Dopaminergic modulation of activity dependent synaptic plasticity in rodent lateral entorhinal cortex

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Contents

1. Abstract

The entorhinal cortex serves as the interface region between allocortical input and the hippocampus, with current theories suggesting that the region is involved in memory encoding, with dopamine serving as a "motivational currency" provided to memories of higher importance to the organism's survival. This study aims to analyse the effects of dopamine on the ability to induce and maintain activity-dependent synaptic plasticity in the lateral entorhinal cortex. Here field recordings of excitatory post-synaptic potentials within rat lateral entorhinal cortex as well as bath application of drugs, including dopamine, were used to analyse their effects following stimulation of layers I and II of the lateral entorhinal cortex. The results indicate that the application of dopamine during paired-pulse low frequency stimulation causes a block of LTD. It was also found that the metaplastic effect of multiple dopamine applications of the same concentration remains in a slice which had been previously depressed by LTD. Results of LTP experiments were inconclusive, with a failure to induce LTP with either a high frequency stimulation or theta burst stimulation protocol. However, switching to a high calcium (10mM) ACSF during HFS improved the success rate of inducing LTP to 44%, with potentiation of the response remaining until the end of the 120-minute experiment. In conclusion, the results surrounding LTD provide further evidence to the theory that dopamine acts as a modulator of synaptic plasticity in the lateral entorhinal cortex (LEC). The LTP data requires further exploration, with use of a wider range of protocols to successfully induce LTP, at which point application of dopamine could be used to examine its effects.

2. Background

2.1 Dopamine, dopaminergic receptors, and signal transduction

Dopamine serves as a key neurotransmitter in the brain, specifically dopamine is vital in the mesolimbic reward pathway, acting as a chemical messenger released from the ventral tegmental area (VTA) in response to successful actions which aid the survival of the organism (e.g. eating, mating) (Adinoff 2004). With specific regard to the reward pathway, dopamine is manufactured in cell bodies of neurons located in the ventral tegmental area (VTA) (Olguín et al., 2016). Research by Slaney et. al (2012) quantified the total concentration of dopamine within the VTA as 4.8 ± 1.5 nM, compared to a concentration of 0.5 ± 0.2 nM within the red nucleus, based on the previous evidence suggesting that dopamine is manufactured within the VTA, this difference in dopamine concentration between the two regions is to be expected.

The mesolimbic pathway, or "reward pathway" contributed to our understanding of reward and pleasure. This pathway is largely dopamine regulated and is a key area of research not only for understanding of addiction, but also in learning behaviour. The pathway originates in largely dopaminergic cell bodies of the central tegmental area (VTA) within the midbrain. From the VTA, axons project to the nucleus accumbens (NAc) within the ventral striatum as well as the amygdala, bed nucleus of the stria terminalis (BNST), lateral septal area, as well as the lateral hypothalamus. The substantia nigra, a highly dopamine rich area, is situated closely to the VTA. The substantia nigra (SN) projects to the dorsal striatum in which motor activity is mediated. The mesolimbic pathway not only mediates the experience of reward for natural rewards, such as food or sex, but this pathway can also be hijacked by drugs of abuse such as alcohol, caffeine, and cocaine. These stimulants typically amplify dopaminergic signals within

the mesolimbic reward pathway by blocking dopamine transporters, essentially preventing the reabsorption of synaptic dopamine leading to a constant activation of postsynaptic neurons, providing a constant feeling of reward, it is this effect which causes addictive tendencies. The origin of the mesolimbic pathway, the VTA, as well as the substantia nigra project to the superficial layers of the entorhinal cortex. It is the supply to the EC by the VTA which likely links the mesolimbic pathway to the entorhinal cortex. As will be explored within this thesis, dopamine modulates synaptic plasticity within the entorhinal cortex and it can be assumed that the mesolimbic pathway's release of dopamine to the entorhinal cortex may modulate learning and memory in relation to rewarding behaviours, such as food and sex (Caruana et al. 2008).

The physiology of dopamine receptors alludes to their function; dopamine is a monoamine catecholamine neurotransmitter, forming one of the seven transmembrane G protein-coupled receptors which regulate motor function, as well as motivation, cognition, emotion, and neuroendocrine secretion (Mishra et al. 2018). It has been indicated that dopamine stimulates the activity and adenylyl cyclase (AC), two forms of dopamine receptors have been characterised based on their ability to regulate cyclic adenosine monophosphate (cAMP). The two dopamine receptor types are D1-like receptors and D2-like receptors. D1 and D2 receptors form the densest population within the central nervous system, with populations of D3, D5, and D4 receptors being incrementally less densely populated.

The types of dopamine receptors can also be categorised based on the mechanism by which they regulate cAMP. D1 receptors act on the G protein subtype Gs to increase intracellular levels of cAMP by activation of adenylate cyclase. D2 receptors, however, act on the Gi subtype G protein, however the intracellular cAMP levels are similarly increased by activation

of adenylate cyclase. Both D3 and D4 receptors lead to a decrease in adenylate cyclase, mediated by the Gi subtype of G proteins, with D5 receptors leading to increase in adenylate cyclase by activation of a Gs subtype G protein.

The function of dopamine receptors is also a factor by which the different receptor types can be categorised. The D1 receptor contributed to locomotion, learning and memory, as well as attention, impulse control, sleep, and regulation of renal function. The D2 receptor contributed to similar function; locomotion, learning and memory, attention, and sleep, but also contributed to reproductive behaviour. D3 receptors regulate locomotion, cognition, attention, impulse control, sleep, as well as the regulation of food intake. The D4 receptor is largely similar in function to the D2 receptor, although not contributing to locomotion and instead regulating cognition. Finally, the D5 receptor encompasses largely cognitive function, including attention, decision making, and motor learning. It is clear from these variety of functions covered by dopamine receptors that dopamine as a neurotransmitter is not solely responsible for locomotion and motor function, but also cognitive function, including learning and memory. Dopamine's contribution to learning and memory, specifically within the entorhinal cortex, is a key focus of this paper.

The signal transduction method of dopamine depends on the type of receptor to which dopamine is binding, the two types being D1-like receptors and D2-like receptors. The group of D1-like receptors includes D1 and D5 receptors, with this receptor type having higher density within the striatum, nucleus accumbens, SN pars reticulata, and the olfactory bulb. D1-like receptors induce adenylate cyclase activity by the activation of G proteins, and cyclic AMP is produced as a secondary messenger. cAMP then leads to the activation of protein kinase A (PKA) as well as protein kinase C (PKC). The activation of both PKA and PKC is caused

by an increase in intracellular calcium, mediated by the activation of phospholipase C. Intracellular calcium increase not only activates PKA and PKC and therefore leads to the activation of various other intracellular proteins, but also induces neurotransmitter release by exocytosis.

D2-like receptors (D2, D3, and D4 receptor subfamilies) typically lead to the inhibition of adenylate cyclase and therefore lead to a reduction of cAMP and PKA. D2 receptors are known to reduce neuronal excitability or reduce the synthesis of DA synthesis and packaging, inhibiting dopamine release. D2 receptors may also contribute to neuronal development of DA neurons during embryonic development.

The variation in the structure, function, and signal transduction of both D1-like and D2-like receptors leads to two distinct possible outcomes for dopamine binding. In the context of this paper, it is possible that the binding of dopamine to either D1 or D2 receptors would have different effects on synaptic plasticity within the entorhinal cortex, with binding to D1 receptors leading to an increase in intracellular calcium, neurotransmitter release, PKA production, as well as PKC production, whereas D2 receptor activation would lead to a largely inhibitory effect, reducing neuronal excitability and reducing levels of PKA and PKC.

Numerous experimental and review papers describe the role of dopamine in reward and behaviour; Berridge and Robinson (1998) define dopamine as a reward currency, providing behavioural value to “food, drink, and sex” but also explain that this reward pathway can be hijacked by drugs of abuse, leading to addictive behaviour. This information provides context and background to the role of dopamine in the brain, but also allows for some inference as to how dopaminergic deficiency in disease will lead to a change in behaviour, for example anhedonia witnessed in depression.

Many papers, such as that of Berridge and Robinson (1998), provide insight into the role of the mesolimbic reward pathway. A poignant research paper by Fouriezos et. Al (1978) assessed the effect of dopamine receptor blockers Pimozide (D2, D3, D4 antagonist) and Butaclamol (D2 antagonist) on lever-press frequencies in hooded rats. Stimulation of the lateral hypothalamus via surgical insertion of an electrode aimed to replicate central dopaminergic brain systems, specifically those of the reward pathway. Fouriezos et. Al's (1978) methods utilised sessions of self-stimulation, with trial groups consisting of those administered with Pimozide and Butalcamol, as well as a current-off trial in which lever pressing produced no current output.

Results showed that there was a significant reduction in self stimulation rates over time in the dopamine receptor blocker trials. No significant difference was found between the pimozide and Butaclamol conditions, however an increase in dose for either drugs produced a significant decrease in response rate. These data and findings suggest that the administration of a dopamine receptor blocker yield an overall decrease in self stimulation behaviour, almost mimicking a current decrease condition, in which the current received by the animal is decreased over time. Therefore, the effect of the dopamine receptor blocker is effective in mimicking a decrease in the reward value of self-stimulation. The behaviour of lever pressing proves to be a rewarding behaviour in that lever pressing causes a release of dopamine within the lateral hypothalamus, thus further supporting the rat to perform said behaviour, the introduction of antagonists which prevent the receptor binding of dopamine within the lateral hypothalamus remove the reward "currency" from lever pressing behaviour, thus reducing the frequency of the behaviour. One might conclude from this that an overall lack or decrease in dopamine concentration within the brain would mean that typically rewarding tasks would lose their "currency" and impact within the mesolimbic reward pathway, thus shifting the

behaviour of the depressed individual to not favour those tasks. Therefore, the focus of this study is the effect of dopamine within memory function and, specifically, how this relates to dopaminergic deficiency in the context of disease such as depression, although this relationship will not be directly examined in this study.

2.2 Entorhinal Cortex

2.2.1 Location

The entorhinal cortex (EC) is located in the temporal lobe and together with the perirhinal cortex (PRC), parahippocampal cortex (PHC) and hippocampal cortex (HC) the medial temporal lobe system is formed (Schultz et al., 2015). The entorhinal cortex's name is derived from the fact it is partially enclosed by the rhinal sulcus. Research interest within the EC (entorhinal cortex) arose within the early 20th century when Ramón Y Cajal discovered the regions connection to the hippocampus in 1902. The entorhinal cortex meets with the olfactory and amygdaloid cortices anteriorly, the piriform cortex laterally, and medially the periamygdaloid cortex and posterior cortical nucleus of the amygdala (Canto et al., 2008). On the ECs medial side are regions belonging to the hippocampal formation or parahippocampal region, for example the amygdalo-hippocampal transition and the parasubiculum. The two other constituents of the parahippocampal region being the perirhinal cortex and parahippocampal cortex border the EC on the lateral and posterior border.

2.2.2 Cellular Composition

The entorhinal cortex receives multimodal and unimodal inputs to the superficial layers, meaning neurons within the entorhinal cortex receive input from either multiple other neurons or one singular neuron, these inputs are then conveyed to the hippocampal formation via neurons in layers II and III of the entorhinal cortex. However, the connection between the EC and hippocampus is not unidirectional as hippocampal area CA1 and the

subiculum return projections to layer II, III, and V of the EC, with the projection to layer V being the densest, further supporting the role of the entorhinal cortex as an interface region between sensory cortical projections and the hippocampus. Layer V of the EC also serves as the origin of cortical and subcortical projections.

Witter et. Al (2017) provide an extensive review of the cellular composition of each layer of the LEC, they describe layer II of the LEC as containing four principal types of cells. Fan cells are the first of these four types, similar in morphology to stellate cells. Most fan cells are reelin positive however some are calbindin positive. Reelin is a glycoprotein responsible for modulation of neuronal migration, whereas calbindin regulates calcium absorption. Layer II also contains pyramidal cells and are largely calbindin positive. The final two cell types are oblique pyramidal cells and multipolar cells. Oblique pyramidal cells are similar in morphology to pyramidal cells and express calbindin, whereas multipolar cells are much more diverse in their morphology and express both calbindin and reelin.

With regard to interneurons, the largest constituent of this group is the parvalbumin (PV) positive interneurons. PV interneurons are GABAergic and contribute to approximately half of all interneurons in the EC, although they are expressed less in LEC layer II compared to MEC (Wouterlood et. Al, 1995). In both LEC and MEC, the distribution of PV-positive neurons forms a gradient, with a greater density closer to the rhinal fissure, decreasing in more ventral regions.

The cellular composition of layer III is relatively unknown compared to layer II. Layer III is largely composed of excitatory pyramidal neurons projecting to both hippocampal CA1 and subiculum. Non spiny pyramidal cells also form a large population within layer III of the EC,

sending axons to the perforant path, inputting to the hippocampus. It is layer II and III of the entorhinal cortex which form the main input to the hippocampus, and are therefore of particular interest in this thesis as it is these inputs which are to be modulated by dopamine and therefor influence memory.

Layer V of the EC is typically subdivided into layer Va and Vb. Layer Va contains large pyramidal neurons, whereas cells in layer Vb are smaller and more uniform in the size of their soma whilst also being more densely packed than cells in Va (Canto et. Al, 2011). Layer Vb of the LEC also contains multipolar neurons as well as a population of GABA-negative interneurons.

2.2.3 Entorhinal-hippocampal connection

It is the extrinsic connectivity of the entorhinal cortex which is of marked interest to researchers. The entorhinal cortex receives a large amount of cortical input, from not only the olfactory cortices but also the amygdaloid cortices. However, the key focus of research appears to fall on the entorhinal cortex connection to the hippocampus, and this focus on the interaction between entorhinal cortex and hippocampus is likely due to the importance of the hippocampus in memory formation and recall, meaning that such research has strong implications in the treatment of memory deficiency conditions such as Alzheimer's disease. Entorhinal fibers typically synapse onto principal neurons of the hippocampus, forming excitatory synapses, as well as forming synapses with inhibitory interneurons (Canto et al., 2008). This suggests that there is direct and abundant connectivity between the entorhinal cortex and hippocampus, but also that the forms of connectivity between the regions are many and varied.

