT025	4/10/2013	28	31	0.25	70%	65

*: WT024-1 and WT024-2 were conducted on the same network but on different days (time between experiments is 800 minutes)

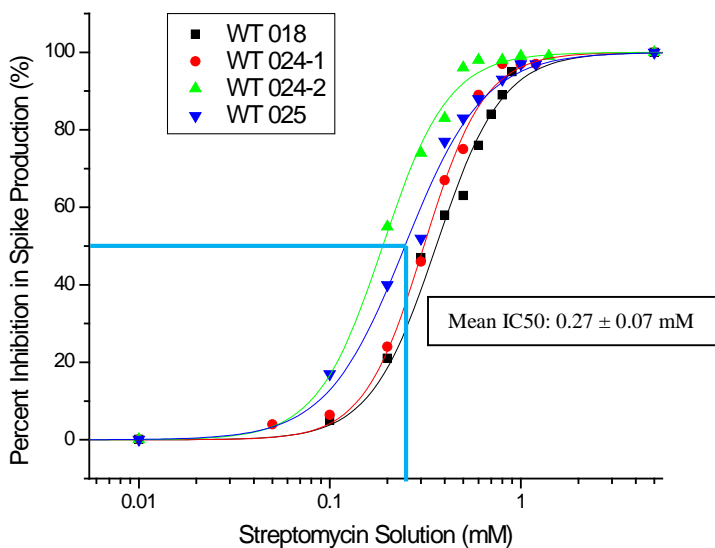


Figure 9. Streptomycin titration curves based on Table 5(n=4). The mean IC₅₀ of Streptomycin is 0.27 ± 0.07 mM.

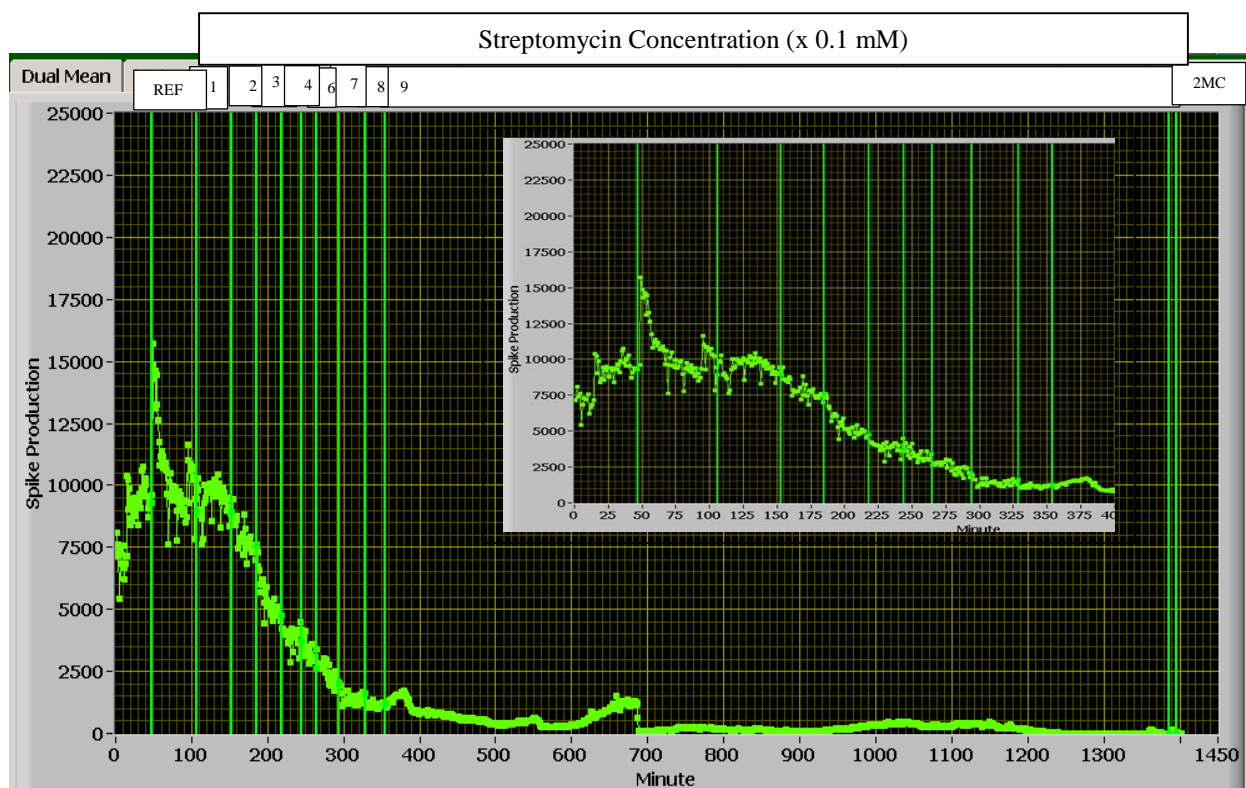


Figure 10. Streptomycin titration followed by long term (1000 minutes) exposure to 0.9 mM streptomycin. Reversibility cannot be shown after 2 medium changes. Insert shows the detail of the first 400 minutes. (WT018).

4.2 Muscimol Titration

Because of its potency and reversibility, muscimol acts as a great test chemical to determine whether streptomycin has an effect on the concentration response of a test substance. In Figure 11, the mean IC₅₀ of muscimol without bicuculline is $0.12 \pm 0.012 \mu\text{M}$ (n=5) and in Figure 12, the mean IC₅₀ of muscimol with 40 μM bicuculline is much lower: $5.24 \pm 1.61 \mu\text{M}$ (n=9)

Table 6. Data of Muscimol Titration Experiments (n=5). Date of experiment, age of the cell culture after being seeded, number of units recorded, IC50 of muscimol, reversibility, and total shutoff time (at 95% shutoff compared to the reference) are shown.

	Date	Age (days)	# of units	IC50 (μM)	Reversibility	Total shutoff time (minutes)
WT039	7/29/13	27	33	0.10	100% (1 wash)	25
WT041-1*	8/26/13	27	32	0.14	92% (1 wash)	25
WT041-2*	8/26/13	27	16	0.12	94% (1 wash)	50
WT049-1**	10/2/13	22	26	0.11	84% (2 washes)	30
WT049-2**	10/2/13	24	25	0.11	64% (2 washes)	25

*: WT041-1 and WT041-2 were conducted on the same network in different days (time between experiments is 700 minutes).

**: WT049-1 and WT049-2 were conducted on the same network on the same day at different time (time between experiments is 40 minutes).

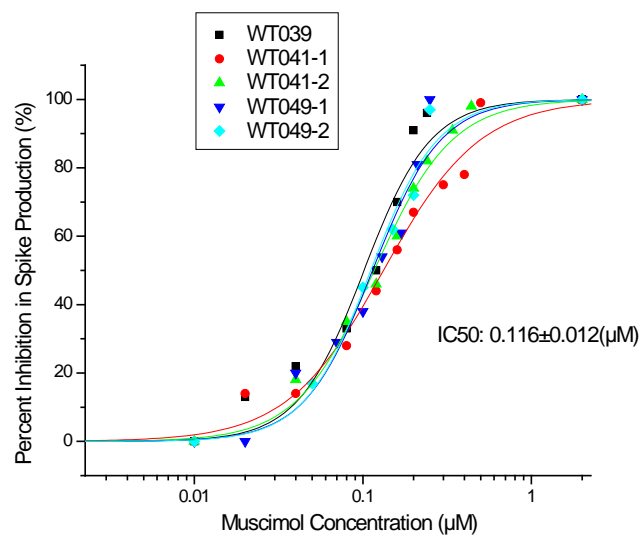


Figure 11. Muscimol Titration Curve without Bicuculline based on Table 6 (n=5). The mean IC50 of muscimol was $0.12 \pm 0.012 \mu\text{M}$.

Table 7. Data of Muscimol Titration Experiments under 40 μM Bicuculline (n=9). Date of experiment, age of the cell culture after being seeded, number of units recorded, IC₅₀ of muscimol, reversibility, and total shutoff time (at 95% shutoff compared to the reference) are shown.

	Date	Age (days)	# of units	IC ₅₀ (μM)	Reversibility	Total shutoff time (minutes)
WT027-1	4/19/13	36	54	6.6	100% (1 wash)	40
WT028-1	4/24/13	42	30	4.0	100% (2 washes)	50
WT031-1*	5/26/13	47	30	3.0	100% (2 washes)	30
WT031-2*	5/27/13	48	25	4.5	0% (2 washes)	30
WT034	7/10/13	36	15	7.3	41% (2 washes)	25
WT050-1**	10/8/13	28	38	5.8	100 % (1 wash)	30
WT050-3**	10/9/13	29	45	4.4	100% (1 wash)	30
WT054-1	10/30/13	36	36	7.5	72% (1 wash)	30
WT056-1	11/12/13	34	60	4.1	100% (1 wash)	25

*: WT031-1 and WT031-2 were conducted on the same network on different days (time between experiment is 620 minutes).

** : WT050-1 and WT050-3 were conducted on the same network on different days (time between experiment is 560 minutes).

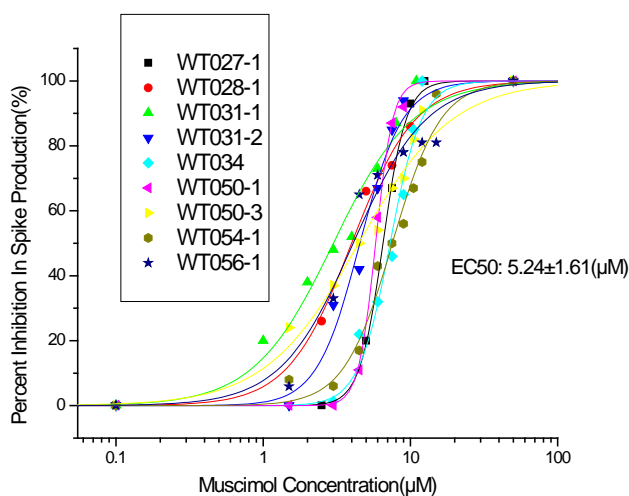


Figure 12. Muscimol Titration Curve with 40 μM Bicuculline (n=9). The mean IC₅₀ of muscimol is $5.24 \pm 1.61 \mu\text{M}$.

4.3 Acute Streptomycin Effects on Muscimol Pharmacology

A muscimol titration was done first as reference and the second muscimol titration was done after cell cultures were exposed to 0.1 mM streptomycin for a period of time (30-70 mins). All of the experiments were done with 40 μ M bicuculline. The streptomycin in the medium was not washed out until a second muscimol titration was finished. In Figure 13, 14, 15, and 16 we can see that after short exposure of streptomycin, the IC₅₀ drops significantly: $46.3 \pm 22.7\%$. Notice that in Figure 16, a muscimol titration was done after washing out the streptomycin but IC₅₀ of muscimol did not recover.

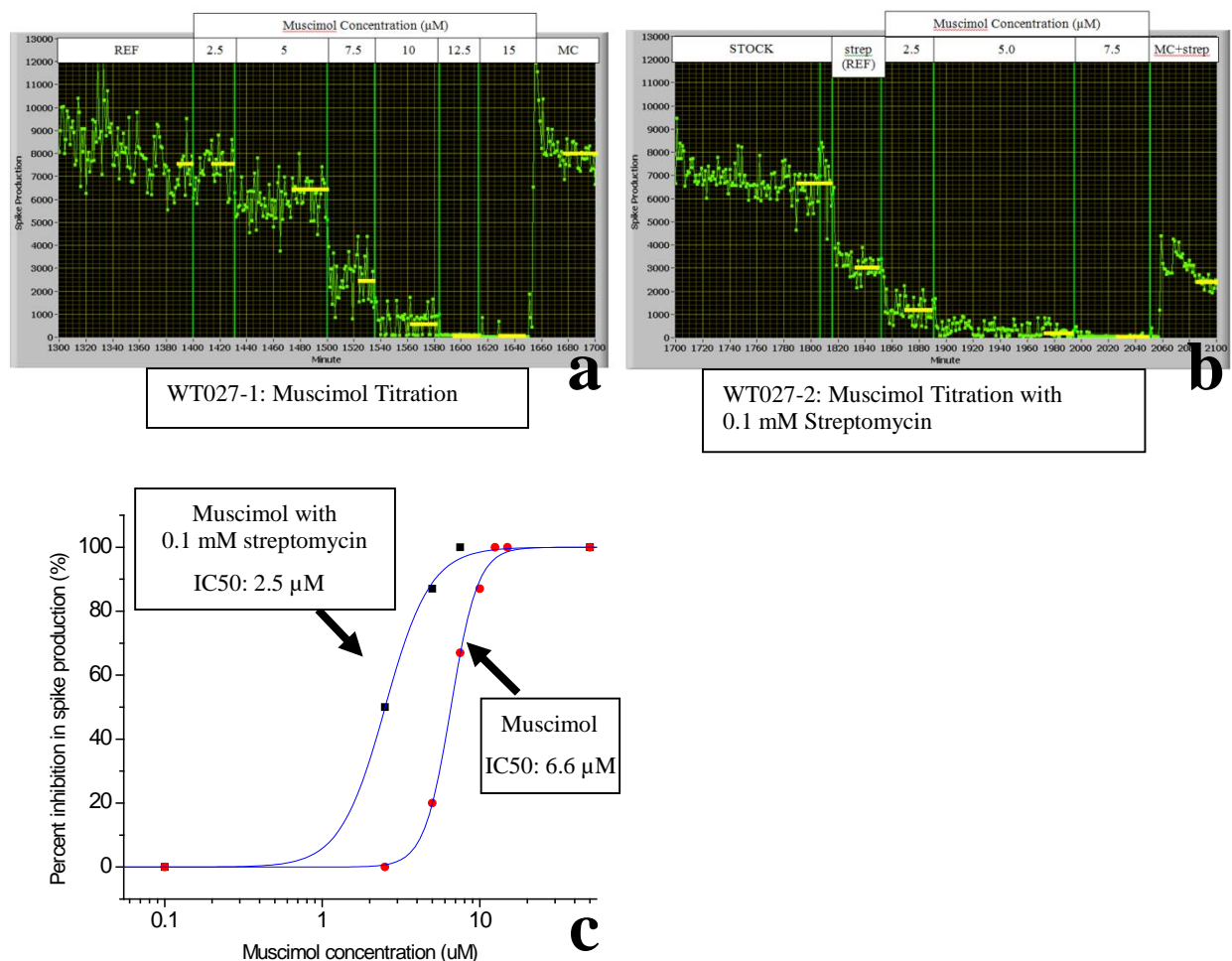
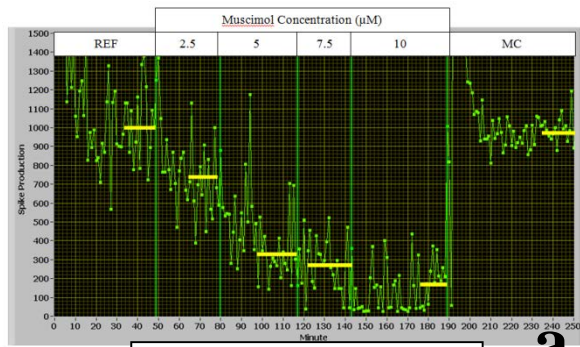
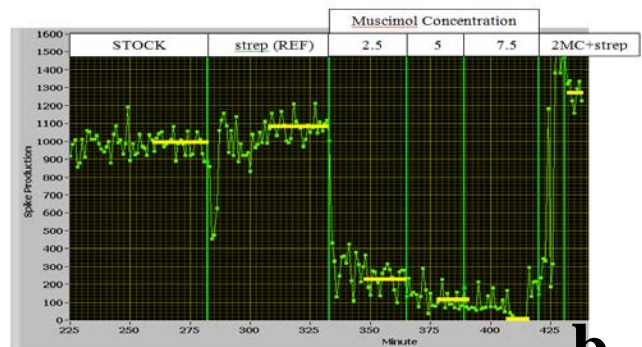


Figure 13. (a) Muscimol titration (WT027-1). IC₅₀ was 6.6 μ M under 40 μ M bicuculline. (b) Muscimol titration with 0.1mM streptomycin (WT027-2). IC₅₀ was 2.5 μ M under 40 μ M bicuculline. (c) Combined dose response curves of WT027-1 and WT027-2. The shift to the left is caused by exposure to streptomycin.



WT028-1: Muscimol Titration



WT028-2: Muscimol Titration with 0.1 mM Streptomycin

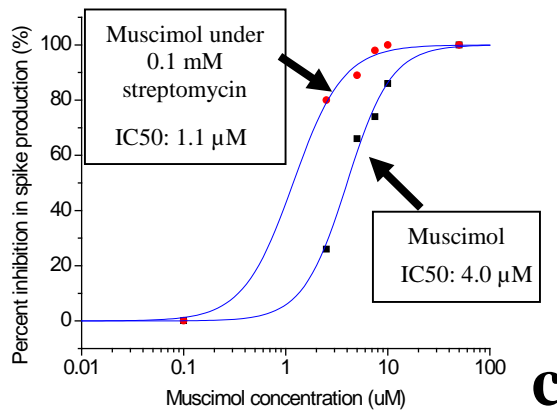
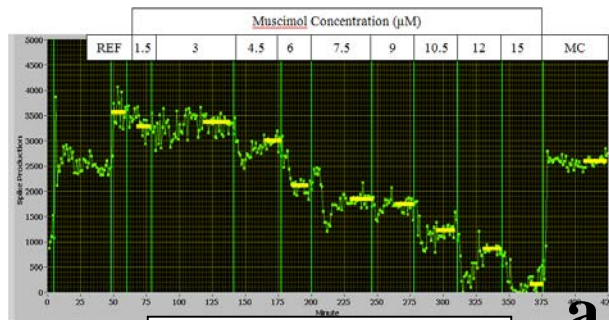
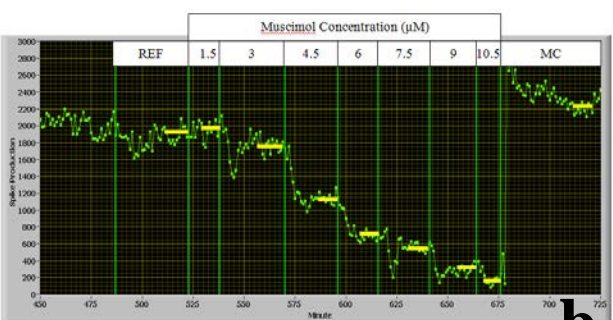


Figure 14. (a) Muscimol titration (WT028-1). IC50 was 4.0 μ M under 40 μ M bicuculline. (b) Muscimol titration with 0.1mM streptomycin (WT028-2). IC50 was 1.1 μ M under 40 μ M bicuculline. (c) Combined dose response curves of WT028-1 and WT028-2. The shift to the left is caused by exposure to streptomycin.



WT054-1: Muscimol Titration



WT054-2: Muscimol Titration with 0.1 mM Streptomycin

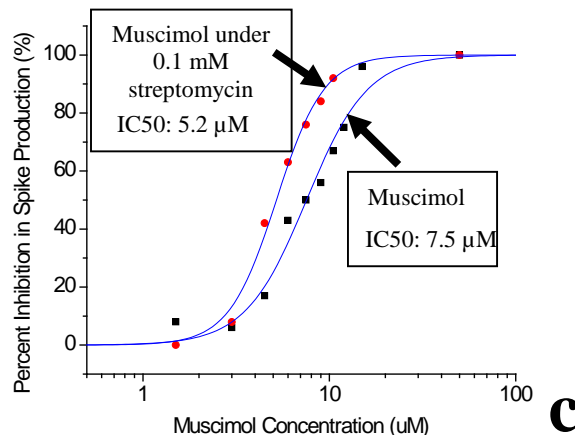


Figure 15. (a) Muscimol titration (WT054-1). IC50 was 7.5 μ M under 40 μ M bicuculline. (b) Muscimol titration with 0.1mM streptomycin (WT054-2). IC50 was 5.2 μ M under 40 μ M bicuculline. (c) Combined dose response curves of WT054-1 and WT054-2. The shift to the left is caused by exposure to streptomycin.

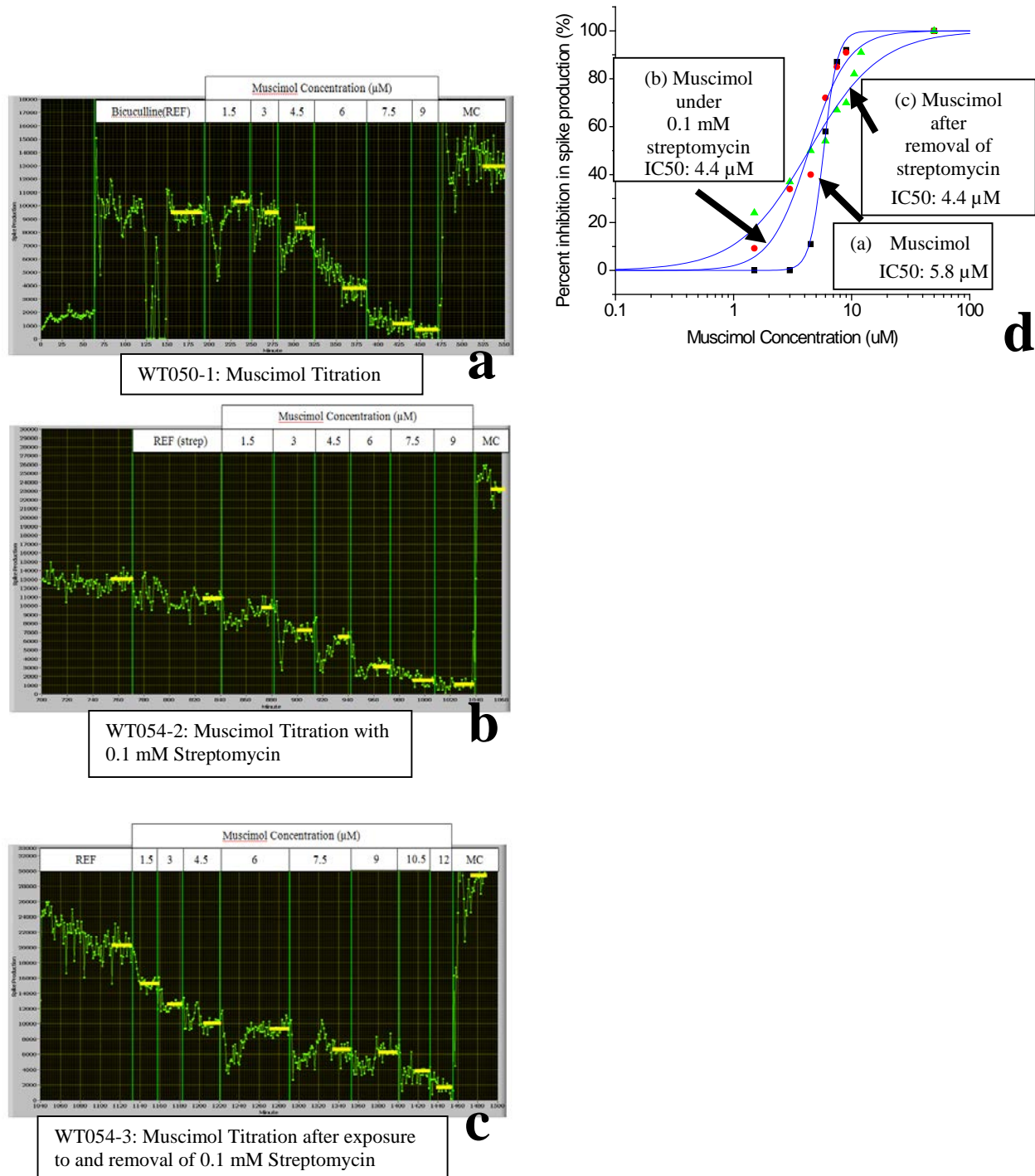


Figure 16. (a) Muscimol titration (WT050-1). IC50 was 5.8 μM under 40 μM bicuculline. (b) Muscimol titration with 0.1mM streptomycin (WT050-2). IC50 was 4.4 μM under 40 μM bicuculline. (c) Muscimol titration (WT050-1) after washing out streptomycin. IC50 was 4.4 μM under 40 μM bicuculline. (d) Combined dose response curves of WT050-1, WT050-2, and WT050-3. The shift to the left is not obvious after exposure to streptomycin since there is a huge slope change. The IC50 of muscimol remained the same after removal of streptomycin.

