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Bidirectional Influence of Epinephrine on Hippocampal LTP via β-Adrenergic Receptors

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Abstract

The formation and storage of memories within the brain remains a subject that is not well understood. The hippocampus has been identified by many studies as a likely center for memory formation (Lynch, 2004), and further research into this subject has begun to suggest that synaptic plasticity in the hippocampus could be partly responsible for the physical changes in the brain, which underlie memory formation. Long Term Potentiation is a form of synaptic plasticity, and is considered to be a physical increase in the strength of connection between neurons or groups of neurons. Much like memories, the duration of a given LTP can last anywhere from minutes to years, depending upon the conditions under which the LTP was induced. Stress, in particular, has been found to either enhance or impair LTP formation, under different conditions. The brain's response to stress, or any kind of emotional arousal, is in part mediated by the release of the hormone epinephrine. This type of "stress memory", or epinephrine-mediated memory formation, is important because it could explain the pathological memory formation that is commonly seen in phenomena such as Post Traumatic Stress Disorder (Korol and Gold, 2008). Epinephrine release in the periphery has been seen to influence LTP in the hippocampus, however epinephrine itself cannot enter the brain. These experiments served to explore the mechanisms by which epinephrine can act to bidirectionally influence hippocampal LTP through activation of β-adrenergic receptors.

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Executive Summary

Memory serves an integral purpose in our lives, yet the mechanisms underlying memory formation are still not well understood. The hippocampus, a small structure within the medial temporal lobes of the brain, is considered to be the center for memory formation. A wide array of clinical and scientific studies have shown that memory formation is disrupted or prevented altogether when the hippocampus is not functioning properly. Although memories are not stored in the hippocampus, per se, this brain structure is crucial to the process by which memories are formed.

Synaptic plasticity is a phenomenon that occurs throughout the brain. Plasticity is a broad that describes the unique ability of neurons to change the strength of their connections to one another over time. Synaptic plasticity is what allows our brain to adapt and change in response to changes in our environment. It has been suggested that synaptic plasticity shapes each person's individual brain by strengthening the connections that are frequently used, and weakening those that are not. Long Term Potentiation (LTP) is a form of synaptic plasticity that can be seen throughout the brain, but has been particularly well studied within the hippocampus. LTP represents an increase in the strength of connection between two neurons or groups of neurons. Many studies of LTP are focused on the hippocampus because LTP is considered to be the cellular mechanism underlying memory formation, though there are pure technical reasons as well. Much like memory, the duration of a given LTP can vary greatly, depending on the conditions under which it was formed. It has been found that stress, in

particular, can have a significant effect on the formation of LTP in the hippocampus. The stress response is mediated, in part, by release of the hormone epinephrine, also known as adrenaline, from the adrenal glands. Epinephrine release into the blood stream has a variety of effects throughout the body. In particular, it has been seen that epinephrine can bidirectionally affect LTP by either enhancing or impairing the formation of LTP within the hippocampus. This epinephrine-mediated "stress memory" or "emotional memory" is significant because it could be responsible for the drastic memory dysfunction that is seen in stress-related conditions such as Post Traumatic Stress Disorder. While it is clear that epinephrine release in the periphery infuences LTP formation within the hippocampus, it is unclear exactly how this occurs.

The blood-brain barrier is a highly selective membrane-like covering, which prevents toxins and unwanted substances that may be circulating in the blood stream from reaching the brain. The blood-brain barrier is an important defense mechanism, and acts to allow only small, hydrophobic molecules to enter the brain. Epinephrine is a relatively large molecule, and as a result is it unable to pass through the blood-brain barrier. This observation calls to question how epinephrine can have such a significant effect within the brain, when it is unable to physically enter the brain. Because epinephrine has been seen to influence LTP, there must exist some mechanism by which epinephrine release in the periphery is translated into a signal that can be received by the brain.

It is thought that epinephrine can have an effect on LTP by stimulating the release of norepinephrine within the brain. Epinephrine release in the periphery

has been found to cause the vagus nerve to begin firing rapidly. This triggers a chain of events, which ultimately results in the release of norepinephrine throughout the brain. Norepinephrine can be released directly into the hippocampus, where it could potentially bind to receptors and influence LTP. Alternatively, norepinephrine could also be released into the amygdala. The amygdala is considered to be the "fear center" of the brain. It comprises a portion of the limbic system, which is thought of as the source of our most primative, emotional responses, such as the "fight-or-flight" response. The amygdala, which rests on top of the hippocampus within the brain, has also been implicated in the process of memory formation. The amygdala contains a variety of adrenergic receptors, which are receptors that selectively bind norepinephrine. Importantly, the basolateral complex of the amygdala (BLA) has many fibers that project to other brain areas, including the hippocampus, and therefore could act to influence hippocampal LTP. In addition, the hippocampus proper has β-adrenergic receptors and may also be the site of LTP modulation by peripheral epinephrine. This project attempted to discern whether hippocampal or amygdalar regulation mediated adrenergic control over epinephrine-enhanced hippocampal LTP.

To examine the role of β-adrenergic receptors in epinephrine-enhancement of LTP, this study used a loss-of-function approach. The β-adrenergic receptor antagonist, propranolol can be used to selectively block activation of β-adrenergic receptors within the brain. Under these circumstances, if β-adrenergic receptors were responsible for mediating epinephrine-enhancement of LTP, it could be predicted that LTP in the hippocampus would not be enhanced by epinephrine

while these receptors are blocked. In order to test this, young, male rats were anesthetized, and stimulating and recording electrodes were implanted within the hippocampus. These electrodes allow for both the delivery of electrical stimulation, as well as measurement of LTP in targeted brain areas. Additionally, each rat was implanted with a cannula, a small tube allowing for direct drug infusion to the recording site.

LTP in the hippocampus can be recorded as electrical activity within the brain, which produces a very characteristic pattern known as an extracellular evoked potential. The strength and durability of any given LTP can then be measured as variation from an established baseline response. To induce LTP, these experiments used an established procedure of high-frequency electrical stimulation (HFS) known to produce LTP within the hippocampus. Before HFS, rats were randomly separated into test groups based on the drug treatment and stimulation protocol that they were to receive. Preliminary experiments were conducted to evaluate the enhancing and inhibiting capabilities of epinephrine under different dosages and stimulation protocols. Additionally, the effectiveness of two different doses of epinephrine was tested to determine the dose used for the main experiment. Within the main experimental group, each test group received a peripheral injection to the intraperitoneal cavity in the abdomen. Additionally, a subset of rats also received a local injection via cannula directly to the hippocampus. Group 1, which was the control group, received injections of peripheral and local saline; this ensured that there was no effect produced by the injections themselves. Group 2 received peripheral epinephrine at a dose

previously established to enhance LTP (0.05 mg/kg) as well as a local infusion of saline; this was the test group for epinephrine enhancement. Group 3, the test group for adrenergic blockade, received peripheral epinephrine as well as the local β-adrenergic antagonist, propranolol. This combination should have served to block the enhancing effect of epinephrine that would be seen with LTP in group 2. After drug treatments were administered, all groups received HFS, after which recordings continued to analyze the influence of each drug treatment on hippocampal LTP formation.

