THE MITIGATING EFFECTS OF LTP ON THE ISCHEMIC HIPPOCAMPAL SLICE

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Abstract

The purpose of this study is to understand how long-term potentiation (LTP) affects the hippocampus after it has undergone ischemia, and whether it has any mitigating effects on cellular damage. By investigating if there are benefits of LTP, possible clinical applications could be used to strengthen LTP connections in the hippocampus to mitigate the effects of ischemic damage. Ischemic damage has been found to destroy cells through multiple pathways, which can result in major damage and memory loss in the hippocampus. There is evidence present in literature describing the pathways of damage ischemia causes (Lipton 2008), the plasticity benefits of LTP (Malenka 1994), and how to induce this LTP (Abrahamsson et al. 2016). In order to investigate the effects of ischemia after LTP has been induced *in vitro*, preliminary data on extracellular recordings of hippocampal slices before and after LTP induction and induction of ischemia were analyzed. The preliminary analysis was done by comparing the slopes of recordings produced by neural signals in response to electrical stimuli. In both the LTP experiment and the ischemia experiment, a baseline was first established based on the slopes of the EPSPs, prior to induction of LTP or Ischemia. In the two ischemia experiments, the slices lost all neural function, then only recovered to an average of only 57.615% of the baseline response after 60 minutes. Contrast to the ischemia experiments, the LTP experiment, demonstrated an increase in neural function, with a 67.278% average higher

response in the last 10 minutes of recording in comparison to the baseline. It is concluded from these experiments that ischemia is damaging to the hippocampal cells, while LTP promotes the cells function. Because of these results, we hypothesize that when LTP is induced prior to ischemia, the slice will recover back to the baseline within 60 minutes after recording.

Introduction

Both ischemia and LTP have major effects on the hippocampus. Ischemia is a state of inadequate blood supply to the hippocampus. LTP is a strengthening of a neural synapse, leading to a stronger response through the synapse when stimulated. It is hypothesized that inducing LTP in the hippocampus will mitigate the damaging effects of ischemia. Much of the effects of ischemia are disastrous to the cell and its ability to maintain memory. Contrary to ischemia, the effects of LTP are usually seen as beneficial in the reinforcement of plasticity, the ability to form new neural connections. Both ischemia and LTP proceed through N-Methyl-D-aspartic acid (NMDA) receptors. NMDA receptors are glutamate receptors, meaning that they are activated when the neurotransmitter, glutamate, attaches to them. Despite LTP and ischemia sharing a common pathway, their combined effects on the hippocampus have not been documented in literature.

A major component of the damage to hippocampal cells, caused by ischemia, happens through NMDA-related calcium influx (Costa et al. 2006). The calcium influx along with arachidonic acid metabolism and free radical production lead to lysosomal permeabilization within hippocampal cells and cathepsin release causing morphological damage of the cell (Lipton 2008).

Calcium influx into the cell is the first step of the cascade of events caused by ischemia, that damages hippocampal cells. The influx occurs through the activation of the receptor that is

found in both the ischemia and LTP pathway, NMDA receptors. Once the NMDA receptors are activated, and the calcium influx occurs, further ischemic events are triggered.

Arachidonic acid metabolism is induced by a calcium influx. Arachidonic acid is an omega-6 polyunsaturated fat. Due to its identity as a fatty acid, it is integral in the formation of membranes in the cell (Tallima and El Ridi 2017). When calcium enters the cell, arachidonic acid is freed from membrane lipids which then leads to the formation of superoxide, a free radical (Lipton 2008).

Free radical production is another component of neural damage. Free radicals are any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital. Free radicals are common within the body and safe, when balanced properly with antioxidants. When free radicals form an excess within our bodies, relative to the amount of antioxidants, a condition known as oxidative stress ensues. Oxidative stress can then damage lipids, proteins, and DNA and trigger several human diseases (Lobo et al. 2010). One of the major free radicals regulated within the human body is superoxide. Superoxide is a form of oxygen with a free radical. When superoxide levels in the cell increase more than usual, they can cause increased lysosomal permeabilization (Lipton 2008).

Calcium influx, arachidonic acid metabolism, and free radical production all ultimately lead to lysosomal permeabilization. This is when the membranes of lysosomes become unable to hold in certain molecules. Lysosomes are organelles present in cells that are responsible for degrading excess material within the cell. When lysosomes become more permeable the degradative substances are released.

Lysosomal permeabilization leads to the release of specific degradative enzymes. A key enzyme that lysosomes use is called cathepsin. This is one of the molecules that is able to cross

the lysosomes membrane after the increased permeabilization. Cathepsins are a type of protease, enzymes the breakdown protein.

The release of cathepsins leads to morphological damage of neural cells. This damage decreases the metabolic efficiency of the cell and inhibits cellular function. The cathepsins cause this damage by degrading the enzymes and structural proteins contained within cells. This is demonstrated in cells that have condensed nuclei, which is an indicator of cathepsin damage (Lipton 2008).

LTP is another neural process that follows an NMDA related mechanism. LTP is a major component in the ability of the hippocampus to form plasticity. LTP is a type of plasticity in which a persistent enhancement of excitatory postsynaptic potentials arises from a high frequency stimulation (Shors and Matzel 1997). How LTP maintains plasticity is still a debated topic, but it is known to involve the formation of proteins that result from plasticity. The mechanism leading to LTP starts with α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, another type of glutamate receptor, stimulating NMDA receptors, leading to an influx of calcium ions (Lüscher and Malenka 2012). With repeated influxes of calcium ions, the signaling mechanisms that result in LTP are activated (Malenka 1994).