Table 8. Data of Muscimol Titration Experiments under 40 μ M Bicuculline. Date of experiment, age of the cell culture after being seeded, number of units recorded, IC₅₀ of muscimol, reversibility, and time between experiments in each row are shown.

	Date	Age (days)	# of units	IC ₅₀ (μ M)	Reversibility	Time between experiments (minutes)
WT027-1	4/19/13	36	50	6.6	1 wash (100%)	155
WT027-2*	4/19/13		49	2.8	2 washes(50%)	
WT028-1	4/24/13	42	30	4.0	1 wash (100%)	93
WT028-2*	4/24/13		30	1.1	1 wash (100%)	
WT050-1	10/8/13	29	44	5.8	1 wash (100%)	707 (between 1 & 2) 105 (between 2 & 3)
WT050-2*	10/9/13	30	44	4.4	1 wash (100%)	
WT050-3	10/9/13	30	44	4.4	1 wash (100%)	
WT054-1	10/30/13	36	37	7.5	1 wash (72%)	112
WT054-2*	10/30/13		22	5.2	1 wash (100%)	

*: experiments exposed to 0.1 mM streptomycin; the exposure time is in Table 9

Table 9. Muscimol IC₅₀ Comparison Data for Pre- and Post- Exposure to Streptomycin (under 40 μ M Bicuculline)

	WT027	WT028	WT050	WT054	Mean	Standard Deviation
IC ₅₀ without Strep (μ M)	6.6	4.0	5.8	7.5	6.0	1.5
IC ₅₀ with Strep (μ M)	2.8	1.1	4.4	5.2	3.4	1.8
Percent IC ₅₀ Decrease (%)	57.6	72.5	24.1	30.7	46.3	22.7
0.1mM Strep Pre-Exposure Time (mins)*	36	51	70	36	48	

*: it refers to the exposure time before muscimol titration; streptomycin didn't get washed out during muscimol titration

A muscimol titration usually takes more than 300 minutes, and one may think that the change of IC₅₀s is not due to the short exposure to streptomycin but to the time factor. In order to shorten the overall experiment time and minimize the time factor, single-point titrations were conducted rather than the full titrations. As it is shown in Figure 17, a single-point muscimol titration was carried out followed by a short exposure (40 minutes) to 0.1mM streptomycin and a

single-point muscimol titration. After washing out the streptomycin, a single-point muscimol titration was conducted to see if streptomycin had any residual effect. Figure 17, Figure 18, Figure 19, and Figure 20 show the result of experiments that use the same method. Table 8 summarizes data from these experiments. In all 4 experiments the streptomycin sensitization was reduced or eliminated after streptomycin was washed out. Short strep exposures do not generate a persistent effect.

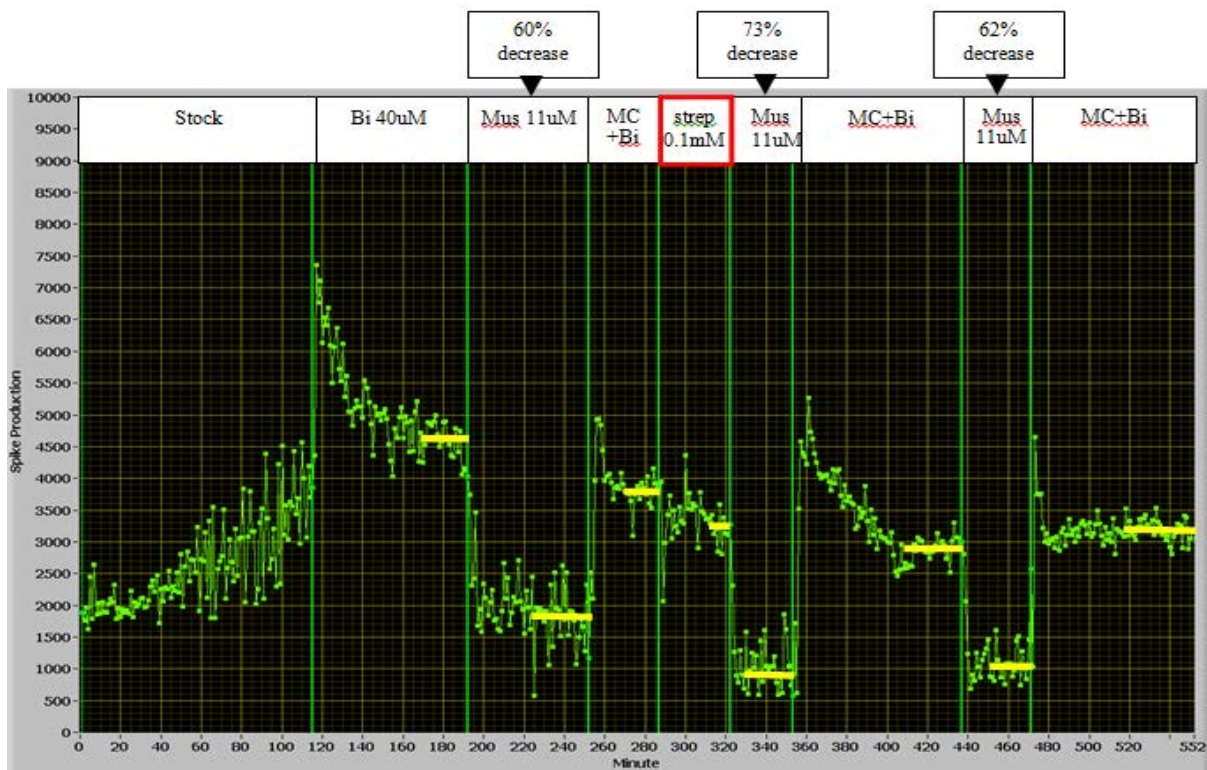


Figure 17. Single-point muscimol titration of 11 μ M followed by short exposure (40 minutes) to 0.1mM streptomycin and a single-point muscimol titration. Another single-point titration was carried out after washing out the streptomycin. Streptomycin exposure lowered the spike production compared to normal titration (73% > 60%). Streptomycin washout returned the single point titration to normal value (60% vs. 62%). (WT065)

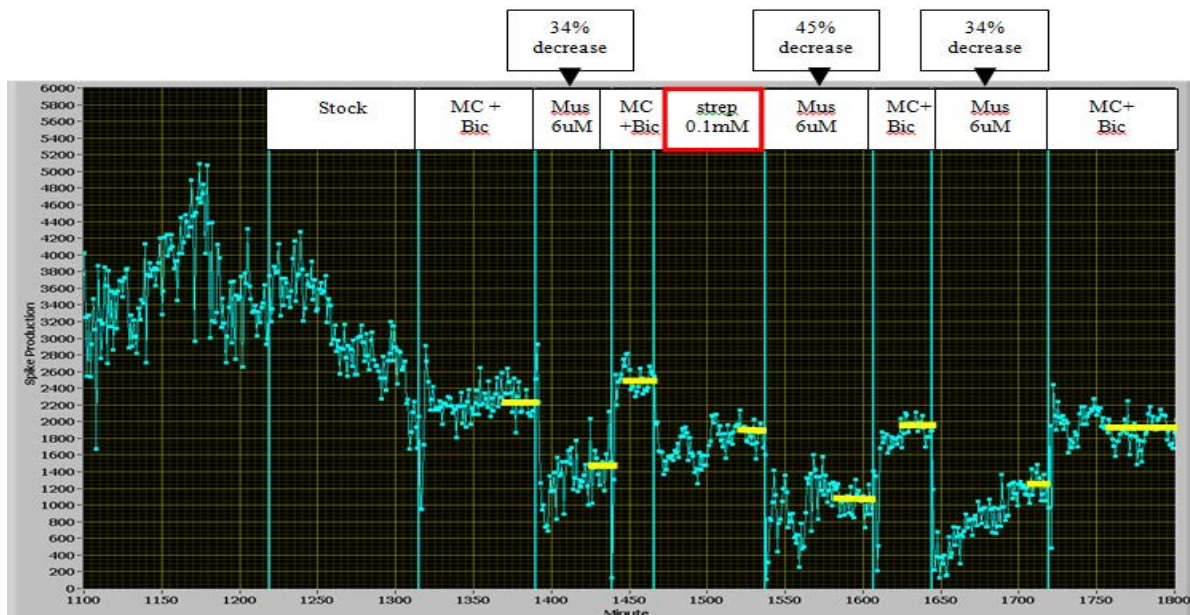


Figure 18. Single-point muscimol titration of 6 μ M followed by short exposure (71 minutes) to 0.1mM streptomycin and a single-point muscimol titration. Another single-point titration was carried out after washing out the streptomycin. Streptomycin exposure lowered the spike production compared to normal titration (45% > 32%). Streptomycin washout returned the single point titration to normal value (32% vs. 32%). (WT073)

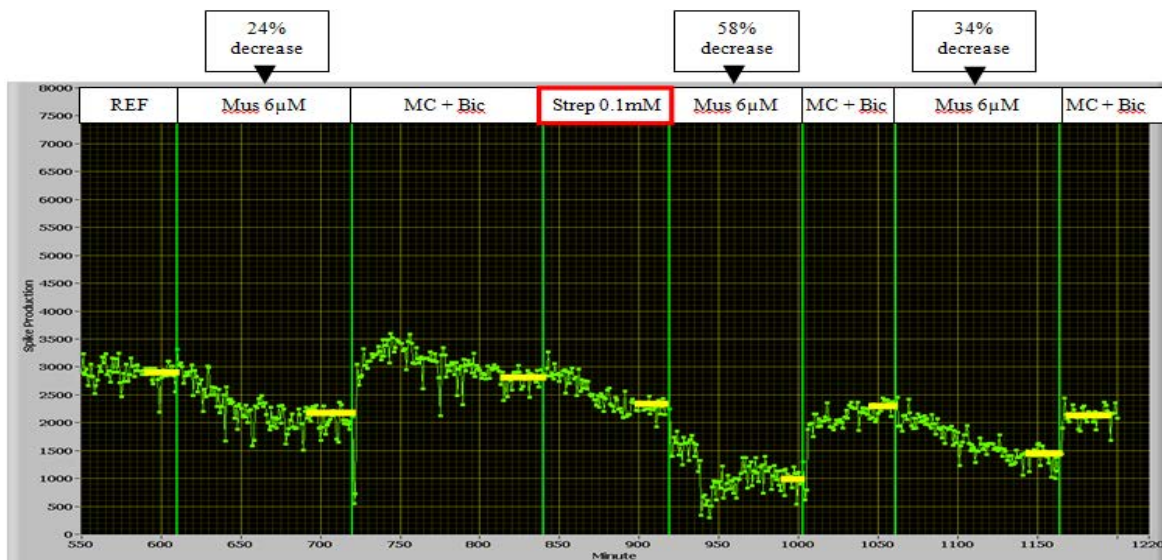


Figure 19. Single-point muscimol titration of 6 μ M followed by short exposure (89 minutes) to 0.1mM streptomycin and a single-point muscimol titration. Another single-point titration was carried out after washing out the streptomycin. Streptomycin exposure lowered the spike production compared to normal titration (57% > 28%). Streptomycin washout returned the single point titration to normal value (28% vs. 39%). (WT089)

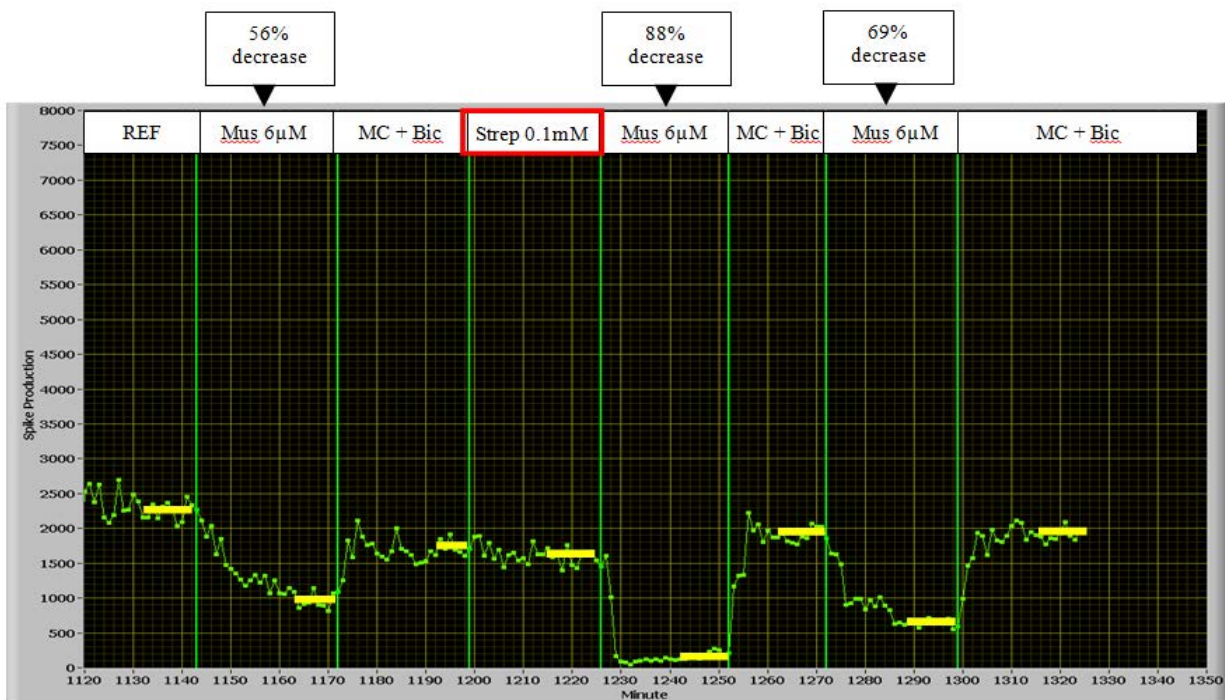


Figure 20. Single-point muscimol titration of 6 µM followed by short exposure (26 minutes) to 0.1mM streptomycin and a single-point muscimol titration. Another single-point titration was carried out after washing out the streptomycin. Streptomycin exposure lowered the spike production compared to normal titration (88% > 69%). Streptomycin washout returned the single point titration to normal value (69% vs. 56%). (88% > 69% & 56%) (WT091)

Table 10. Data of Singe-Point Muscimol Titration Experiments under 40 µM Bicuculline (n=4). Date of experiment, age of the cell culture after being seeded, number of units recorded, % decrease of reference, % decrease of strep exposure, and % decrease of internal control are shown each row are shown. “Reference % decrease” indicates the spike production % decrease after adding muscimol without pre-exposure to streptomycin. “Strep exposure % decrease” indicates the spike production % decrease after adding muscimol with pre-exposure to streptomycin. “After wash out % decrease” indicates that after streptomycin was washed out, the spike production % decrease of a single addition of muscimol.

	Date	# of units	Age (days)	concentration of muscimol (µM)	Reference % decrease	Strep exposure % decrease	After wash out % decrease
WT065	3/26/14	46	36	11	60	73	62
WT073	4/30/14	42	42	6	34	45	34
WT089	9/30/14	29	21	6	24	58	34
WT091	10/13/14	17	19	6	56	88	69

CHAPTER 5

DISCUSSION

5.1 Streptomycin Toxicity

Streptomycin has been routinely supplemented to culture media to prevent bacterial growth (Schantz & Ng, 2004). Martínez-Liarte pointed out in his paper that at the routine concentration (100 units/ml penicillin and 100 micrograms/ml streptomycin), tyrosinase activities and melanin content increased with time during the first 24-48 hours, and decreased cell viability is seen (Martínez-Liarte JH, 1995). However, he also pointed out that the adverse effect is minimal and the antibiotics should still be used in cell culture.

In this study, in Figure 9 we showed that streptomycin can stop all neural activity with the mean IC₅₀ of 0.27 ± 0.07 mM. Noticeably, comparing to Figure 2b, which was conducted in 2007 in this lab by Dayne Hollmuller, we can see that the present experiments and his experiments have very similar IC₅₀ of streptomycin (0.27 and 0.33 mM respectively). The IC₅₀ is only slightly higher than the recommended concentration of 0.17 mM (<http://www.atcc.org/products/all/30-2300.aspx>). At 0.17 mM streptomycin, spike production in my studies was lowered by approximately 20%. This inhibition effect should not be neglected.

The recoverability of activity from streptomycin was tested at the end of every titration: two medium washes were applied to most cell cultures. As it is shown in Table 5, except for experiment WT018, which has longer shutoff time (1000 minutes), the rest of the cell culture with shorter shutoff time (<100minutes) has partial or full recoverability. It can be concluded that short exposures to streptomycin with short shutoff times are reversible. However, we cannot conclude that a long shutoff time will lead to cell death because a single experiment does not provide enough evidence. The reversibility for long shutoff time was not tested because such

concentrations (0.9 mM) are five times higher than the recommended concentration (0.17 mM). Therefore, no further long shut off experiments were conducted.

In Table 5 of page 21, the streptomycin titrations of the experiment WT024-1 and WT024-2 were carried on the same neuronal network but on different days. It is noticed that the IC50 drops significantly (0.31 \rightarrow 0.19 mM) as well as the units (27 \rightarrow 19). The possible explanation is that the network was rather young, 21 days old, and the units are still unstable. Osmolarity and pH instability could also have affected the cell culture.

5.2 Muscimol Titration

In Figure 11 and Figure 12, we can see that the mean IC50 of muscimol without bicuculline is $0.12 \pm 0.01 \mu\text{M}$ (n=5), and the mean IC50 of muscimol with 40 μM bicuculline is higher: $5.2 \pm 1.6 \mu\text{M}$ (n=9), as the result of bicuculline being a competitive antagonist of GABA_A receptors. The none-bicuculline titration with muscimol has shown high consistency; the coefficient of variation is 8.3. In Figure 11, all the dose-response curves almost overlap with each other. Also, the none-bicuculline titration with muscimol showed similar IC50 as those reported by Rijal-Oli (0.12 ± 0.01 versus $0.014 \pm 0.05 \mu\text{M}$) (MS thesis, UNT 2008). On the other hand, the 40 μM bicuculline results from both studies varied widely (5.2 ± 1.6 versus $19.4 \pm 3.5 \mu\text{M}$). This discrepancy is presently unexplained. However, Table 7 shows that all muscimol IC50 under 40 μM bicuculline were relatively consistent in this study. The mean IC50 was $5.2 \pm 1.6 \mu\text{M}$. The standard deviation of 1.61 μM yields a coefficient of variation of 30.7. This represents a high variability in the data. Given that the streptomycin statistics analysis used only experimental data under 40 μM bicuculline, this variability is of concern. Because of the high variability in the data, the paired t-test was selected. Based on this statistical test, the

streptomycin sensitization of muscimol titrations, which was the main focus of this study, should still be valid. As long as the bicuculline exposure was at the same concentration, I have assumed that the streptomycin effect is independent.

The main reason why bicuculline was heavily used in spite of the bigger variation it caused is that it can stabilize and stimulate cell culture. Cell cultures could not be used in the study if they did not have regular native activity and high spike rates. By treating cell cultures with bicuculline, many cell cultures became usable in this study.

As it is shown in Table 6 and Table 7, half of the experiments show full reversibility after 1 to 2 washes. And except for 2 experiments, the rest of experiments show more than 50% recovery. We can see that high reversibility is prevailing.

5.3 Acute Streptomycin Effects on Muscimol Pharmacology

As shown in Figure 1 and Table 1, Rijal-Oli's MS thesis pointed out that early exposure (on day 5) to normal pen-strep concentration (0.17 mM) for 48 hours shifted dose-response curve to the left, indicating increased sensitivity on day 27. In my study, instead of having early exposure, cell cultures were exposed to less streptomycin (0.1mM) for 30 to 70 minutes right before the muscimol titration. And as shown in Figure 13, 14, 15, and 16, sensitization was demonstrated. In Table 9, we can see that IC₅₀'s values were decreased 46.3%. A paired t-test was performed, to test whether there was significant change. The null hypothesis of the paired t-test was that the mean IC₅₀ of muscimol before streptomycin exposure was equal to the mean IC₅₀ of muscimol after streptomycin exposure. In Figure 20, an R program analysis shows the result of paired t-test: p-value is 0.0142 which means the IC₅₀ of muscimol became significantly lower (*p<0.05) after acute exposure to streptomycin.

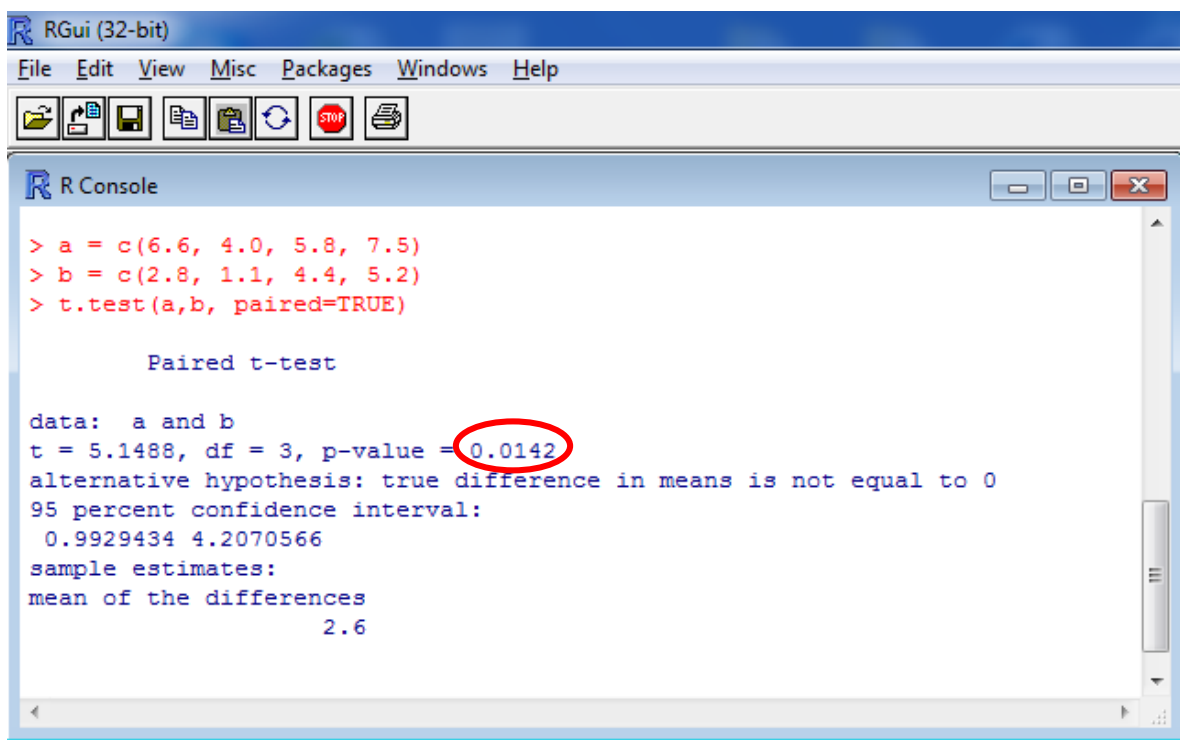


Figure 21. Comparison of Statistics from R program: Muscimol IC₅₀'s of pre- and post-exposure to streptomycin (under 40 μ M bicuculline) (n=4) *p<0.05

As it is shown in Table 8, 7 out of 9 experiments show full reversibility after one medium change. The rest two experiments have 50% and 72% of reversibility. In Figure 13c, 14c, and 15c, the dose-response curves of pre- and post-streptomycin have similar slopes in each experiment. However, in Figure 16d, not only did pre- and post-streptomycin have distinct slopes, the IC₅₀ of muscimol didn't recover after washing out. The reason is unexplained currently. However, time factors may be the cause since the total experiment time was over 24 hour.

Experiments of single-point titration were conducted to minimize time factors. In Table 10 of page 31, 4 experiments show sensitization after acute exposure to streptomycin. A paired t-test in Figure 21 shows the significant EC₅₀ sensitization after short exposure to streptomycin (p<0.05). The null hypothesis is that there is no difference in % decrease of spike production

between the muscimol titration of pre- and post- exposure to streptomycin. As an internal control, additional muscimol titration was conducted after washing out streptomycin. In Figure 22, the result of paired t-test shows no significant difference in % decrease of spike production between the muscimol titration before exposure to streptomycin and the internal control after streptomycin washout ($p > 0.05$). This suggests the acute streptomycin effect is not persistent.