The results of this project showed that, unexpectedly, it could not be fully demonstrated that epinephrine-enhancement of LTP in the hippocampus was blocked when β-adrenergic receptors were blocked by propranolol. However, the main effect observed in the results was that epinephrine does act to bidirectionally influence hippocampal LTP formation. The mechanisms underlying epinephrine-modulated hippocampal LTP were revealed to be more complex than was initially thought. Further, it became clear after examining the results that individual animals could exhibit very different reactions to the same stimulation protocol and dose of epinephrine. Overall, this led to the conclusion that individual animals can respond differently to the same stressor, a phenomenon known as the stress-response phenotype. This stress-response phenotype could have implications for the broader application to PTSD, as not all individuals who undergo traumatic experiences will develop this condition. Understanding the mechanisms by which epinephrine acts to influence LTP in the hippocampus could help illuminate the larger mechanisms underlying memory formation as a whole. Because memory

formation under stressful conditions often results in disruptive conditions such as Post Traumatic Stress Disorder, it is important to understand, in particular, how stress can act to influence memory formation. The findings of these experiments are significant because they contribute not only to our knowledge about stress memory, but also to our understanding of individual differences in memory formation as a whole.

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Advice to Future Honors Students

Having been working on this project for the better part of two years, my only advice to future honors students working in Biology is this: keep at it. The direction of this project changed course more times than I can count, and each time we simply regrouped, pushed onwards, and continued to explore new possibilities. It can be incredibly frustrating to work on a project for months, and then find that your results are not what you expected, but the data do not lie. Often, these confounding moments that provided the most insight and inspired new hypotheses. Further, I found that I was most deeply involved in the project when I made an effort to actively immerse myself in the field. Read papers; find out what other people in the field have been accomplishing, science is constantly moving and you'll do your best work when you try keep up. Most of all, start early!

Introduction

The mechanisms underlying the formation and storage of memories have long been a subject of interest to neuroscientists. Experience-dependent changes in the strength of connections between neurons have been implicated as a biological mechanism that may underlie long-term memory. Specifically, persistent increases and decreases in the strength of synapses, known as long term potentiation (LTP) and long term depression (LTD), respectively, exhibit many parallels to long-term memory and may play an important role in the storage of memories (Matynia et al., 2002). LTP is defined as a physiological increase in the strength of connections between neurons or groups of neurons. This form of synaptic plasticity occurs throughout the brain, but has been particularly well studied in the hippocampus. This area of the brain is an ideal electrophysiological model, as its highly layered structure makes it very easy to study. Further, the hippocampus has been associated with the processes underlying learning and memory (Sutherland et al., 2011).

Much like memory, the duration of a given LTP is variable (Malenka et al., 2004), depending on the conditions under which it was induced. Stress, in particular, is a condition that has been seen to have a profound impact upon LTP formation within the hippocampus (Liang et al., 1985; Joels and Krugers, 2007). Previous studies have identified that the intensity and duration of the stressor present at the time of memory formation is one factor that can largely impact the resulting memory (De Boer et al., 1990; Gold, 1989). The stress response is mediated, in part, by the release of the adrenal hormone epinephrine into the

bloodstream. It has been found (Maggio and Segal, 2010) that under stressful conditions, LTP in the hippocampus can be enhanced by the presence of peripheral epinephrine, however the mechanisms by which this occurs are not well understood.

Epinephrine is a relatively large, lipophilic molecule that is unable to pass through the blood-brain barrier. As a result, epinephrine does not directly enter the brain, yet it has been seen to have a significant effect upon memory formation. For example, several studies have found that administration of peripheral epinephrine shortly before a training event can enhance memory for that task (Gold and van Buskirk, 1976; Gold et al. 1982; McGaugh et al., 1996). In one particular study, it was found that rats trained on an inhibitory avoidance task, where a mild foot shock was followed by injection of epinephrine, remembered as well as rats that were given a much stronger foot shock (Gold, 1989). These and other studies have extensively demonstrated the enhancing effects, both behaviorally and electrophysiologically, of epinephrine on memory.

The results of these studies have indicated that epinephrine is capable of converting a weak memory into a stronger, more durable form. Further, studies with aged rats (2-years-old) have shown that epinephrine has the ability to prevent the rapid forgetting that is characteristic in older rats (Gold, 1989; Korol and Gold, 2008). When trained on the same task, old and young rats are both capable of learning, however the aged rats generally forget more rapidly.

Memory formation and forgetting are intrinsically linked processes within the brain. The hippocampus has been used as a model to study these processes, as

it is thought to play a role in long term information storage (Kuhl et al., 2010). For example, it has been found that patients with damage to the temporal lobes, where the hippocampus is located, often experience problems related to memory formation (Blake et al., 2000). There are parallel studies in animal models showing that damage to the hippocampus or disruption of its normal function impairs some types of learning and memory, and leads to more rapid forgetting.

LTP has been implicated as a mechanism by which long term memory formation could occur or perhaps a model paradigm to demonstrate long term cellular memory. Particularly, it has been found that when the late phase of hippocampal LTP is blocked, performance on spatial memory tasks is impaired (Oikawa, 2012). Furthermore, there are demonstrations that motor memory and forgetting are tightly correlated to increases and decreases in synaptic strength in the motor cortex (Rioult-Pedotti et al., 2000). Thus, changes in synaptic strength may be the general properties of experience-induced brain changes involved in both memory formation and forgetting. The ability of peripheral epinephrine to prevent rapid forgetting suggests that epinephrine may also play an important role in age-related memory loss (Rene, 2008). These findings indicate that epinephrine is likely to be involved in the cellular mechanisms underlying memory formation, and could be important for converting new memories into durable, long-lasting ones.

Epinephrine has the ability to bidirectionally influence memory formation, acting either to enhance or to impair memory and its correlates (Joels and Krugers, 2007). Many studies with epinephrine have observed an inverted-U

relationship between epinephrine and memory (Gold, 1989). In these cases, intermediate dosages (0.01-0.1 mg/kg) of epinephrine produced enhancement of memory response, while both higher and lower doses produced no significant effect (Gold, 1989). This enhancing dose of epinephrine has been seen to both improve memory retention in training tasks, as well as enhance LTP of evoked responses within the hippocampus (Gold, 1989, Korol and Gold, 2008). In the Korol and Gold study (2008), it was found that compared to saline control treatments, an enhancing dose of epinephrine (0.1 mg/kg) caused LTP in the hippocampus to be significantly elevated for as long as responses were assessed (96 hrs). Further, emotional arousal and stress can enhance LTP within the hippocampus (Tully and Bolshakov, 2010). Emotionally arousing events stimulate the release of norepinephrine throughout the brain, which can act to lower the threshold for LTP within the hippocampus (Hu et al., 2007), providing one possible mechanism for arousal's actions. Though the enhancing effects of epinephrine on both LTP and memory is most widely reported, it is important to emphasize that stress, perhaps through changes in peripheral epinephrine can act to influence memory formation bidirectionally, impairing and enhancing retention.

Similar to effects on memory, stress can also either enhance or impair the formation of hippocampal LTP. For example, the presence of behavioral stress, such as a foot shock, during training impaired LTP formation in the CA1 region of the hippocampus (Joels and Krugers, 2007). Further, the Joels and Krugers (2007) study proposed an alternate mechanism to epinephrine by which stress

could induce changes in hippocampal LTP involving the release of corticosteroids in response to stress. Injections of corticosterone, both *in vitro* and *in vivo*, reduced LTP formation within the hippocampus in a dose-dependent manner (Joels and Krugers, 2007). This effect of corticosterone also followed an inverted-U dose-response curve, aligning well with data showing that stress can up-or-down-regulate LTP formation within the hippocampus. However, the ability of epinephrine to influence both task-related memory and LTP provides a strong model for understanding the role of epinephrine in modulating memory formation under emotionally arousing conditions.

Epinephrine plays a major role in the formation of memories during emotionally arousing events in humans (Cahill and Alkire, 2003). For example, administration of peripheral epinephrine before a memory test improved memory in a dose-dependent manner in young adults (Cahill and Alkire, 2003). This observation has implications for problems such as post-traumatic stress disorder (PTSD), a condition characterized by persistent and often disturbing memories that occur after an emotional or traumatic event (Sundin et al., 2010). The problematic memories that are associated with PTSD are induced under conditions of high stress, and result in strong memories that are extremely resistant to decay (Roozendaal et al., 2009). In these circumstances, it is possible that memory formation could be overly enhanced by the body's neuroendocrine stress response. Because epinephrine is capable of converting a transient form of LTP into a relatively durable one, it is possible that epinephrine could contribute

to the necessary conditions for the formation of the type of non-decaying memories that are seen with PTSD (see Korol and Gold, 2008).