It is important to note that the complex spike burst of CA1 pyramidal cells enables the induction of LTP during theta frequency synaptic stimulation in the CA1 region of hippocampal slices maintained in vitro, because this theta frequency synaptic pattern can be imitated (Thomas et al. 1998). Complex spike bursts are signal patterns the brain sends to induce LTP, in the CA1 region of the hippocampus, which is the region that we test in. Imitating these signals can be done through intermediate stage theta burst stimulation (TBS), which helps induce LTP in vitro and enhance memory (Stäubli et al. 1999). Theta burst stimulation is a high frequency electrical

stimulation that is used to imitate complex spike bursting. Complex spike bursting are patterns present in the hippocampus that induce LTP and are thought to be related to memory (Thomas et al. 1998).

It must be noted that there is inconsistency in the projected conjoining of LTP and ischemia. Ischemia and LTP begin with stimulation of NMDA receptors. This activation of NMDA receptors then allows ischemia and LTP to occur (Filippini 2016 et al.). Because ischemia and LTP both proceed through NMDA receptors, there could be a compounding effect instead of a mitigating effect. This means that when combined, LTP and ischemia could do more damage to neural cells then if just ischemia occurred.

In order to fill the gap present in literature between the relationship of ischemia and LTP an experiment must be done to determine their effects upon one another. By seeing if the responses of hippocampal cells remain relatively stable after inducing LTP, despite the cells having undergone ischemia it can be proposed that LTP has protective effects on hippocampal cells. The unit of analysis for the responses will be millivolts. If the cell is damaged despite the induction of LTP then it will be noted that LTP has little or no protective effects against ischemia.

Methods

The hippocampal slices used in this study are from rats 2-3 months in age so that skull hardness does not become a problem during dissection. If the skull is too hard, dissection time can proceed too long, resulting in slices that are unable to respond to stimuli due to neural damage. The hippocampus is obtained through the use of a guillotine and subsequent dissection using metal spatulas. The hippocampus is then sliced using a McIlwain tissue chopper. This specific chopper is used because slices must be cut thin enough to be able to distinguish the

different layers of the hippocampus, which cannot be done by hand. The slices are placed in a petri dish filled with high magnesium buffer solution, to imitate the cells in vivo environment. The high magnesium concentration inhibits neural activity which helps in the recovery time of preventing excitatory neural damage. This petri dish is placed in an ice bath in order to prevent further trauma damage occurring to the slices. From the petri dish, the slices are then placed in a 100 mL beaker, on top of mesh netting that has a solution of high magnesium buffer solution at a level just slightly above the netting. The beaker has been warmed in a large water bath to 34°C and the solution has had carbogen run through it for at least 15 minutes prior to the placement of slices. Carbogen is a gaseous mixture of 95% oxygen and 5% carbon dioxide, which simulates in vivo concentrations, in order to maintain the slices physiological responses. The temperature also corresponds to hippocampal cell environment temperature in the body. After the placement of slices, carbogen is bubbled in the solution for 45 minutes in order to normalize the slices to their environment and allow them to recover. They must recover because the trauma caused by dissection can cause them to go in to shock and not be able to produce responses.

During the 45 minutes required for incubation, a 250 mL Erlenmeyer flask is filled with low magnesium/high calcium buffer solution and warmed in a small circular water bath to 35°C. The low magnesium/high calcium buffer promotes synaptic activity which is why it is used for recording. This solution is then pumped through the tubing system connecting the solution and brain slice chamber, where the recordings will be take. A flow rate of 2-4 mL/min is established. This flow rate allows for enough solution to maintain slices health, without disturbing them too much, which would lead to damage.

Once the 45 minutes required for incubation have passed, the mesh netting along with the slices are transferred to another 100 mL beaker containing low magnesium/high calcium buffer solution, which has been warmed in the same large water bath at 34°C and bubbled with carbogen for at least 15 minutes. The slices then incubate in the second chamber for 45 minutes.

While the slices are incubating, the computer program LabChart is set up to check for excess electrical noise affecting the slice chamber located in the faraday cage. The stimulus electrode and recording electrode are placed into solution in the brain chamber and a stabilization around 0 mV is looked for. Electrical noise stabilization is needed, because if it is not stabilized, responses can be hard to distinguish from the electrical noise. The stimulus electrode is then stimulated to ensure that an electrical signal is being sent and that the recording electrode is picking it up. The stimulus artifact should be about 1V in height in order to properly stimulate the hippocampus. When the system is found to be working properly, and the slices have incubated for at least 45 minutes, then recording can begin.

The recording begins with proper placement of the electrodes. Both electrodes should be in the dendritic layer, also known as the stratum radiatum (SR) of the CA1 region. This is the layer where the dendrites of neurons are, which are used to normalize the synaptic responses. The electrodes should be placed 1-2 SRs away from each other. The stimulus electrode should be on the side closer to the CA2 region. The stimulus electrode and recording electrode are arranged in this format because the hippocampus naturally emits signals in a pathway that goes from CA2 to CA1. In order to induce LTP through TBS, deliver trains of 4–5 pulses at 100 Hz every 200 ms, repeated 10–75 times (Abrahamsson et al. 2016).