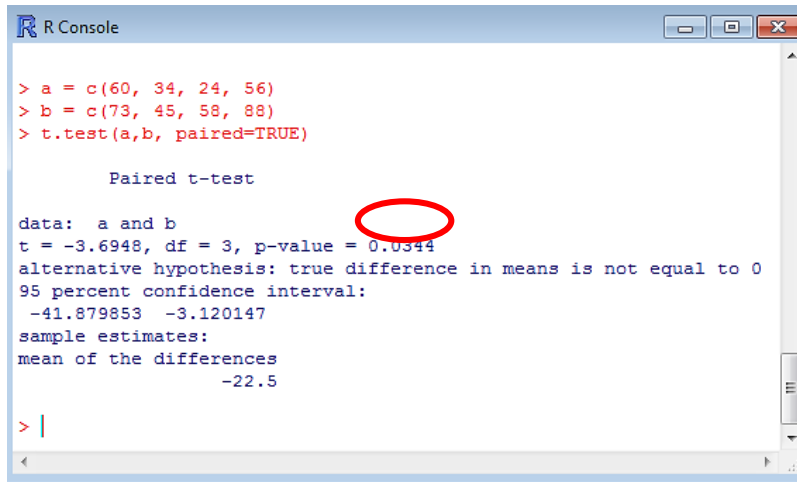


Figure 22. Statistics from R program: comparison of muscimol IC₅₀'s between pre- and post-exposure to streptomycin (under 40 μ M bicuculline) (n=4) * $p < 0.05$

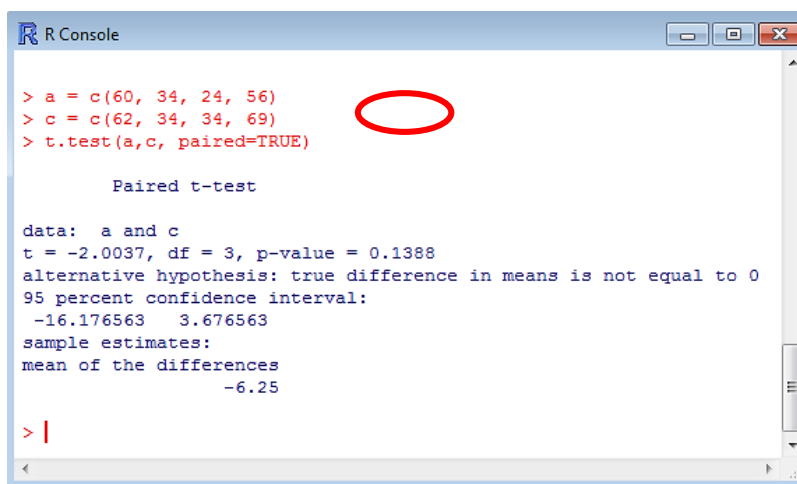


Figure 23. Statistics from R program: comparison of muscimol IC₅₀'s between pre-exposure to streptomycin and internal control (under 40 μ M bicuculline) (n=4) * $p > 0.05$

APPENDIX A
EXPERIMENT LOG

Exp #	Date	MMEP#	Age	Chemicals	Purpose	Result	Notes
17	1/23/ 2013	4A158	35	Streptomycin	Recoverability after exposure to streptomycin	N/A	Contamination
18	2/06	4A386	49	Streptomycin	Recoverability after exposure to streptomycin	Activity failed to recover after exposure to 0.9 mM streptomycin for 17 hours	N/A
19	2/27	4A394	28	Streptomycin	Recoverability after exposure to streptomycin	N/A	Contamination and off-target neurons
20	3/06	4A415	21	Streptomycin	Recoverability after exposure to streptomycin	N/A	Very low density
21*	3/07	4A427	21	Streptomycin	Recoverability after exposure to streptomycin	Full recoverability is shown without bicuculline (IC50: 0.38 mM)	N/A
22	3/20	4A223	21	Streptomycin	Recoverability after exposure to streptomycin	N/A	No processes
23	3/27	4A200	28	Streptomycin	Recoverability after exposure to streptomycin	Streptomycin seems less effective (may have expired)	Streptomycin solution may have expired (made 2/5)
24*	4/03	4A771	21	Streptomycin	Recoverability after exposure to streptomycin	Under 40mM bicuculline; full recoverability; cell culture became more sensitive in 2 nd exp. (IC50: 0.32, 0.19 mM)	N/A
25*	4/10	4A431	28	Streptomycin	Recoverability after exposure to streptomycin	Full recoverability couldn't be shown (only 70%) (IC50: 0.24 mM)	Ex8 and Ex9 are in 3/13 batch
26	4/17	4A436	35	Streptomycin + Muscimol	Dose-response curve shifting effect	N/A	Very few channels
27*	4/18	4A307	36	Streptomycin + Muscimol	Dose-response curve shifting effect	After being exposed to streptomycin, IC50 is dropped from 6.6 to 2.8 μ M	N/A
28*	4/24	4A581	42	Streptomycin + Muscimol	Dose-response curve shifting effect	After being exposed to streptomycin, IC50 dropped dramatically from 4.0 to 1.1 μ M	More data points are needed.
29	5/1	4A398	48	Streptomycin + Muscimol	Dose-response curve shifting effect	Muscimol dose-response curve was created, but it was too late (2AM) to do another one with strep; and it became an unstable cell	Lower concentration of muscimol is made.

						culture on the next day.	
30	5/8	4A245	28	Streptomycin + Muscimol	Dose-response curve shifting effect	N/A	MEA has too many flatlines (16)
31*	5/26	4A523	49	Muscimol	Dose-response curve	Full recovery in first part, but fail in second part. (IC50: 3.0, 4.5 μ M)	N/A
32	6/26	4A439	22		Dose-response curve	N/A	No activity
33	6/26	4A30	22	N/A	Dose-response curve	N/A	Contamination; no activity
34*	6/26	4A253	22	Muscimol	Dose-response curve	Full recoverability is shown under 40 μ M bicuculline (IC50: 7.3 μ M)	N/A
35	7/15	4A386	28	N/A	Dose-response curve	N/A	Contamination; no activity
36	7/17	4A461	43	Muscimol	Dose-response curve	N/A	Unstable activity
37	7/22	5C819	32	N/A	Dose-response curve	N/A	Unstable activity
38	7/24	4A427	22	N/A	Dose-response curve	N/A	no activity
39*	7/29	4A118	27	Muscimol	Dose-response curve	Full recoverability is shown without bicuculline (IC50: 0.1 μ M)	N/A
40	8/8	4A431	32	N/A	Dose-response curve	N/A	Contamination; no activity
41*	8/26	4A38	27	Muscimol	Dose-response curve	2 experiments have shown full recoverability without bicuculline (IC50: 0.14, 0.12 μ M)	N/A
42	9/3	5C138	34	Muscimol	Dose-response curve	N/A	No activity
43	9/3	4A207	21	Muscimol	Dose-response curve	N/A	Unstable activity
44	9/10	4A417	55	Muscimol	Dose-response curve	N/A	Unstable activity
45	9/10	4A474	56	Muscimol	Dose-response curve	N/A	Contamination
46	9/17	4A356	36	Muscimol	Dose-response curve	N/A	Unstable activity
47	9/24	4A336	70	Muscimol	Dose-response curve	N/A	No activity
48	9/25	4A70	71	Muscimol	Dose-response curve	N/A	Unstable activity
49*	10/2	4A417	22	Muscimol	Dose-response curve	2 experiments showed full recoverability without bicuculline (IC50: 0.11, 0.11 μ M)	N/A

50*	10/8	4A324	28	Streptomycin + Muscimol	Dose-response curve shifting effect	3 experiments are conducted (IC ₅₀ : 5.8, 4.45, 4.35 μ M (with strep))	N/A
51	10/15	4R3	35	Muscimol	Dose-response curve	N/A	Low activity
52	10/15	4R49	35	Muscimol	Dose-response curve	N/A	Contamination
53	10/29	4A291	49	Muscimol	Dose-response curve	N/A	No activity
54*	10/30	4A412	36	Streptomycin + Muscimol	Dose-response curve shifting effect	2 experiments are conducted (IC ₅₀ : 7.54, 5.20 μ M (with strep))	N/A
55	11/5	4A159	27	Muscimol	Dose-response curve	N/A	Unstable activity
56*	11/12	4A390	34	Muscimol	Dose-response curve	3 experiments were conducted (IC ₅₀ : 4.09, 3.12 (DMEM6), 3.19 μ M (DMEM6))	DMEM6 medium was used in 2 experiments (Pre-exposed to pen-strep)
57	11/19	4A441	50	Muscimol	Dose-response curve	N/A	Unstable activity
58*	11/26	4A437	63	Muscimol	Dose-response curve	Under 20 μ M bicuculline; 1 experiment was conducted (IC ₅₀ is 1.54 μ M)	N/A
59	2/5/2014		29	Muscimol	Dose-response curve shifting effect	N/A	No activity
60	2/5	4A191	29	Muscimol	Dose-response curve shifting effect	N/A	No activity
61	2/5	5C437	29	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Unstable activity
62	2/12	5C282	36	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Unstable activity
63	3/14	5C680	24	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Unstable activity
64	3/19	5C495	29	Muscimol	Dose-response curve shifting effect	N/A	Unstable activity
65*	3/26	5C966	36	Muscimol + Bicuculline	Dose-response curve shifting effect	Exposure to strep (40 mins) lowered spike production more (sensitization); under 40 μ M bicuculline	72% compared with non-exposure (61, 62%)

66	4/2	5C548	28	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	No activity
67	4/9	5C557	21	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Unstable activity
68	4/16	5C551	28	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Unstable activity
69	4/23	5C865	35	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Unstable activity
70	4/25	5C495	37	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Unstable activity
71	4/25	4D26	36	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Unstable activity
72	4/30	5C448	28	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	No activity
73*	4/30	5C403	42	Muscimol + Bicuculline	Dose-response curve shifting effect	Exposure to strep (71 mins) lowered spike production more(sensitization); under 40µM bicuculline	47% compared with non-exposure (38, 37%)
74	4/29	5C448	14	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Unstable activity
75	5/14	5C631	28	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Unstable activity
76*	5/20	5C39	34	Muscimol + Bicuculline	Dose-response curve shifting effect	Exposure to strep (84 mins) lowered spike production more(sensitization); under 40µM bicuculline and 100µM quinolinic Acid	62% compared with non-exposure (17, 24%)
77	5/21	5C646	21	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Low activity
78	5/21	5C94	21	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Unstable activity
79	5/23	5C94	23	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Unstable activity
80	5/25	5C23	39	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Low activity

81	5/25	5C559	39	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Unstable activity
82	5/27	5C134	27	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	No activity
83	5/27	5C985	41	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Unstable activity
84	5/28	5C574	42	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Unstable activity
85	9/22	5S300	53	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Low activity
86	9/22	4A485	40	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Low activity
87	9/22	5C417	53	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Low activity
88	9/30	4D119	48	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	No activity
89	10/1	5C713	21	Muscimol + Bicuculline	Dose-response curve shifting effect	Exposure to strep (89 mins) lowered spike production more(sensitization); under 40μM bicuculline	58% compared with non-exposure (24, 34%)
90	10/10	4A179	30	Muscimol + Bicuculline	Dose-response curve shifting effect	N/A	Low activity
91	10/13	4d8	19	Muscimol + Bicuculline	Dose-response curve shifting effect	Exposure to strep (26 mins) lowered spike production more(sensitization); under 40μM bicuculline	88% compared with non-exposure (69, 59%)

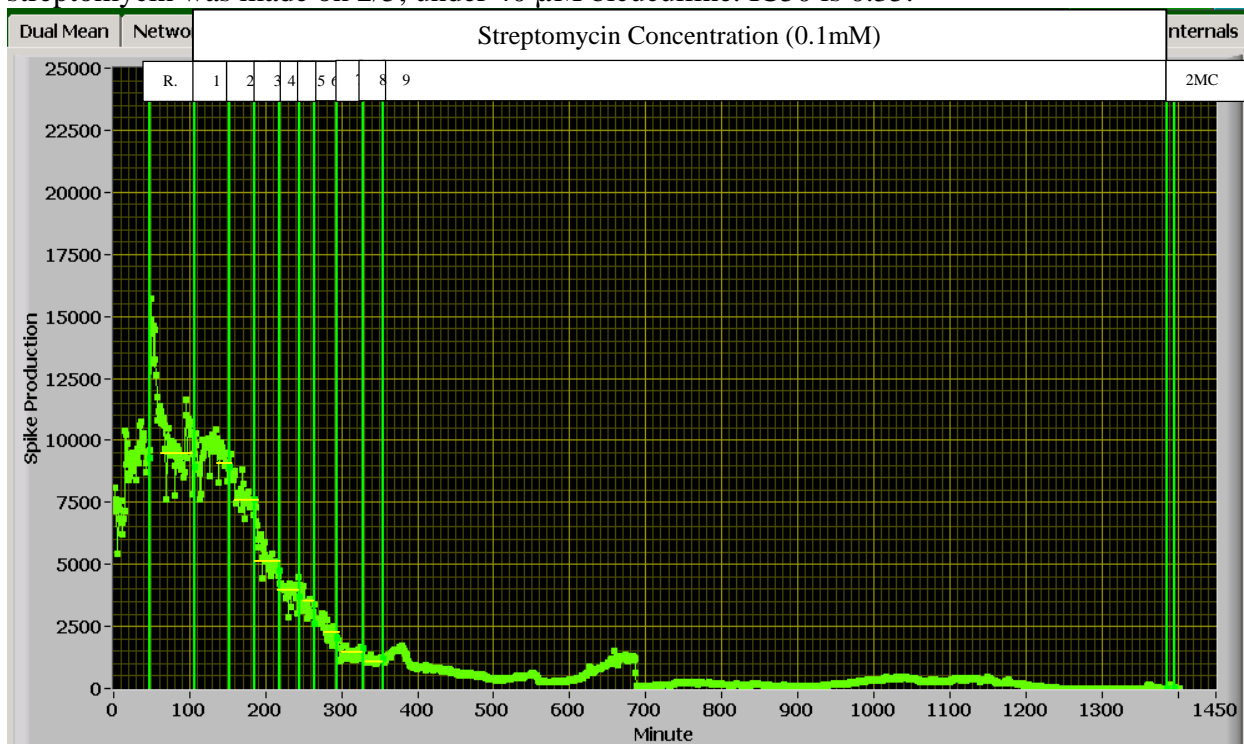
(Tissue: Frontal Cortex and Auditory Cortex)

Experiments that have * after experiment number: experiments that I used in data analysis

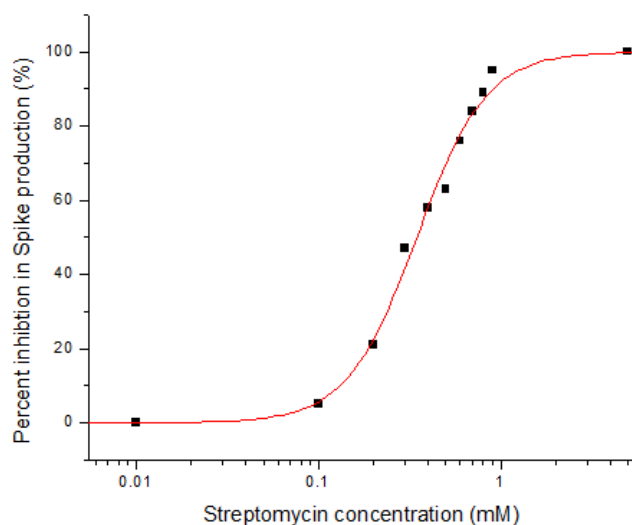
APPENDIX B

STREPTOMYCIN TITRATION

Ex 1 (WT018 2/6/2013): No recovery after overnight exposure to 0.9 mM streptomycin; fresh streptomycin was made on 2/5; under 40 μ M bicuculline. IC50 is 0.35.

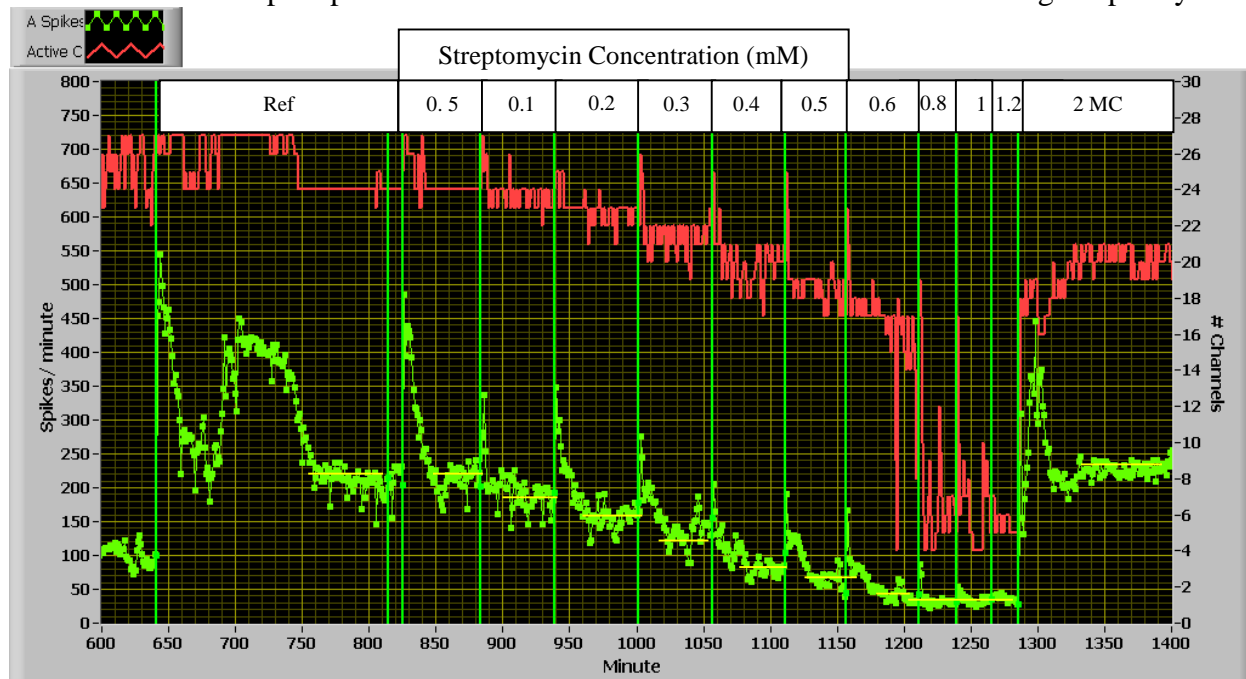


Molarity(mM)	REF	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	MC
Spike Production	9500	9000	7500	5000	4000	3500	2300	1500	1000	500	0
Percent Decrease(%)	N/A	5	21	47	58	63	76	84	89	95	100

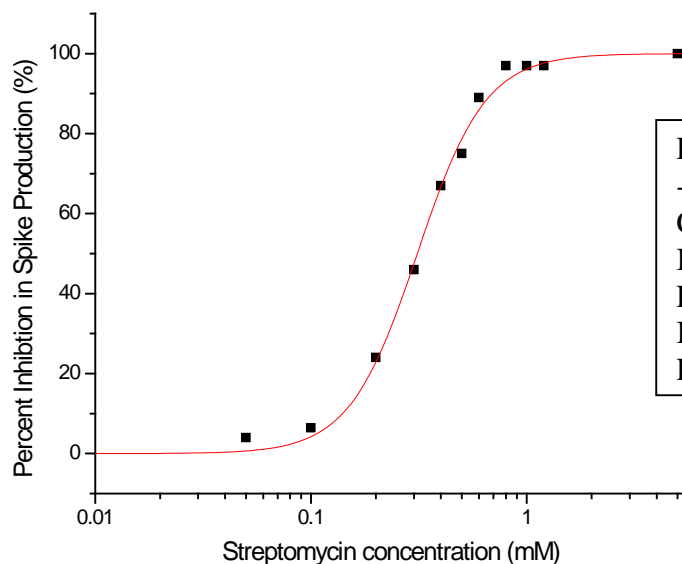


Parameter	Value	Error
Chi ² /DoF	12.93633	
Initial(A1)	0	0
Final (A2)	100	0
IC50 (x0)	0.36037	0.01099
Power (p)	2.51444	0.17969

Ex 2 (WT024 part 1 04/03/2013): Under 40 μ M bicuculline, full recovery is shown after 2 medium change (right after experiment); IC₅₀ is 0.31mM. In the beginning of each step, sudden increase of spike production is shown due to the disturbance while adding streptomycin.

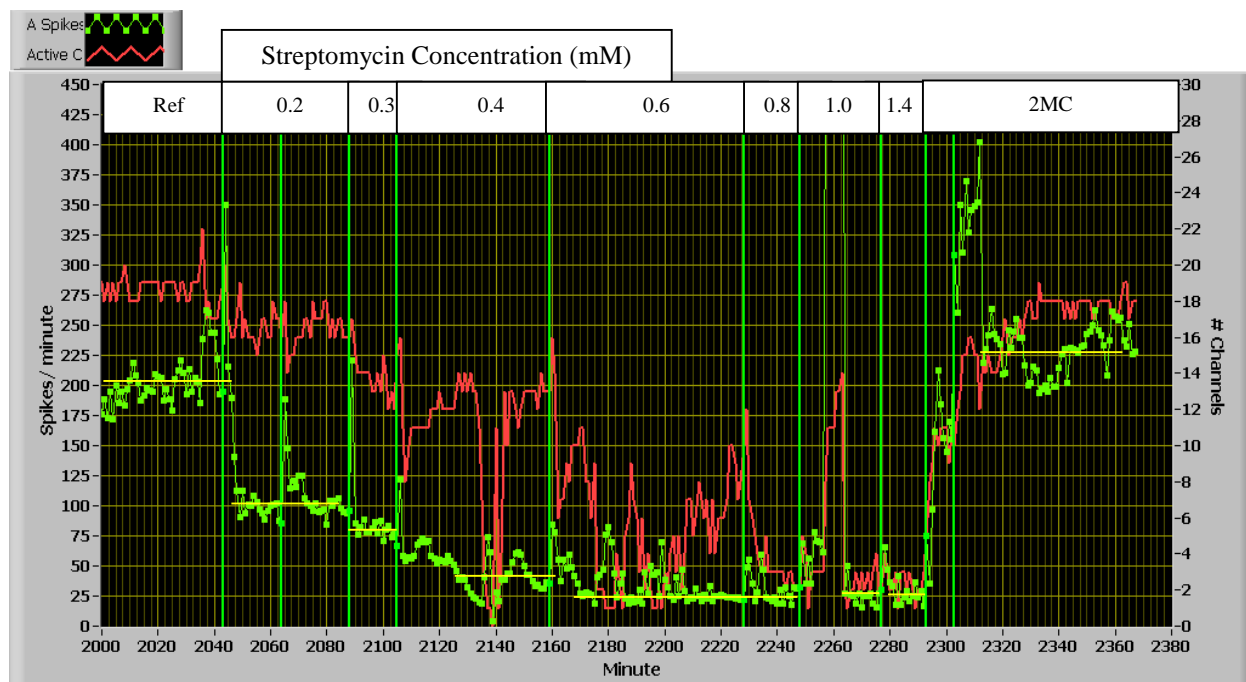


Molarity (mM)	REF	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.8	1.0	MC
Spike Production	5000	5200	4680	3810	2720	1652	1262	551	170	160	4500
Percent Decrease (%)	N/A	-4	6.4	24	46	67	75	89	97	97	10

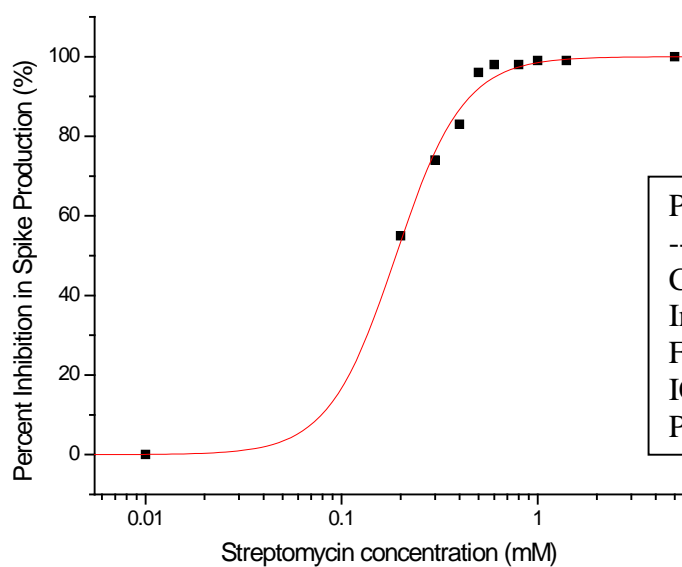


Parameter	Value	Error
Chi ² /DoF	5.92868	
Initial(A1)	0	0
Final (A2)	100	0
IC ₅₀ (x0)	0.3109	0.00657
Power (p)	2.75546	0.14622