A notable feature of the entorhinal-hippocampal connectivity is that of the topographic layout of entorhinal connections. The topographic mapping of the entorhinal cortex projections

along the long axis of the hippocampal projection contribute to the spatial mapping of the hippocampus. For example, neurons in the dorsal region of the hippocampus and, therefore, the dorsal region of the entorhinal cortex exhibit firing patterns and properties that represent small areas of the environment. Cells in ventral regions of the hippocampus and entorhinal cortex exhibit firing fields in relation to larger areas of the environment. This is notable due to the relationship between this topographical mapping and the behaviour exhibited in response to lesions (Steffenach et al., 2005), (Kjelstrup et al., 2002). Lesions of the dorsolateral band of the entorhinal cortex in rats lead to a total deficiency in spatial memory retention of a water maze task, not only this but lesions of the ventromedial entorhinal cortex band lead to a reduction in defensive behaviour in rats; the findings in Steffenach's 2005 paper reinforce the previous findings by Kjelstrup in 2002, in which they also found that ventral hippocampal lesions lead to a reduced fear expression during an elevated plus water maze task, as well as a decreased stress response when the rat was confined to a bright lit chamber. It is also expected, based on this research, Kjelstrup's research, that a lesion to the EC's input to the hippocampus would cause a total inability to store or recall information.

The importance of these findings is found in the strong implications which the papers provide with regards to the entorhinal-hippocampal connection. It is clear from these papers, which take different stances in terms of the location of the lesion (for example in Kjelstrup's paper the lesion was made in the hippocampus, whereas Steffenach created the lesion in the entorhinal cortex) that the connectivity between the entorhinal cortex and hippocampus is required for typical behaviour. This suggests that it is not the role of solely one of these regions to control behaviours such as spatial memory and fear, but rather the interface and innervation between the two regions with the EC serving as a relay between the sensory information innervated from the cortex and the storage of episodic and contextual memory

within the hippocampus. A Further point to be made is that the entorhinal cortex, as suggested by the papers mentioned previously, is not simply a region concerned with spatial memory formation and retention, but also other behaviours such as fear and stress responses. It is vital to consider this when conducting any form of research into the region, as any treatments which may come from research must consider the vast spectrum of behaviour which might be affected.

2.2.3 Basic Function of the Entorhinal Cortex

The entorhinal cortex remains as one of the most poorly understood areas of the brain, although it is largely accepted that the entorhinal cortex is pivotal in memory formation, very little is truly known as to how the area contributes to memory. The entorhinal cortex serves as a relay within the brain between the cortex and hippocampus, necessary for the formation of long-term memory and the declaration of those memories. Canto et al. (2008) provide a deeper understanding of the structure of the entorhinal cortex both intrinsically and in terms of projections to and from the EC. Canto analogises the entorhinal cortex as a station within a large city, not only allowing for people to leave or enter the city, but also for incoming and outgoing people to interact. Applying this to the role of the entorhinal cortex in memory specifically, sensory inputs from a vast array of locations (visual, olfactory etc.) are contextualised within the entorhinal cortex into a “story” which can then be outputted to the hippocampus for memory storage. Such a region within the brain is vital in memory formation and recall, as animals we rely on our senses to understand our environment, and the formation of contextualised memories is vital in retaining and recalling important memories. Applying this to the role of dopamine within the brain, merging the function of the entorhinal cortex to its large dopaminergic input, we can understand that dopamine may affect the contextualisation of memories within the EC, perhaps the dopamine “reward currency” applies a hierarchy to memory, with behavioural actions which release a greater amount of

dopamine (e.g. eating, mating etc) being more favourable and memorable within the entorhinal cortex, making such memories easier to recall. This appears to be supported from an evolutionary perspective of our brain development, as actions which ensure survival and continuation of genetic information would be more important to remember and recall. It is the function of the entorhinal cortex and its known dopaminergic input which will serve as the focus of research, aiming to analyse the effect that dopamine may have on the relay properties of the region.

2.3 Plasticity

2.3.1 Mechanisms underlying Plasticity

As mentioned previously, the two most common forms of plasticity are long-term potentiation and long-term depression, LTP and LTD respectively, these forms are replicated within research as they are believed to underlie memory encoding. This introductory section will provide some insight into the synaptic mechanisms underlying these forms of plasticity and provide research which aims to modify these mechanisms.

2.3.1.1 Long Term Potentiation

In development of the hippocampus LTP takes many forms, in early development LTP is characterized by an increase in the release of glutamate from the presynaptic bouton, this early form of plasticity appears to be initiated by an activity-dependent loss of G protein-dependent Kainate receptor (KAR) function postsynaptically, ultimately resulting in the maturation of excitatory transmission (Lauri et al., 2006). As the hippocampus continues to develop the activity of the synapse increases, however the replacement of high conductance AMPA receptors with low conductance AMPA receptors at this point ultimately results in a decrease in synaptic transmission. In this instance it is morphology and affinity of the AMPA

receptor which mediates the plastic effects, rather than an overall reduction or increase in the number of receptors.

By 14 days of age in rats, the majority of LTP is mediated by a replacement of low conductance AMPA receptors for high conductance receptors, this either occurs by modifying existing low conductance receptors (Benke et al., 1998) or by swapping low conductance receptors for high conductance receptors (Terashima et al 2004, Plant et al 2006). The other form of synaptic plasticity is caused by an increase in the number of AMPA receptors, as opposed to any change in their affinity or conductance.

LTP events in hippocampal CA1, notably not entorhinal cortex, are dependent on N-methyl-D-aspartate receptors (NMDARs) within young animals, acting in a signal cascade resulting in the phosphorylation of AMPA receptors (Citri and Malenka, 2007). This occurs due to periods of high frequency trains of stimulation, known as tetanisation, or paired-pulse stimulation (PP). Following these physiological stimulation periods, the resultant depolarisation relives a voltage-dependent Mg^{2+} block of the NMDA receptor. Calcium influx through the membrane is now possible due to the removal of the block, it is this calcium influx which initiates the signalling cascade causing phosphorylation of AMPA receptors by calcium/calmodulin dependent protein kinase II (CamKII), either increasing their conductance or exchanging and inserting high conductance receptors. It is vital to note that although LTP is strictly caused by an increase in conductance of the AMPA receptor, the series of biochemical events which cause an increase in conductance are NMDA receptor mediated (Citri and Malenka, 2007). Therefore, when considering LTP from a pharmacological standpoint, a block of either the AMPA or NMDA receptor would prevent LTP occurring due to the block of activity overall, also any drug which activates the NMDA receptor is likely to be more viable physiologically as it

would more accurately imitate the mechanism by which LTP is induced, rather than artificially increasing AMPA receptor conductance by the use of drugs.

Another consideration to be made is that the form of plasticity induced, either LTD or LTP, is likely to be NMDA receptor subunit dependent. Bartlett et al (2007) conducted research into the blockade of NMDA receptors in hippocampal region CA1, using NVP (highly selective for the GluN2A subunit of NMDA receptors) and Ro 25-6981 (GluN2B selective). The IC_{50} value for Ro suggests that it is effective in inhibiting the GluN2B subunit by 50% at 9nM whereas its effective concentration for a 50% inhibition of the GluN2A subunit is 52μM. With regard to the IC_{50} value of NVP, it is 50% effective at 0.27μM for the GluN2A subunit and 29.6μM for the GluN2B subunit. Bartlett found that NVP was able to successfully block LTP and LTD whereas Ro 25-6981 only managed to block LTP. NVP was able to reduce the effect of LTP by 63% at 0.1μM whereas Ro was only able to reduce LTP by 45% at 5μM. With regards to LTD, at 5μM Ro had no significant effect on the long-term depression of the response, however at 0.2-0.4μM NVP was able to blockade LTD on a concentration-dependent basis. This research evidences the theory that LTP and LTD induction may be subunit dependent. The results collected by Bartlett et. Al (2007) are comparable to the IC_{50} values of the respective NMDA receptor antagonists, the 5μM concentration of Ro far exceeds the IC_{50} value detailed above and should elicit a 50% inhibition of the GluN2B subunit. As there was no significant effect of Ro on LTD one can infer that the mechanism underlying LTD induction and maintenance is not dependent on the GluN2B subunit of NMDA receptors and is instead dependent on the GluN2A subunit, as evidenced by the effective block of LTD by NVP at between 0.2μM and 0.4μM. As for LTP induction, Ro was effective in blocking LTP by 45% at 5μM and NVP was effective in causing a 63% block at 0.1μM. Comparing again to the IC_{50} values, it is apparent that LTP is dependent on both GluN2A and GluN2A subunits, evidenced by Bartlett et. Al's

(2007) findings as NVP is effective in blocking both LTD and LTP at low concentration, whereas Ro is only effective in blocking LTP at comparability high concentration.

In order to induce long term potentiation experimentally in Entorhinal Cortex slices, various stimulation methods may be used in field recordings to elicit a long-term potentiation response. One such method is theta burst stimulation, low currents are applied to the slice via a bipolar electrode in a burst pattern, often repeated numerous times in the form of “trains”. The number of trains required can be dependent on the brain region being electrophysiologically recorded, and the particular slice being used (for example, the age of the animal from which the slice was produced). One paper outlining the method states that each burst of the theta burst protocol should be either 4 or 5 pulses, with the number of trains being between 10 and 75 (Abrahamsson et al., 2016).

The reason for the efficacy of theta burst stimulation (TBS) in inducing LTP is due to the ability of the protocol to overcome the feedback inhibition which is typically induced by a singular stimulation. Larson and Munkácsy (2015) show that repeated bursts of stimulations, repeated at a frequency of around 5Hz results in the LTP response due to maximal activation of the NMDA receptor which, as mentioned above, underlies the potentiation mechanism. The common choice to repeat bursts at 5Hz frequency when stimulating is to align with the endogenous theta wave rhythm which is present throughout the hippocampus and the rest of the brain, and therefore represents physiological firing patterns.

Linking back to the entorhinal cortex, and the reasoning for inducing LTP in this region, research has found that theta burst stimulation within the entorhinal brain area can improve performance in memory and recognition tasks (Titiz et al., 2017). In human participants,

entorhinal stimulation via microelectrode and thus the induction of LTP lead to an improved ability by the patient in recognising previously viewed photographs of portraits, as well as being able to more easily distinguish and reject similar lures. This *in vivo* data along with data mentioned above in rodents shows that long term potentiation is clearly vital in memory formation, not only during brain development but also later in life upon the introduction of novel stimuli and new learning scenarios. It is therefore vital that we, as researchers, understand how dopamine (the neurotransmitter heavily implicated in the reward pathway) may affect long term potentiation, as will be studied here. It is also important to consider the effects which pharmaceutical and recreational drugs may have on the induction of LTP, and whether a drug's ability to hijack the reward pathway is carried through to the ability of said drug to either inhibit or enhance memory formation and recall.

Another method used to induce LTP is high frequency stimulation, consisting of a consistent tetanisation train of pulses (often around 100) applied at a frequency of 100Hz (Grover et. al, 2009). As both of these methods are widely used it is important to consider both as viable options to induce LTP, however many papers have shown that theta burst stimulation produces the optimal magnitude of potentiation in rodent hippocampus (Grover et al., 2009). Interestingly, Grover's findings also suggest that LTP induction may be dependent on the postsynaptic channels and receptors as it was found that theta burst LTP was NMDAr dependent whereas high frequency LTP was more dependent on L-type calcium channels.

LTP is not a novel concept, Bliss and Lømo discovered the method for inducing LTP in 1973. Their method involved the dentate area of the hippocampus in rabbits, stimulating a frequency of 15hz for 15-20 seconds, this method was known as tetanus and on 26% of occasions lead to an overall potentiation in responses that would last from 1 hour to 3 days.

The potentiation of the response was characterised by 3 factors; increase in the synaptic amplitude and population spike amplitude, latency reduction of the population spike, and reduction in the variability of the population spike (Bliss and Lømo, 1973). In the event that the potentiation observed was long lasting then there would also be an increase in the excitability of cells postsynaptically, as well as an increase in the extracellular current flow produced by the synapse. Bliss and Gardner Medwin's research provided an early understanding of LTP within the brain and the role that it may play within memory, an increase in the sensitivity of a synapse would also lead to an increase in the ability of that neuron to contribute to the formation of new memories and possibly the recall of old memories.

When performing cursory research into LTP within the entorhinal cortex, it was evident that very few were carrying out LTP assays in entorhinal cortex directly, often the method for inducing LTP in entorhinal cortex involved stimulation of axons within the hippocampus in order to induce LTP, not the entorhinal cortex (Leutgeb, Frey and Behnisch, 2003). Other studies instead record the presence of LTP within areas such as the hippocampus or amygdala after stimulation of the entorhinal cortex, rather than recording the presence of LTP in the EC itself (Yaniv et al., 2003).

2.3.1.2 Long Term Depression

The inverse of LTP, LTD, is categorised by a long-term decrease in synaptic response amplitude (Luscher and Malenka, 2012). LTD is typically induced by prolonged low frequency stimulation of 1Hz, often an LTD protocol will consist of 900 pulses, lasting 15 minutes (Cheong et al., 2002). This is in clear contrast to the protocols used to induce LTP, which are often only lasting not much more than a second, with high frequency bursts of stimulation. From the protocol which are used to induce the two forms of plasticity, we can infer that LTD requires a more long-lasting stimulation physiologically and is likely a process with a slower onset. LTP on the

other hand requires rapid stimulation and most likely occurs with fast onset, such a fast onset is likely to be required in memory formation, as research shows LTP is involved in. There are currently two possible forms of LTD induction, these being NMDAR-dependent LTD and mGluR LTD.