The responses are captured and processed through LabChart. A proper pulse height stimulation is found in LabChart prior to inducing LTP. This is done by starting at a pulse height

of 2V and increasing it by 2V every minute until the response does not increase further. When this happens, the pulse height that last increased response intensity is used. This pulse height is used so that the slice is not over stimulated, leading to neural damage.

A baseline must be established using the pulse height that was found. This is done by measuring the slope of the rising phases of the EPSPs (Figure 1). When the slopes are consistent for 20 minutes, when stimulating every 2 minutes, the baseline response can be used, and the experiment can progress. The baseline is measured, and all subsequent response are related to it by field Excitatory Post Synaptic Potential percentage (fEPSP%), where the baseline is 100%. fEPSP% helps in measuring how large of a response there was in relation to the baseline response that was established prior to LTP or ischemic induction. This measurement is used, because it will describe the relative health of the slice before and after ischemia.

After the baseline is established, LTP is induced through TBS. 60 minutes after responses reach a consistent baseline, Ischemia is induced through oxygen/glucose deprivation and nitrogen profusion. This is done by cycling solution through the slice chamber that does not contain glucose, and then closing the oxygen tube, and using a tube connected to a tank of nitrogen gas instead. After 5 minutes ischemia is stopped and carbogen and regular recording solution are reintroduced into the system and the slice is left to sit for 60 minutes. The fEPSP% of the slice is then observed for 60 minutes. The slice is then tested again, but with no LTP induction, and the fEPSP%'s are compared using a t test of means to see whether there was a significant difference in fEPSP% 60 minutes after reintroduction of carbogen. This will describe whether there was a significant recovery in health over time for the LTP induced slices.

Results

Preliminary studies were conducted on the effects of LTP and Ischemia separately. Ischemia Experiment 1 used a stimulus intensity of 14V and showed a 16% increase in the baseline deviation over the 20-minute baseline (figure 2b). The slice recovered through a linear trend (R^2=0.88) with 0.5335% increased recovery per minute following ischemia, and a 25.3% total recovery in relation to the baseline (Figure 2c). Ischemia experiment 2 was carried out using a stimulus intensity of 5V. The 20-minute baseline demonstrated a 19% decline in baseline deviation (Figure 3b). The recovery followed a linear trend (R^2 = 0.82), with a 1.52% increase recovery per minute and an 89.9% total recovery (Figure 3c). When the ischemia experiments were averaged, the resulting recovery followed a linear trend (R^2 = 0.91) and recovered at 0.9871% per minute. The average total recovery after 60 minutes for the two experiments was 42.37% ± 32.30%. When LTP was induced without any ischemia occurring, the baseline demonstrated a linear trend (R^2 = 0.68) that increased 0.9838% per minute (Figure 5b). The baseline deviation increased by 9%. The post LTP induction experiment itself also demonstrated a linear trend (R^2 = 0.80), with recovery increasing at a rate of 3.3398% per minute (Figure 5c). The LTP induction resulted in synaptic responses jumping to a 67.278% average increase in fEPSP% in the last 10 minutes of recording.

Discussion

Preliminary studies on LTP and ischemia demonstrated results showing promise for the hypothesis that LTP will inhibit ischemic damage in hippocampal cells. From these results, is specifically hypothesized that when LTP is induced prior to Ischemia the slice will recover back to the baseline by 60 minutes post ischemia recording, meaning that the fEPSP% will reach 100% within 60 minutes after recording has begun following induction of ischemia. It was also projected that when LTP is induced, recovery will begin at the same time as it did for

the ischemia experiments with no LTP induction. Ischemia decreased the function of cells enough that they only recovered to an average of 42.37% of their pre-experimental health 60 minutes after the ischemia was induced. This highlight and confirms that ischemia's previously mentioned cellular pathway is devastating to cellular health. Opposite to ischemia's damaging properties, LTP was shown to increase neural function by 67.278% in the hippocampal cells. When these two results are compared LTP is better in promoting neural function than ischemia is in destroying neural function, and this is why a recovery back to baseline health is hypothesized. An experiment in which LTP is induced before ischemia is induced must be done in the future in order to prove the validity of this hypothesis.

There are a few shortcomings with the preliminary study data. The ischemia experiments had a standard error of 32.30%. This standard error is rather large, and more experiments should be done in order to gauge the effects of ischemia without so much ambiguity in the exact percentage that ischemia decreases neural function. The large standard error could have arisen because in ischemia experiment 1 the baseline response was still increasing before ischemia was induced. If the responses were instead allowed to maximize before induction of ischemia, the slice could have been more resilient to ischemic damage and recovered to a higher fEPSP%. There was also only one LTP experiment carried out, so more must be done in the future so that the promotive effects on neural activity can be demonstrated without high variability. This is necessary because strong LTP should be used so that no confounding variables can be attributed to increased recovery.

If the data lays out as it is hypothesized to, then there will be major conclusions that can be drawn. The original hypothesis that LTP induction will mitigate the effects of ischemic damage in the hippocampus will not be able to be rejected statistically. This has implications on

further understanding of LTP and Ischemia. It will mean that their mechanisms are not independent from one another. With this knowledge further studies can be done to specifically highlight how they affect each other mechanistically, instead of if they affect each other. This experiment could also have real world implications. It could be possible to find ways to utilize LTP in stroke prone individuals, which would mitigate the damage on neural cells, extending their length and enjoyment of life.