Ex 3 (WT024 part 2 04/03/2013): More sensitive to muscimol ($IC_{50}:0.19<0.32$); Full recovery after 2 medium change; under $40\mu M$ bicuculline

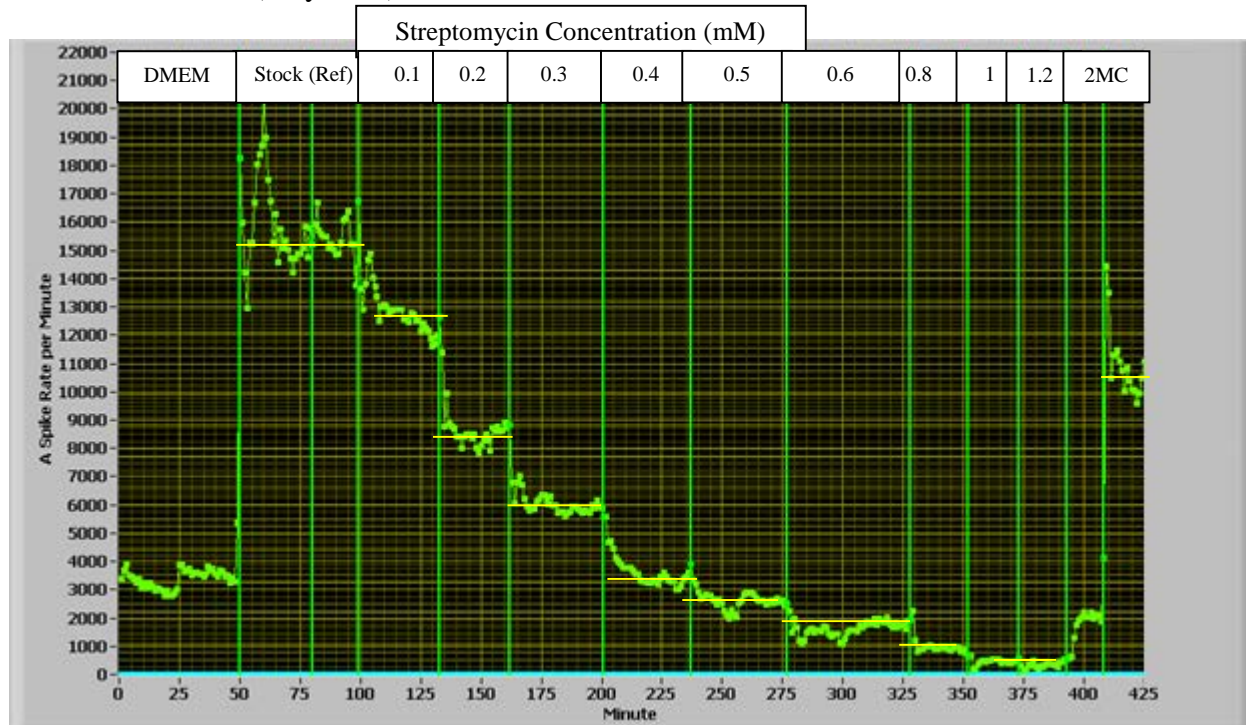


Molarity(mM)	REF	0.2	0.3	0.4	0.6	0.8	1.0	1.4	2MC
Spike Production	3800	1700	975	650	150	75	75	50	3825
Percent Decrease	N/A	55%	74%	83%	96%	98%	98%	99%	0%

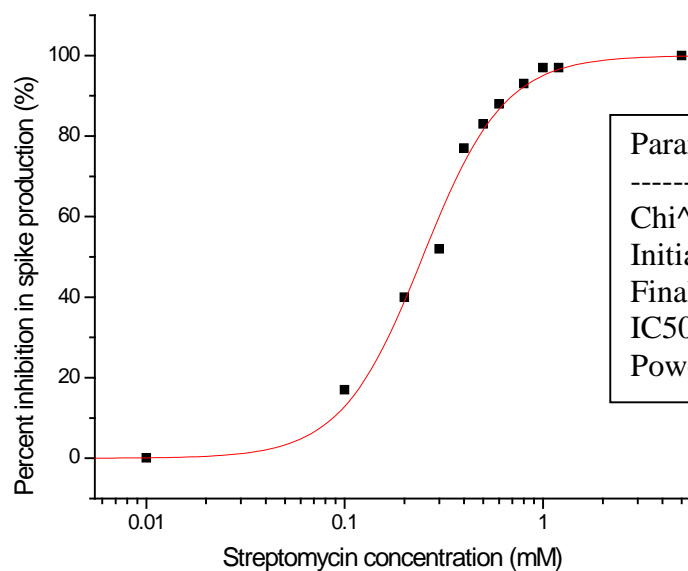


Parameter	Value	Error
Chi ² /DoF	6.00778	
Initial (A1)	0	0
Final (A2)	100	0
IC ₅₀ (x0)	0.1891	0.00757
Power (p)	2.51821	0.21802

Ex 4 (WT025 04/10/2013): IC 50 of streptomycin is around 0.24mM. Full recoverability couldn't be shown (only 70%). Not under bicuculline.



Molarity (mM)	REF	0.1	0.2	0.3	0.4	0.5	0.6	0.8	1.0	1.2	MC
Spike Production	15000	12500	9000	6000	3500	2500	1800	1000	500	400	10500
% Decrease	N/A	17	40	52	77	83	88	93	97	97	30

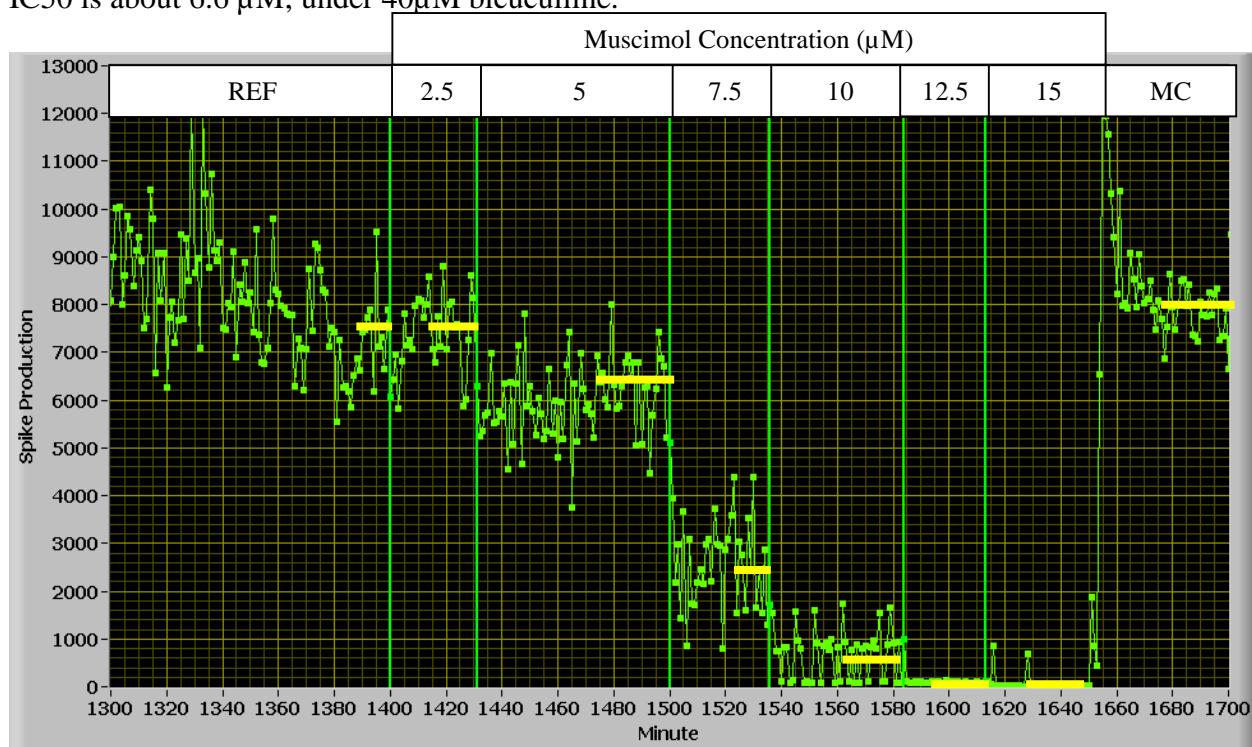


Parameter	Value	Error
Chi ² /DoF	11.60243	
Initial(A1)	0	0
Final (A2)	100	0
IC50 (x0)	0.24725	0.00951
Power (p)	2.11103	0.15202

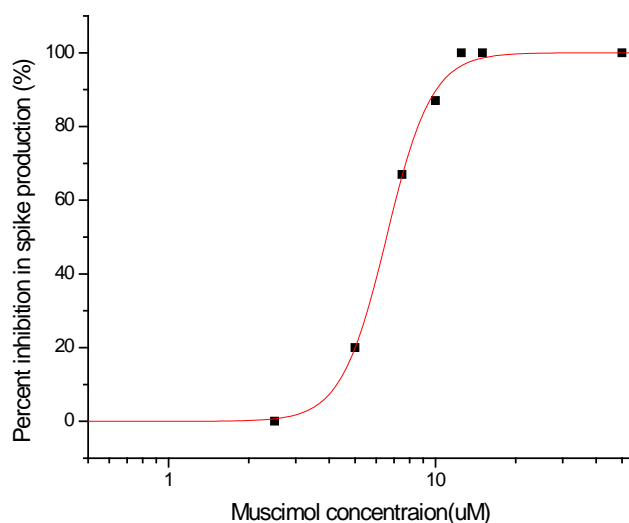
APPENDIX C

MUSCIMOL DOSE-RESPONSE CURVE

Ex 5-1 (WT027 part 1 4/18/2013): Dose-response curve of muscimol is created.
 IC50 is about 6.6 μM ; under 40 μM bicuculline.

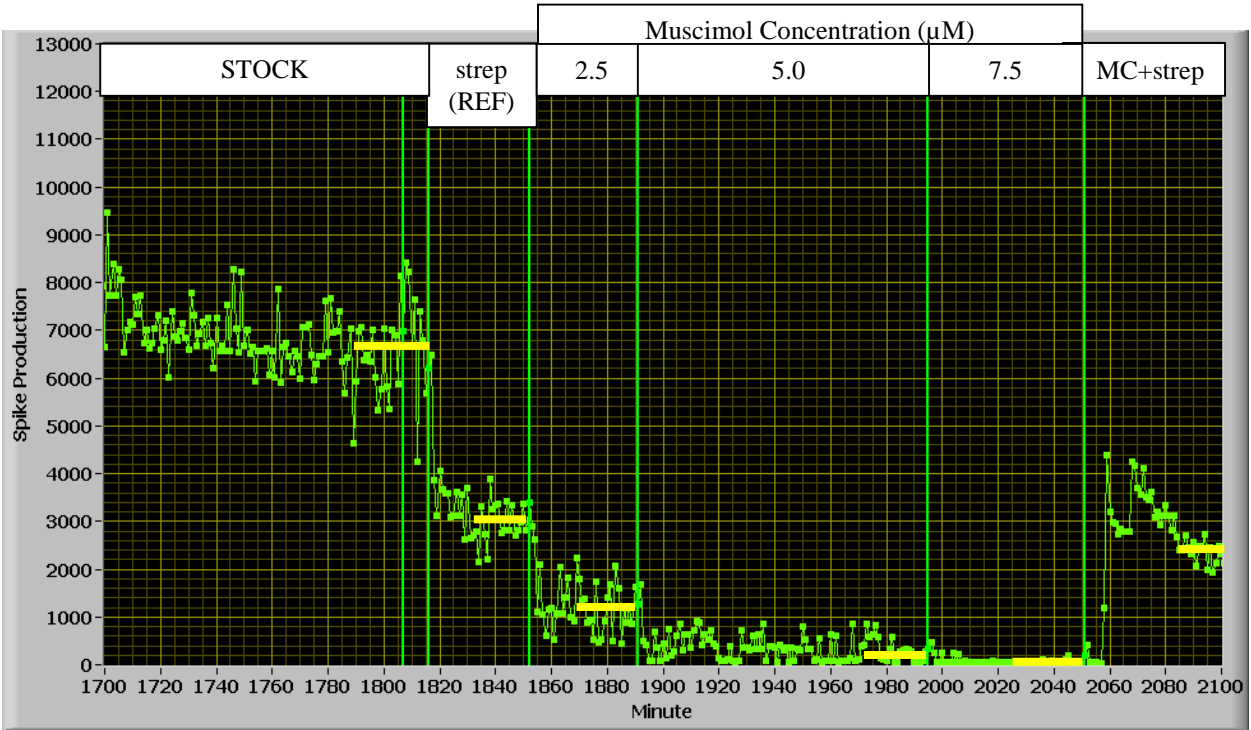


Molarity(μM)	REF	2.5	5	7.5	10	12.5	15	MC
Spike Production	7500	7500	6000	2500	500	0	0	8000
% Decrease	N/A	0	20	67	93	100	100	+5.8

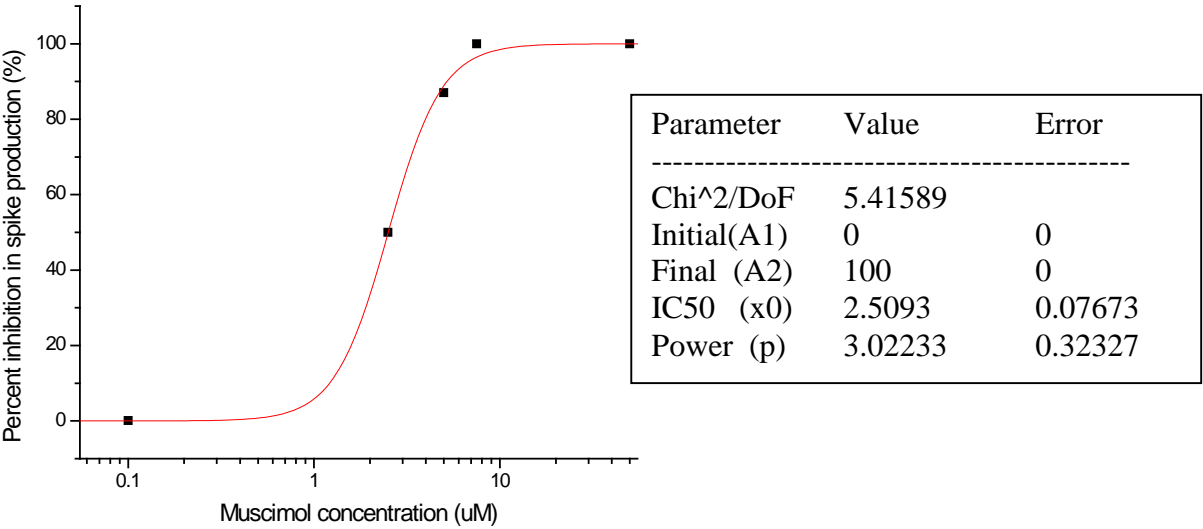


Parameter	Value	Error
Chi ² /DoF	3.73548	
Initial(A1)	0	0
Final (A2)	100	0
IC50 (x0)	6.55902	0.08661
Power (p)	5.13555	0.28128

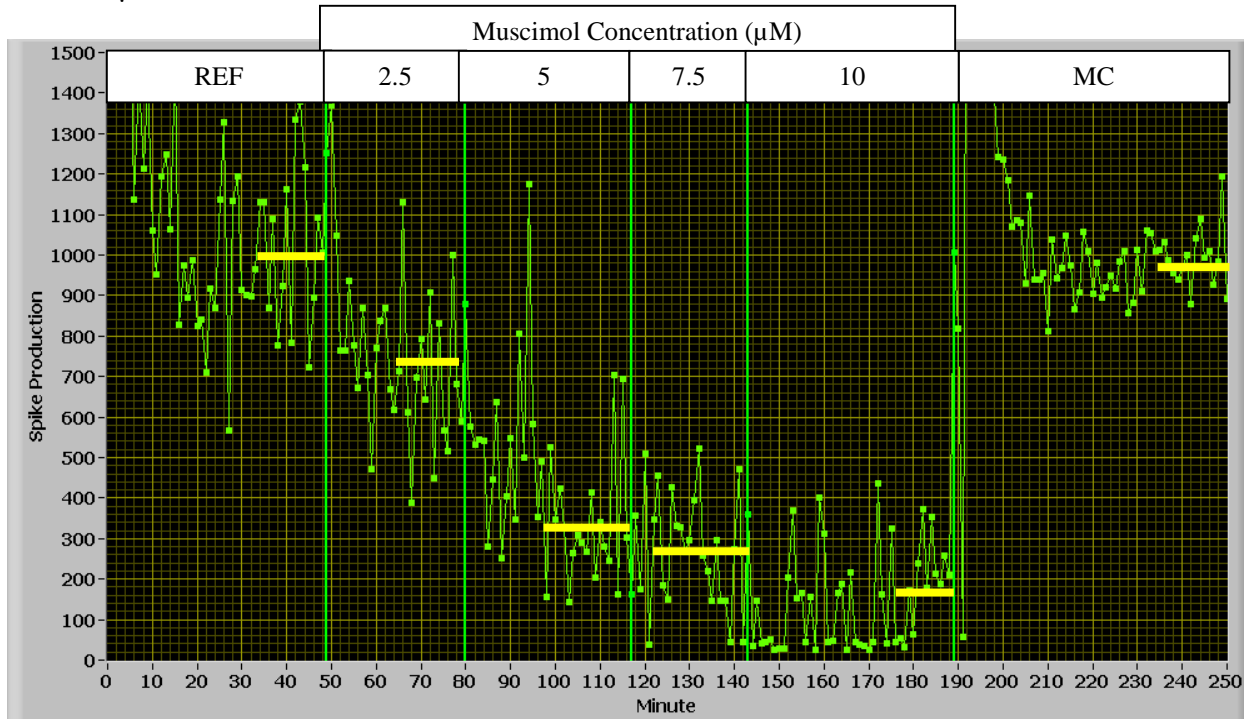
Ex 5-2 (WT027 part 2 4/18/2013): Dose-response curve of muscimol under 0.1mM streptomycin and 40μM bicuculline (0.1 mM is the recommended concentration) is created. IC 50 is dropped from 6.6 to 2.8 μM in the same cell culture on the same day.



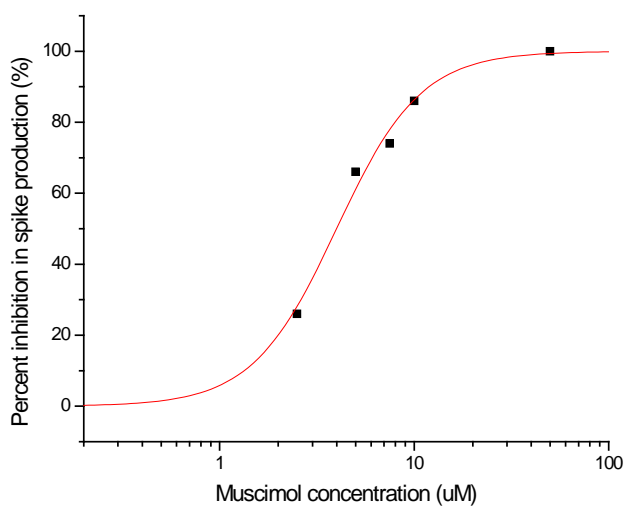
Molarity(mM)	0	(strep)REF	2.5	5	7.5	MC+strep
Spike Production	6500	3000	1500	400	0	2500
Percent Decrease	N/A	N/A	50%	87%	100%	17%



Ex 6-1 (WT028 part 1 4/24/2013): Dose-response curve of muscimol is created. IC50 is 4.0 μM under 40 μM bicuculline.

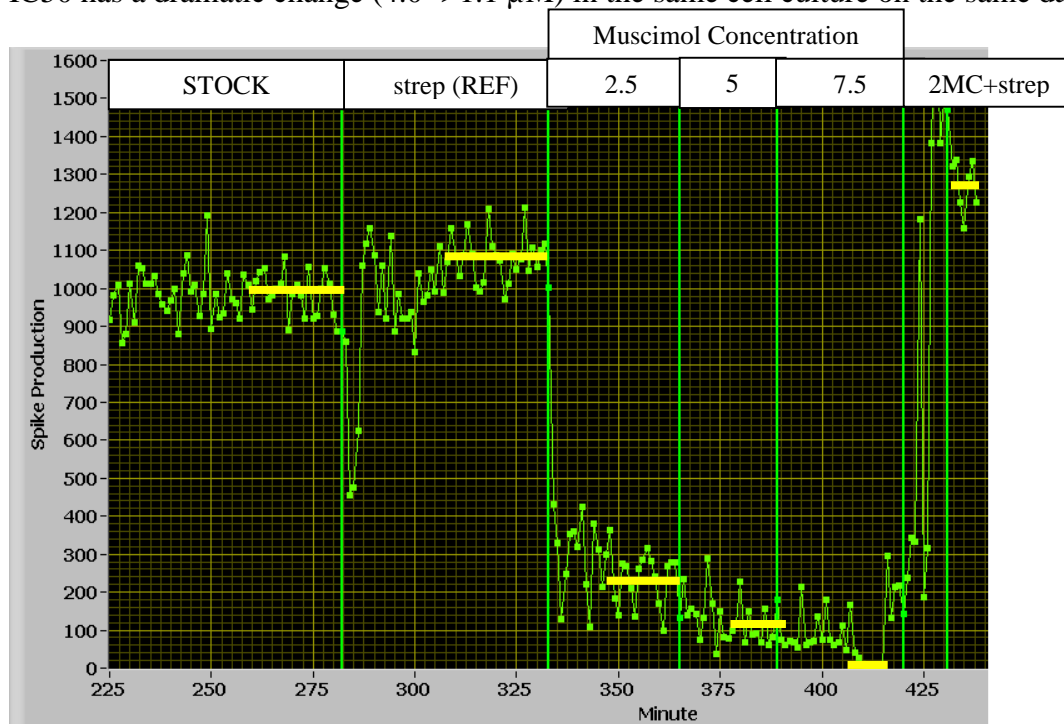


Molarity(μM)	REF	2.5	5	7.5	10	MC
Spike Production	1000	740	340	260	180	960
Percent Decrease	N/A	26%	66%	74%	82%	4%

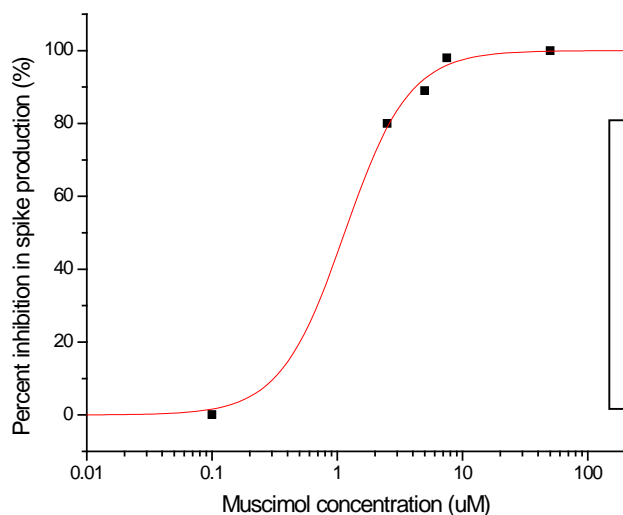


Parameter	Value	Error
Chi ² /DoF	11.21251	
Initial(A1)	0	0
Final (A2)	100	0
IC50 (x0)	3.98969	0.18983
Power (p)	2.00678	0.19004

Ex 6-2 (WT028 part 2 4/24/2013): Dose-response Curve of muscimol under 0.1 mM streptomycin and 40 μ M bicuculline (0.1 mM is the recommended concentration) is created. IC50 has a dramatic change (4.0 \rightarrow 1.1 μ M) in the same cell culture on the same day.