The molecular mechanism underlying NMDAR dependent LTD is still at the centre of some intrigue and question, a long-standing theory suggests that LTD is dependent on calcium ions within the synapse, and more specifically the neuronal calcium sensor (NCS) protein hippocalcin (Palmer et al., 2005). The influx of calcium ions into the synapse results in the activation of hippocalcin, forming a complex with AP-2 (adaptor protein 2) and eventually displacing the NSF (N-Ethylmaleimide-Sensitive Fusion protein) protein which typically stabilises the GluA2 subunit of the AMPA receptor. It is this displacement which ultimately leads to the recruitment of clathrin, responsible for the formation of cellular vesicles (Royle, 2006). The internalisation of AMPA receptors results in the desensitization of the post-synapse in response to low frequency stimulation, meaning that the synapse yields a reduced response to stimulation. Huber et. Al (2000) explain the PP-LFS paradigm and its ability to induce mGluR dependent LTD within the hippocampus. They found that Paired pulse low frequency stimulation at a frequency of 1Hz with a 50ms inter-pulse interval for a duration of 15 minutes effectively produced mGluR dependent LTD. The mechanism for mGluR mediated LTD relies on an increase in intracellular $Ca^{2+,}$ leading to an activation of postsynaptic $mGlu_{1\ and}$ $mGlu_5$ receptors. The activation of these group I mGluRs activates the phospholipase C (PLC) pathway, releasing intracellular Ca^{2+} from stores, activating protein kinase C (PKC). PICK1 (protein interacting with C kinase 1) forms a complex with NCS-1, a Ca^{2+} sensor for LTD. PICK1 also binds to PKC-α, phosphorylating the GluA2 subunit of AMPARs, dissociating ABP-GRIP, eventually causing the removal of AMPARs from the synapse, causing LTD. The calcium influx

caused by PP-LFS activates the mGluR-dependent LTD signalling cascade by increasing intracellular calcium.

The information above is taken from research within the hippocampus, however research since has suggested that LTD induction within the entorhinal cortex, as is the focus of this study, is also NMDA receptor mediated. Research by Kourrich and Chapman (2003) used field recording within the superficial layers of the entorhinal cortex, as well as a paired-pulse low frequency stimulation protocol (as mentioned previously) to induce LTD. Kourrich and Chapman (2003) found that bath application of the NMDAR inhibitor d-2-amino-5-phosphonovalerate (APV) resulted in a blockade of long-term depression. Recordings in the absence of APV when administered with paired pulse low frequency stimulation (PP-LFS) elicited a 77% ± 3.0% reduction in response amplitude 5 minutes after the protocol was applied, this response depression remained at 90.1 ± 2.7% after 60 minutes of follow up. In the presence of APV the level of depression was not significant from baseline levels 30 minutes after application, resulting in an overall reduction in response amplitude of only 99.3 ± 5.4%. Therefore, application of APV resulted in a significant block of the long-term depressive effect of PP-LFS.

Kourrich et al. (2008) outlined further experiments which aimed to understand the postsynaptic processes underlying LTD in the entorhinal cortex, instead of using field recording protocols, Kourrcih utilised whole cell recording in order to individually record from cells which had been filled with various pharmacological solutions via the recording electrode. First Kourrich aimed to verify the validity of the NMDA receptor dependency theory which is common in discussion the postsynaptic LTD mechanism, presence of a calcium chelator BAPTA within the recording electrode, and filling layer II entorhinal cortex cells with BAPTA resulted in a successful block of LTD induction by PP-LFS. The presence of BAPTA prevents the increase

of calcium postsynaptically. The results of BAPTA application were consistent with a block of LTD by the NMDA receptor antagonist APV, therefore suggesting that LTD appears to be NMDA receptor mediated, causing an increase in postsynaptic calcium. Kourrich also conducted experiments to test the role of protein phosphatases in LTD induction, more specifically the calmodulin-dependent protein phosphatase calcineurin. This was tested by the presence of cyclosporin A or FK506 in the recording electrode. After administration of a PP-LFS protocol, cyclosporin appeared to display a partial block of LTD, with response amplitudes decreasing to 82.4% compared to 58.6% in control conditions, however no significant difference was found between these two conditions. FK505 however, resulted in a complete block of LTD, the efficacy of this block was verified by the fact that the solution was not bath applied, and was instead administered via the recording electrode, meaning the drug could only act postsynaptically, specifically on calcineurin.

2.3.2 Plasticity and memory

Plasticity is often separated into two distinct types, Long term depression (LTD) and long-term potentiation (LTP) (Citri and Malenka, 2007). Long term depression refers to a long-lasting decrease in synaptic response, LTP is the inverse of this, showing a long-lasting increase in synaptic response. The extent of LTD or LTP can be recorded in electrophysiology as a decrease, or increase respectively, in the response amplitude.

Chapman's 2008 review paper underlines some of the key characteristics of plasticity in the entorhinal cortex, most notably the hypothesised role of plasticity in memory processing. Researchers have shown that the presence of prenatal malnutrition impedes the ability of the EC to undergo plastic changes, and this has been linked to an adult memory deficit (Hernández et al., 2008). Regarding memory processing, Lipton and Eichenbaum (2008) aimed to explain the role of the EC in memory, specifically spatial memory. Their findings show that the

mechanism of the medial entorhinal cortex in forming episodic memories, meaning the amalgamation of sensory information to form a distinct memory "episode", is converse to that of the mechanism in which the hippocampus encodes spatial information; the hippocampus encodes events representative of the location in which they occurred whereas the entorhinal cortex segregates the sequential information into episodic memory. It is concluded that the combination and relay between the two regions combines both forms of memory processing, providing a holistic memory episode. It has also been postulated that the degeneration of the EC in ageing, and subsequently the reduced ability of the EC to undergo plasticity, results in a reduced learning ability as we age (Bevilaqua et al., 2008). The research presented within this review supports the aim and rationale for this study, in which experiments conducted in rodent lateral entorhinal cortex will aim to analyse the effect of drugs on the ability of the region to undergo synaptic plasticity, and thus postulate the effect that these drugs, such as dopamine, may have on the encoding of episodic memory.

Episodic memory, as mentioned previously in reference to Lipton and Eichenbaum's research (2008), refers to the storage of memory with regard to the context in which it was first experienced. It is a phrase commonly referred to when discussing the role of plasticity in the entorhinal cortex and hippocampal formation. Lipton and Eichenbaum's paper devises a possible explanation, drawing on anatomical cellular imaging evidence for the mechanism underlying the formation of contextual episodes in the entorhinal cortex and hippocampus. Two streams of neocortical inputs are involved in the formation of such memories; "what" information originating from neocortical input to perirhinal cortex, and "where" information comprised of neocortical input to the parahippocampal cortex (in rodents: postrhinal cortex). Lipton goes on to explain that the two streams of "what" and "where" information remain segregated and are only combined and encoded together within the hippocampus. Efferents

from the hippocampus then return to the cortical area from which the information originated, therefore, upon presentation of an object as a memory cue a circuit is completed in which the hippocampus recovers the contextual information from either the parahippocampal or medial entorhinal area. It is because of this circuit between the hippocampus and entorhinal cortex that context is provided to memory. The anatomical explanation provided here leads to some explanation with regard to the role of the entorhinal cortex in episodic memory processing, and how the rich innervation of the region contributes to its role in not only the formation of memory but also recall. It is likely that the dopaminergic input to the entorhinal cortex (Caruana and Chapman, 2008) may have some involvement in memory processing carried out by the relay between the EC and hippocampus. Experiments will aim to explore this interaction and provide insight into the biochemical factors which affect memory encoding.

2.3.3 Neuromodulation of synaptic plasticity

A novel area of plasticy research concerns the interactions between plastic effects within the brain, whether this be the interaction between LTD and LTP, or the effects of repeated LTD or LTP protocols during experiments. A key focus of this research is the effect of physiological neurotransmitters, such as dopamine, on plasticity. A 2008 paper by Caruana and Chapman outlined the effects of dopamine on postsynaptic potentials in the lateral entorhinal cortex. They found that low concentrations of dopamine (1µM) lead to a facilitation of field excitatory postsynaptic potentials (fEPSPs) to 132.7 **±** 4.4% of baseline levels, this effect was able to be reversed after 15 minutes of washout in ACSF, however this experiment alone clearly shows that dopamine is capable of causing short term effects in lateral entorhinal cortex. Although these effects may not be long term, it is highly likely that these neurotransmitter effects may interfere with activity-dependent plasticity such as LTD and LTP. Caruana and Chapman (2008) also found that high concentrations of dopamine (such as 50µM) lead to a depression of synaptic responses to 38.5 **±** 5.8% of baseline. This shows that dopamine is capable of having

either facilitative or depressive effects on a concentration-dependent basis, and that concentration dependence may point to some form of neurotransmitter coding mechanism in entorhinal cortex neuronal excitability.

Their research also aimed to provide some evidence as to the mechanism underlying dopamine's effects on lateral entorhinal cortex synaptic potentials. Further experiments showed that application of the D2 receptor antagonist Sulpiride lead to a non-significant depression of postsynaptic responses, suggesting that the depressive effect of dopamine is D2 receptor mediated. However, in the presence of Sulpiride the decrease in response was found to be close to statistical significance following data analysis, therefore another mechanism, not D2 receptor mediated, appears to contribute to suppression of responses.

Another paper by Caruana et. al (2007) shows how dopamine appears to modulate the ability of the entorhinal cortex to undergo plastic effects, such as LTD and LTP. Pre-treatment with the dopamine reuptake inhibitor GBR12909 lead to a block of LTD and LTP induction. The researchers hypothesise that the role of dopamine within the entorhinal cortex may be to prevent activity-dependent plasticity during reward-based learning or when processing novel stimuli. This links to the neuromodulation of activity dependent synaptic plasticity, as neurotransmitters within the entorhinal cortex, such as dopamine, are able to control the ability of the region to undergo plastic changes. This would make sense behaviourally as, during stimulation of the reward pathways via dopamine, any long-term depression or potentiation of synaptic responses is prevented in order to provide a greater "reward currency" tag to the stimuli. From the standpoint of evolutionary psychology, this would support the idea that stimuli which invoke the release of dopamine within the reward pathway, such as finding food or a mate, would require higher salience within our memory.

3. Introduction

The role of this research in the field of lateral entorhinal cortex research is to assess the ability of dopamine to modulate the induction and maintenance of activity dependent synaptic plasticity as well as test the efficacy of various stimulation protocols in inducing activity dependent plasticity. Previous research in conjunction with my own provided results to suggest that repeated dopamine applications cause a reduction in the suppression of fEPSP caused by dopamine in subsequent applications, this thesis will aim to expand on this finding and assess the interaction between LTD induction and this effect. The results from these experiments support the results from Caruana et. Al in previous papers, showing that dopamine is capable of exhibiting a block, or at least a reduction, of LTD. The role of LTD in the metaplastic mediation of dopaminergic suppression will also be assessed here, previous research documents that within learning and memory, dopamine at low concentration enhances the ability of the striatum to undergo LTP rather than LTD (Giordano et al., 2017), and this provides evidence to suggest that activity dependent plasticity and dopaminergic input are metaplastic in nature. Further aims include quantifying the conditions and protocols necessary to induce LTP within the lateral entorhinal cortex of rats, varying stimulation protocols as well as application of the GABA antagonist Picrotoxin and increasing extracellular calcium.

4. Methods

4.1 Preparation of Physiological Solutions

A 10X stock solution of ACSF was prepared from (in mM) 2.50 KCl, 17.0 D-glucose, 124.0 NaCl, 1.43 KH2PO4, 26.0 $NaHCO_3$. This stock solution was then refrigerated before dilution for use in electrophysiological recordings and slice preparation. Upon dilution of the ACSF stock in deionized water, 2mM Calcium Chloride and 2mM Magnesium Chloride solution was also added into the diluted solution and the solution was then oxygenated with 95% O_2 5% CO_2. The use of ACSF is electrophysiological recording is vital to ensure the survival of neurons throughout recording, maintaining osmolarity of the solution as well as buffering pH, by improving the survival of neurones throughout recording this improves recording stability and allows for much longer experiments to be performed. Although not accurate to physiological conditions, ACSF allows for stable recording *in vitro.*

In a similar process, a stock of cutting solution was prepared containing (in mM); 234.0 Sucrose, 26.0 $NaHCO_3$, 10.0 D-Glucose, 2. KCl, 1.43 KH2PO4, as well as 3mM MgCl2, and 1mM CaCl2. The stock solution was then bubbled using 95% O_2 5% CO_2 for 10 minutes prior to storage in a refrigerator as well as during use in the dissection process. The purpose of cutting solution is to ensure the integrity of rat brain slices during the dissection process, as well as serving as a high osmolarity solution which prevents cytolysis of dopaminergic cells (Ting et al., 2018).

4.2 Dissection and Entorhinal Cortex Slicing

Sprague Dawley rats aged between 14 and 21 days (P14-P21) were used in experimentation, all rats used during experiments and the method described below were handled in accordance with ASPeL regulations. Throughout all experiments, rats used were aged been p14 and p21

due to the greater ability of younger rat brains to undergo LTD or LTP compared to that of older rats. For the purpose of all results detailed in this thesis, N refers to the number of rats used in each experimental group, although multiple slices and thus multiple experiments were performed from each rat brain, multiple experiments within each experimental group were not performed on the same rat.

The rat was first sacrificed via schedule 1 protocol of cervical dislocation. The head was then removed, and an incision was made in the skin surrounding the top of the skull to remove it, this allowed for clear access to the skull. A pair of fine scissors were then used to make two incisions on either side of the spinal cord. Following this, the skull was cut open dorsally using scissors, and the two halves of the skull were separated from the brain using tweezers. At this point the brain was removed from the skull using a spatula and placed into a beaker of oxygenated cutting solution at a temperature of 4°C. The oxygenation of the solution allows for a constant supply of oxygen to the slice, preventing the death of cells which are to be recorded from, similarly the low temperature of the solution slows metabolism within the slice, ensuring the health of cells within the slice and, therefore, the stability of recording.

The entorhinal cortex was isolated by “blocking” of the brain with a straight razor blade. First, filter paper was doused in cutting solution and the brain was placed on top of the filter paper. Taking a straight razor, the cerebellum was first removed from the brain, followed by the frontal cortex. The brain was then flipped forwards to remove the top portion of the brain, then being flipped forward again to split the brain into two hemispheres.