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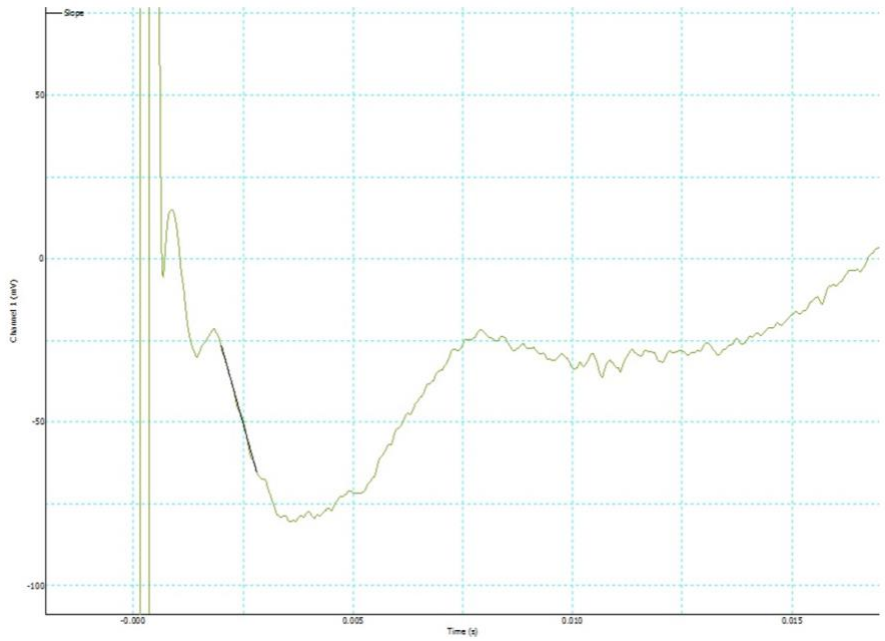

Figure 1. shows an EPSP and how the slope of the EPSP is measured. The black line is where the rising phase of the EPSP is located and indicates where the slope is taken.

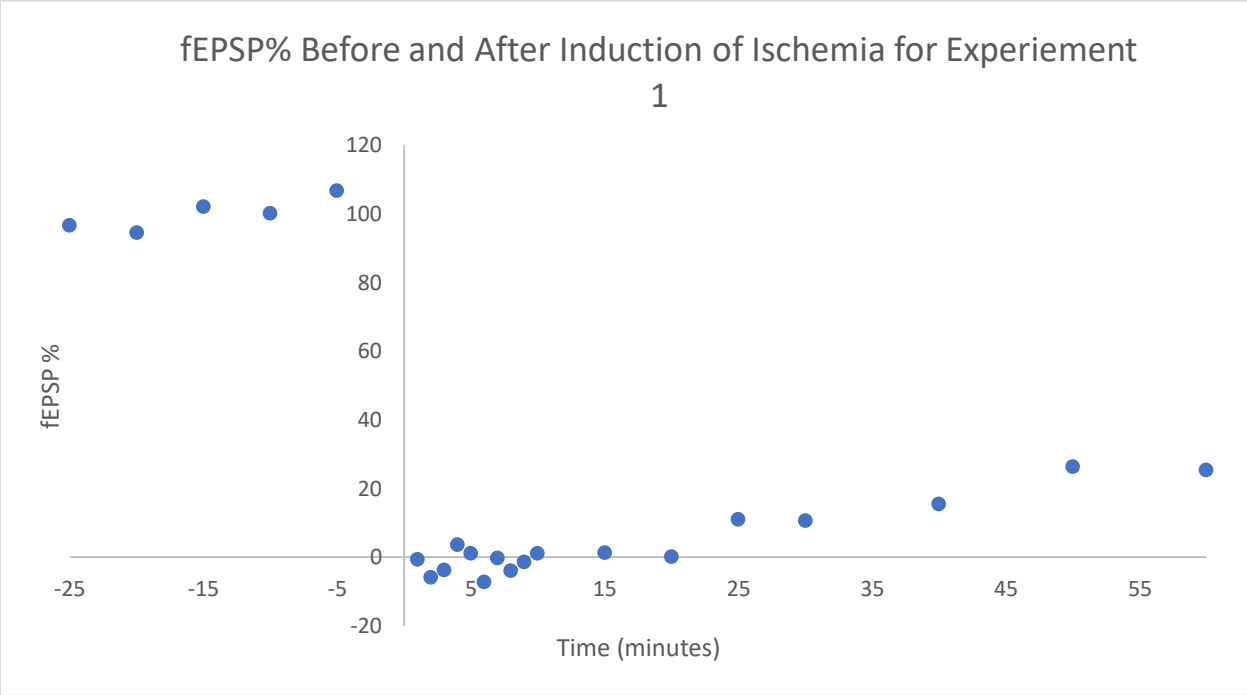

Figure 2a. shows a full ischemia experiment, where 0 minutes is the time that ischemia is induced, and everything before that is establishment of a baseline.