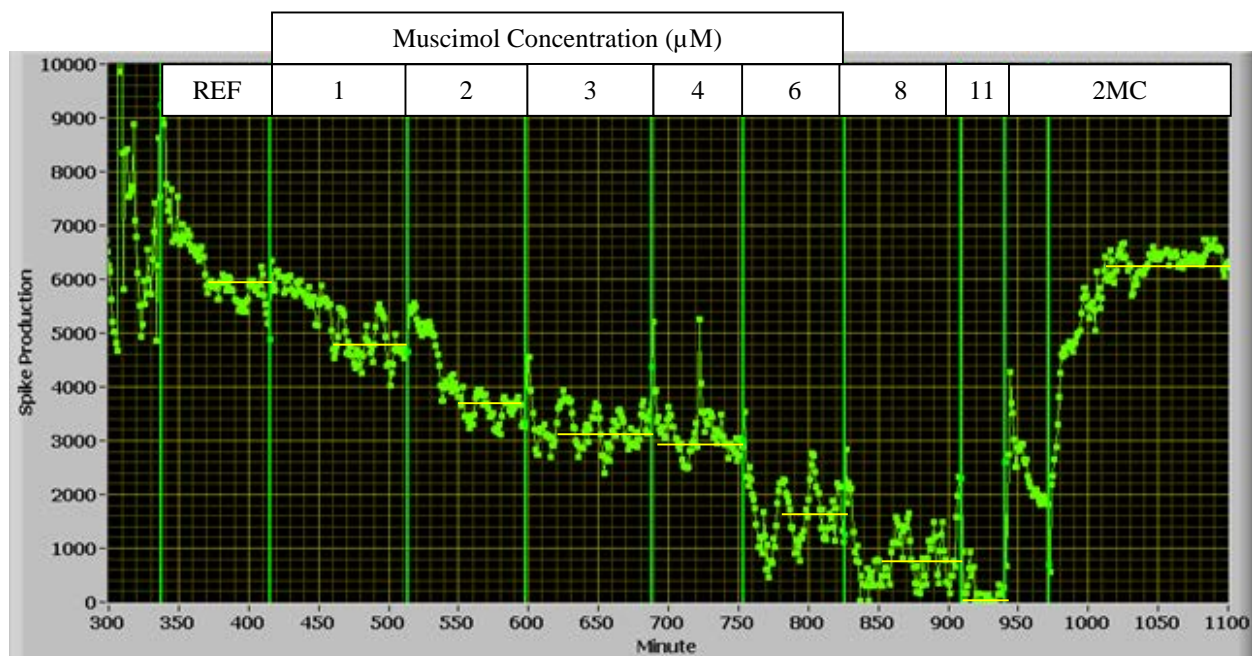


Molarity(μ M)	0	strep (REF)	2.5	5	7.5	2MC+strep
Spike Production	1000	1080	220	120	20	1280
Percent Decrease	N/A	N/A	80%	89%	98%	-19%

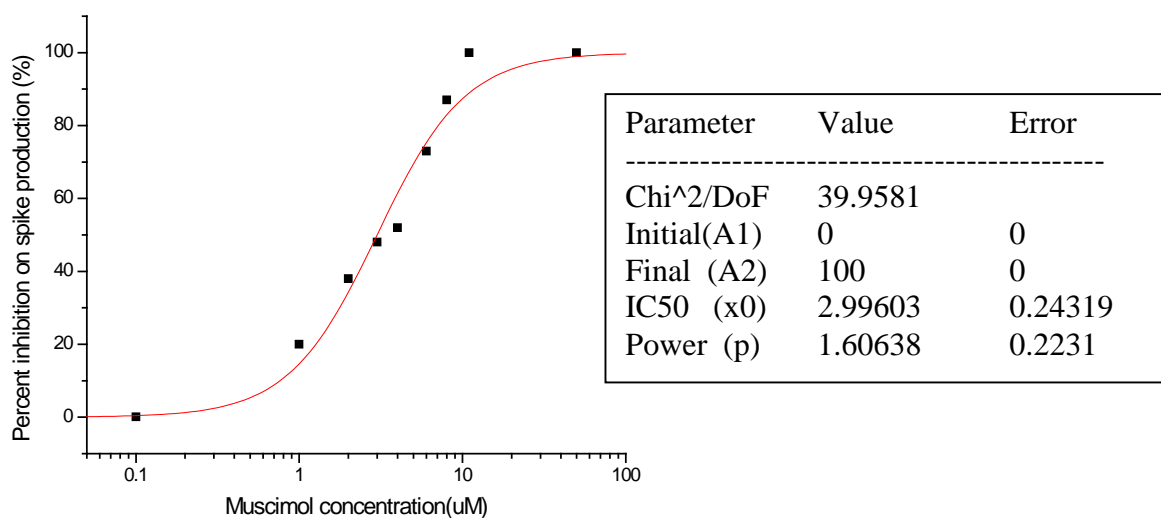


Parameter	Value	Error
Chi ² /DoF	6.23778	
Initial(A1)	0	0
Final (A2)	100	0
IC50 (x0)	1.14376	0.21394
Power (p)	1.68723	0.31477

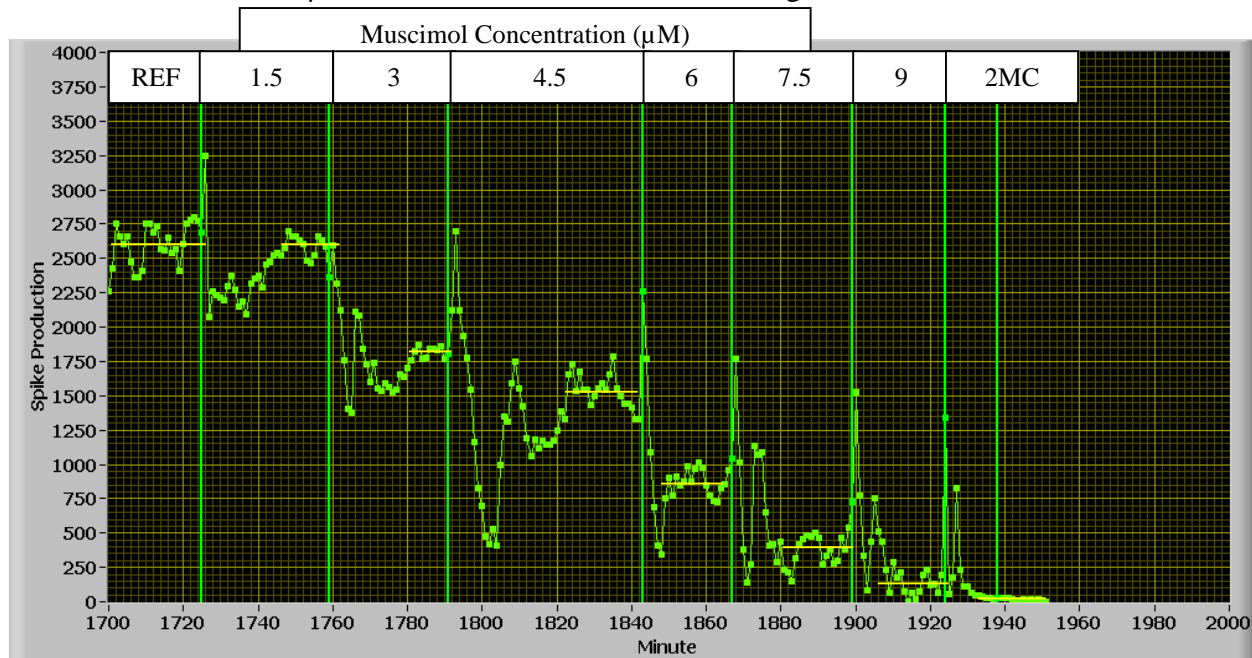
Ex 7-1 (WT031 part 1 5/26/2013): Dose-response curve of muscimol is created under 40 μ M of bicuculline. IC₅₀ is 3.0 μ M.



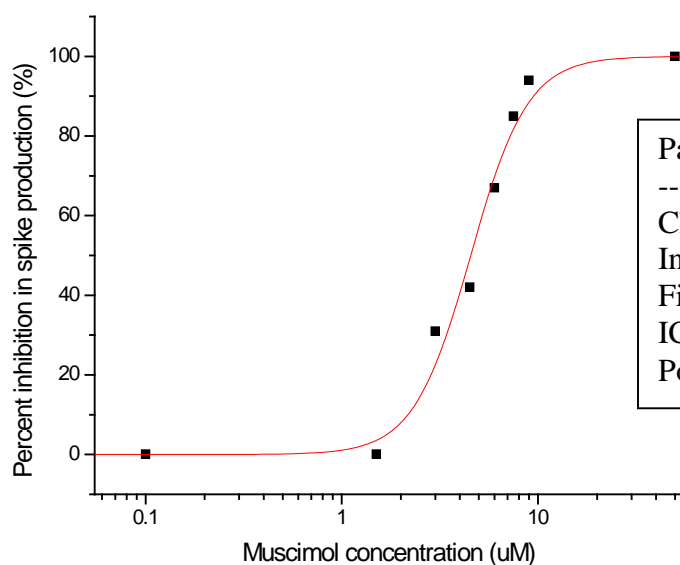
Molarity (μ M)	REF	1	2	3	4	6	8	11	MC
Spike Production	6000	4800	3700	3100	2900	1600	800	0	6200
Percent Decrease	N/A	20%	38%	48%	52%	73%	87%	100%	-3%



Ex 7-2 (WT031 part 2 5/26/2013): Dose-response curve of muscimol is created under 40 μ M of bicuculline. IC₅₀ is 4.5 μ M. No recover after 2 medium changes.

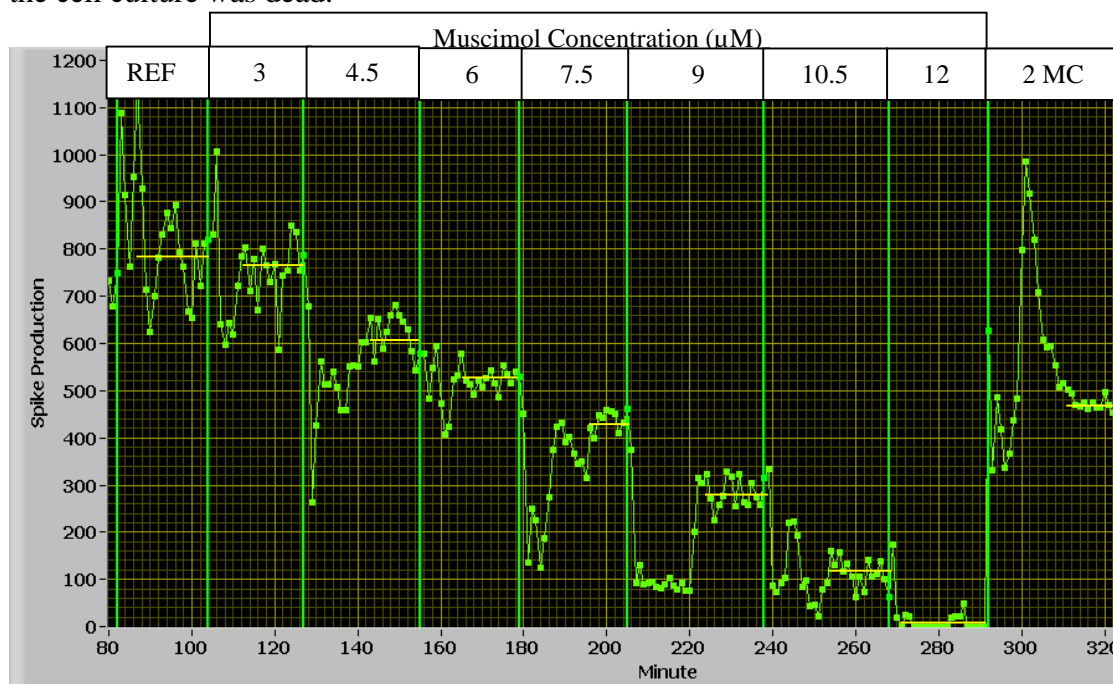


Molarity(μM)	REF	1.5	3	4.5	6	7.5	9	2MC
Spike Production	2600	2600	1800	1500	850	400	150	0
Percent Decrease	N/A	0%	31%	42%	67%	85%	94%	100%

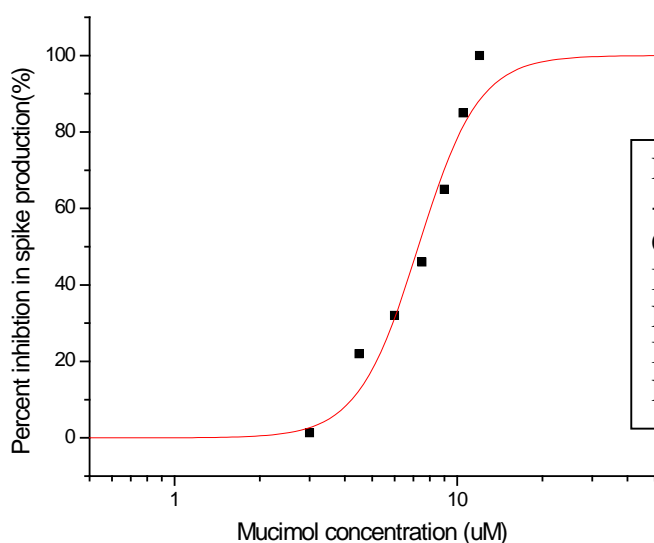


Parameter	Value	Error
Chi ² /DoF	30.97447	
Initial(A1)	0	0
Final (A2)	100	0
IC50 (x0)	4.5392	0.21265
Power (p)	2.98447	0.40332

Ex 8 (WT034 7/10/2013): Dose-response curve of muscimol is created under 40 μ M of bicuculline. IC₅₀ is 7.3 μ M. Some recovery was shown after 2 medium changes, but eventually the cell culture was dead.

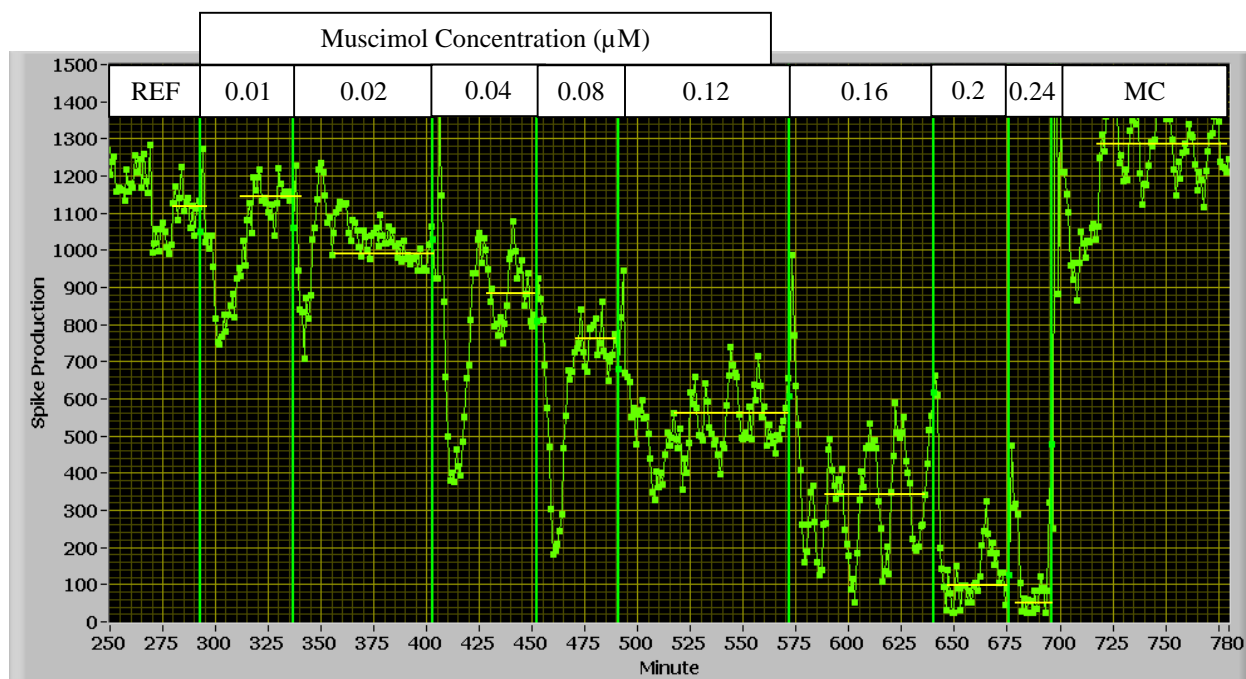


Molarity(μ M)	REF	3	4.5	6	7.5	9	10.5	12	2MC
Spike Production	780	770	610	530	420	280	120	0	460
Percent Decrease	N/A	1.3%	22%	32%	46%	64%	85%	100%	41%

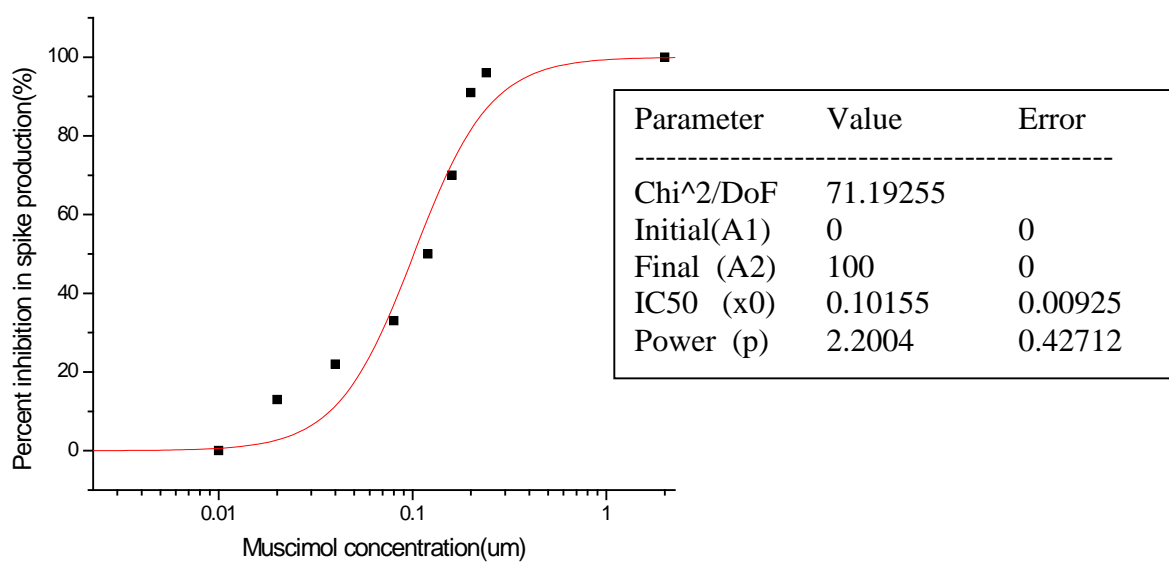


Parameter	Value	Error
Chi ² /DoF	53.01604	
Initial(A1)	0	0
Final (A2)	100	0
IC ₅₀ (x0)	7.27914	0.29902
Power (p)	4.06019	0.65837

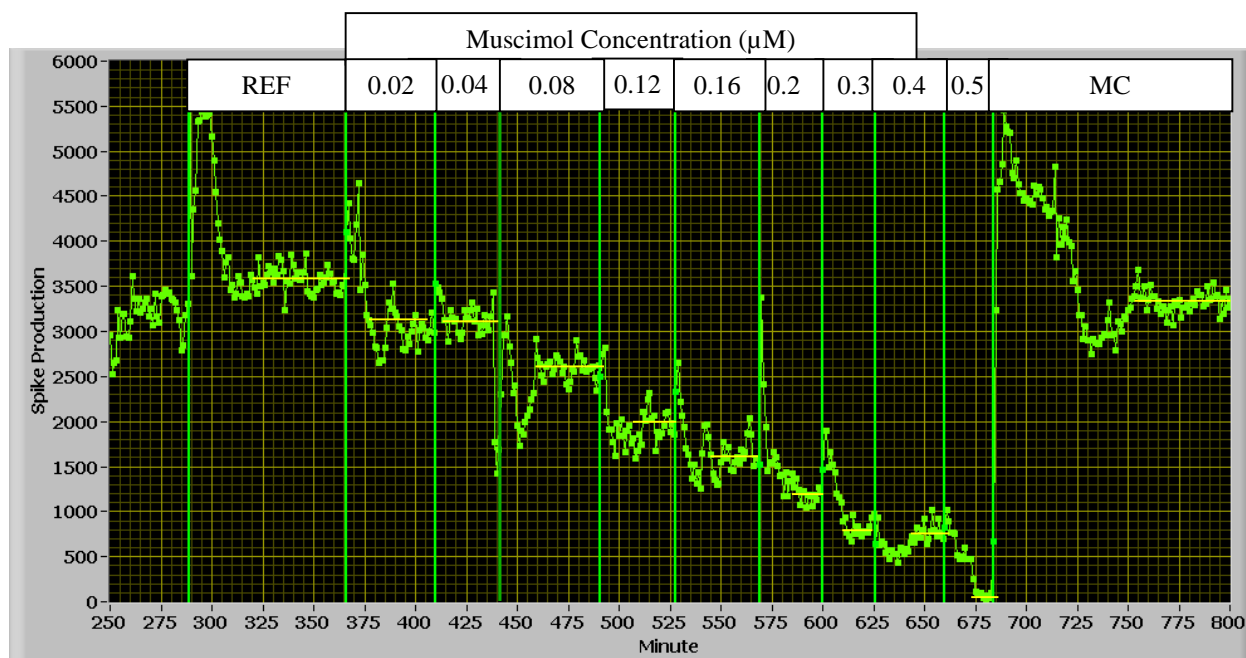
Ex 9 (WT039 7/29/2013): Dose-response curve of muscimol is created under NO bicuculline. IC50 is 0.10 μ M. Full recovery was shown after one medium change.