Although the influence of epinephrine on memory processing has been demonstrated by a variety of studies, the mechanism by which it does so remains unclear. As mentioned above, epinephrine is not readily taken up into the brain, as it does not cross the blood-brain barrier. However, one possible mechanism is that epinephrine release in the periphery could result in elevated levels of glucose within the brain. Recent studies have found that epinephrine may act to enhance memory by triggering the release of hepatic glucose stores into the blood stream (Gold and Korol, 2014). Epinephrine release in the periphery can stimulate hepatic adrenergic receptors, initiating the breakdown of glycogen to glucose, which is then released into the blood (Sutherland and Rall, 1960). There is a variety of evidence that supports the idea that the effects of epinephrine on memory could be mediated by increases in circulating glucose levels (Gold, 1995, 2005, McNay and Gold, 2002, Korol and Gold, 2007). Much like epinephrine, glucose has been seen to enhance memory in a dose-dependent manner that follows an inverted-U shaped curve (Korol and Gold, 2008). In fact, changes in circulating blood glucose levels have been associated with the efficacy of epinephrine and glucose on enhancing memory. Further, direct infusions of glucose into specific brain regions are capable of enhancing memory (Ragozzino et al., 1998, Canal et al., 2005). Alternatively, a different mechanism has been proposed by which epinephrine release could influence memory.

Epinephrine can be released into the blood stream from the adrenal medulla in response to arousal. This neuroendocrine response activates the vagus nerve, which fires rapidly to activate the locus coerulus neurons of the brain stem, which in turn stimulate the release of norepinephrine diffusely throughout the brain (King II and Williams, 2009, Chen and Williams, 2012). As a result, the release of epinephrine in the periphery ultimately results in the release of norepinephrine into many areas of the brain, including the hippocampus and amygdala (Liang et al., 1985). The amygdala is a collection of nuclei that rests in the medial temporal lobe above the hippocampus. This structure is also considered to be involved in regulating the effects of stress on memory formation. For example, animal studies have indicated that both acute and chronic stress can induce functional changes in specific amygdala nuclei (Roozendaal et al., 2009). These morphological changes may underlie the behavioral changes that can be seen in conditions such as PTSD.

Whether arousal acts to modulate neural plasticity through humoral or circuit processes, it is likely that central adrenergic activation may translate the peripheral response into a central response. The basolateral complex of the amygdala (BLA) may play an important role in modulating the effects of stress upon memory consolidation (McGaugh, 2000). Adrenergic receptors found throughout the amygdala are capable of binding norepinephrine. Specifically, stress hormone-induced activation of β-adrenergic receptors (β-ARs) in the BLA can be associated with enhanced memory for inhibitory avoidance tasks in rats (Roozendaal et al., 2009). The BLA has been found to influence memory

formation through its numerous efferent connections to other brain areas. This interconnectivity could explain the BLA's role in memory consolidation, as it contains fibers that both directly and indirectly project to the hippocampus (Pikkarainen et al., 1999). Similar findings in human studies support these results, and indicate that both norepinephrine and amygdala activity are required for the enhanced memory consolidation that often accompanies the stress response (Cahill et al., 1994, Cahill et al., 1995).

The activation of β-ARs by norepinephrine in the hippocampus is an attractive mechanism by which epinephrine could act to modulate memory formation within the brain. β-ARs are cell surface receptors which play an important role in many areas of the body. These receptors function to activate adenylyl cyclase via coupling to G-proteins (Liggett, 2000). β-ARs are expressed throughout the body, and influence critical sympathetic responses in the cardiovascular, pulmonary, metabolic, and central nervous systems. β-AR antagonists, commonly referred to as beta-blockers, are capable of effectively blocking the action of these receptors within the body for a variety of purposes.

Similar to the bidirectional effect of epinephrine on LTP that was demonstrated in rats (Korol and Gold, 2008), β-AR antagonist action has also been shown to follow an inverted U-shaped dose-response in smooth muscle (Calabrese, 2001). This similarity between epinephrine and β-AR action within the body supports the conclusion that epinephrine could act to influence bodily processes via β-ARs and adrenergic receptors as a whole. Further, it has been shown that β-AR activity decreases with age (Tuttle, 1996). If epinephrine does

act to influence memory formation through activation of β-ARs, this observation could possibly explain the rapid forgetting that is characteristic in aged subjects.

It is clear that epinephrine is capable of bidirectionally influencing hippocampal LTP, in a dose-dependent manner, although the mechanisms by which it does so remain unclear. The stress response, accompanied by the release of epinephrine in the periphery has a profound impact upon both memory and LTP. One possibility is that epinephrine activates β-AR in the amygdala, which in turn modulates hippocampal synaptic plasticity. Another possibility, though not mutually exclusive, is that β-AR signaling in the hippocampus proper is responsible for the enhanced LTP. By examining the role of β-AR activation in the hippocampus, this study has explored the relationship between peripheral epinephrine and enhancement of LTP within the hippocampus in an attempt to illuminate the underlying mechanisms. We found that overall, a large amount of variability exists between individual responses to stress, and the mechanisms underlying epinephrine-enhancement or impairment of hippocampal LTP are very complex. Moreover, while developing our stimulation protocols, we found that the epinephrine dose interacts with the amount of stimulation used to induce LTP to produce bidirectional modulation of LTP durability, much like the interaction between arousal level and epinephrine effects on memory formation. These findings point to important neurochemical events during and after activation that may play a role in long-lasting synaptic plasticity and information storage.

Methods

Animals

Young adult male Sprague-Dawley rats, age 3-4 months were used in these experiments. Rats were housed individually on a 12:12 light-dark cycle, with *ad lib* access to food and water throughout the experiment

Surgery

Surgeries were conducted in a manner similar to those outlined in previous studies (Korol and Gold, 2008). Monopolar stimulating and recording electrodes were constructed by hand from Teflon-coated stainless steel wire, 114 μm in diameter. Approximately 1 mm of Teflon coating was stripped from the tip of each stimulating electrode. Grounding electrodes were constructed from stainless steel jeweler's screws soldered to Teflon-coated stainless steel wire. Stainless steel screws were also used to provide anchorage for the headcap.

Prior to the start of surgery, each rat's head was shaved and disinfected with betadine. Each rat was also given a non-steroidal anti-inflammatory and analgesic drug (Flunixin, 0.001 mg/kg) as well as penicillin (0.0003 mg/kg) prior to the start of surgery. Before and during surgery, each rat was anesthetized with isofluorane (induction with 5.0% isofluorane at 500mL/min for approximately 10 minutes, during surgery 250mL/min flow rate with isofluorane between 1.8-4.2%) for stereotaxic implantation of stimulating and recording electrodes in the medial perforant pathway (8.1 mm posterior to bregma, 4.2 mm lateral to midline, ~3.5 mm ventral to dura) and hilus of the dentate gyrus (3.5 mm posterior to bregma, 2 mm lateral to midline, ~4 mm ventral to dura), respectively.

A subset of rats was also implanted with a 6 mm cannula, attached to the recording electrode, to allow for direct injection of drugs at the recording site. Under physiological control, the depth of both stimulating and recording electrodes was adjusted stereotaxically during surgery to maximize the extracellular evoked potential. Three screws were attached to the surface of the skull, one on either side of the midline just anterior to bregma, one along the midline posterior to lambda, and served as anchors for the headcap. An additional four screws were attached to the surface of the skull, one on either side of the midline just posterior to bregma, one on either side of the midline at the back of the skull posterior to lambda. These served as reference electrodes.