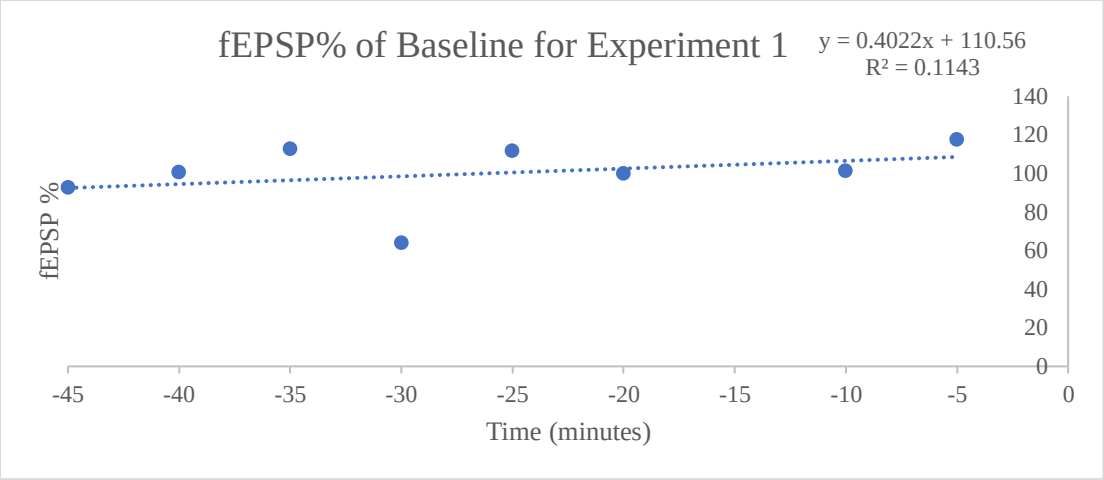

Figure 2b. is the establishment of a baseline prior to LTP induction in Experiment 1. The baseline increases in a linear trend described by the equation y = 0.4022x + 110.56 (R^2 = 0.1143). There is a 16% increase in fEPSP% described by this equation over establishment of the baseline.

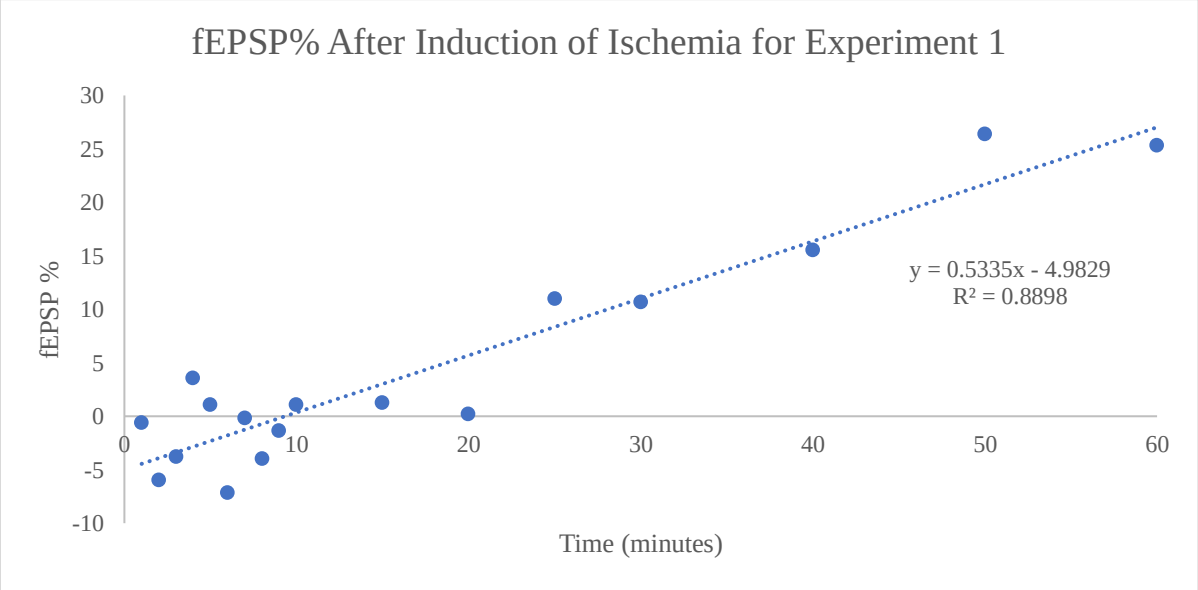

Figure 2c. describes the recovery of the hippocampal slice after induction of ischemia. Recovery begins around 20 minutes, and gradually increases. The data is explained by the line y = 0.5335x - 4.9829 (R^2 = 0.8898), with an 0.5335% increase in recovery per minute, and a total recovery of 25.31% of the baseline.