The vibratome was prepared prior to the dissection process, with ice placed around the slicing chamber and a piece of sylgard super-glued to the slicing plate in order to ensure that the movement of the vibratome blade did not move or misalign the brain during slicing. Once the brain had been blocked, the two hemispheres were super glued to the slicing plate with the brain oriented horizontally, the entorhinal cortex of each hemisphere facing inwards. The slicing plate could then be secured into the slicing chamber, with cutting solution being added and oxygenated. The blade was also secured to the vibratome and the start and end points for slicing were found. The start point just before the blade reaches the brain, and the end point just as the blade reaches the sylgard. The Vibratome was set to acquire slices of 340µM in thickness, and slices were taken from the most ventral section of the entorhinal cortex to the most dorsal. The 340µM thickness of slices is chosen due to the requirement for a large number of healthy, connected neurons within the slice that are able to be stimulated for recording. Slices were collected from the vibratome by first using a scalpel to gently cut them free of the blade, and then picked up using a glass transfer pipet, before being transferred to a glass chamber containing oxygenated ACSF atop a hotplate which maintained the solution temperature at 31°C. The slices were placed atop a wire mesh which ensured flow of ACSF over the entire slice. Slices were then allowed to rest for 60 minutes before use in recording, this time allows the slice to gradually reach physiological temperature prior to recording.

4.3 Recording of Entorhinal Cortex Synaptic Potentials

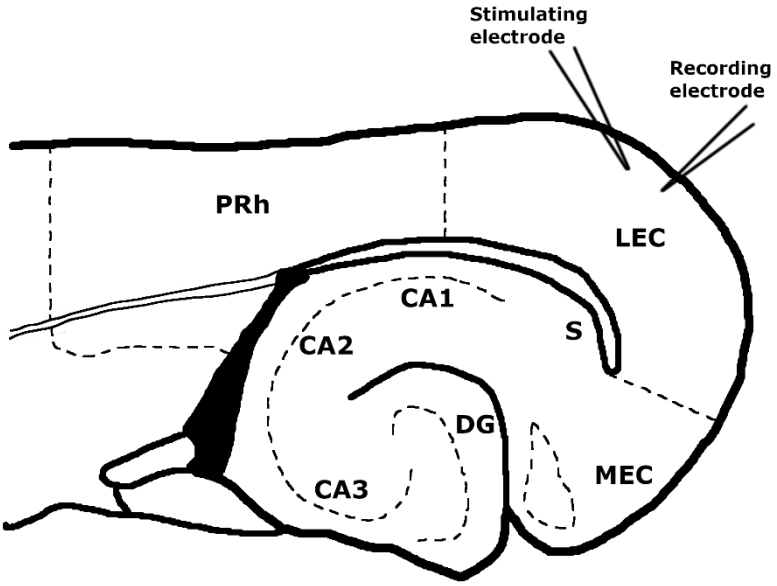

Figure 1. Location of stimulating and recording electrodes within the lateral entorhinal cortex of rat brain slice. The correct location of the LEC was consistently verified through use of a stereotaxic atlas. The stimulating electrode was placed within the peripheral layers across the layer I-II border, with the recording electrode placed within layer II, placed approximately 1.0 to 2.0mm rostral of the stimulating electrode. Abbreviations; (LEC) Lateral entorhinal cortex, (MEC) Medial entorhinal cortex, (PRh) Perirhinal cortex, (DG) Dentate gyrus. (S) Subiculum.

Once slices had been rested in ACSF for 1 hour they were transferred to a recording chamber containing a nylon mesh, which allowed the constant perfusion of oxygenated ACSF over the entire slice. A tungsten bipolar stimulating electrode was then lowered into the slice with the position of the electrode verified visually using a dissection microscope. The correct recording location was verified by reference to a stereotaxic brain atlas. The stimulating electrode was placed spanning the layer I-III border. Recording electrodes consisted of a borosilicate glass capillary, filled with ACSF and mounted into a recording head stage. Contact between the head stage chloride-coated silver wire and the ACSF within the capillary was ensured by filling the electrodes completely. Synaptic recordings were taken relative to a silver chloride reference pellet which was placed within the recording bath, a short distance from the slice.

When finding responses, the recording electrode was positioned within the superficial layer II of the entorhinal cortex and the stimulating electrode within the layer I/II border, with the correct spacing of the two electrodes within the slice maintained in order to ensure that the fibre volley of the response was not larger than the response itself. The positioning of the electrodes in this manner was to record the change in fEPSP amplitude specifically within layer II of the lateral entorhinal cortex, this is the area of interest for this study due to the layer II neurons projecting to the hippocampus and are therefore most likely the neurons which contribute to the formation of episodic memory. It is these neurons which are the target for analysis in relation to synaptic plasticity.

A stimulation of a set amplitude was administered every 20 seconds indefinitely, using a DS3 isolated constant current stimulation (Digitimer, UK), with the amplification current set between 20-200μA, with the evoked synaptic response digitised and recorded. Temperature of the recording bath was maintained using a water bath heated to approximately 40°C with a thermostatically controlled, in-line heating element maintain the temperature of perfused ACSF, and thus the recording bath temperature, to between 31°C and 32°C, a temperature sensor within the recording bath allowed for verification of this temperature throughout recording. Tis temperature was used to slow the metabolic demand of neurons, thus allowing for the survival of cells and greater stability of recording particularly during longer experiments. Perfusion of ACSF and drugs e.g. dopamine was carried out using a peristaltic pump, with the flow rate set to 1.6ml/minute, with all external solutions (including ACSF, Dopamine, and AP5) bubbled with 95% O_2 5% CO_2 prior to and during use in experimentation.

4.3 Induction of activity dependent synaptic plasticity

4.3.1 Long Term Potentiation (LTP):

In this project we aimed to assess both the activity-dependent and dopaminergic regulation of plasticity, for the former of these aims we utilised stimulation protocols which were programmed into the WinLTP software in order to be automatically administered when desired. Long term potential ion (LTP) is one form of activity-dependent plasticity which we aimed to assess in this study. We used two methods to induce long term potentiation within the entorhinal cortex. The first of which being Theta-burst stimulation (TBS) involving 10 trains of 4 pulses, each pulse having a 10ms inter pulse interval. The second protocol tested involved high Frequency stimulation (HFS) in which 100 pulses were delivered at a frequency of 100Hz, with a protocol length of 1 second. All protocols used were performed following a stable 30-minute baseline period, preventing any false negative or positive results in terms of increase or decrease in fEPSP amplitude which may have been caused by a run-up in baseline recording.

4.3.2 Long Term Depression (LTD):

The protocols used in order to induce LTD of entorhinal cortex synaptic responses involved stimulation with 900 pairs of pulses at a frequency of 1Hz with 30 milliseconds inter pulse interval (IPI) making the total protocol length 15 minutes. This method of inducing LTD has been previously verified by Bouras and Chapman (2003), Caruana et. Al (2007) and the frequency of pulses, as well as the inter pulse interval, and protocol duration has been found to be effective in inducing LTD. This protocol would typically be induced following a 30-minute baseline and the response was allowed a 60-minute follow-up period to evaluate whether LTD had been successfully induced.

4.4 Pharmacological modulation of entorhinal cortex synaptic responses

A variety of solutions and drugs were used to modulate synaptic responses recorded in the entorhinal cortex. These solutions were applied via bath application, being pumped over the slice as per the ACSF application. During experiments the tubing leading to the ACSF used could be changed do a different solution. A reservoir of around 1ml was maintained prior to the recording bath in order to ensure than the swapping of tubing did not cause any air bubbles to enter the bath and cause the electrodes or slice to move, improving the stability of recording, particularly during long experiments.

Dopamine solution was prepared using a 10X stock of Dopamine prepared in ACSF which could then be diluted further in ACSF as per requirement for each experiment. The stock Dopamine solution was kept refrigerated at around 4°C, with stock solutions only being retained for 7 days before being disposed of and a new solution made. The diluted dopamine solution also contained sodium metabisulfite, an antioxidant which prevented oxidation of the dopamine during application. Due to the photosensitivity of dopamine, solutions were prepared in the dark, with a cover placed over the recording equipment and the solution itself during the application process in order to minimise any exposure to light. Solutions were placed in a water bath heated to 44°C and the water bath was then covered when using dopamine solutions, bath temperature was set to 44°C to allow for loss of heat throughout the tubing leading to the recording bath. All solutions were also oxygenated whilst in the thermostatically controlled water bath with 95% O_2 5% CO_2. The water bath coupled with a thermostatically controlled in-line heater ensured the temperature of the solution within the recording bath remained at around 31°C throughout recording. Similarly, AP5 solution was prepared from a 10X stock solution, from which a 50μM solution was prepared for use during experimentation in ACSF.

4.5 Data Handling and Analysis

All data was collected using WinLTP software package version 2.30, in which the peak amplitude of each recorded trace was recorded every 20 seconds. The fEPSP data was then transferred to GraphPad prism data analysis software in which each 20 second peak amplitude recording was averaged over 1-minute periods in order to be plotted and presented. The averaged peak amplitude data were then normalised to the 30-minute baseline period of recording, with the resulting data being in the form of a percentage of the baseline data. The amplitude of fEPSPs was analysed due to both LTD and LTP producing either a decrease or increase in fEPSP amplitude, respectively. Statistical analysis of the collected data could then be performed via either T-test, or repeated measures ANOVA in the case of LTP data, comparing the fEPSP amplitude (%) between conditions. Sample traces of fEPSP responses were signal averaged using WinLTP reanalysis software (Version 2.30), as well as the average of every 3 sweeps produced. Traces were then converted to an exportable ABF (Axon binary file) type and viewed in ClampFit (Version 10.7), sweeps for selected time points were then exported into Adobe Illustrator (2019) for final presentation.

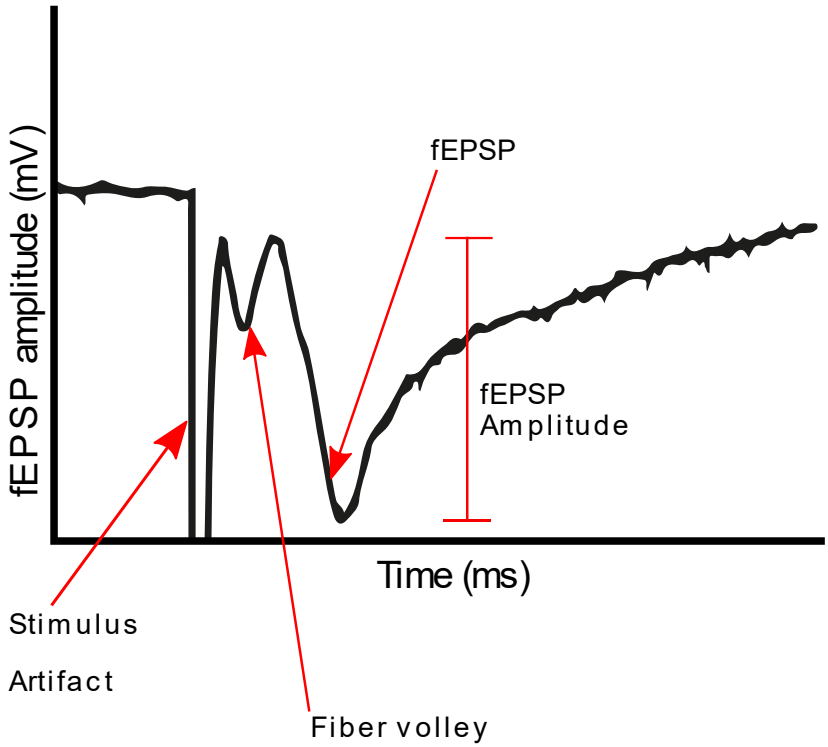

Figure 2. Visual representation of fEPSPs collected throughout all experiments. The fEPSP amplitude either reduces or increase in the event of LTD or LTP respectively, and it is the fEPSP amplitude which as consistently analysed through experiments. The fEPSP amplitude was typically analysed as a percentage of the baseline recording period of 30 minutes, and thus any change in fEPSP amplitude was provided in the form of a percentage change. The stimulus artifact represents the action potential produced in initial response to stimulation and is not representative of the response potential, therefore it is not considered in analysis. The fiber volley is caused by the compounded action potentials of axons in the stimulated region, and its amplitude typically increases with stimulation intensity. The final slope is the fEPSP (field excitatory postsynaptic potential), representing the change in membrane potential postsynaptically.

5. Results

5.1 Control Data

Control data indicates that materials used produce stable recordings. Vehicle control results indicate that sodium metabisulfite has no effect on fEPSP amplitude.

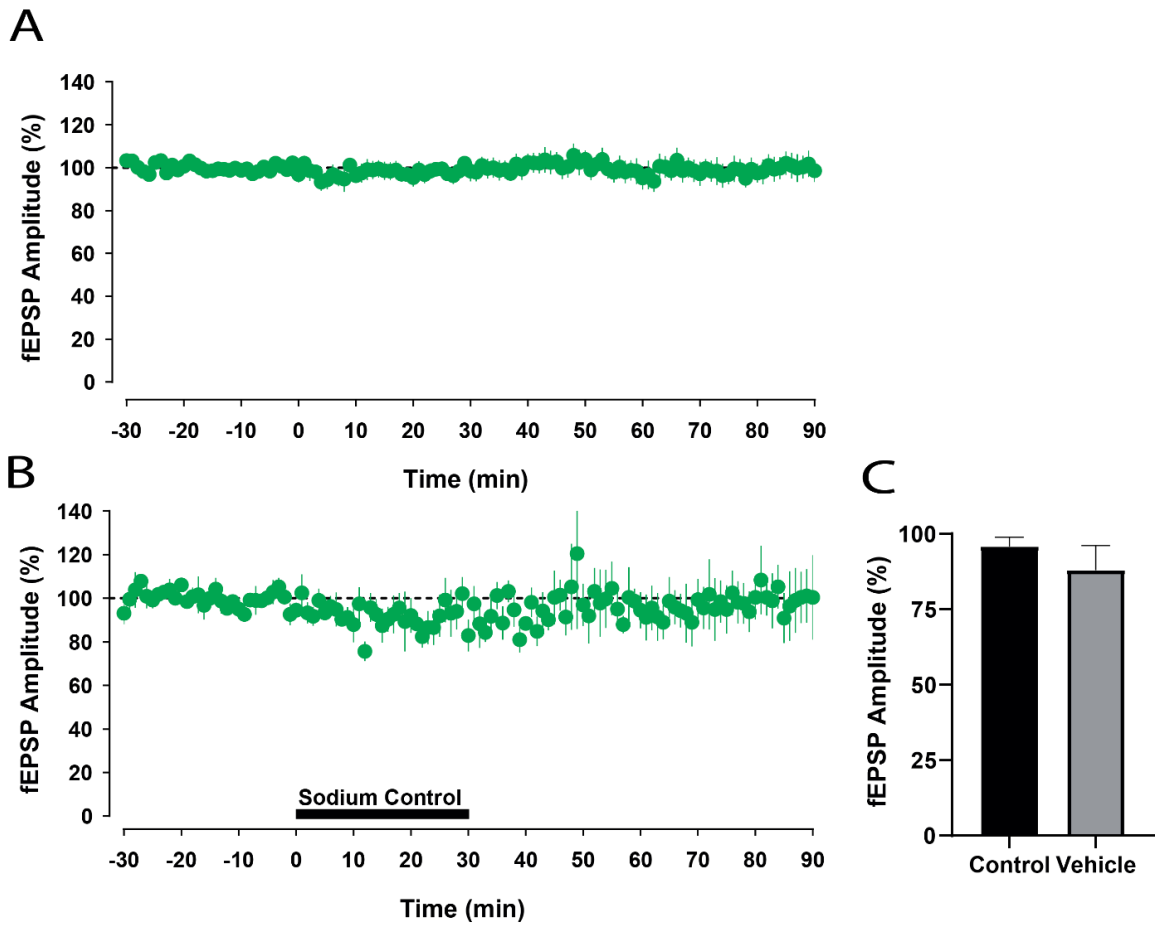

Figure 3. (A) Depicts the control data collected through all experiments (n=10). The fEPSP data collected was then averaged to each minute and normalised to a percentage of the first 30-minute baseline. These data represent the 2-hour control data, which utilised results from longer controls, up to 5 hours. (B) Sodium metabisulfite control data (n=4) indicates that sodium metabisulfite vehicle has no significant effect on fEPSP amplitude.