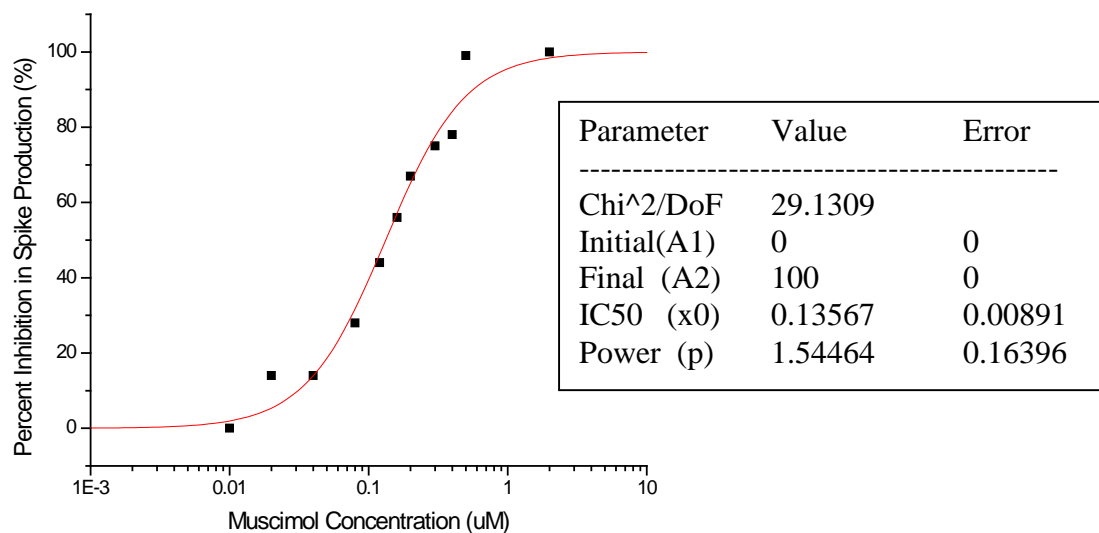
Molarity (μ M)	REF	0.01	0.02	0.04	0.08	0.12	0.16	0.20	0.24	MC
Spike Production	1125	1150	980	880	760	560	340	100	50	1280
Percent Decrease	N/A	0%	13%	22%	33%	50%	70%	91%	96%	0%



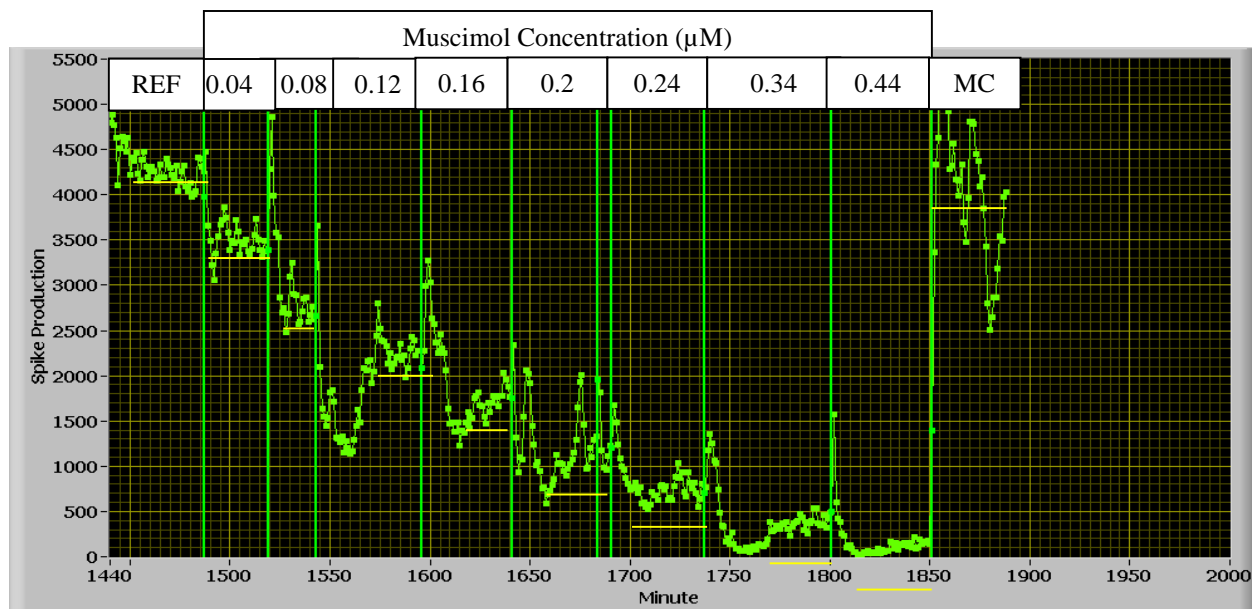
Ex 10-1(WT041-1 8/26/2013): Dose-response curve of muscimol is created under NO bicuculline. IC50 is 0.14 μ M. Almost full recovery was shown after one medium change. Second dose-response experiment was carried on the second day.



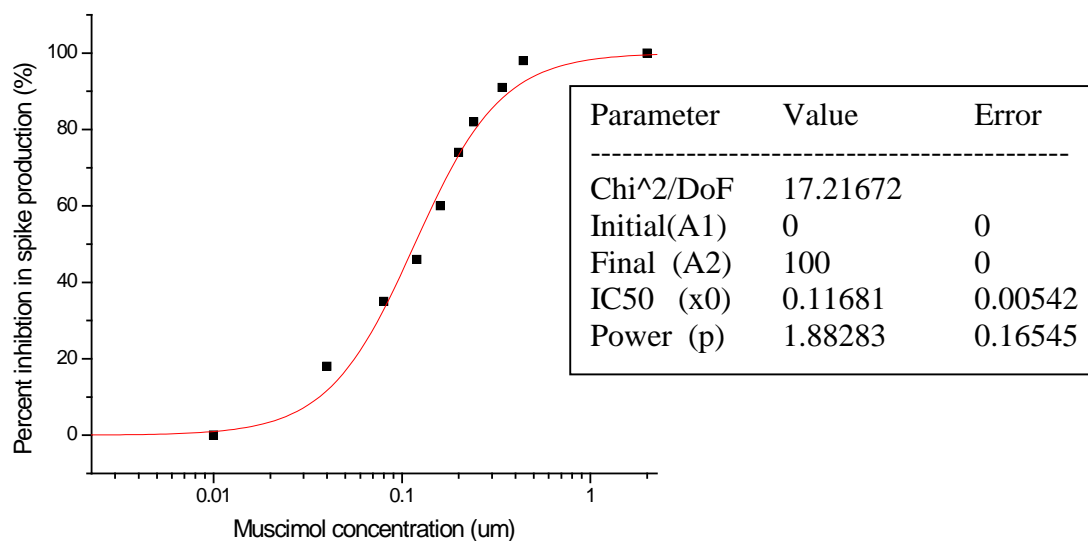
Molarity (μ M)	REF	0.02	0.04	0.08	0.12	0.16	0.20	0.30	0.40	0.50	MC
Spike Production	3600	3100	3100	2600	2000	1600	1200	750	700	50	3300
Percent Decrease	N/A	14%	14%	28%	44%	56%	67%	79%	81%	99%	8%



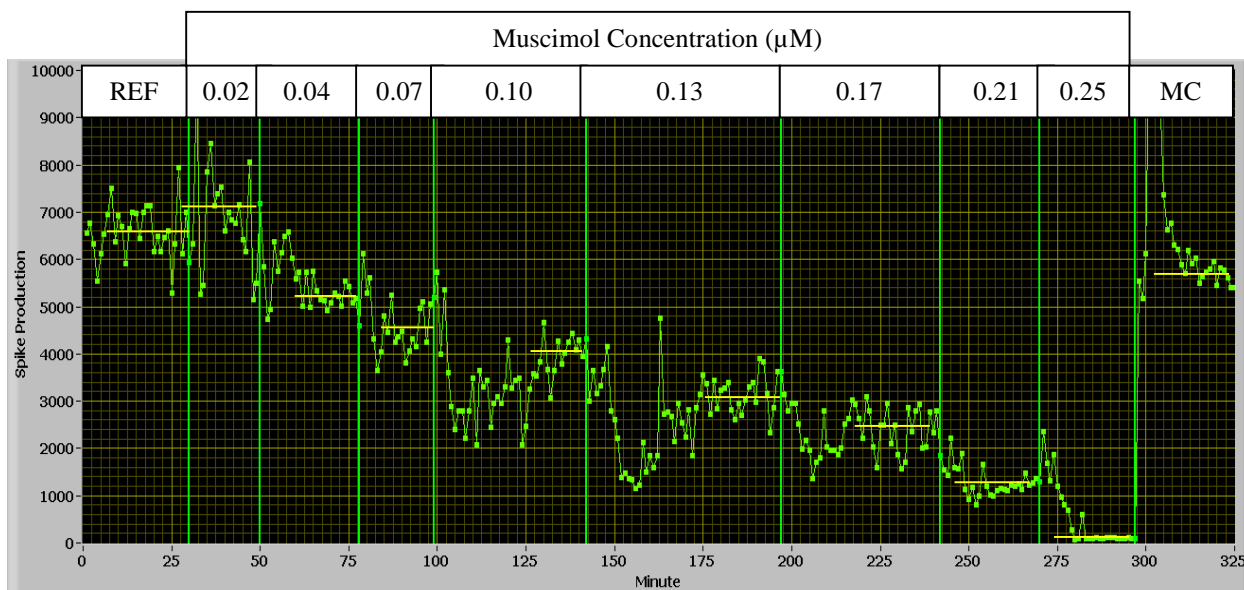
Ex 10-2 (WT041-2 8/27/13): Dose-response curve of muscimol is created under NO bicuculline. IC₅₀ is 0.12 μ M. This is the second part of experiment, but interestingly, the spike production of reference was increased from 3500 to 4250. Almost full recovery was shown after one medium change.



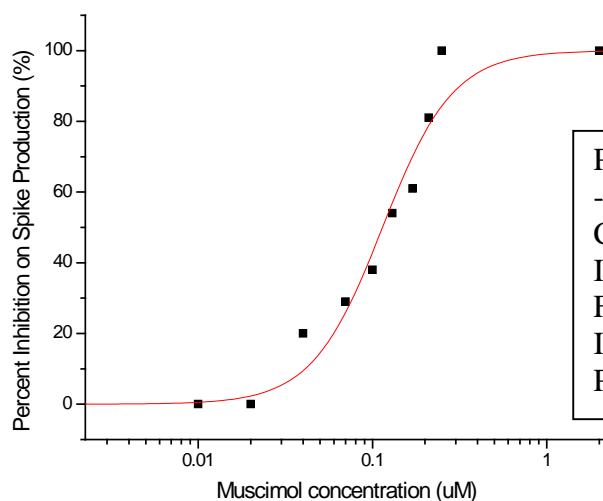
Molarity (μ M)	REF	0.04	0.08	0.12	0.16	0.20	0.24	0.34	0.44	MC
Spike Production	4250	3500	2750	2300	1750	1100	750	400	100	4000
Percent Decrease	N/A	18%	35%	46%	60%	74%	82%	91%	98%	6%



Ex 11-1 (WT049-1 10/2/13): Dose-response curve of muscimol is created under NO bicuculline. IC50 is 0.11 μM . Almost full recovery was shown after one medium change.

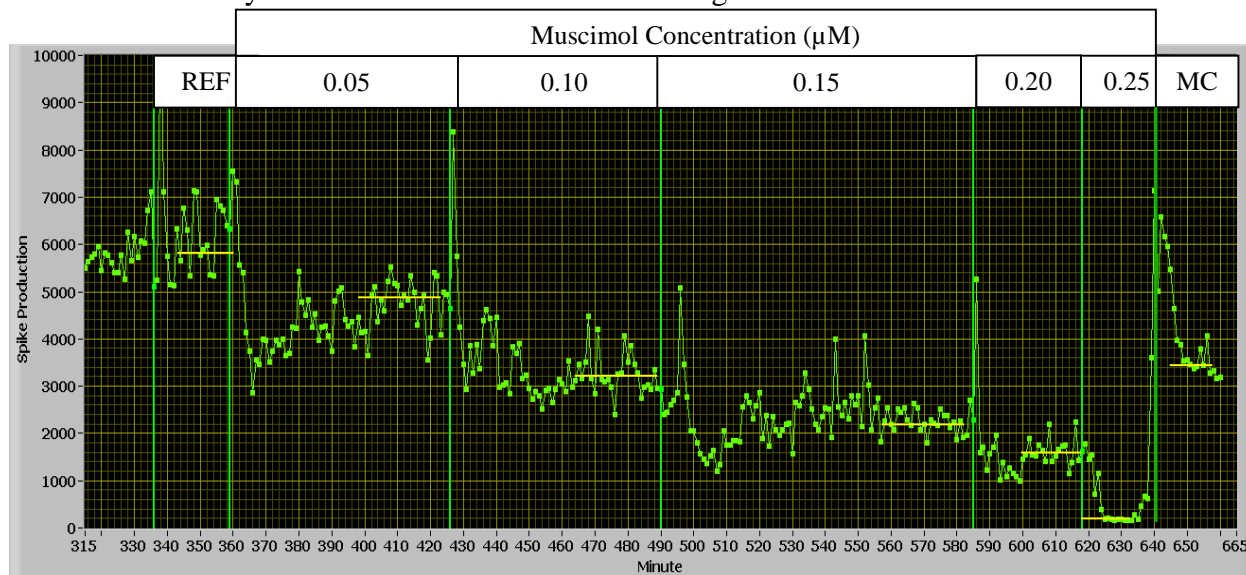


Molarity(μM)	REF	0.02	0.04	0.07	0.10	0.13	0.17	0.21	0.25	MC
Spike Production	6500	7000	5200	4600	4000	3000	2500	1250	0	5750
Percent Decrease (%)	N/A	+7.6	20%	29	38	54	61	81	100	16

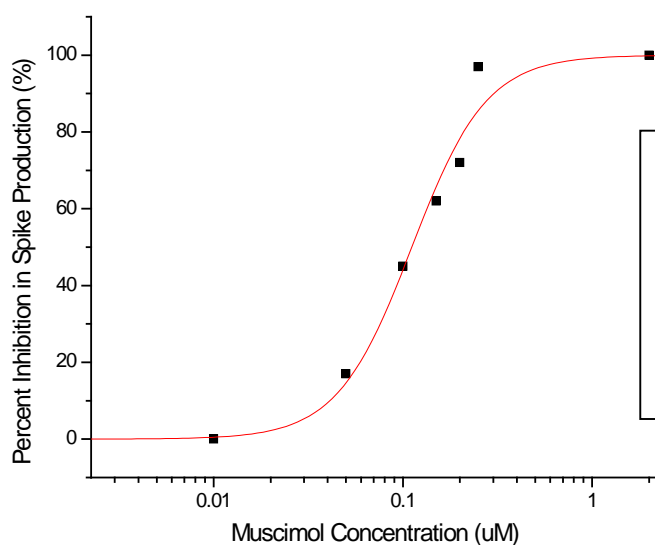


Parameter	Value	Error
Chi ² /DoF	61.56863	
Initial(A1)	0	0
Final (A2)	100	0
IC50 (x0)	0.11409	0.00842
Power (p)	2.15041	0.36201

Ex 11-2 (WT049-2 10/2/13): Dose-response curve of muscimol is created under **NO** bicuculline. IC50 is 0.11 μ M. This is the second part of experiment that was carried on the same day of Ex 14-1. Half recovery was shown after one medium change.

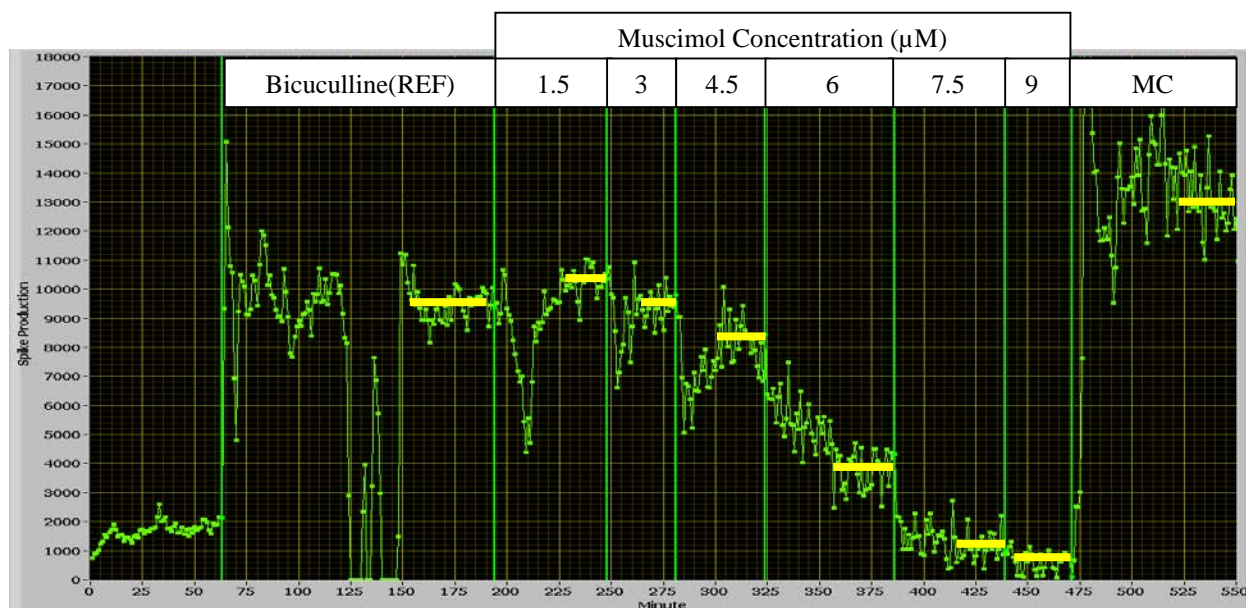


Molarity(μ M)	REF	0.05	0.10	0.15	0.20	0.25	MC
Spike Production	5800	4800	3200	2200	1600	200	3400
Percent Decrease	N/A	17%	45%	62%	72%	97%	64%

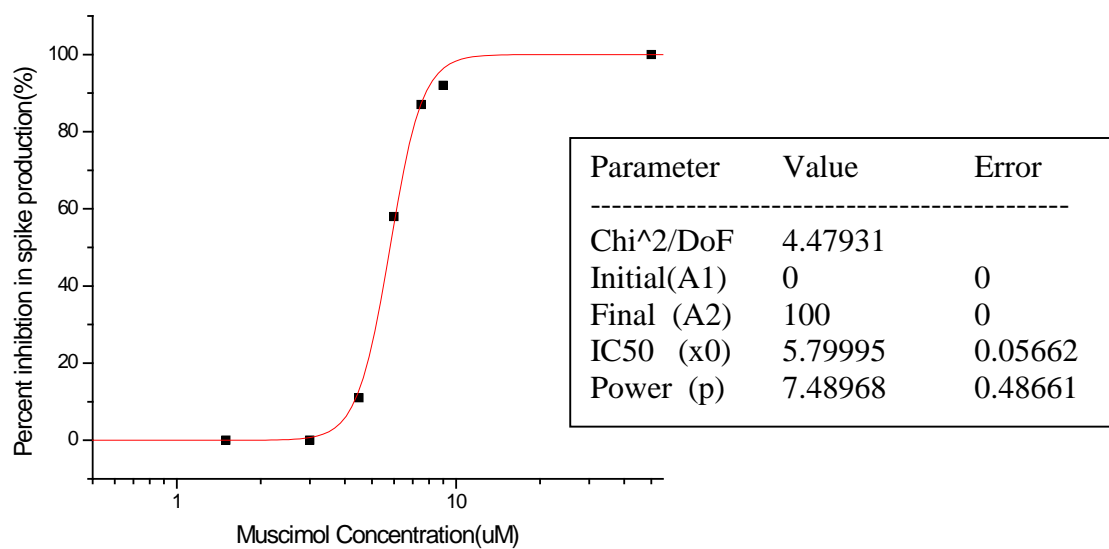


Parameter	Value	Error
Chi ² /DoF	38.55901	
Initial(A1)	0	0
Final (A2)	100	0
IC50 (x0)	0.11143	0.00803
Power (p)	2.20163	0.34997

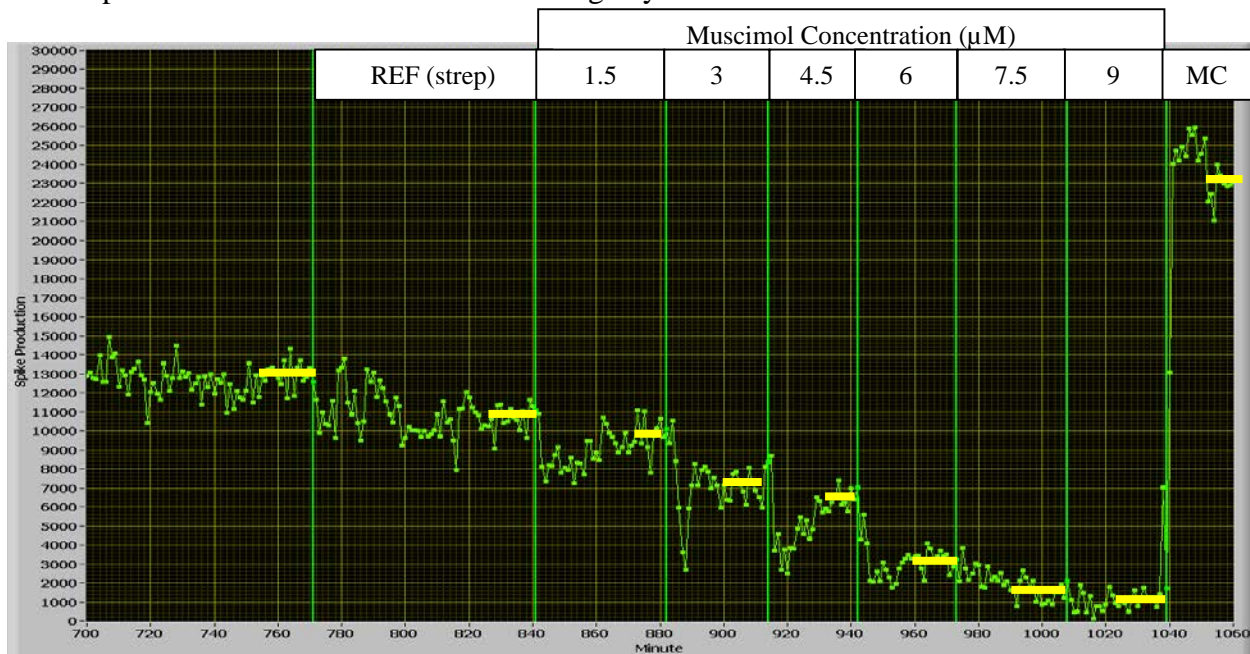
Ex 12-1 (WT050 10/8/13): Dose-response curve of muscimol is created under 40 μ M bicuculline. IC50 is 5.80 μ M. Full recovery was shown after one medium change.



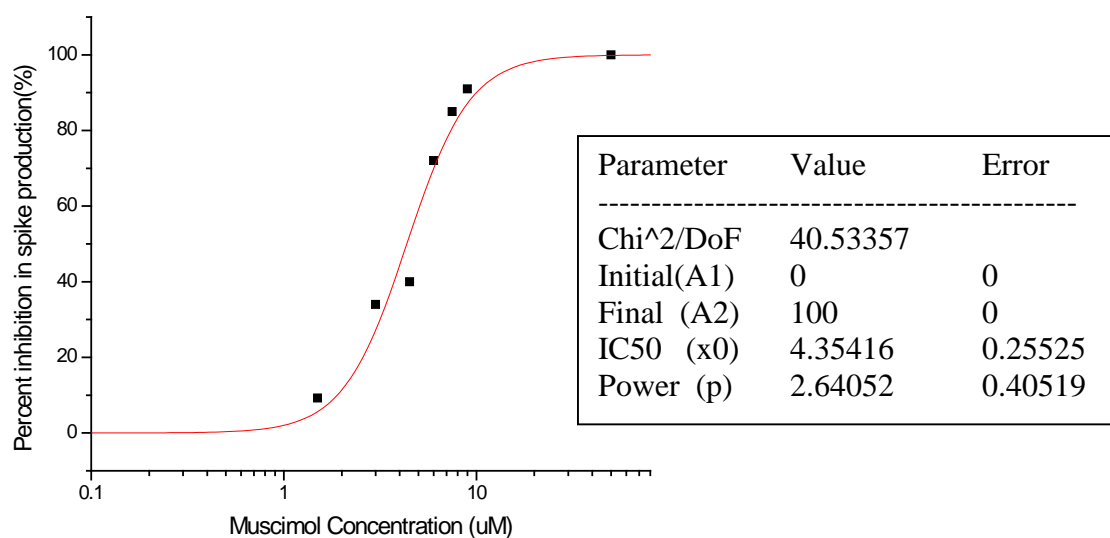
Molarity(mM)	REF	1.5	3	4.5	6	7.5	9	MC
Spike Production	9500	10400	9500	8500	4000	1200	800	12800
Percent Decrease (%)	N/A	+9.4	0	11	58	87	92	+35



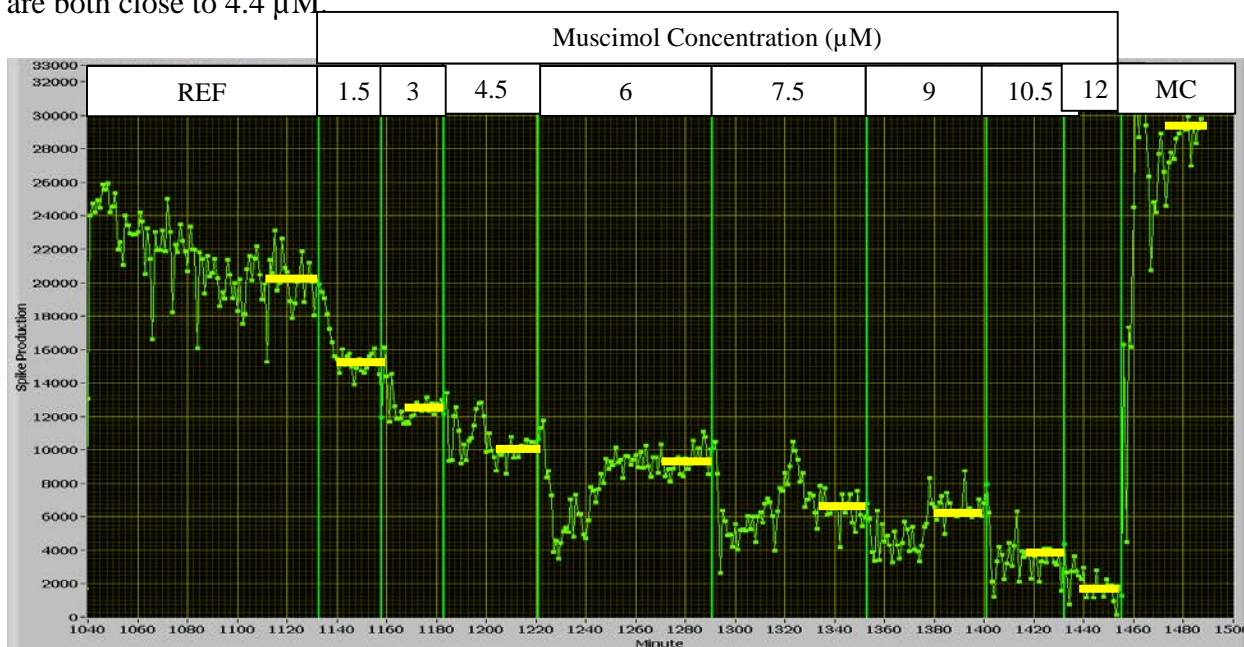
Ex 12-2 (WT050 10/9/13): Dose-response curve of muscimol is created under 40 μ M bicuculline and 0.1mM of streptomycin. IC₅₀ is 4.4 μ M. Full recovery was shown after one medium change. This experiment is conducted on the following day of Ex15-1.