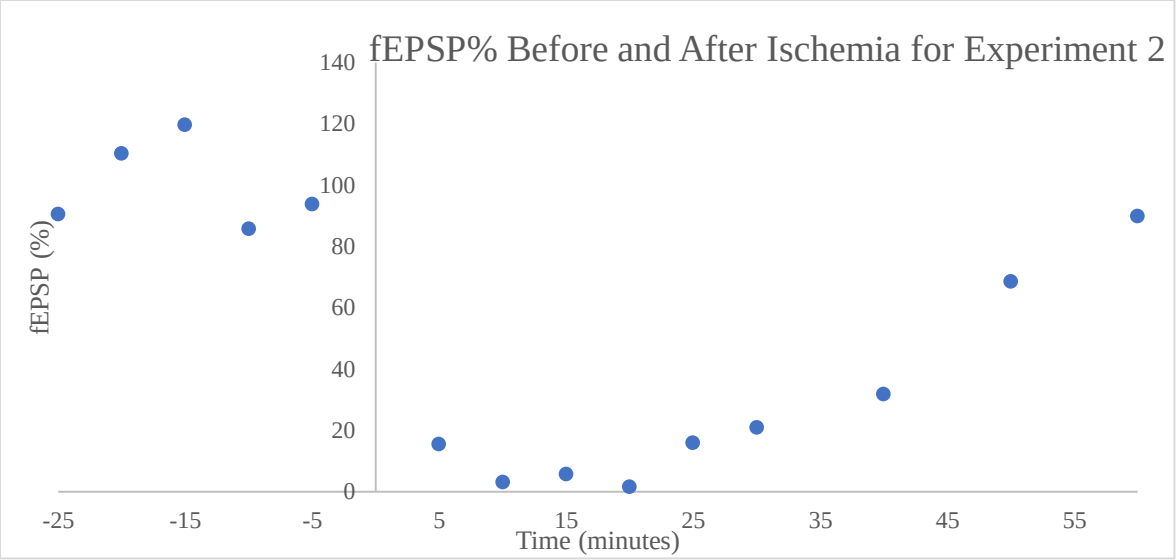

Figure 3a. demonstrates the entire ischemia experiment for experiment 2. The establishment of a baseline is shown before 0 minutes, which is the time that ischemia was induced.

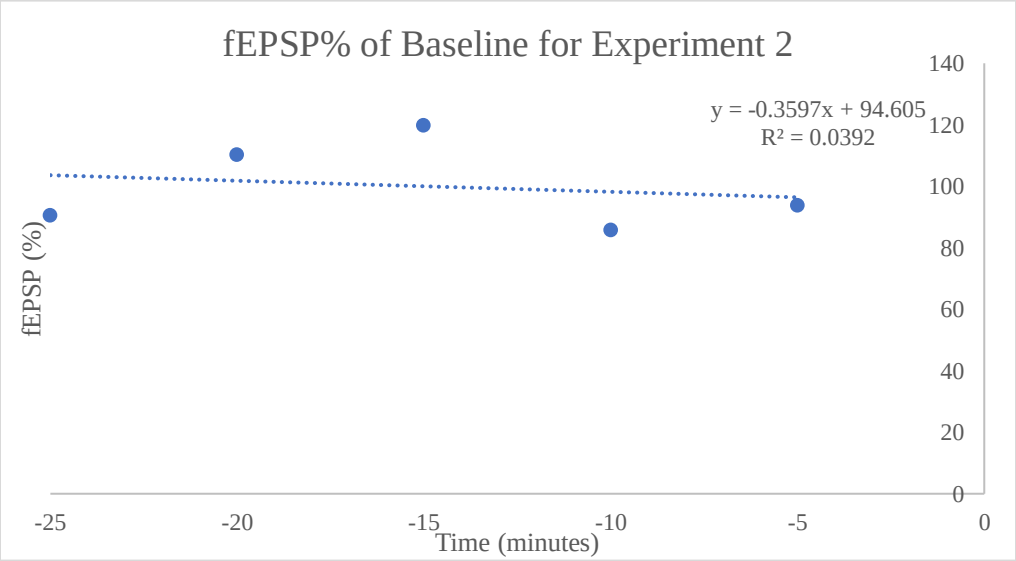

Figure 3b. is the baseline that was established prior to induction of ischemia for experiment 2. The baseline is explained by the equation y = -0.3597x + 94.605 (R^2 = 0.0392), which shows a 19% decrease in the baseline leading up to induction of ischemia.

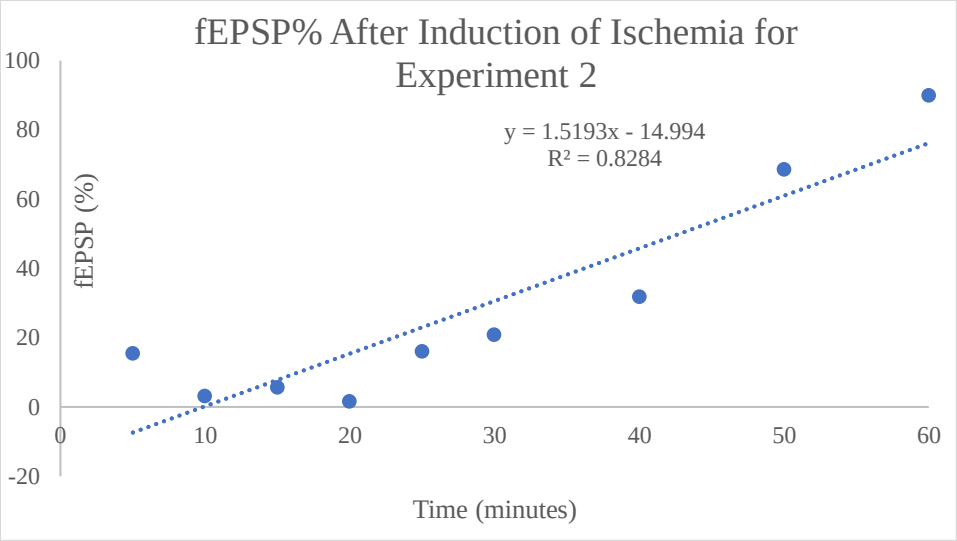

Figure 3c. is the fEPSP% responses of the hippocampal slice following induction of ischemia in experiment 2. Recovery begins around 20 minutes. The experiment follows the equation y = 1.5193x - 14.994 (R^2 = 0.8284) and increases at about 1.5193% per minute. The total recovery of the slice is 89.92% of the baseline.