All electrode wires were connected to male, gold Amphenol pins. These pins were inserted into a 9-pin plug that was firmly mounted to the skull using dental acrylic. Post-surgery, antibacterial ointment (Bacitracin) was applied to the incision. Analgesic (ibuprofen, 2.35 mL in 500 mL of water) was also given for 24 hours post-surgery to reduce pain. Rats were allowed to rest in their home cages for one week post-surgery before the start of electrophysiological recordings.

Electrophysiological Recording

During recording sessions, each rat remained in its home cage, which was placed inside of a shielded recording chamber. Electrodes in the headcap were connected via a plug that held the stimulating and recording leads. All leads were attached to a commutator that was suspended from the frame above the recording

chamber. This setup allowed the rats to move freely within their home cage during recordings, and also served to minimize pressure on the headcap. Recording was performed at the same time daily between the 900 and 1200 hr of each day (early during the rat's light cycle) to eliminate any possible confounds produced by circadian rhythms.

Minimum and maximum stimulation values were determined for each rat and were used to create an input/output (I/O) curve (Figure 1). Ten stimulation values were used in the I/O tests. These ranged from an intensity that produced a threshold field EPSP response (minimum stimulation value) to one at which no further increase in EPSP slope could be detected (maximum stimulation value). The eight intervening intensities were generated and were separated by equal increments. Three test stimulations that were biphasic current pulses of 200 μs duration were delivered to each perforant pathway every 20 s, alternating between the left and right hemispheres every 10 s.

I/O functions serve multiple purposes. First, they are used to ensure that the slope of the EPSP increases linearly in response to increasing stimulation values to demonstrate that small changes in EPSP can be detected during the experimental procedure. Input/output functions also served to establish a baseline stimulation value at 40% of the maximum stimulation in the linear range of the input/output function, thereby allowing detection of both increases and decreases in response magnitude. Baseline stimulation intensities ranged from 150 μA to 2mA. Finally, these curves can be used to measure changes in synaptic strength at intensities other than those used during daily test recordings. Immediately

following the I/O curve recording, 30 test pulses at baseline stimulation intensity were delivered to each pathway. Responses to these 30 test pulses were used to quantify the main dependent measures for analysis throughout the study.

Stimulations and recordings were made with (Signal 4.0, Cambridge Electronic Design) and delivered with Grass S48 stimulators through constant current isolation units so any small change in resistance or conductance in the stimulating electrodes would not produce change in stimulation intensity given to the perforant pathway. The evoked potentials recorded from the dentate gyrus were amplified (200-500 times), and filtered (0.3 Hz High pass, 3kHz low pass) with Grass amplifiers (Model P511K) to allow maximal detection of the synaptic and action potential component of the field potential responses. Responses were sampled at 10 kHz by computer, saved and analyzed offline using Matlab (MathWorks, 2011). Because evoked responses were 10-15 msec in length (Figure 1A), this sampling frequency provided excellent waveform resolution (10 points/ msec) for quantification.

Daily recordings continued until a stable baseline, defined as < 5% change in mean response size from day to day could be determined for the evoked response (typically about 4 days). Once a stable baseline was achieved, the rats were randomly separated into test groups based upon the drug treatments that were administered prior to the high-frequency stimulation (HFS) known to produce LTP.

Experimental Groups:

Experiment 1: Enhancing and Impairing effects of Epinephrine

Group 1: small HFS-vehicle control

Within this test group (N=6), each rat received a peripheral saline injection prior to stimulation with a small HFS protocol (one set of ten 400 Hz trains of five pulses each (1 x 10 x 5)).

Group 2: small HFS- low epinephrine

This group (N=7) received the same small HFS stimulation protocol (1 x 10 x 5) paired with a peripheral injection of epinephrine at the enhancing dose (0.01 mg/kg).

Group 3: large HFS-vehicle control

This group (N=9) received a peripheral injection of saline combined with a larger HFS protocol (two sets of twelve 400 Hz trains of five pulses each (2 x 12 x 5)).

Group 4: large HFS- moderate epinephrine

This group (N=7) also received the larger stimulation protocol (2 x 12 x 5), as well as a larger dose of peripheral epinephrine (0.1 mg/kg) to demonstrate the impairing effects of epinephrine under these conditions.

Experiment 2: Dose-dependent influence of epinephrine

Group 1: Moderate HFS- vehicle control

Animals in this control group (N=6) received a peripheral injection of saline, as well as a moderate stimulation protocol known to produce LTP (one set of ten 400 Hz trains of ten pulses each (1 x 10 x 10)).

Group 2: Moderate HFS-moderate epinephrine

This test group (N=8) received the same moderate stimulation protocol (1 x 10 x 10) as well as an intermediate dose of peripheral epinephrine (0.1 mg/kg).

Group 3: Moderate HFS- high epinephrine

This test group (N=4) also received the same moderate stimulation protocol (1 x 10 x 10), in combination with a higher dose of peripheral epinephrine (0.3 mg/kg)

Experiment 3: Role of β-ARs in mediating epinephrine's effects on hippocampal LTP

Group 1: Low HFS- vehicle control (central)- vehicle control (peripheral)

This control group (N=4) received both local (intrahippocampal) as well as peripheral saline injections, combined with the original stimulation protocol used in experiment 1 (1 x 10 x 5).

Group 2: Low HFS- vehicle control (central)- low epinephrine (peripheral)

This test group (N=5) received the same stimulation protocol (1 x 10 x 5), as well as a local saline injection. Additionally, animals in this group received peripheral injections of epinephrine at the enhancing dose (0.01 mg/kg) in an effort to establish the enhancing effects of epinephrine under these conditions.

Group 3: Low HFS-propranolol-epinephrine

This test group (N=8) received the same stimulation protocol (1 x 10 x 5) combined with local injections of propranolol (0.5 μg/μL) and peripheral injection

of epinephrine at the enhancing dose (0.01 mg/kg). All rats received peripheral (intraperitoneal) injections prior to HFS, while a subset of rats also received local (intrahippocampal) injections via a 6 mm cannula ten minutes prior to systemic injections. This protocol ensured that the β-Adrenergic receptor antagonist had sufficient time to act upon receptors within the brain before any systemic injections of epinephrine.

Once all treatments were administered, the rats were allowed to rest in their home cages for 30 minutes; a post-injection baseline recording was taken before all groups were given HFS. Three additional recording sessions (I/O and baseline) followed HFS, after which the rat was returned to the vivarium. Six hours after HFS, the rat was subjected to one more recording session. After HFS, daily test recordings continued to evaluate the magnitude of LTP, which gave us a measure of durability.

Waveform and Data Analysis

Responses were analyzed from all treatment groups, and were evaluated using two parameters: EPSP slope and magnitude of population spike (Figure 1A). The degree of LTP is often monitored using these parameters because increases in the slope of the EPSP and magnitude of the population spike reflect increases in the extracellularly recorded synaptic event and increases in the number and synchrony of cells firing action potentials, respectively (Taube and Schwartzkroin, 1988). Changes in these values were extracted from the waveforms for ten recording sessions: pre-HFS, immediately post-HFS (t0), 20

minutes post-HFS (t20), 40 minutes post-HFS (t40), 60 minutes post-HFS (t60), 6 hours post-HFS (t6hr), 24 hours post-HFS (t24hr), 48 hours post-HFS (t48hr), 72 hours post-HFS (t72hr), and 96 hours post-HFS (t96hr). Responses were analyzed based upon overall change in both the slope of the EPSP and the magnitude of the population spike. Statistical tests were conducted for EPSP slope and PS area independently to determine if LTP occurred in test groups and if treatment groups differed across time points throughout the recording session within treatment groups. Effects of drug injection were also assessed to ensure that there was no significant change in responses due to the injection itself. Repeated measures ANOVA were conducted to compare effects within and between test groups. Significant ANOVA results were followed with post-hoc Dunnett's test using baseline values as the control data point.