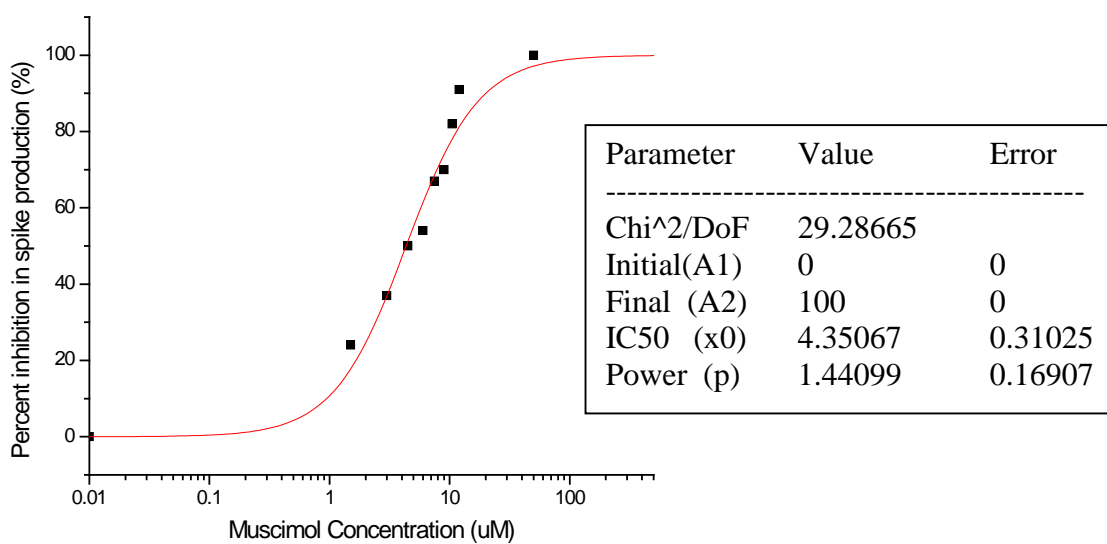
Molarity(mM)	Pre-strep	strep	1.5	3	4.5	6	7.5	9	MC
Spike Production	13000	10900	9900	7200	6500	3000	1600	1000	23000
Percent Decrease	N/A	N/A	9.2%	34%	40%	72%	85%	91%	+77%



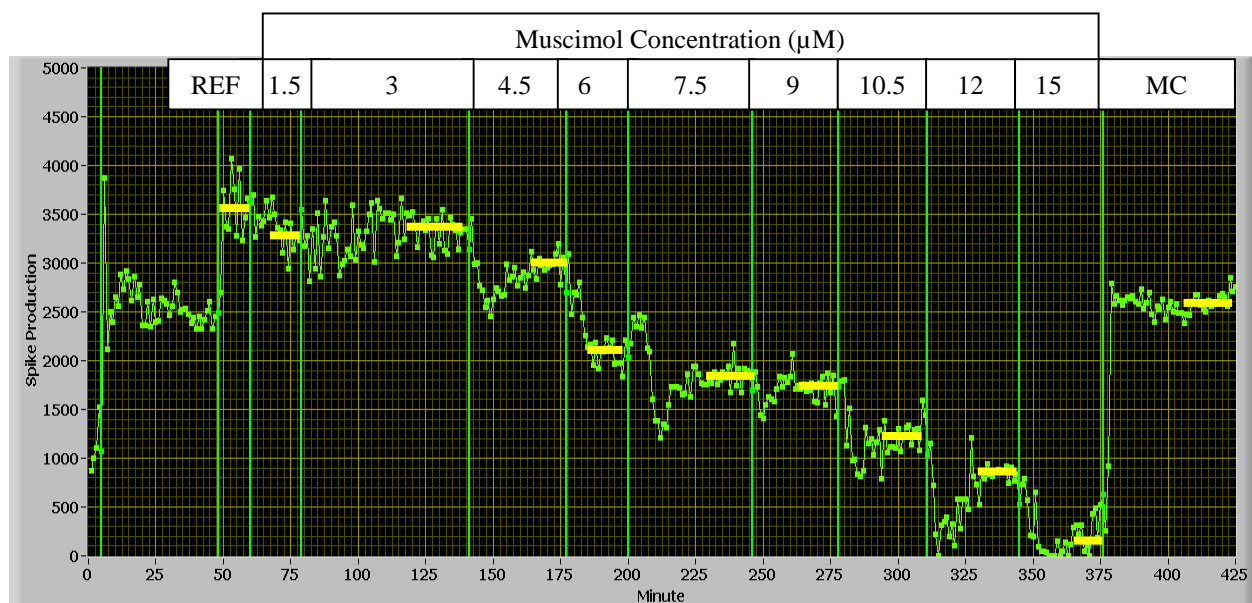
Ex 12-3 (WT050 10/9/13): Dose-response curve of muscimol is created under 40 μ M bicuculline and without streptomycin. IC₅₀ is 4.4 μ M. Full recovery was shown after one medium change. This experiment is conducted on the same day of Ex 15-2. Notice that IC₅₀ is very similar: they are both close to 4.4 μ M.



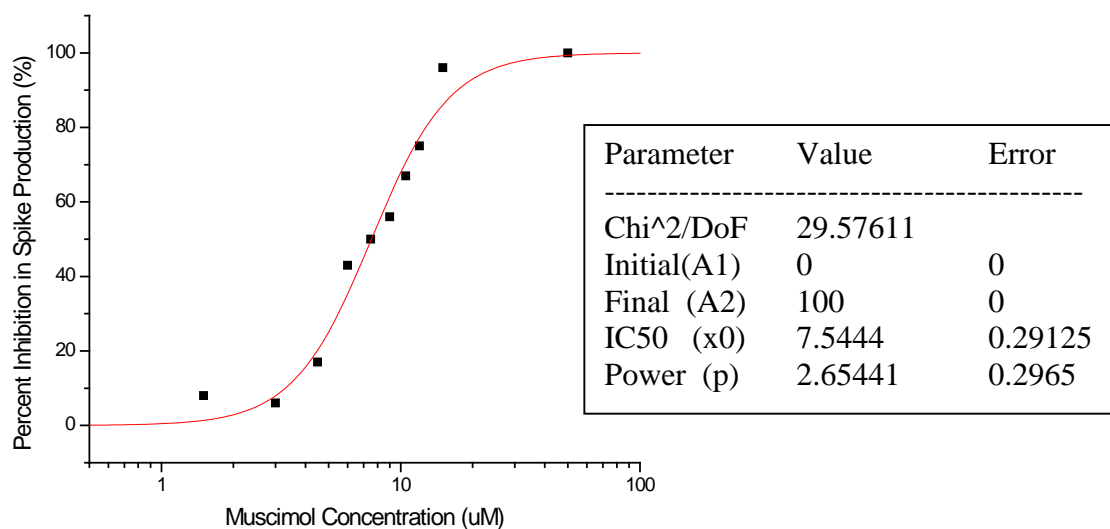
Molarity (mM)	REF	1.5	3	4.5	6	7.5	9	10.5	12	MC
Spike Production	20100	15200	12600	10000	9200	6600	6100	3700	1800	28800
Percent Decrease	N/A	24%	37%	50%	54%	67%	70%	82%	91%	+43%



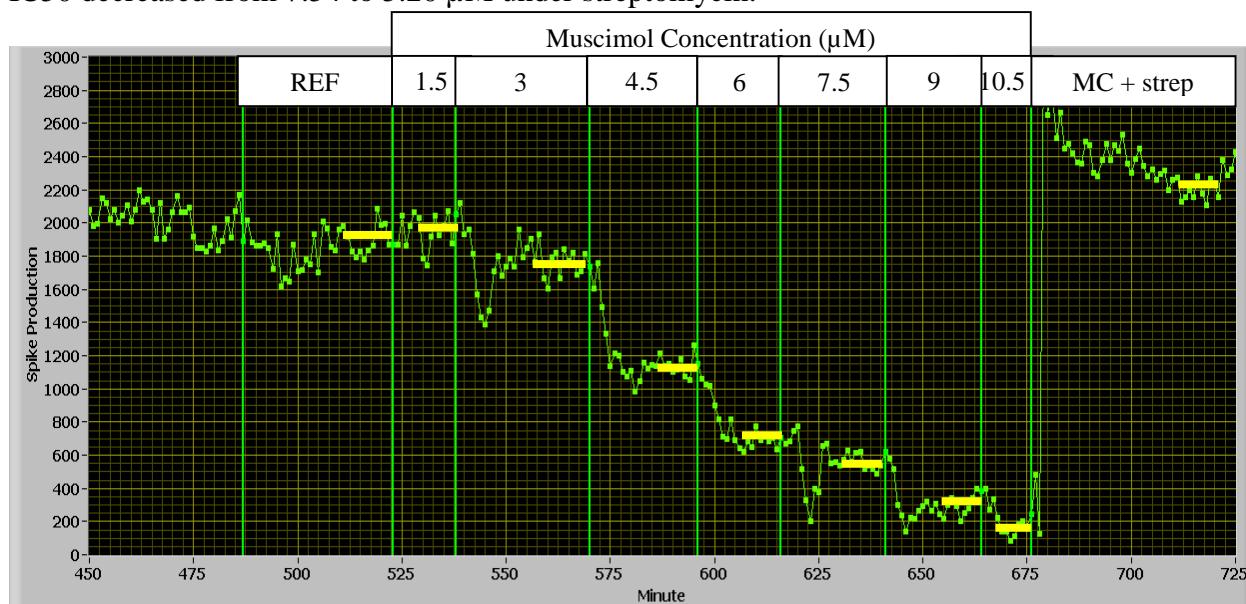
Ex 13-1 (WT054 10/30/13): Dose-response curve of muscimol is created under 40 μ M bicuculline. IC50 is 7.5 μ M. Partial recovery was shown after one medium change.



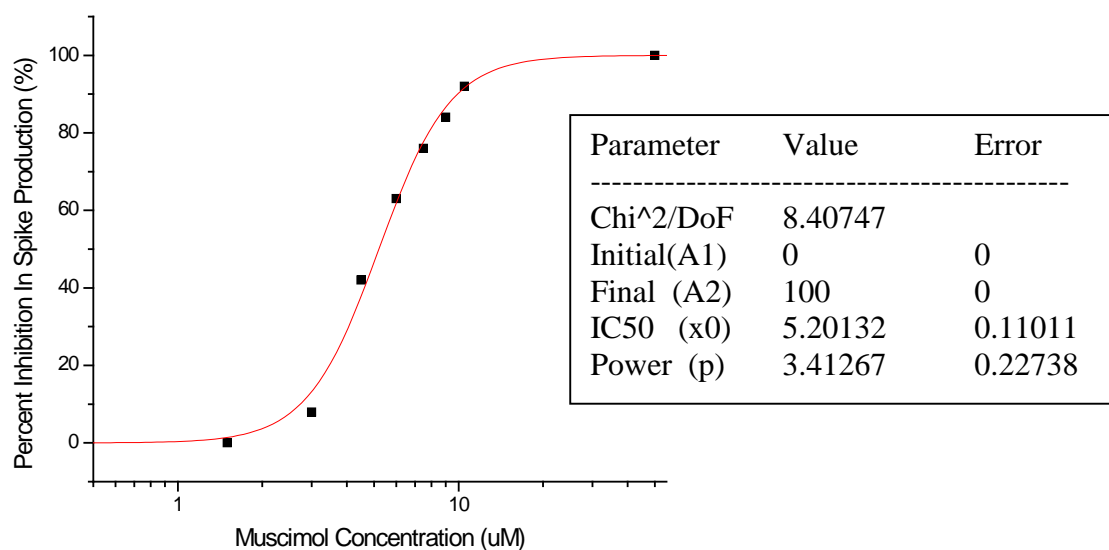
Molarity (mM)	REF	1.5	3	4.5	6	7.5	9	10.5	12	15	MC
Spike Production	3600	3300	3400	3000	2050	1800	1600	1200	900	150	2600
Percent Decrease	N/A	8%	6%	17%	43%	50%	56%	67%	75%	96%	28%



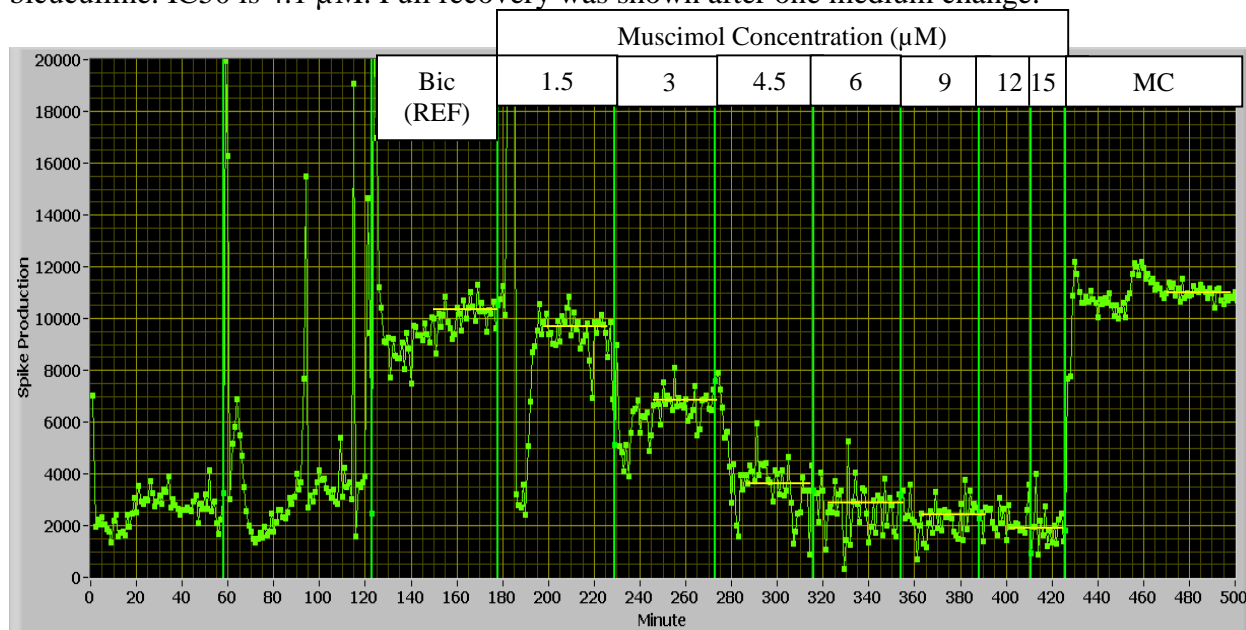
Ex 13-2 (WT054 10/30/13): Dose-response curve of muscimol is created under 40 μ M bicuculline and 0.1 mM streptomycin. IC₅₀ is 5.20 μ M. Full recovery was shown after one medium change. This experiment is conducted on the same day of Ex 13-1, and it's shown that IC₅₀ decreased from 7.54 to 5.20 μ M under streptomycin.



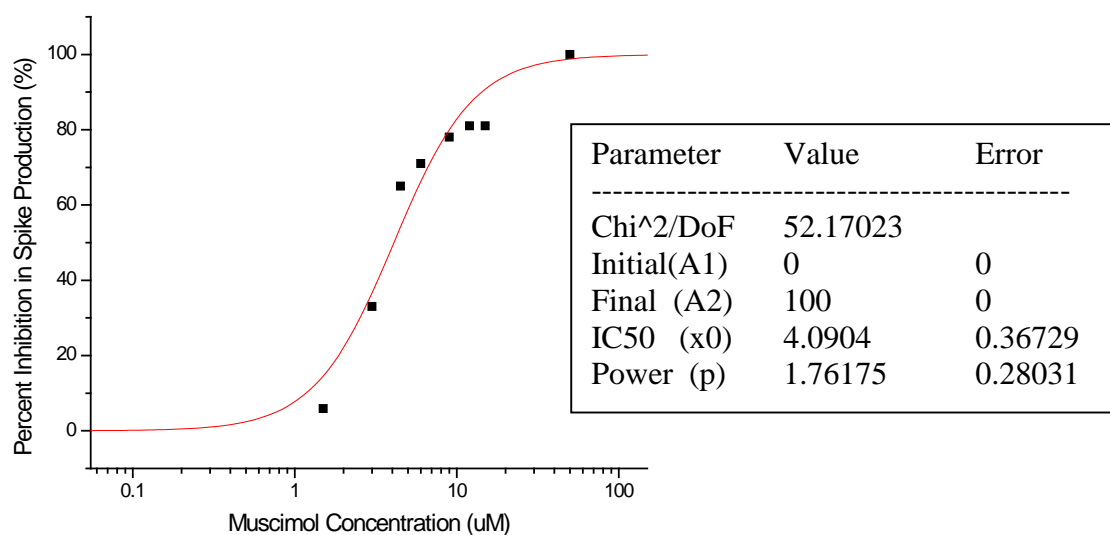
Molarity(mM)	REF	1.5	3	4.5	6	7.5	9	10.5	MC
Spike Production	1900	1950	1750	1100	700	450	300	150	2200
Percent Decrease (%)	N/A	+2.6	7.9	42	63	76	84	92	+15



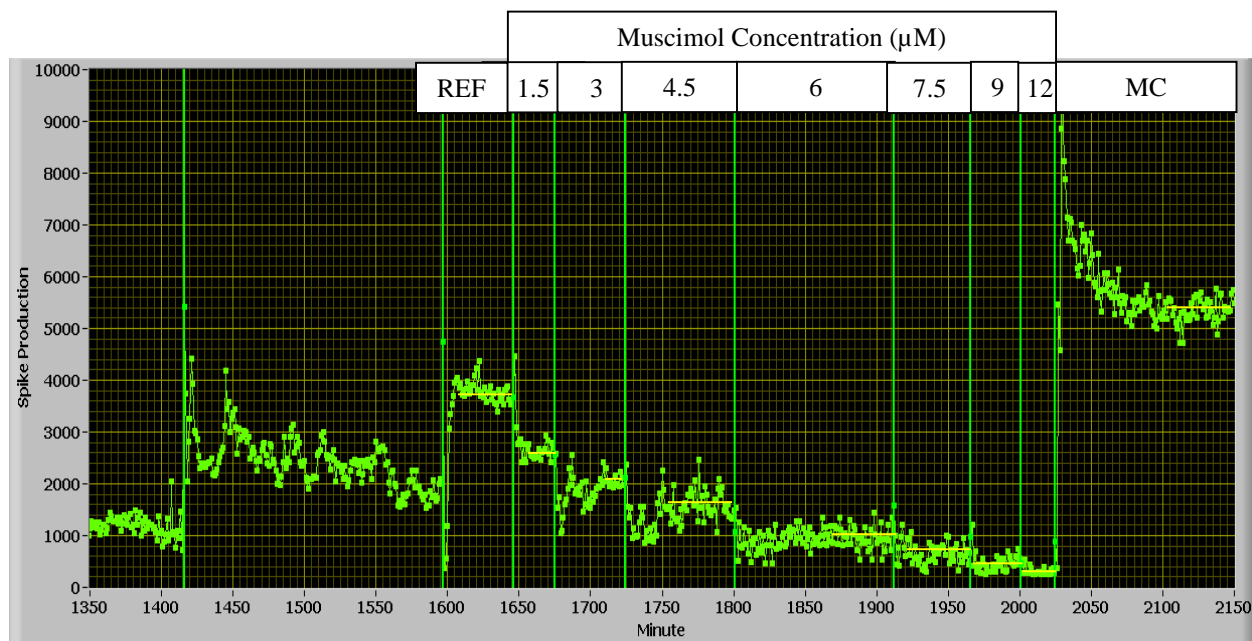
Ex 14-1 (WT056 11/12/13): Dose-response curve of muscimol is created under 40 μM bicuculline. IC50 is 4.1 μM . Full recovery was shown after one medium change.



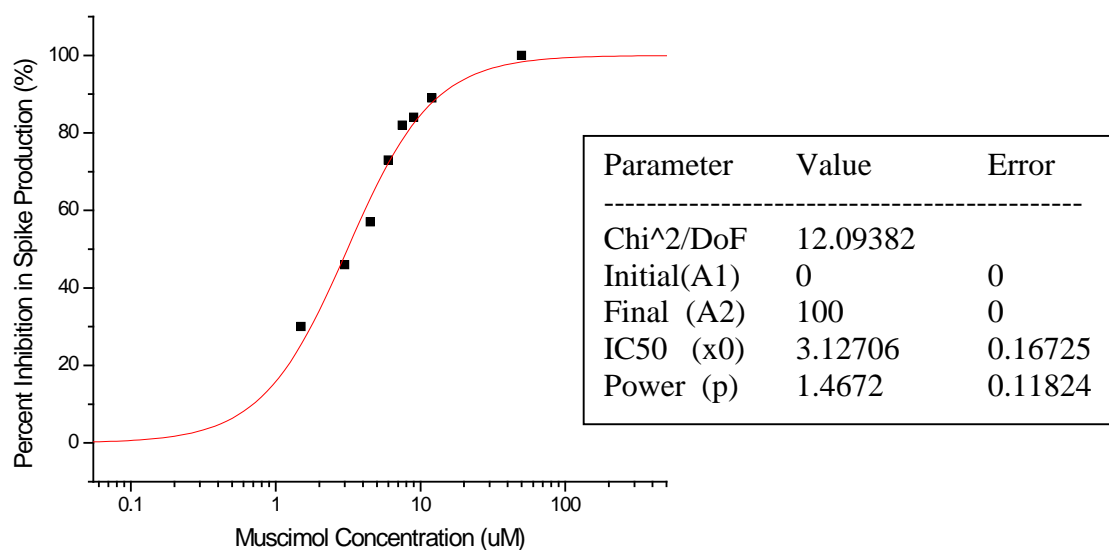
Molarity(mM)	REF	1.5	3	4.5	6	9	12	15	MC
Spike Production	10200	9600	6800	3600	3000	2200	1980	1980	11000
Percent Decrease	N/A	5.9%	33%	65%	71%	78%	81%	81%	+7.8%



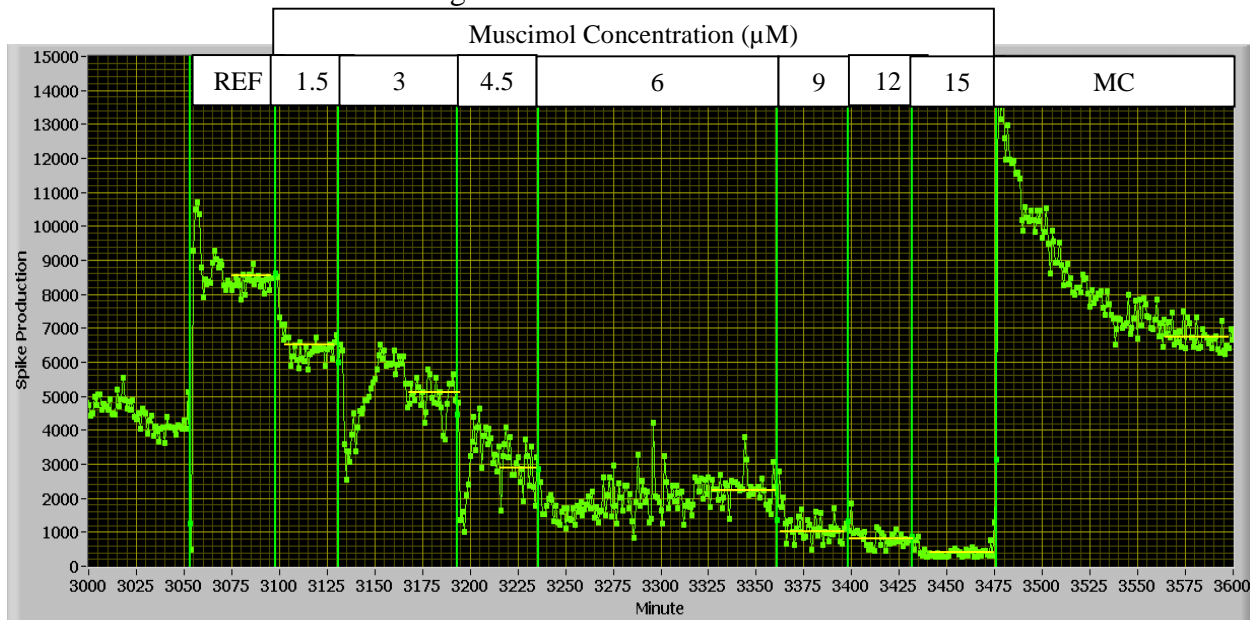
Ex 14-2 (WT056 11/13/13): Dose-response curve of muscimol is created under 40 μ M bicuculline. IC₅₀ is 3.12 μ M. Full recovery was shown after one medium change. This experiment and the following experiment were conducted in DMEM6 medium.