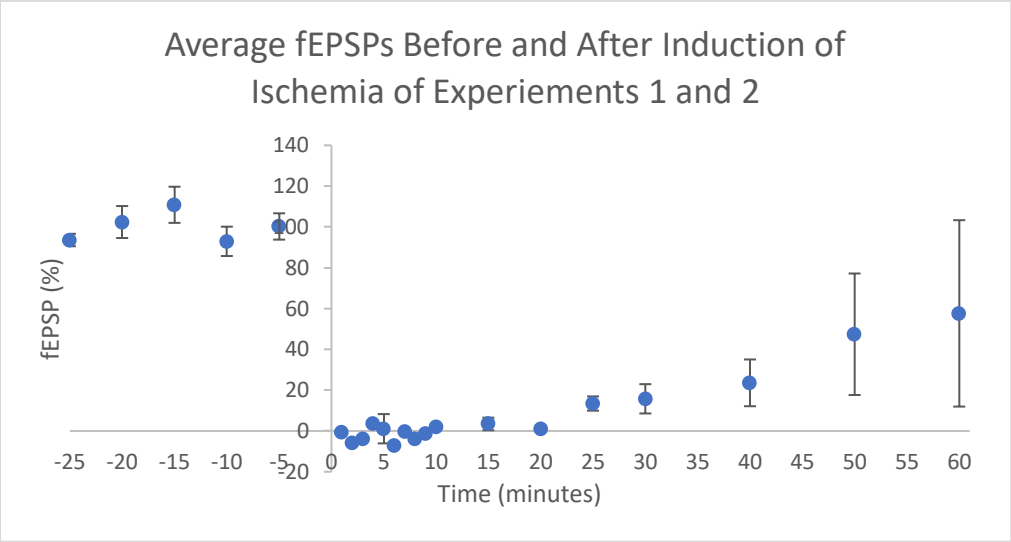

Figure 4a. explains the average neural responses from Experiment 1 and Experiment 2. Both experiments show recovery beginning at about 20 minutes after induction of ischemia. The standard error increases as time after recovery progresses.

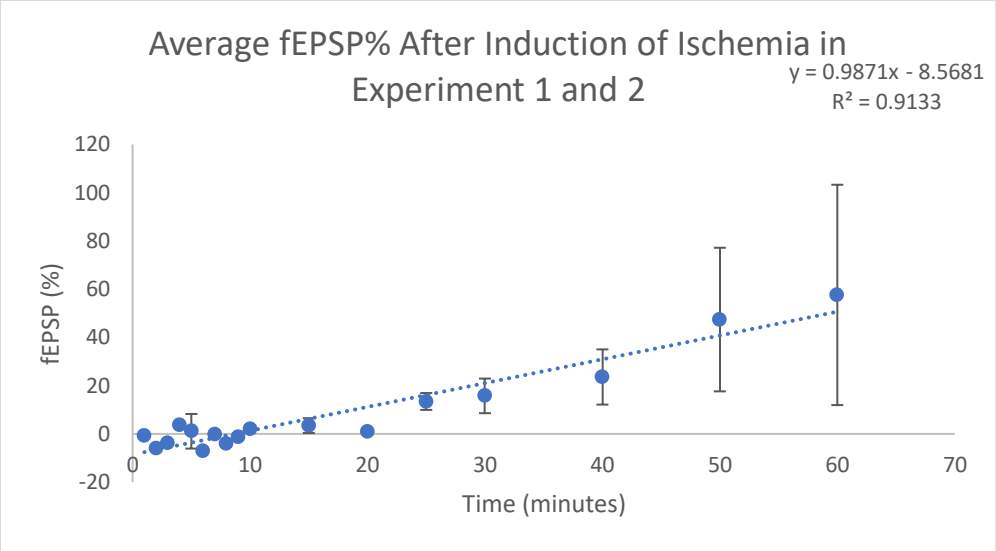

Figure 4b. is a representation of the average responses of Experiment 1 and Experiment 2 following induction of LTP. The responses follow a linear trend with the equation of y = 0.9871x - 8.5681 (R^2 = 0.9133). There is an average increase in recovery of 0.9871% per minute. The total average recovery is 42.37% ± 32.30%.