Throughout data collection for both experimental chapters control data was also collected for baseline comparison in statistical analysis. In the case of Repeated measures ANOVAs, these comparisons were time matched such that the stability of the response over time would not contribute to a false positive in terms of statistical significance. Throughout all experiments a consistent factor was the use of Sodium Metabisulfite in any solutions containing dopamine. The sodium metabisulfite acted as an antioxidant which prevented the oxidation of dopamine

during its application period (Caruana and Chapman, 2008). Had dopamine been degrading whilst bath application onto the slice was occurring, its suppressive effect on fEPSP amplitude would be reduced, presenting itself as a desensitisation of the slice to the dopamine. Partial degradation of dopamine may still occur, even in the presence of sodium metabisulfite, and this must be considered throughout experimentation. To examine and ensure that sodium metabisulfite had no significant effect on fEPSP amplitude during a 30-minute application period a number of vehicle controls were carried out. In this case a solution mimicking the exact composition and volume of bath-applied dopamine was created, with dopamine absent, this would leave just ACSF and Sodium Metabisulfite in solution, which was then bath applied for 30 minutes as normal. The control data (n=10, meaning 10 rats used) is shown in figure 3, with the vehicle (sodium metabisulfite) control data in 1B (n=4). The mean fEPSP amplitude of the 30-minute application period between 0 and 30 minutes was then compared between the control and vehicle data sets using a T test, the results of which are shown in the form of a bar graph (fig.2c). The result of an unpaired T-test found no significant difference between the fEPSP amplitude of the control and vehicle conditions during the 30 minute application period ($t(12)=1.128$; $p=0.2816$). Therefore, one can conclude that Sodium Metabisulfite has no significant effect on fEPSP amplitude and would not contribute to any depressive effect observed in the presence of dopamine. It is vital to analyse the effect that a vehicle may have during an experiment as the effect witnessed may be accentuated by the vehicle's presence. If the statistical analysis yielded a significant difference between control and vehicle data, then the depressive effect of Sodium Metabisulfite would be quantified and considered in the results of any dopamine conditions.

5.2 Long term depression

Paired Pulse Low Frequency Stimulation (PP-LFS) is successful in inducing long term depression (LTD) in lateral entorhinal cortex.

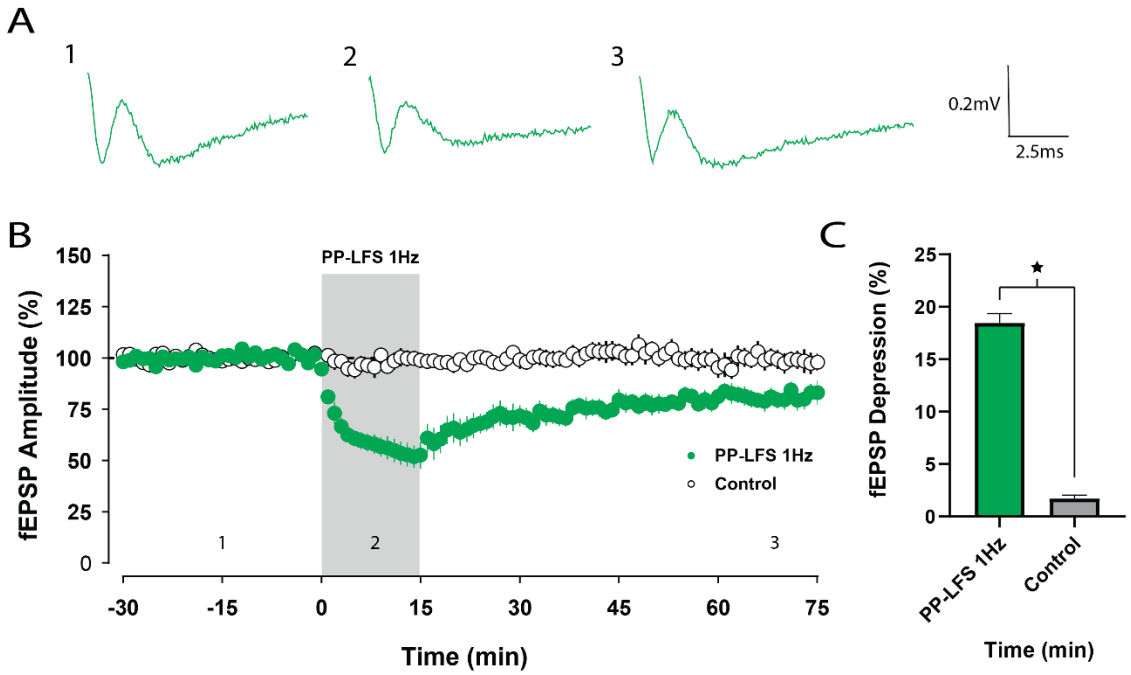

Figure 4. (A) Sample traces depicting the signal averaged sweeps from time points denoted in B. (B) Graph representing data from PP-LFS experiments as well as interleaved control experiments. Experiments consisted of a 30-minute baseline period, at which point the PP-LFS protocol was delivered. This protocol lasted 15 minutes, after which a further 60 minutes of recording was collected. (n=15) (C) The average fEPSP depression (%) during the last 5 minutes of the experiment was then compared to time matched control data and found to be significantly different to the time-matched control data at a 95% confidence interval. Showing that PP-LFS successfully induces LTD within the lateral entorhinal cortex.

The first group of experiments conducted aimed to assess whether PP-LFS (Paired-Pulse Low Frequency Stimulation) could induce LTD in the LEC. Previous research (Kourrich et al., 2008) has shown that a stimulation protocol consisting of 900 pulses at a frequency of 1Hz induced LTD in sensory inputs to the superficial layers of the LEC, and this experiment aimed to extend this research**.** Figure 4B provides a graphical representation of the averaged fEPSP amplitude through the 105-minute-long experiment. As the recordings taken throughout the experiment are taken every 20 seconds, each point displayed in 2B represents 1 minute of recording, thus each point depicts the average of 3 recordings. However, during the 15-minute PP-LFS

application period each point represents an average of 60 recordings. These data show that PP-LFS is an effective method in producing long term depression (LTD) of fEPSP amplitudes within slices from rat lateral entorhinal cortex, as well as producing a model for the effect that is expected from this protocol. 2B exhibits a decrease of 52.84% ± 6.077 in response amplitude at the 15-minute time period as a result of PP-LFS, following this suppressive effect the response amplitude begins to return to baseline levels over the course of 45 minutes, before plateauing at an amplitude level which is 16.52 ± 1.034% lower than time-matched control data. The average suppression of fEPSP amplitude during the 70-75 minute time point (18.06 ±1.034) was found to be statistically significant than that of the control data (1.542 ± 1.034) when compared using an unpaired T test ($t(8)=15.98$; $p=<0.0001$), with 8 representing the number of degrees of freedom for this test.

The aim of this experiment was to assess the effect of PP-LFS on fEPSP amplitudes in lateral entorhinal cortex, showing that it is an effective method in inducing LTD. The results of this condition also provide a model for LTD within rat lateral entorhinal cortex. Experiments which rely on the induction of LTD before other assays may be performed can be compared to this model in order to qualify whether the PP-LFS was successful in inducing LTD.

Application of 100μM causes a suppression of fEPSP amplitude. Application of 100μM Dopamine post-activity dependent LTD causes responses to return to the depressed level.

Following initial experiments, focus turned to attempting to prevent this effect, or experiment with how the induction of LTD can be neuromodulated by dopamine. The next condition aimed to assess the effect of dopamine on synaptic responses in lateral entorhinal cortex slices. As described in the methods section, dopamine of known concentration, in this instance 100μM, was washed onto the slice in the recording bath for 30 minutes, preceded by a 30-

minute baseline. A 30-minute baseline is consistently used across all experiments, the objective of this baseline is to ensure that recording is flat and stable before performing any assays on a certain slice. This prevents any false positives in terms of changes in amplitude which may have been due to a slight run-up or run-down in the baseline. Figure 5B depicts a graph of fEPSP amplitude as a percentage over time. Interleaved control data (n=10) are overlaid by the experimental data in which 100uM DA was applied to the slice for 30 minutes (n=7), in this experiment dopamine was applied to 7 slices collected from 7 different rats, essentially N here represents 7 rats, meaning experiments were not repeated within the same animal. As with 2B a 60-minute washout period was conducted following the DA application to fully assess any long-term effects which may occur.

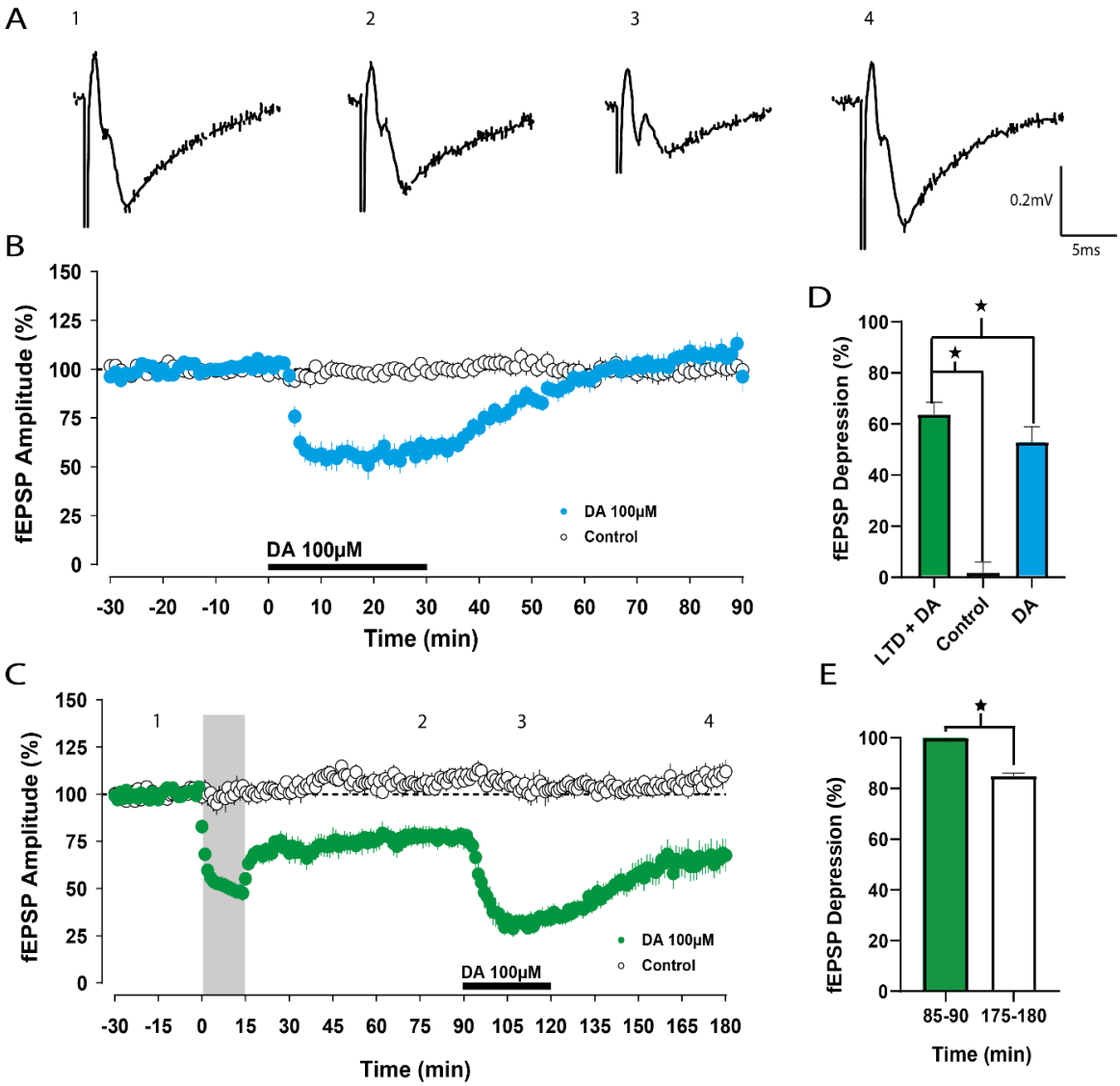

Figure 5. (A) Sample traces of synaptic responses collected from the time points denoted in B. (B) Single application of 100uM dopamine caused a depression of fEPSP amplitude during the 30-minute application period. (n=7) (C) LTD induction followed by a 60-minute washout period, after which 100uM dopamine was applied. The results of these 3 experiments were then analysed and compared using a one-way ANOVA (D), analysing the peak fEPSP depression during the first 15 minutes of the dopamine application period. (n=10) (E) Results of an unpaired T -test comparing the fEPSP depression at the 85-90 minute and 175-180-minute time periods of 3C, indicating that fEPSP amplitude returns to below the depressed level caused by the PP-LFS protocol.