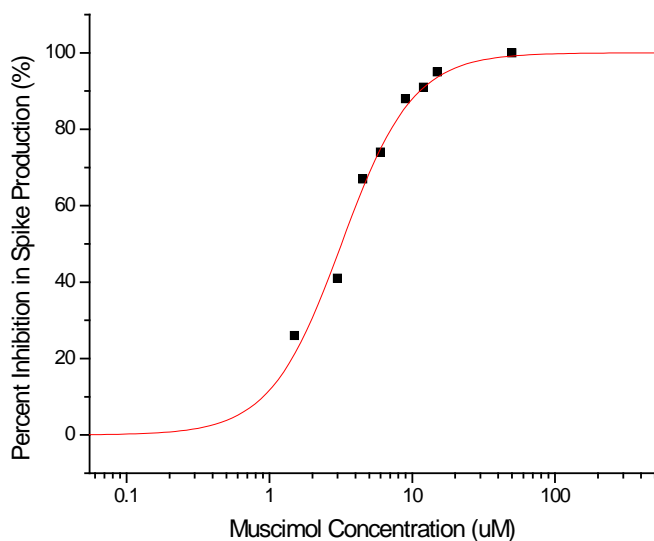
Molarity(mM)	REF	1.5	3	4.5	6	7.5	9	12	MC
Spike Production	3700	2600	2000	1600	1000	650	600	400	5400
Percent Decrease	N/A	30%	46%	57%	73%	82%	84%	89%	+46%



Ex 14-3 (WT056 11/14/13): Dose-response curve of muscimol is created under 40 μ M bicuculline. IC50 is 3.19 μ M, which is very close to previous result, 3.12 μ M. Partial recovery was shown after one medium change. DMEM6 was used as medium.

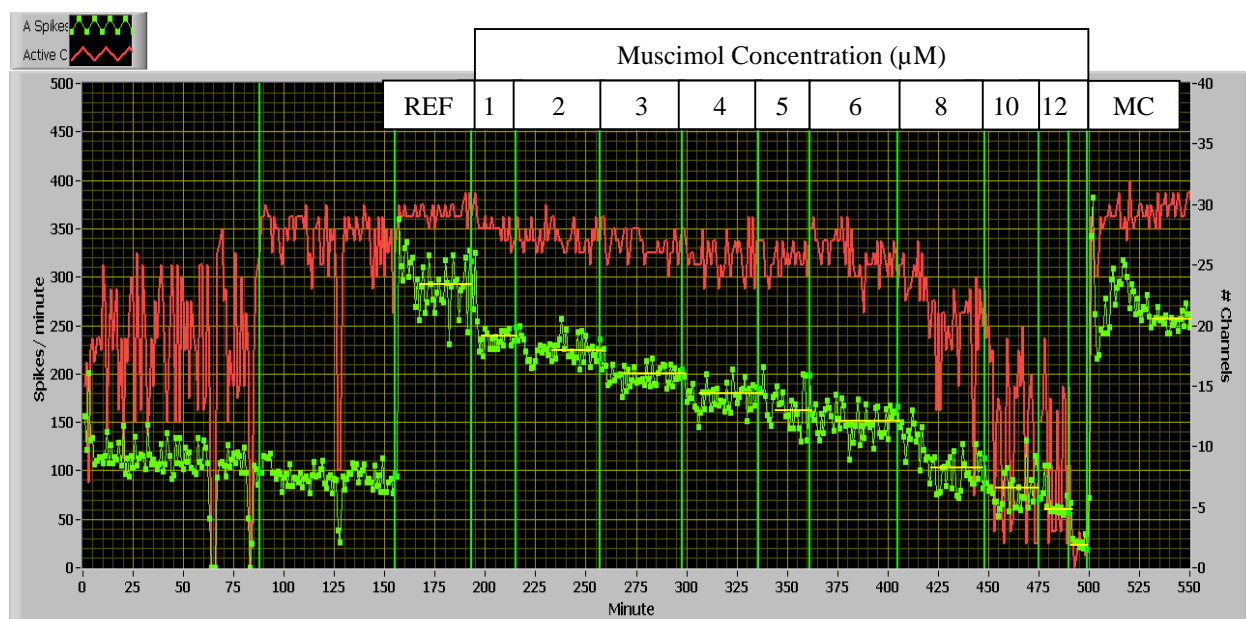


Molarity(mM)	REF	1.5	3	4.5	6	9	12	15	MC
Spike Production	8500	6500	5000	2800	2200	1000	800	400	6600
Percent Decrease	N/A	26%	41%	67%	74%	88%	91%	95%	22%

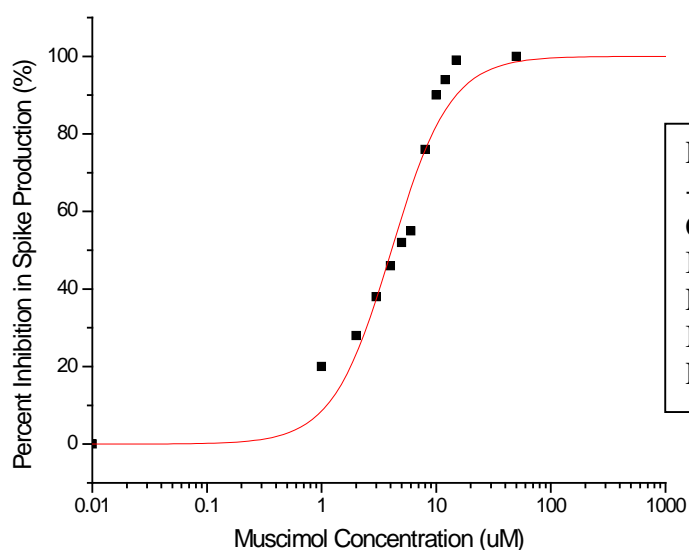


Parameter	Value	Error
<hr/>		
Chi ² /DoF	11.02994	
Initial(A1)	0	0
Final (A2)	100	0
IC50 (x0)	3.19441	0.14585
Power (p)	1.73935	0.13227

Ex 15 (WT058 11/26/13): Dose-response curve of muscimol is created under 20 μM bicuculline. IC50 is 4.07 μM . Partial recovery was shown after one medium change.



Molarity (mM)	REF	1	2	3	4	5	6	8	10	12	15	MC
Spike Production	8410	6720	6075	5200	4500	4000	3750	2000	800	480	50	7800
Percent Decrease	N/A	20%	28%	38%	46%	52%	55%	76%	90%	94%	99%	7%



Parameter	Value	Error
Chi ² /DoF	53.42138	
Initial(A1)	0	0
Final (A2)	100	0
IC50 (x0)	4.06957	0.31226
Power (p)	1.69061	0.23139

APPENDIX D

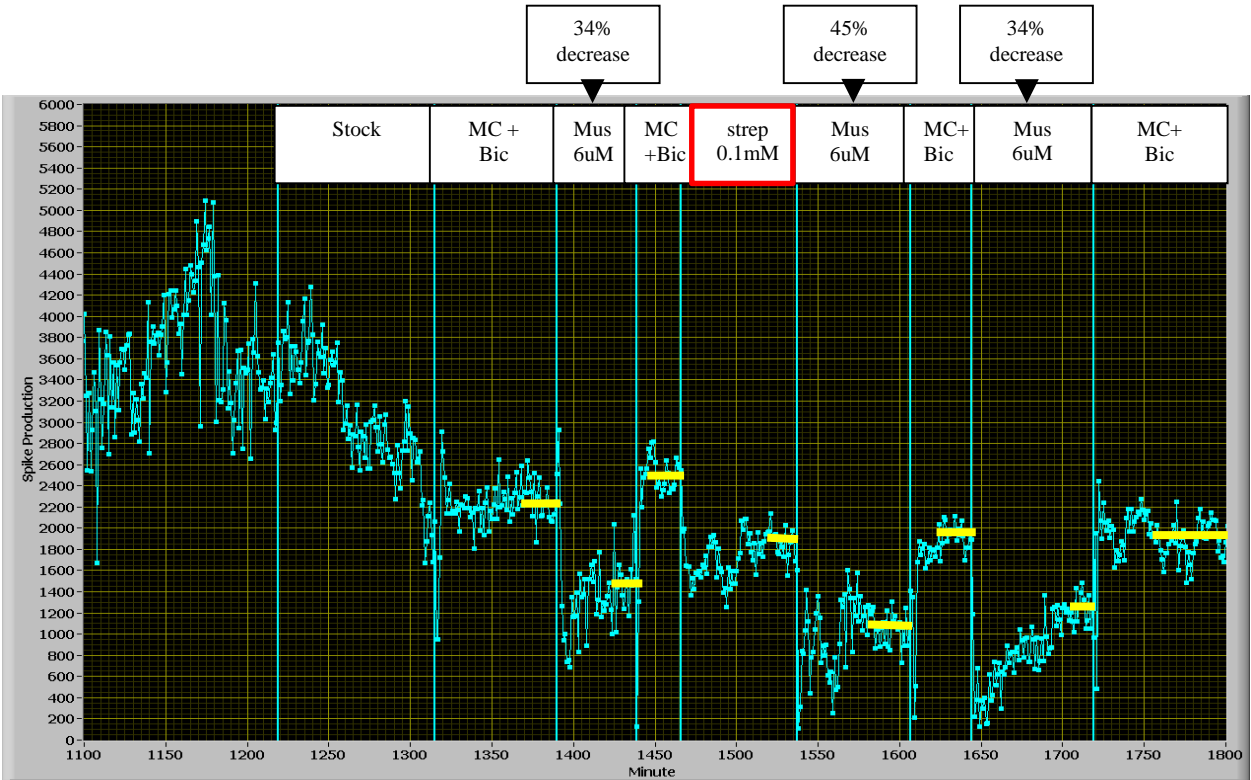
STREPTOMYCIN SENSITIZATION EXPERIMENTS

Ex 16 (WT065 3/19/14): Instead of creating dose-response curve, I added same amount of muscimol (11 μ M) each time to show that if short exposure to 0.1mM streptomycin (34 mins) will cause sensitization. As a result, the spike production of cell culture decreased more after a short exposure to streptomycin (73%: 60, 62%). This experiment was under 40 μ M bicuculline.



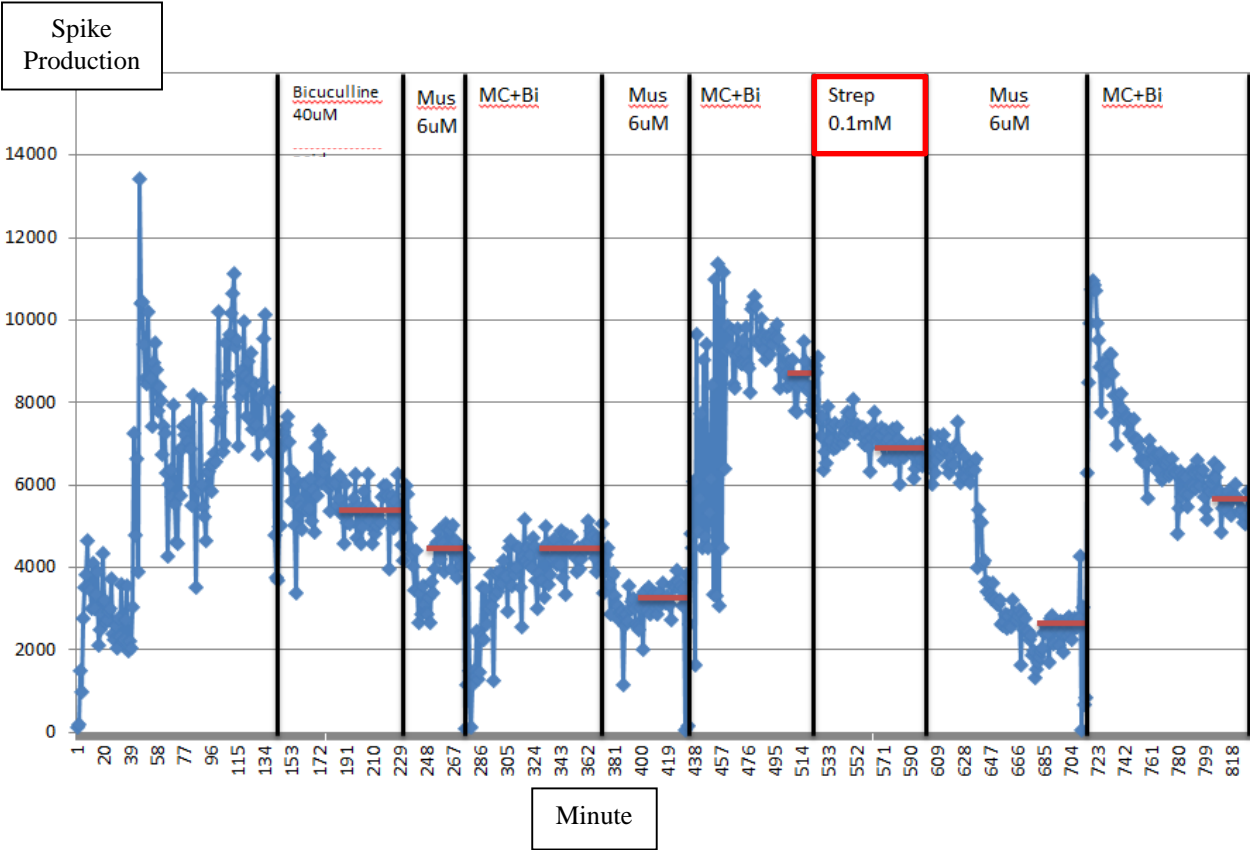
	Reference (Bicuculline 40 μ M)	Muscimol 11 μ M	MC + Bicuculline	streptomycin 0.1mM	Muscimol 11 μ M	MC + Bicuculline	Muscimol 11 μ M	MC + Bicuculline
Spike Production	4600	1850	3800	3350	900	2900	1100	3200
Percent Decrease	N/A	60%	N/A	(10%)	73%	N/A	62%	N/A

Ex 17 (WT073 3/19/14): Instead of creating dose-response curve, I added IC50 amount of muscimol (6μM) to show that if short exposure to 0.1mM streptomycin (71 mins) will cause sensitization. As a result, the spike production of cell culture decreased more after a short exposure to streptomycin (45%: 34, 34%). This experiment was under 40μM bicuculline.



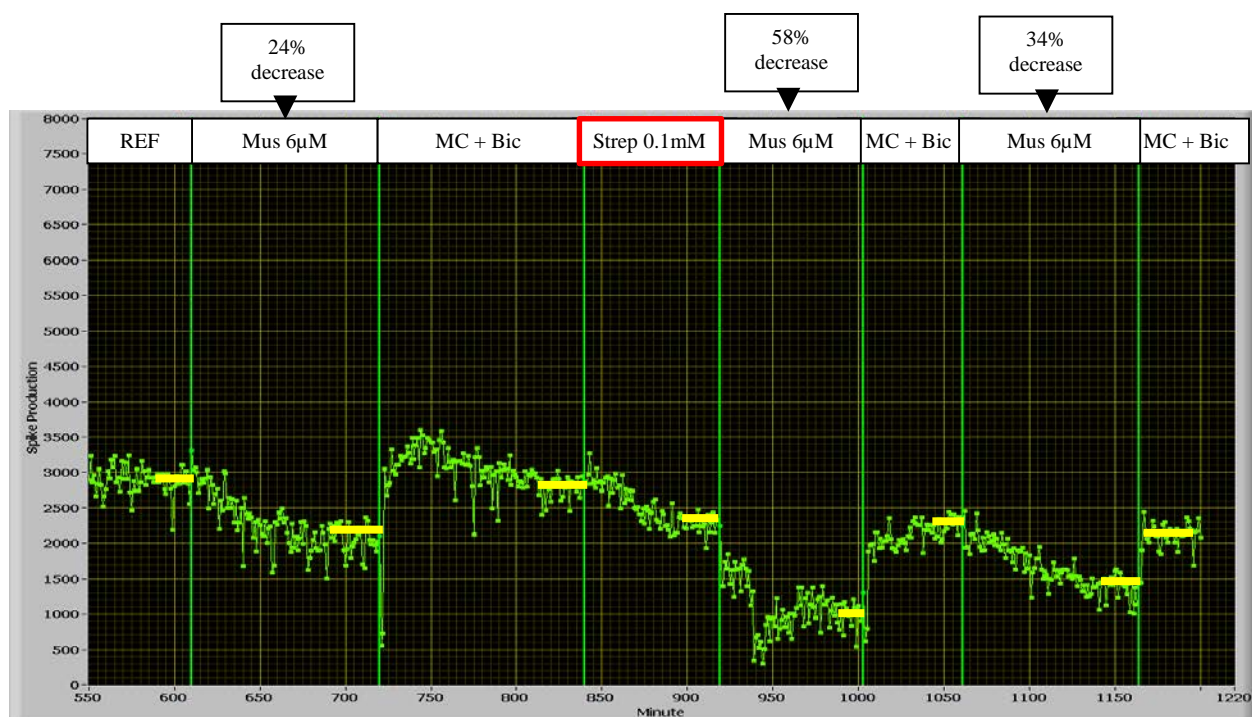
	Reference (Bicuculline 40μM)	Muscimol 6μM	MC + Bicuculline	Streptomycin 0.1mM	Muscimol 6μM	MC + Bicuculline	Muscimol 6μM	MC + Bicuculline
Spike Produciton	2200	1450	2500	1900	1050	1900	1250	1900
Percent Decrease	N/A	34%	N/A	(22%)	45%	N/A	34%	N/A

Ex 18 (WT076 5/23/14): I added IC50 amount of muscimol (6μM) to show that if short exposure to 0.1mM streptomycin (84 mins) will cause sensitization. As a result, the spike production of cell culture decreased more after a short exposure to streptomycin (62%: 17, 24%). This experiment was under 40μM bicuculline and 100μM quinolinic acid.



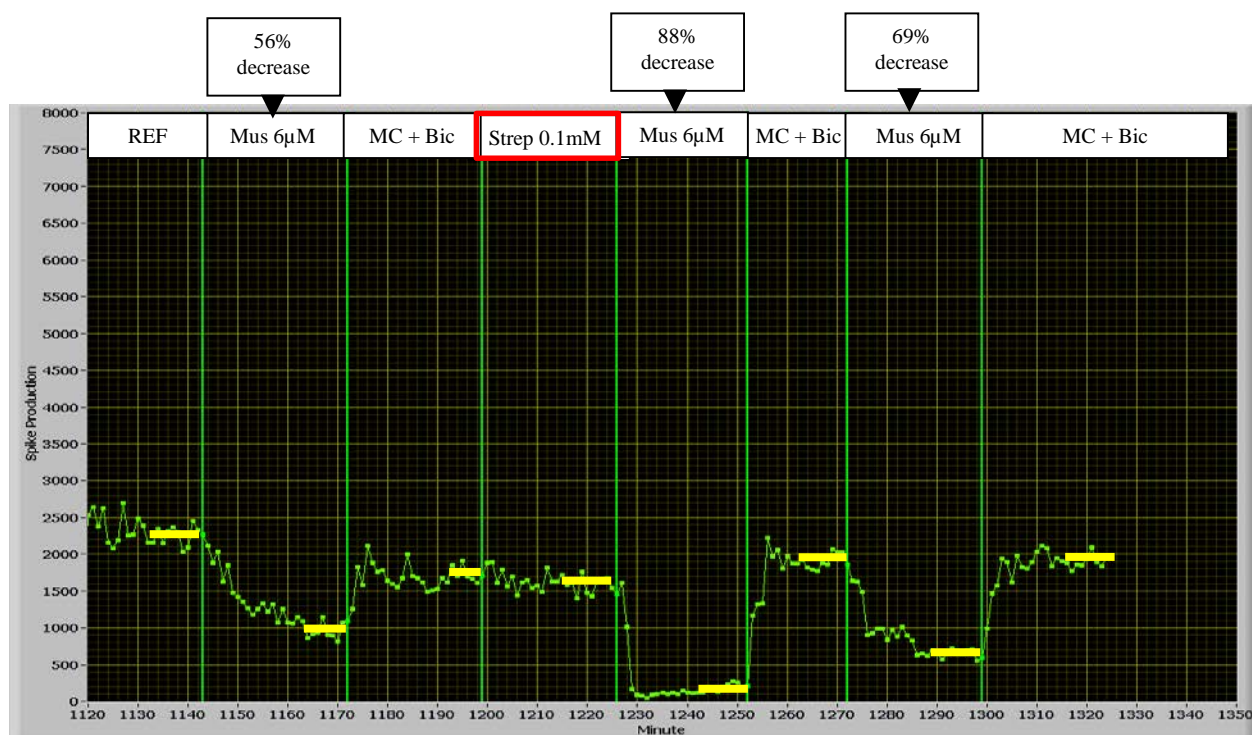
	Reference (Bi 40uM+ QA 100uM)	Muscimol 6μM	MC + Bi + QA	Muscimol 6μM	MC + Bi + QA	Streptomycin 0.1mM	Muscimol 6μM	MC + Bi + QA
Spike Produciton	5400	4500	4500	3400	8400	6900	2600	5600
Percent Decrease	N/A	17%	N/A	24%	N/A	(18%)	62%	N/A

Ex 19 (WT089 10/2/14): I added IC50 amount of muscimol (6 μ M) to show that if short exposure to 0.1mM streptomycin (89 mins) will cause sensitization. As a result, the spike production of cell culture decreased more after a short exposure to streptomycin (58%: 24, 34%). This experiment was under 40 μ M bicuculline.



	Reference (Bicuculline 40 μ M)	Muscimol 6 μ M	MC + Bicuculline	Streptomycin 0.1mM	Muscimol 6 μ M	MC + Bicuculline	Muscimol 6 μ M	MC + Bicuculline
Spike Production	2900	2200	2800	2400	1000	2300	1500	2200
Percent Decrease	N/A	24%	N/A	(14%)	58%	N/A	34%	N/A

Ex 20 (WT091 10/13/14): I added IC50 amount of muscimol (6 μ M) to show that if short exposure to 0.1mM streptomycin (26 mins) will cause sensitization. As a result, the spike production of cell culture decreased more after a short exposure to streptomycin (88%: 69, 56%). This experiment was under 40 μ M bicuculline.



	Reference (Bicuculline 40 μ M)	Muscimol 6 μ M	MC + Bicuculline	Streptomycin 0.1mM	Muscimol 6 μ M	MC + Bicuculline	Muscimol 6 μ M	MC + Bicuculline
Spike Production	2250	1000	1750	1600	200	1950	600	1950
Percent Decrease	N/A	56%	N/A	(8.6%)	88%	N/A	69%	N/A

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