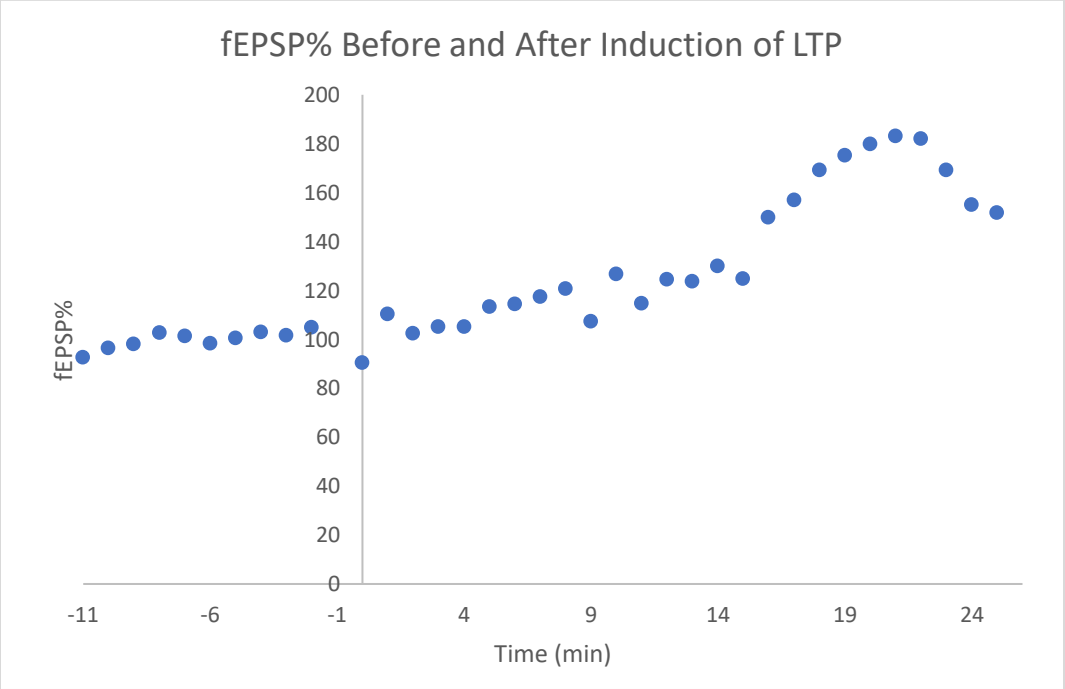

Figure 5a. shows the effects of LTP on neural activity. A baseline is established, and then neural function increases steadily from the time of LTP induction, which occurs at 0 minutes.

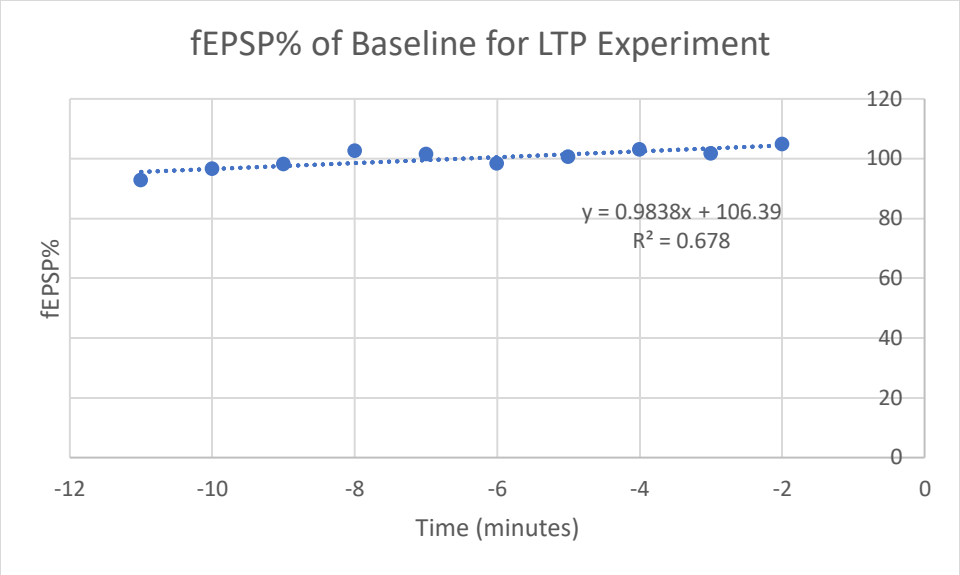

Figure 5b. is an exhibition of the baseline established prior to induction of LTP. The baseline increases by 9% prior to LTP induction, which is explained by the equation y = 0.9838x + 106.39 (R^2 = 0.678).

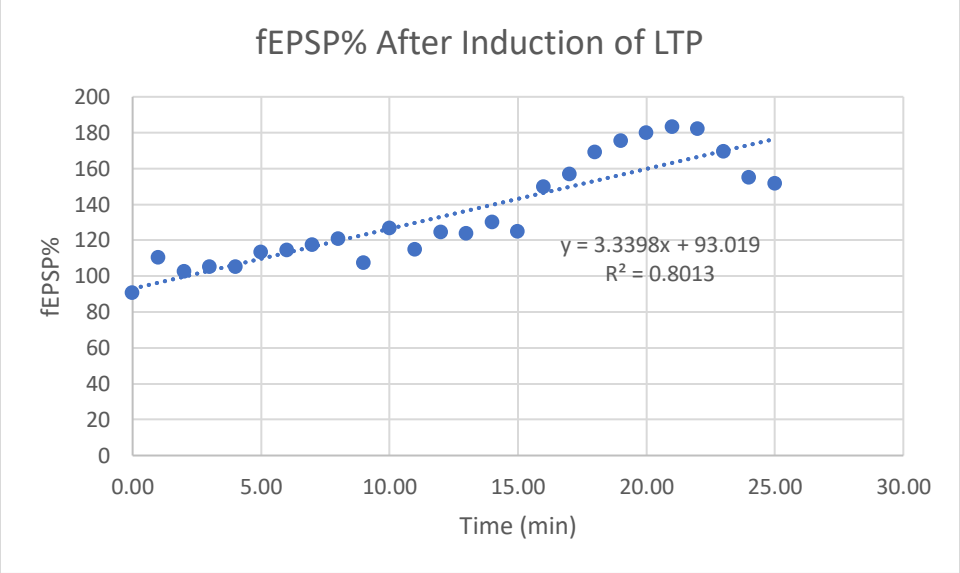

Figure 5c. is an indication of the increased neural activity following LTP induction. There is a 3.3398% increase in fEPSP% every minute, which is explained by the equation y = 3.3398x + 93.019 (R^2 = 0.8013). The average potentiation increase in the last 10 minutes of recording is 67.278%.