The results of this condition show that 100uM DA causes a 52.84 ± 6.1% peak suppression of fEPSP amplitude, however the effect showed a rapid return to baseline following the application period with no long-term depression of response amplitude. This suggests that any plastic effects which are occurring due to dopamine are not long term, a contrast to the effects of the PP-LFS protocol.

The progression from the experiment in figure 4 and figure 5B aimed to assess how the induction of LTD through PP-LFS affects the peak suppression of dopamine at the concentration of 100uM. In 4C, following 30 minutes of baseline, a PP-LFS protocol was administered for 15 minutes as had been performed in previous experiments. In this case the washout period was 75 minutes, and during this time averaged data shows that LTD was successfully induced, with a depression of fEPSP amplitude maintained at around 25% between 75 - 90-minute time period. Comparing to figure 3B, the level of depression within this experiment aligns with the expected level for successful LTD.

The analysis performed in figure 5E compares the fEPSP depression in the 85-90 minute time period in 4C to the final 5 minutes of 5C, the results of this unpaired T-test indicate that there is a significant difference between the fEPSP depression at these two time points ($t(10)=10.98$, $p=<0.0001$). However, the notable feature of this comparison is that application of dopamine following activity-dependent LTD causes the dopamine depressed responses to return to a level which is lower than the original depression. Contrary to 5B, in which the depressed responses return to baseline levels. Further experimentation may be required here, extending the time-course of the experiment, specifically dopamine the washout period, to test if the depressed responses, caused by dopamine, eventually return to baseline levels or if they remain at the level of depression seen at 85 minutes in 5C. It is possible that the depressed level seen in figure 5C at 180 minutes is due to the washout period not being long enough for the response to return to the LTD level, extending the washout period would verify this.

Application of 30μM Dopamine during PP-LFS causes a block of LTD induction.

The interaction between PP-LFS and dopamine has been observed previously by Caruana et. al 2007 in which applying dopamine to the slice during PP-LFS protocol blocked the LTD effect which was typically induced. The following experiment aimed to replicate and support this finding, inducing LTD under the presence of 30uM Dopamine which would be bath applied for 30 minutes following a 30-minute baseline. 15 minutes into the DA application period, PP-LFS would be carried out. It was then possible to compare the fEPSP depression percentage during the last 5 minutes of the experiment between the PP-LFS + DA, and control condition in which baseline recording was taken for 210 minutes with no dopamine applied or PP-LFS protocol. The results of an unpaired T test found that there was a significant difference between the mean fEPSP depression of the PP-LFS + DA (8.449%) and control (-1.552%) condition ($t(8)=6.564$; $p=0.0002$) at the 95% confidence interval. A comparison could then be made, using a one-way ANOVA, the peak suppression during the last 5 minutes of control data, PP-LFS, and PP-LFS + DA data sets, in order to quantify the difference, and the significance of the difference, between PP-LFS only and PP-LFS + DA conditions. This analysed the effect that dopamine has regarding blocking the LTD effect caused by the PP-LFS protocol. The results of the ANOVA are shown in 4F indicating that there is a significant difference between not only the control and experimental groups, but also between the LTD and LTD+DA groups. The mean difference between PP-LFS and PP-LFS + DA was 9.614% ± 1.353, and this difference was significantly different at the 95% confidence interval ($t(12)=7.107$; $p=<0.0001$). The results of this ANOVA conclude that application of 30uM dopamine causes an effective, although not complete as indicated by 6E, block of LTD when induced by PP-LFS, supporting previous research by Caruana et. al (2006).

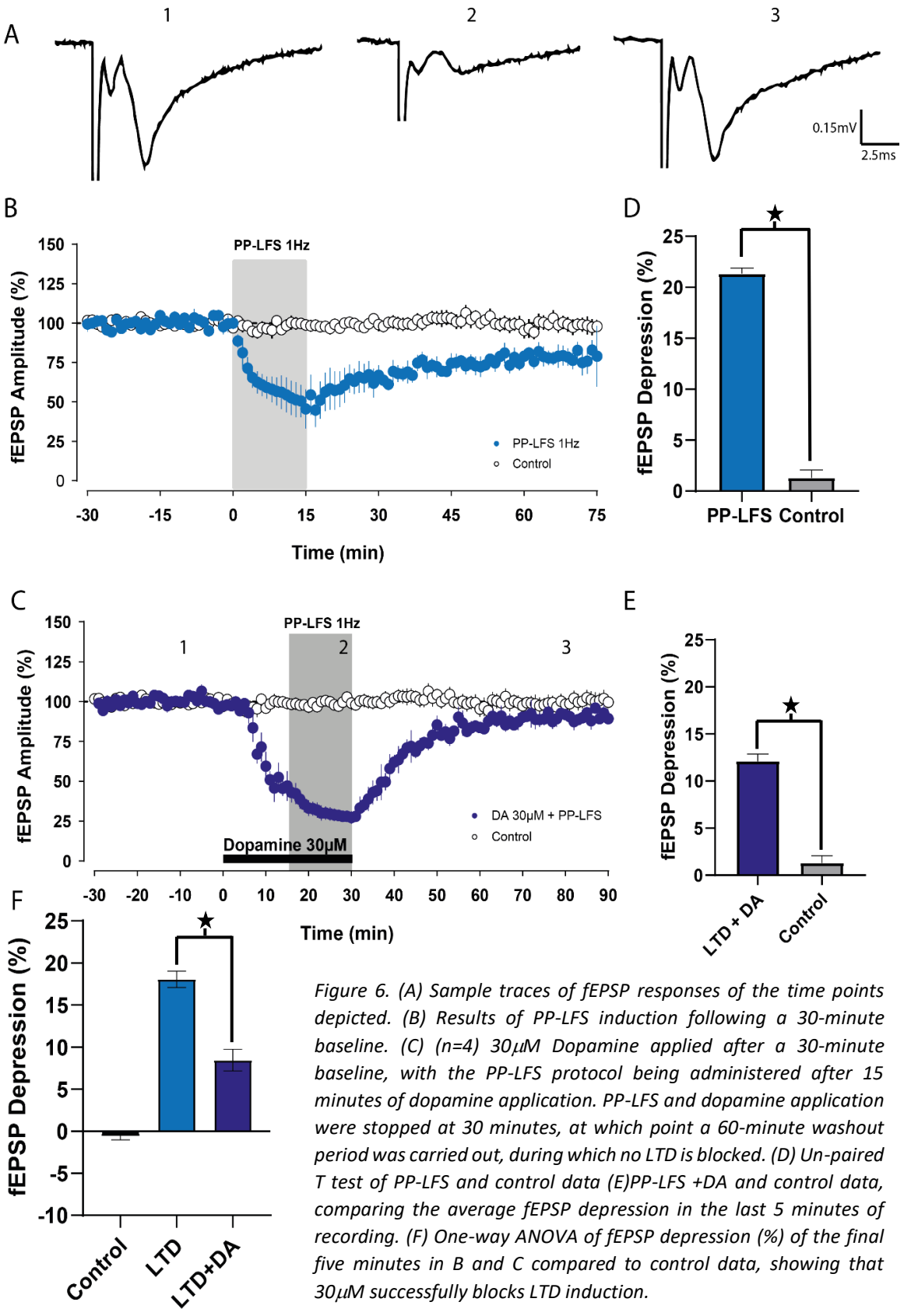

Figure 6. (A) Sample traces of fEPSP responses of the time points depicted. (B) Results of PP-LFS induction following a 30-minute baseline. (C) (n=4) 30µM Dopamine applied after a 30-minute baseline, with the PP-LFS protocol being administered after 15 minutes of dopamine application. PP-LFS and dopamine application were stopped at 30 minutes, at which point a 60-minute washout period was carried out, during which no LTD is blocked. (D) Un-paired T test of PP-LFS and control data (E)PP-LFS +DA and control data, comparing the average fEPSP depression in the last 5 minutes of recording. (F) One-way ANOVA of fEPSP depression (%) of the final five minutes in B and C compared to control data, showing that 30µM successfully blocks LTD induction.

LTD induction prior to successive dopamine applications yields no significant difference in fEPSP depression during dopamine application, contrary to previous experiments in the absence of LTD.

The results from previous experiments guided the future direction of experimentation, specifically toward the interaction between dopamine application and the induction of long-term depression by method of PP-LFS. Parallel research to this project within the lab found that repeated applications of the same concentration of dopamine resulted in a decrease in the peak suppression of each application. Essentially, the repeated application of dopamine to a slice with washout periods between application causes the effect of the dopamine to diminish, acting as a form of dopamine-mediated metaplasticity or desensitisation as opposed to the activity dependent plasticity detailed above. The aim of this experiment was the analyse the effect of prior induction of activity dependent LTD on the DA-mediated desensitisation, inducing LTD within a 120-minute time period, then applying 100uM dopamine for 30 minutes twice, followed by a 60-minute washout in each instance (7B). Results of this experiment would indicate whether there is an interaction between LTD induction and dopaminergic suppression of synaptic responses. The graph shown in figure 7B outlines the experiment overall, with 7A depicting sample sweeps from labelled time points, those being the baseline period, the depressed response following PP-LFS, and each dopamine application.

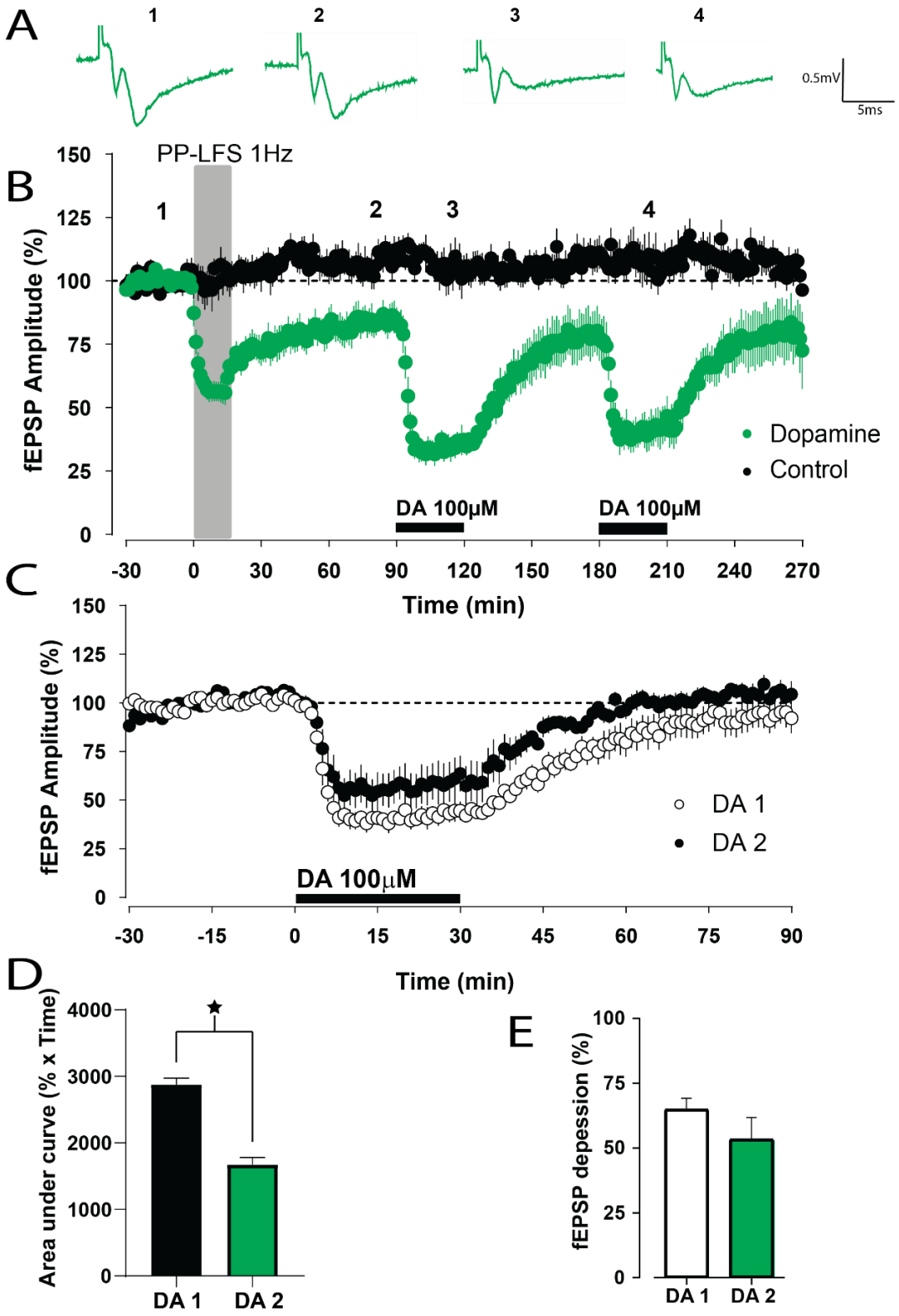

Figure 7. (A) Sample traces from labelled time points. (B) (n=6) Following a 30-minute baseline, LTD was induced using PP-LFS, if this effect remained after a 60-minute washout period then 100uM dopamine was applied, allowed a 60-minute washout period, and then applied again, before a final washout period. The peak fEPSP suppression during DA application was then compared between the two application periods using an un-paired T test (E), further analysis was performed comparing the area under the curve (AUC) of the application periods in C with an unpaired T-test (D) yielding a significant difference in AUC between the two application periods, however no significant difference was found in fEPSP depression between applications.

Initial exploratory experiments within this data set appeared to show that the induction of LTD prior to the double application of dopamine blocked the effect seen in previous experiments, in which the peak dopamine-mediated suppression would be reduced during the second application. Therefore, the focus of these experiments was the application periods themselves. These application periods shown superimposed in Figure 6C yielded no significant difference between the peak suppression of fEPSP amplitude between DA application 1 (64.87% ±4.373) and DA application 2 (53.41% ±8.424); $t(5)=2.557$, $p=0.0508$. Although the result of this T-test yields no significant difference; statistically it should be reported that $p=0.0508$, close to significant difference.