All procedures were approved by Syracuse University's IACUC and followed federak guidelines for the use and care of animals in research.

Results

In general, the results of this study showed that epinephrine has variable effects on hippocampal LTP, depending upon the amount of stimulation and dose of epinephrine. Results from Experiment 1 showed that, compared to saline controls, a low dose (0.01 mg/kg) of epinephrine combined with a small stimulation protocol (1 x 10 x 5) enhanced potentiation of the EPSP slope (Figure 2A, B). Conversely, epinephrine at a larger dose (0.1 mg/kg) combined with a larger stimulation (2 x 12 x 5) blocked potentiation of the slope of the EPSP (Figure 2C, D). These initial results formed the basis for my independent work as they indicated that epinephrine, under different conditions, can either enhance LTP or impair LTP.

In Experiment 2, different dosages of epinephrine were tested under identical stimulation protocols (1 x 10 x 10) to evaluate the dose-dependency of the epinephrine response (Figure 3). An intermediate dose of epinephrine (0.1 mg/kg) was capable of enhancing hippocampal LTP, as seen by increases in the slope of the EPSP. Rats treated with saline exhibited very stable responses, with no significant potentiation of the EPSP slope occurring during any of the recording sessions following HFS. Within the intermediate epinephrine group (0.1 mg/kg) LTP was enhanced following HFS, as seen by increases in the slope of the EPSP, on average. However, while the average data for this group showed a general enhancement of hippocampal LTP, responses from individual animals indicated that this dose was capable of either enhancing or impairing hippocampal LTP. A second, higher dose (0.3 mg/kg) of epinephrine was also tested in this

experiment. The results from the higher dose of epinephrine fit well with the established inverted-U dose-response curve. Specifically, there was initial enhancement immediately afte HFS that seemed to decline at 20 min post-HFS until 24 hrs later when responses were potentiated again.

Finally, in Experiment 3 rats treated with both local and peripheral saline showed no increase in the slope of the EPSP any of the time points after low levels of HFS (Figure 4A). Responses from rats treated with local saline and peripheral epinephrine at 0.01 mg/kg, the previously established enhancing dose in rats without cannula, did not show the anticipated enhancement of EPSP slope (Figure 4B). Although there were subtle increase in the EPSP slope just after HFS, responses in this group actually became depressed from 20 min to 6 hrs post-HFS, mirroring the impairing effect that was seen in the 0.3 mg/kg epinephrine group from Experiment 2. Rats in the group that received both local propranolol (0.5 μg/μL) and peripheral epinephrine (0.01 mg/kg) demonstrated stable responses on average, with no significant potentiation of the EPSP slope visible during any of the recording sessions (Figure 4C).

Because responses across rats seemed variable, we compared two separate groups of animals that were tested under the same dose of epinephrine (0.01 mg/kg) and stimulation protocol (Figure 5). Group 1(Figure 5A) was tested under these conditions in Syracuse, while Group 2 (Figure 5B) had been tested previously in Illinois. Further, rats in Group 1 had been implanted with intrahippocampal cannulae, which could have served to confound the results. These results, when compared, show a strikingly different effect of epinephrine.

Under similar conditions, in separate groups of animals, epinephrine could be seen to influence hippocampal LTP in very different ways.

While epinephrine did not significantly enhance LTP in these experiments, upon further examination, an interesting trend became apparent within the epinephrine test group. Frequently, in the epinephrine group, that the slope of the EPSP was depressed, compared to its baseline value, in the recording sessions that immediately followed injections and HFS. However, it was often the case that the EPSP slope recovered in the later time points. This trend seemed to indicate that epinephrine actually had an impairing effect upon LTP in the hippocampus in these rats. Baseline responses for all rats were established to be stable before the day of HFS, however the results from the day of HFS were somewhat confounding. A significant decrease in the slope of the EPSP was seen, on average, from the initial baseline measurement to the post-injection baseline measurement, indicating that some effect could have been produced by the injection itself. Overall, when compared to the initial baseline measurement, there was no significant potentiation of the slope of the EPSP seen at any of the time points after HFS. As expected, no significant potentiation of the slope of the EPSP was seen at any time point for the saline control group. When compared with the saline controls, it appeared as though some slight potentiation occurred in the epinephrine group at time zero. However, this result was not significant, and only appeared to be potentiated compared to the depressed response that was seen with the post-injection baseline.

While no significant potentiation occurred in either group, closer examination revealed that there was a difference in responses between the epinephrine test group and saline controls. LTP responses for the saline group remained relatively stable at all time points recorded. Animals in the epinephrine group, however, exhibited a large amount of variability in both the slope of the EPSP and the magnitude of the population spike. Across all animals in the epinephrine test group, the slope of the EPSP was depressed, generally from the t20-t6hr recording sessions, and recovered to around baseline levels in recording sessions about 24 hours after the initial experiment. Additionally, the overall waveforms varied greatly for recording sessions following treatment with epinephrine. Conversely, the slope of the EPSP, magnitude of the population spike, and overall waveforms remained relatively stable throughout all recording sessions for the saline group. This effect could be seen in individual rats, as well as within the groups as a whole. Based on these observations, responses were grouped into three general categories: epinephrine-enhanced, epinephrine-impaired, and stable responses (Figure 6A, B, and C, respectively). These results, while unexpected, could serve to provide insight on the bidirectional effect of epinephrine on hippocampal LTP. The variability that was seen from rat to rat in these experiments supports the idea that different individuals could respond differently to stress, a factor that could have served to confound the results. Overall, the results of these experiments have provided further evidence for the bidirectional effect of epinephrine upon hippocampal LTP, and could serve to illuminate the existence of individual differences in response to stress.

Discussion

Overall, the role of β-ARs in mediating epinephrine's influence upon hippocampal LTP could not be determined. It could not be established through these experiments that epinephrine was capable of significantly enhancing hippocampal LTP responses at the dose given (0.01 mg/kg), which had previously been established as an enhancing dose. Instead, the results of this study supported the idea that epinephrine can act either to enhance or impair the LTP response, depending on the dose given. The bidirectional effects of epinephrine that were explored in these experiments could imply that individual responses to the same stressor can vary greatly, an observation which has implications for the broader picture of PTSD onset.

It has been established that epinephrine release in the periphery stimulates the release of norepinephrine in many brain areas, including release into the hippocampus and amygdala (Gold and van Buskirk, 1978, Miyashita and Williams, 2004). These experiments explored the possibility that epinephrine in the periphery could act to influence processes within the brain through noradrenergic activation of β-ARs in the hippocampus. For the purposes of these experiments, hippocampal LTP was induced via stimulation to the perforant pathway, and responses were recorded from neurons in the dentate gyrus. It has been previously established that the amygdala appears to mediate the formation of LTP in the dentate gyrus (Frey et al., 2001). Specifically, previous studies have found that lesions to the BLA reduced overall LTP produced by HFS in the dentate gyrus (Ikegaya et al., 1995). The BLA, as discussed earlier contains

afferent fibers that project to many areas of the brain, including the hippocampus. The interconnectivity of this region could allow the amygdala to exert some influence upon the formation of LTP within the hippocampus. Similar studies have demonstrated that lesions to the amygdala also serve to diminish performance in memory tests, supporting the modulatory role of the amygdala in memory formation as a whole (McGaugh, 2004). Further, the use of β-ARs to block adrenoreceptor function inhibited the enhancing effect of epinephrine, and other hormonal and pharmacological treatments on memory (McGaugh, 2004). Finally, it has also been shown that β-ARs mediate the modulation of novelty-induced changes in LTP (Straube and Frey, 2003). Given this evidence, it was expected that when β-ARs were blocked with the adrenergic receptor antagonist propranolol (0.5 μg/μL), the enhancing effect of epinephrine on LTP in the dentate gyrus would also be blocked.

While the results do not show the significant enhancement of LTP by epinephrine observed in the past, several factors could have served to confound these results. First, it cannot be demonstrated that no effect was produced due to the local (intrahippocampal) injections themselves. In the epinephrine test group, the slope of the EPSP was seen to significantly decrease from the baseline to post-injection time point, indicating that the injection itself may have influenced the response. Additionally, it is possible that the presence of the 6 mm cannula was disruptive to the brain tissue, and any injections could have served to further disrupt function in these areas. Epinephrine was not found to produce enhanced hippocampal LTP compared to saline controls at any of the time points recorded

(up to 96 hrs post HFS). However, when compared with the relatively stable responses in the saline controls, it can be seen that hippocampal LTP responses were influenced by the presence of peripheral epinephrine. The effective enhancing dose of epinephrine (0.1 mg/kg) that had been previously established (Korol and Gold, 2008; Gold et al., 1984) was determined to be too large to be within the enhancing range for epinephrine in previous trials; therefore a lower enhancing dose (0.01 mg/kg) was utilized in these experiments. This lower dose was not confirmed to be capable of significantly enhancing hippocampal LTP responses by the results of these experiments. Enhancement of these responses should have been seen by an increase in both the slope of the EPSP and the magnitude of the population spike, with enhancement lasting as long as 96 hours post treatment. These primary findings were unable to establish that epinephrine at the tested dose is capable of producing enhancement of both the magnitude and duration of hippocampal LTP in rats with cannulae. Due to the difficulty incurred in producing enhancement of LTP by epinephrine, it was therefore difficult to assess the enhancing capability of epinephrine, and the mechanisms by which this could occur. However, the inability to enhance hippocampal LTP with epinephrine in these experiments served as a basis to explore further the bidirectional influence that epinephrine has been seen to exert upon hippocampal LTP formation.

While our experiment could not establish epinephrine-enhancement of LTP, and therefore could not determine a role for β-ARs, it has been shown that peripheral epinephrine is capable of enhancing LTP within the hippocampus, and

further, this enhancement of LTP can be blocked by administration of the β-AR antagonist, propranolol. These results seem to indicate that β-ARs do play a significant role in modulating the effects of peripheral epinephrine on hippocampal LTP. Unfortunately, this effect could not be demonstrated in these experiments, indicating that there could be other mechanisms at play. In support of this theory, if peripheral epinephrine does act to induce changes in hippocampal LTP solely through activation of β-ARs, then it would follow that activation of these β-ARs should be sufficient to produce enhanced LTP in the hippocampus. In an earlier phase of this study (results not shown), this hypothesis was tested using local infusion of the β-AR agonist, clenbuterol. In the absence of peripheral epinephrine, β-ARs in the hippocampus were activated with clenbuterol, in the hopes of producing enhancement of hippocampal LTP in a manner that was consistent with the epinephrine-mediated enhancement of LTP that had previously been demonstrated. These experiments with clenbuterol produced some unexpected results. Potentiation that was induced with HFS and clenbuterol was different from that seen in previous trials in that it produced significant potentiation of the population spike, but did not significantly potentiate the slope of the EPSP. This lack of potentiation in the slope of the EPSP suggests that mechanistic differences could exist between epinephrine-induced and norepinephrine-induced potentiation, including the possibility that enhancement of LTP by epinephrine is not mediated entirely by norepinephrine.

Alternatively, there are several sub-types of β-Adrenergic receptors, and clenbuterol is a β-2 specific adrenergic receptor agonist. It is possible that

norepinephrine-mediated enhancement of LTP also involves β-1 adrenergic receptors, which are not activated by clenbuterol. In fact, β-1 adrenergic receptors have been associated with ERK activation, a protein kinase that could play an important role in producing long-lasting LTP (Smolen, 2006). Overall, it was seen that β-adrenergic receptors are at least partly involved in the mediation of epinephrine-enhancement of LTP and, receptor subtype specificity may determine the components of the evoked responses that are modulated. Propranolol is a non-selective β-AR antagonist, therefore the blockage of potentiation in both the slope of the EPSP and magnitude of the population spike that was seen with propranolol is compatible with the theory that epinephrine enhancement of hippocampal LTP could be dependent on activation of more than one β-AR subtype. These differences in β-1 versus β-2 regulation of LTP may point to different cellular and molecular mechanisms including the signaling pathways and the circuit characteristics involved in the synaptic plasticity. The results of these experiments, however, suggest that neurotransmitter systems other than noradrenergic signaling that could play important roles.

One alternative mechanism by which epinephrine in the periphery could influence hippocampal LTP formation involves the release of glucose from hepatic stores. As discussed earlier, epinephrine release in the periphery triggers a cascade of events, one of which involves the conversion of glycogen to glucose, followed by release of this hepatic glucose into the blood stream (Sutherland and Rall, 1960). Further, systemic injections of glucose can act to enhance memory in a variety of tasks (Korol and Gold, 2007; Gold, 2008, 2014). Glucose can enhance

memory function in a manner that is very similar to epinephrine, as it also enhances in a dose-dependent manner, according to an inverted-U dose-response curve (Korol and Gold, 2008). This alternative theory as to how epinephrine influences hippocampal LTP could serve to explain some of the results seen in this study, as noradrenergic activation of β-ARs may not be the only relevant consequence of peripheral epinephrine release.

As outlined in the results, an intermediate dose of epinephrine (0.1 mg/kg) often enhanced hippocampal LTP, while a larger dose (0.3 mg/kg) produced unreliable LTP, which was seeming depressed from 20 mins to 24 hrs post-HFS. The bidirectional effect of epinephrine that was demonstrated in these experiments was clearly dose-dependent, following the inverted-U curve that has been established for epinephrine (Gold, 1989). Additionally, the intermediate dose (0.1 mg/kg), which was expected to be enhancing, could impair LTP formation in certain cases. Based on these results, it was hypothesized that the range at which epinephrine acts to enhance hippocampal LTP is very small (0.01-0.1 mg/kg). Further, in these cases where the 0.1 mg/kg dose acted to impair LTP, it is likely that endogenous stress levels at the time of injection created endogenous epinephrine levels that, when combined with the epinephrine dose injected, created a very high level of circulating epinephrine that was outside of the enhancing range. This effect could be accounted for in future studies by measuring concentrations of epinephrine in the blood stream to determine that the correct dosage was given.

The most striking trend that emerged from within the epinephrine test group results was the incredible variability in the responses. While the saline responses were seen to remain stable throughout, animals that were given the same dose of epinephrine could be seen to have entirely different responses. This observation supports the hypothesis that individual differences in stress response exist between different subjects.