From this analysis further analysis on the application periods needed to be performed, in this instance an area under the curve (AUC) analysis was used to quantify if there was any statistically significant difference between the two application periods in terms of area under the curve. Due to the nature of the method of data collection, the AUC analysis was modified to consider negative peaks, the total area under the curve of application period 1 was 2877 ± 96.48 (fEPSP amplitude (%) x Minutes), whereas the area under the curve for application period 2 was 1675 ± 105.8 (fEPSP amplitude (%) x Minutes). These areas were then statistically compared by method of a T test to check for statistically significant difference between the two, the results of which found there to be a significant difference in the area under the curve between dopamine application period 1 and 2; $t(10)=7.764$, $p=<0.0001$. The result of this T test does not support initial theories that the effect witnessed when performing multiple applications of dopamine could be blocked by first inducing LTD (7E). Therefore, this experiment shows that there is a significant difference in the area under the curve between the first and second DA application.

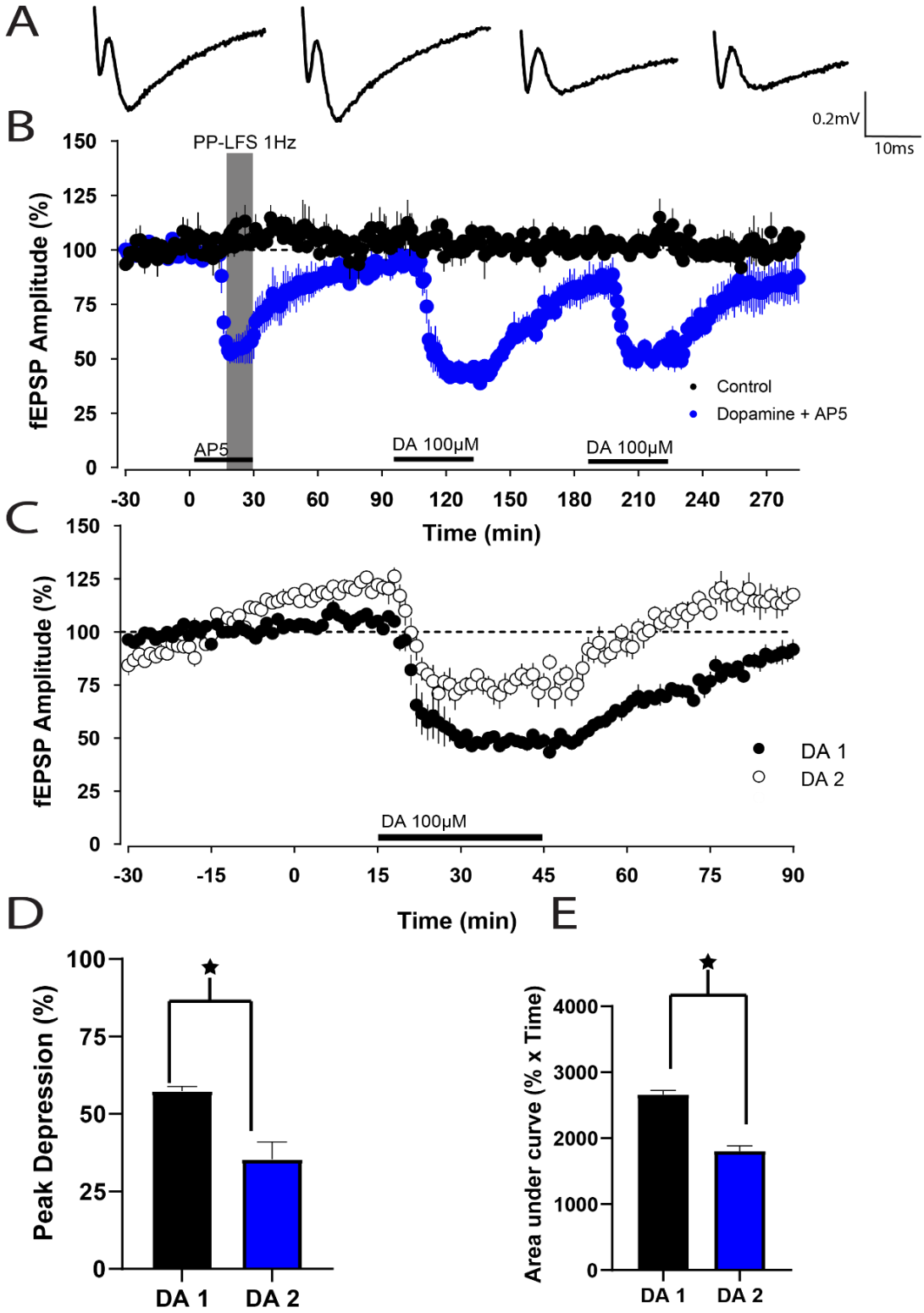

Figure 8. (A) Sample traces from labelled time points. (B) (n=5) With the same procedure as the multiple dopamine applications in figure 5, with APV solution applied 15 minutes before and during the PP-LFS protocol, blocking LTD induction. The peak fEPSP suppression during DA application was then compared between the two application periods using an un-paired T test (D), further analysis was performed comparing the area under the curve (AUC) of the application periods (E). Blocking LTD with APV had no effect on the difference in AUC seen previously, however did elicit a significant difference in fEPSP depression not exhibited when LTD had been induced.

Application of 10μM AP5 to block LTD prior to dopamine application yields significant difference between dopamine applications with regard to both peak depression and AUC analysis.

In order to further analyse the effect that LTD may be having on the dopamine-mediated plasticity seen in rat lateral entorhinal cortex this experiment aimed to observe the effect that a block of LTD induction would have on the result presented in figure 7. Using the NMDA receptor antagonist AP5, the final experiment within this chapter concerned the blocking of LTD prior to the double application of 100uM dopamine. The methodology for this experiment drew inspiration from previous research which utilised AP5 to block LTD (Wong and Gray, 2018). A single application of AP5 before and during PP-LFS was considered to be the optimum method for application, as with previous experiments AP5 was administered following a 30-minute baseline to ensure stability of recording and slice health. Notably, AP5 was highly efficient in blocking the effect of PP-LFS, reducing the fEPSP amplitude suppression which had been observed in figure 7.

The data for application periods 1 and 2 were then compared (figure 8B), with the data renormalised to the -30 to 0-minute time period. In this case, the AUC analysis showed that the total area of DA 1 (2663 ± 62.07 fEPSP amplitude x Minutes) was greater than that of DA 2 (1804 ± 80.52 fEPSP amplitude x Minutes), and after comparing these data with a T-test a statistically significant difference was found $t(8)=8.449$; $p= <0.0001$. Examining the peak suppression data, the lowest fEPSP amplitude during the first 15 minutes of dopamine application, of DA 1 showed a 57.40% ± 1.503 decrease in fEPSP amplitude, and DA 2 displayed a 35.40% ± 5.591 decrease in fEPSP amplitude, these results indicate that the peak suppression of DA 1 is lower than that of DA 2 ($T(4)=4.340$; $p=0.0123$). Such a result would serve as supporting evidence to the previous experiments which showed the same result

without LTD induction. As there was no significant difference between the peak suppression in figure 7E, it would appear as though the blocking of LTD with AP5 has led to a significant difference in peak fEPSP suppression which would typically be synonymous with double applications in the absence of prior LTD. However, both figures C and D show that, with regard to AUC, that there is no significant difference between the two application periods regardless of LTD.

Summary of Long-Term Depression results

In this experimental chapter the aim was to quantify the suppressive effect of both PP-LFS and dopamine and examine their effects on synaptic potentials in rat entorhinal cortex. By applying PP-LFS and known concentrations of dopamine, their plastic effects within the slice could be quantified. These assays provide quantifiable evidence that PP-LFS is an efficient method of inducing LTD in rat lateral entorhinal cortex. However, when pairing the two assays, such as LTD during DA application or LTD prior to DA application, it was found that dopamine is capable of blocking the effect of PP-LFS and reducing the fEPSP suppression (%) by around 10% compared to the PP-LFS only protocol (Fig.6C). This finding presents novel evidence supporting findings by Caruana et. al (2007). Figure 5 presents the key finding that inducing LTD followed by dopamine application causes the dopamine depressed response to return to 10.98% below the depressed level, caused by activity-dependent LTD. It is clear from these results alone that the neuronal mechanisms underlying both activity dependant and dopamine dependant plasticity are interacting, specifically in the case of LTD induction prior to the application of dopamine.

5.3 Long term potentiation

High Frequency stimulation (HFS) was found to be incapable of inducing Long-Term potentiation (LTP)

The first experiments within this chapter concerned the refinement of methods in order to induce LTP within rat lateral entorhinal cortex slices. Due to varying views from papers surrounding LTP induction all experiments were carried out, at least in the early stages, with two different forms of tetanus. The first involved stimulation of the slice with 100 pulses at a frequency of 100Hz, known as high frequency stimulation or HFS. The graphical analysis shown in figure 7A indicates the time point at which the HFS protocol was applied, this being directly following a 30-minute baseline period. The average fEPSP amplitude between 3 different time points for each replicate were compared to control data using a repeated measures (RM) ANOVA, the results of which are shown graphically in 8B. The time points chosen aimed to wholly represent any effects which may be occurring as a result of HFS, these time points being 1-minute post-tetanus, the 26-30-minute period, and the last 5 minutes of the experiments (86-90 minutes). Selecting these time points considered that, in the absence of long-term potentiation, short term potentiation (STP) or post-tetanic potentiation (PTP) may be present. PTP occurs due to an increase in neurotransmitter release following the high frequency protocol administered and is considered to be largely presynaptic (Bao, Kandel and Hawkins, 1997), thus would not be the focus of analysis as it is the postsynaptic potentiation of synaptic responses which this experiment aims to induce.

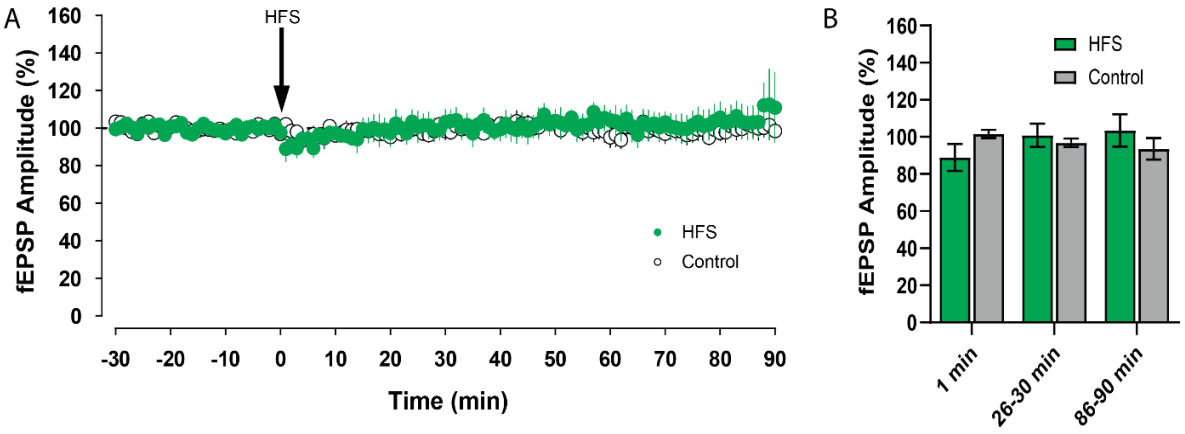

Figure 9. (A) Graphical presentation of data collected from high frequency stimulation (HFS) experiments (n=6), the HFS protocol was initiated at the point labelled following a 30-minute baseline. fEPSP amplitude data was then averaged and normalised to a percentage of the 30-minute baseline. (B) results of a repeated measure two-way ANOVA comparing the 1 minute, 26-30 minute, and 86-90-minute time periods to time matched control data. HFS alone was unsuccessful in inducing LTP.

From the RM ANOVA of the 3 different time points it was concluded that there is no statistically significant difference between the HFS condition and control data, meaning that the HFS protocol, in this instance, was not successful in inducing any form of potentiation ($F(1,5)=0.007, p=0.9339$). Multiple post hoc comparisons were performed in the form of a Tukey test which revealed a 12.65% ± 5.843 decrease in fEPSP amplitude at the 1 minute time point between the HFS and control condition, however this result was found to be not statistically significant ($p=0.5760$). Between 26 and 30 minutes the difference in fEPSP amplitude between the HFS and control condition was found to be an increase of 3.975% ± 5.843, and this was found to be not statistically significant ($p=>0.9999$), similarly at the 86-30 minute time period the 9.948% ± 5.843 increase in amplitude was found to be not statistically significant ($p=0.8517$).