The stress response triggers a cascade of events within the body, involving both the locus coeruleus-norepinephrine system, which has been studied here, and the corticotrophin releasing hormone system (Ellis et al., 2006). These two functionally integrated circuits interact with one another to mediate the stress response in each individual. It has been theorized that the stress response is not a straight-forward mechanism that acts in the same manner for all individuals, but rather there are several stress-response phenotypes, which guide development of stress reactivity (Ellis et al., 2006). In this case, individuals react differently to the same stressor, due to differences in genetic expression and environmental factors. For example, it has been found that the level of maternal care received during development can alter the expression of genes that regulate both behavioral and physiological responses to stress (Meaney, 2001). Further, it was demonstrated in a different study that rats can be categorized as either “high responding” or “low responding” based on their level of stress-induced exploration in a novel environment (Kabbaj et al., 2000). These phenotypes did not change throughout the course of the study, further cementing the observation that the stress-response phenotype is developed early in life, and stems from both genetic and

environmental factors. This stress-response phenotype has also been shown in humans. One study, in particular, surveyed women before and after they received colonoscopies, and found that the level of anxiety measured before the test was a direct indicator of the severity of emotional reaction that was incurred upon diagnosis (MacLeod and Hagan, 1991). This model of stress-reaction phenotypes not only fits well with the variable data that were seen in this experiment, but it could also have implications for the broader picture of this project, to understand PTSD. While many people experience PTSD after traumatic life events, it is not a universal phenomenon. That is, not every person who undergoes a traumatic event will develop PTSD. The existence of multiple stress-response phenotypes could explain why, in the case of PTSD, certain individuals are more likely to develop this condition than others.

The results of these experiments were complex and require further investigation, but they confirm earlier studies showing that epinephrine does play a role in modulating both LTP and memory formation. Further, upon examination of the individual responses, interesting trends became apparent which could underlie the bidirectional nature of epinephrine's effect upon hippocampal LTP formation. Epinephrine has been shown to influence memory according to an inverted-U dose-response curve (Korol and Gold, 2008). According to this model, if the injections themselves were particularly stressful to the rats, then endogenous epinephrine levels could have skewed the results. If the animals were already experiencing elevated epinephrine levels at the time of injection, then it is very possible that the dose of epinephrine given was not well controlled. As a result, it

is possible that under stressful injection conditions, rats could have had higher levels of peripheral epinephrine than was thought, which could have put them well beyond the range of the established enhancing dose. Further, the different stimulation protocols tested could have served to activate different signaling pathways, resulting in different amounts of norepinephrine release within the brain.

As a whole, the study of epinephrine-mediated enhancement of LTP is very significant as this mechanism could play a large role in the pathology of conditions such as PTSD. While it is difficult to base conclusions on the results of this study, my findings served to illustrate the bidirectional nature of epinephrine's influence on hippocampal LTP. Further work on the mechanisms underlying this phenomenon could illuminate the role of peripheral epinephrine in mediating both LTP and memory formation as a whole and in conditions where stress interacts with memory and other aspects of brain function to impair optimal performance.

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Appendix:

Figure Legends:

Figure 1. Characterization of the evoked response. **A)** Typical evoked response shows the change in voltage over time after stimulation **B)** Input/Output function: the magnitude of the evoked response varies in accordance with increasing stimulation input. **C)** Typical change in evoked response that was seen following HFS that produced significant potentiation. Note the increased slope of EPSP and magnitude of population spike following HFS (Red) as compared to baseline (black).

Figure 2. Epinephrine modulates LTP under different stimulation protocols. **A)** No potentiation of EPSP with saline and small stimulation protocol. **B)** Enhancement of potentiation with small dose of EPI and small stimulation. **C)** Potentiation of EPSP with saline and large stimulation protocol. **D)** No enhancement of EPSP with large dose of EPI and large stimulation.

Figure 3. Average changes in slope of EPSP for EPI and saline treatments. **A)** Intermediate dose of EPI (0.1 mg/kg) generally produces potentiation of EPSP slope **B)**. Large dose of EPI (0.3 mg/kg) does not produce potentiation at all time points. **C)** Saline produces stable response.

Figure 4. Average change in slope of EPSP for EPI, saline, and propranolol + EPI treatments. **A)** Treatment with EPI failed to produce enhancement of EPSP seen in figure 2. **B)** Treatment with saline produces stable EPSP response. **C)** Treatment with propranolol + EPI produces stable EPSP response.

Figure 5. Average effect of EPI (0.01 mg/kg) on different animal groups. **A)** Data collected from Syracuse, EPI (0.01 mg/kg) in which rats have cannulae do not produce significant enhancement of potentiation. **B)** Previous data collected from Illinois, in which rats do not have cannulae, EPI (0.01 mg/kg) enhances potentiation.

Figure 6. Individual examples of EPSP slope that characterize the epinephrine-enhanced, epinephrine-impaired, and stable groups. **A)** Variable effect of epinephrine (0.1 mg/kg). EPI-enhanced (left) EPI-impaired (right). **B)** Epinephrine at high dose (0.3 mg/kg) produces unreliable LTP **C)** Saline produces stable response.

Figures:

Figure 1

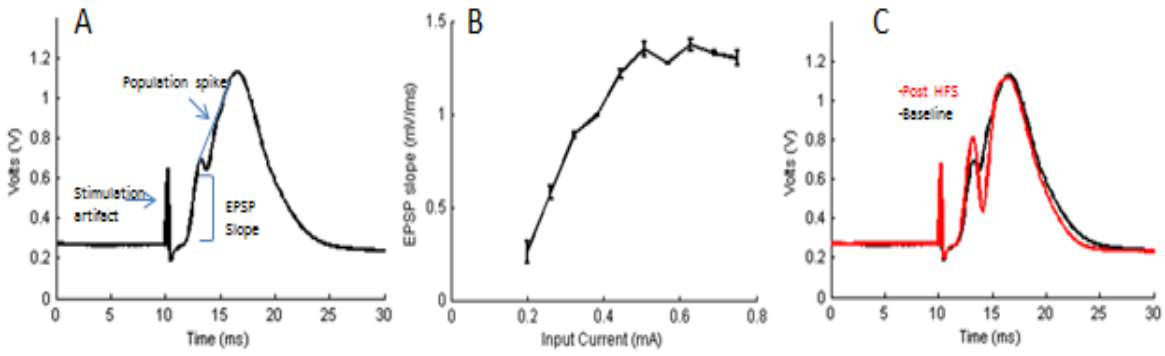


Figure 2

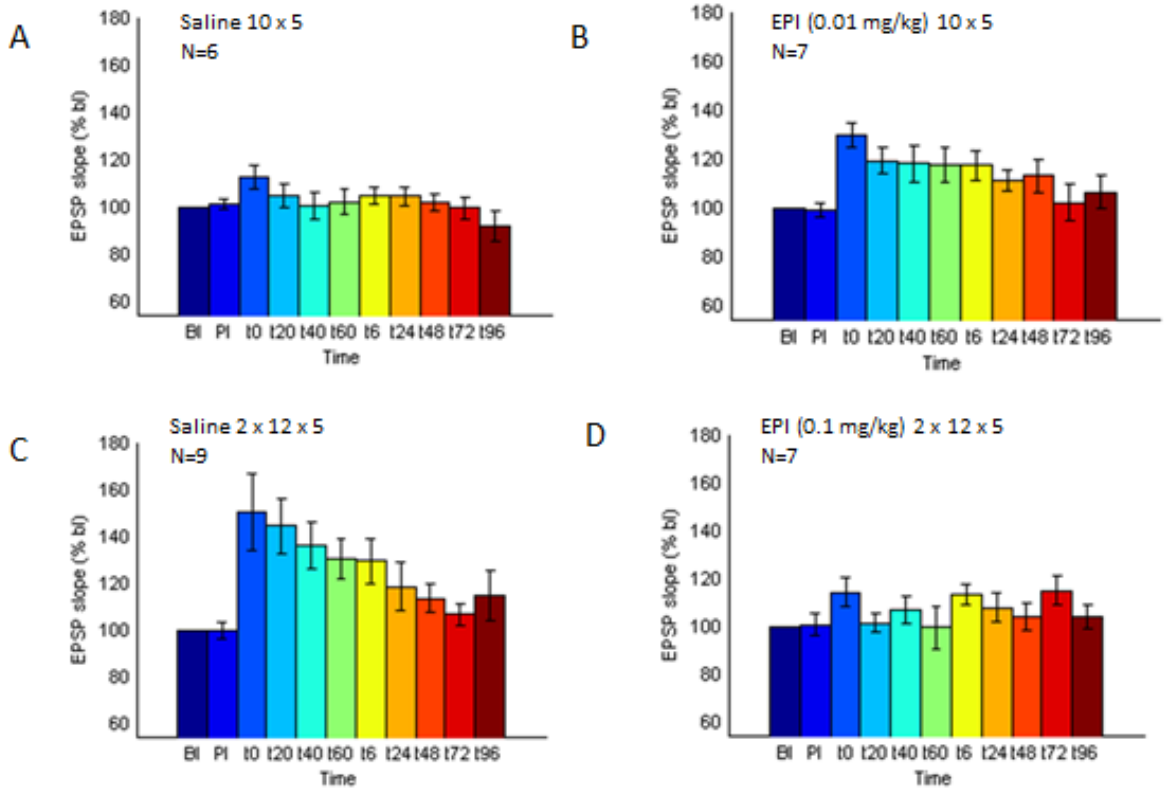
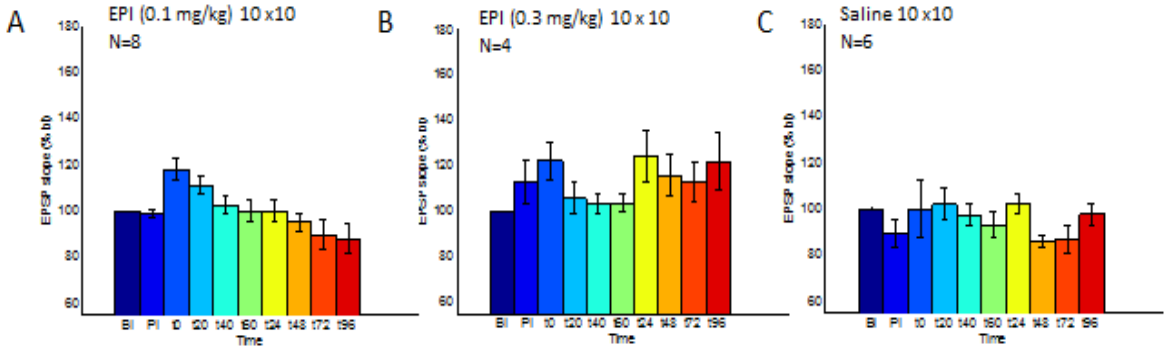
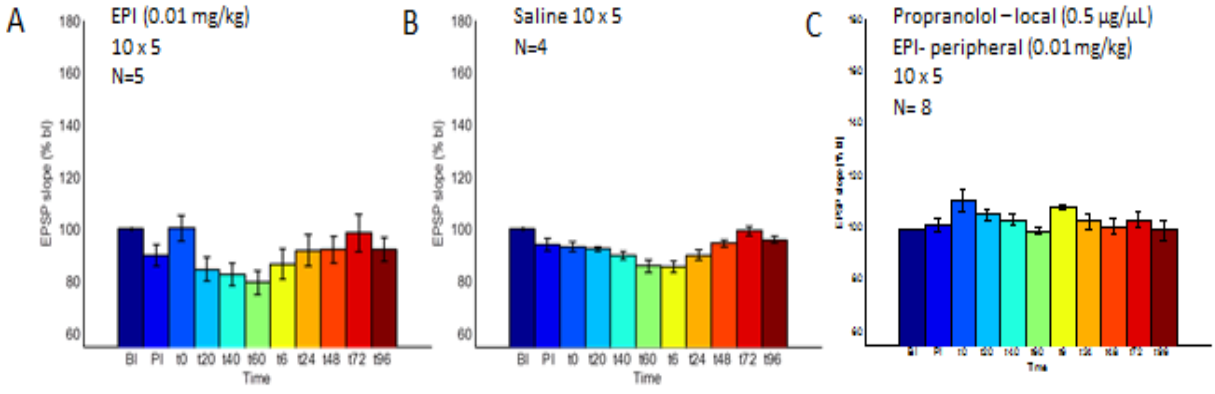


Figure 3



Repeated Measures ANOVA:		
Group Effect	F(2,16)=1.47	p=0.26
Time Effect	F(8,128)=1.95	p=0.058 (approaching significance)
Interaction	F(16,128)=1.34	p=0.18

Figure 4



Repeated Measures ANOVA:		
Group Effect	F(2,5)=0.25	p=0.79
Time Effect	F(9,45)=1.15	p=0.35
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Figure 5

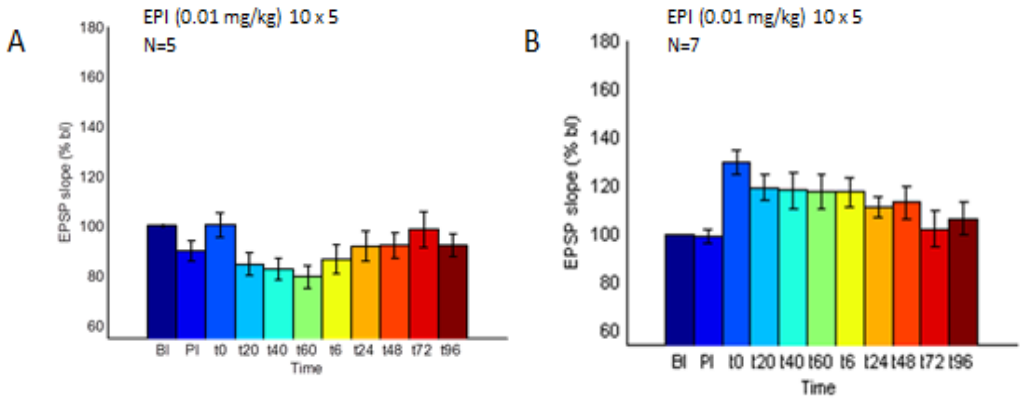
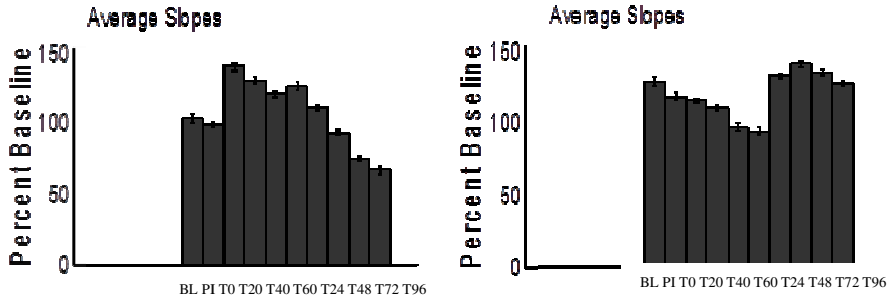


Figure 6

A



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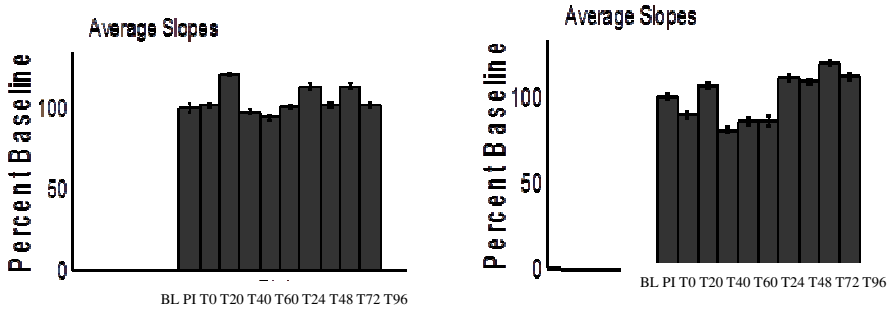

C