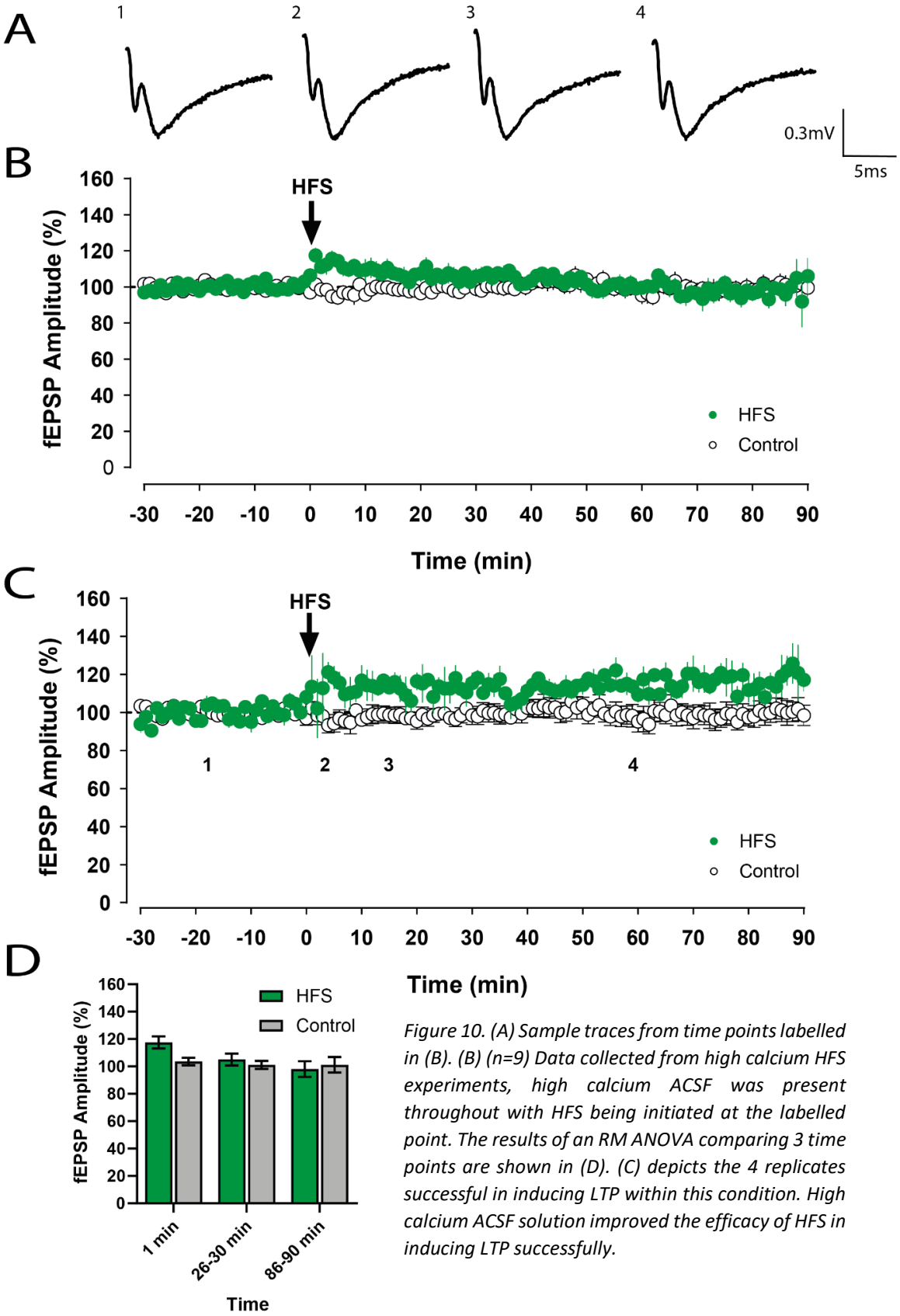

Figure 10. (A) Sample traces from time points labelled in (B). (B) (n=9) Data collected from high calcium HFS experiments, high calcium ACSF was present throughout with HFS being initiated at the labelled point. The results of an RM ANOVA comparing 3 time points are shown in (D). (C) depicts the 4 replicates successful in inducing LTP within this condition. High calcium ACSF solution improved the efficacy of HFS in inducing LTP successfully.

10mM Calcium ACSF improved the efficacy of High Frequency Stimulation in inducing Long-term potentiation in lateral entorhinal cortex.

It was clear from these first experiments that high frequency stimulation alone would not be sufficient in inducing LTP or even STP, therefore the focus changed to combining the HFS protocol with various chemical compounds or changing the conditions in which the experiments were performed. In this first instance, the variable of extracellular calcium concentration was increased to 10mM, 2.5x that of the usual concentration for all previous experiments. The high calcium ACSF solution was applied for the duration of the experiment, as it was found that the amplitude of synaptic responses was, typically, higher in high calcium solution compared to normal ACSF. Therefore, completing the entire experiment under high calcium conditions meant that there would be no false positive results caused by changing to high calcium ACSF during or after tetanisation, the effect of high frequency stimulation alone would cause any potentiation of the response.

The results of the experiment combining HFS stimulation with high calcium ACSF are shown in Figure 10B with the point of HFS induction labelled. From the graphical analysis one would infer that HFS has caused potentiation of fEPSP amplitude at 1 minute post-tetanus, however the results of a post hoc Tukey's multiple comparisons test showed that the mean difference between HFS and control conditions (13.88% ± 5.140) is not statistically significant (p=0.1774). The mean difference between the HFS and control conditions between 26-30 minutes (3.935) was also found to be not significant (p=0.9776), as well as the 86-90 minute period for which the mean difference was found to be -3.090, (p=0.9996). The overall results of the repeated measures ANOVA comparing the high calcium HFS group to the control group also indicated no statistically significant difference overall ($F(1,8)=0.5338$, $p=0.4859$). However, the RM ANOVA comparing the three time points found the total variation between HFS time points to

be statistically significant ($F(1.5,11.7)=34.60$, $p=<0.0001$). Further comparisons using Tukey's multiple comparisons test found the mean difference in fEPSP amplitude between the 1-minute and 30-minute time period to be 12.42% ± 2.990, and this difference was found to be statistically significant ($p=0.0260$), as well as being significantly different to the 90-minute time period (19.44 ± 3.42, $p=0.0041$). These results indicate that, overall, the increase in fEPSP amplitude 1-minute post tetanisation is significantly different to the fEPSP amplitude at the two other time points during the experiment, suggesting that some STP has occurred. When analysing these data it became apparent that, in the case of some replicates, this assay was capable of inducing long term potentiation (the average data of these replicates are shown in figure 10C however in this case it is important to consider all replicates as a whole in order to understand the efficacy of this method and therefore the entire data set was considered in statistical analysis.

GABA Antagonist Picrotoxin paired with high frequency stimulation unsuccessful in inducing long-term potentiation in lateral entorhinal cortex.

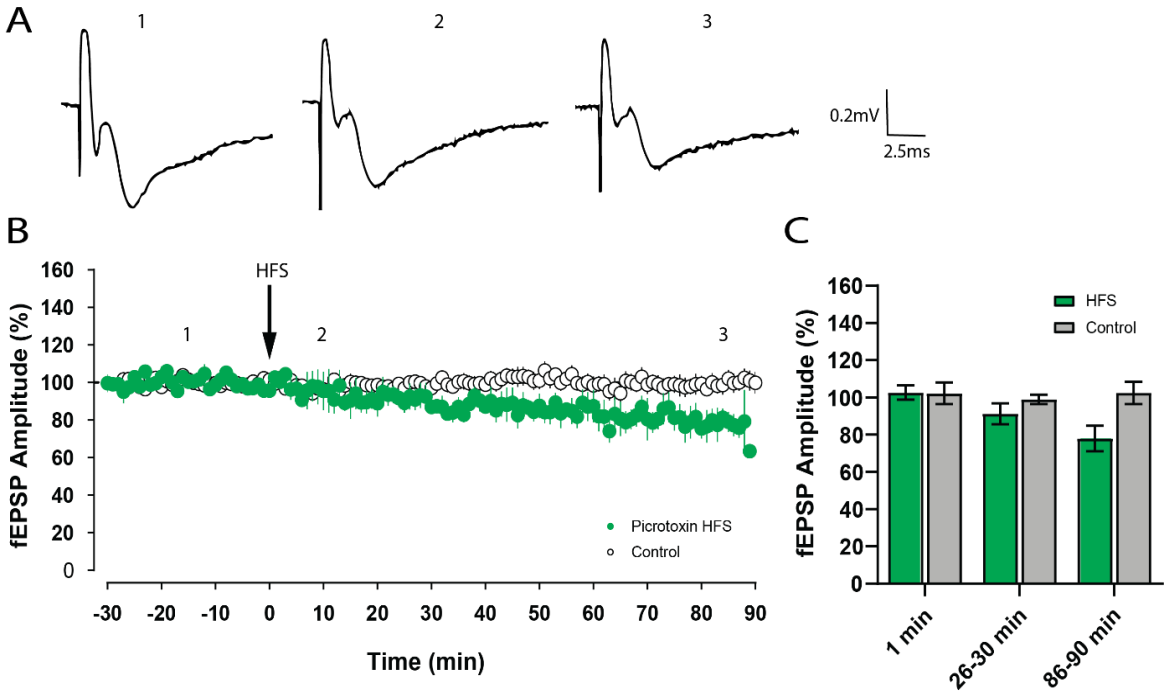

Figure 11. (A) Sample traces depicting synaptic responses collected from the time points denoted in B. (B) (n=3) Graphical presentation of collected data from HFS experiments in the presence of Picrotoxin throughout experimentation. (C) Results of a RM ANOVA comparing the 1 minute, 26-30, and 86-90-minute time periods between the Picrotoxin HFS and control conditions indicating that the application of Picrotoxin coupled with HFS was unsuccessful in inducing LTP.

The final experimental assay conducted in the HFS condition was the application of the non-competitive GABA antagonist Picrotoxin throughout the experiment, the aim of picrotoxin application was to reduce the level of GABAergic inhibition within the slice, which could be preventing the induction of LTP (Kotak, Mirallave, Mowery and Sanes, 2017). The results of this experiment are displayed in figure 11. Overall RM ANOVA results found there to be no significant difference between the HFS and control groups ($F(1,2)=1.651$, $p=0.3276$). Post hoc comparisons in the form of a Tukey's test were utilised to compare the difference between HFS and control data at each time point shown in 9C. At 1-minute post-tetanisation there was found to be a mean difference in fEPSP amplitude of 0.3749% ± 4.986, however this difference was found to be not statistically significant ($p=>0.9999$). Similar results were found for the 26-

minute period in which the mean difference of -7.770% ± 7.943 was found to be not significant (p=0.8936). The final time point of 86 minutes post-tetanisation yielded a -24.48% ± 12.32 difference between conditions, also found to be not significant at the 95% confidence interval (p=0.5343). The only statistically significant result from this data set proved to be the mean difference between the 26 minute and 86-minute time point in the HFS condition (13.27% ± 1.575, p=0.0485). At the 95% confidence interval this result is barely statistically significant, and with only 3 replicates within the condition it is difficult to draw any conclusion from this result. Therefore, this experiment shows that picrotoxin paired with HFS yielded no significant difference in fEPSP amplitude.

Theta-burst stimulation protocol produces no significant increase in fEPSP amplitude at 1, 26, or 86 minutes post-tetanisation.

The second method that I found literature to support its efficacy in inducing LTP is theta burst stimulation, this protocol involves the delivery of 10 trains of 4 pulses delivered of a 20 second time period, each pulse lasted 0.1ms with a 75ms delay between pulses. TBS experiments were laid out the same as HFS experiments, although the TBS protocol takes longer than the HFS protocol. Figure 11 shows the results of the TBS protocol condition, comparing figure 11 to 8 visually gives rise to a clear difference in the two experiment post-tetanus, with TBS showing no change in fEPSP amplitude whilst HFS caused a depression of synaptic response. RM ANOVA results overall indicate no significant difference between conditions at the 95% confidence level, ($F(1,6)=0.2387$, $p=0.6425$). The test also found no significant difference between time points ($F(1.2,7.0)=2.127$, $p=0.1901$). Finally, comparing the time points between TBS and control conditions also yielded no significant difference ($F(2.0,11.6)=0.9248$, $p=0.4207$). The post hoc Tukey's test results for the TBS condition concur that there is no significant difference between the control and TBS condition at either the 1 minute

(t(11.65)=0.8784; p=>0.9999), 26-30 minute (t(10.18)=0.3075; p=>0.9999), or 86-90 minute period (t(10.28)=0.5033; p=>0.9999). Therefore, Theta burst stimulation had no effect on fEPSP amplitude in rat entorhinal cortex slices in these experiments.

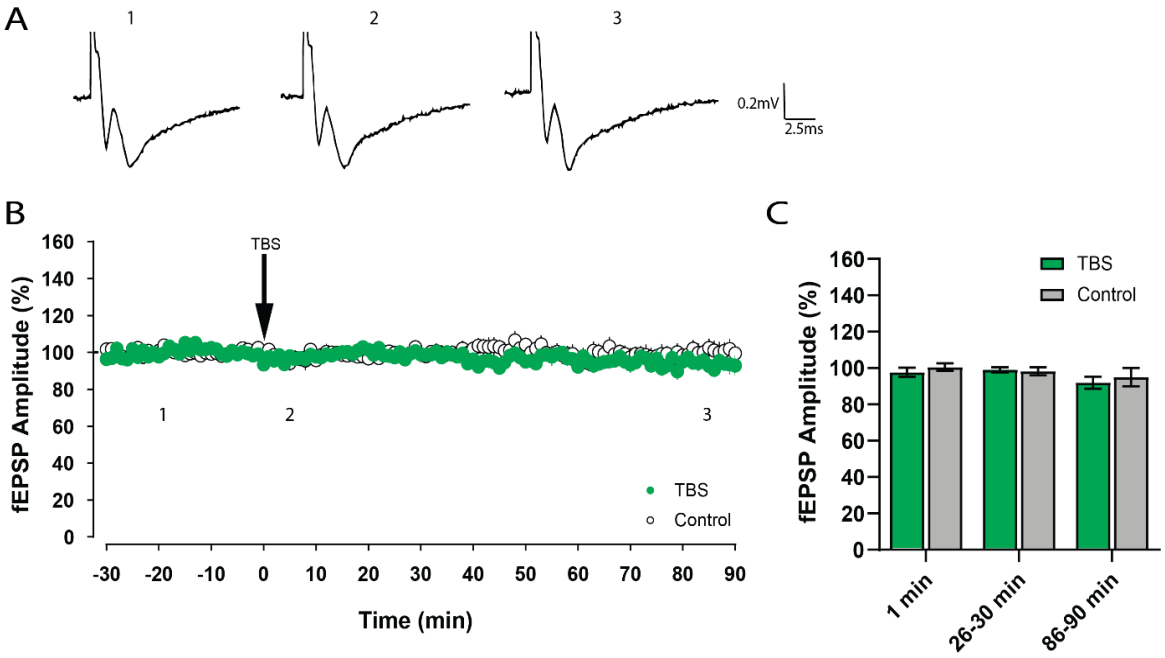

Figure 12. (A) Sample traces collected from the time points denoted in B. (B) Graphical presentation of data collected from theta burst stimulation (TBS) experiments (n=7), the TBS protocol was initiated at the point labelled. fEPSP amplitude data was then averaged and normalised to a percentage of the 30-minute baseline. (C) RM ANOVA comparison between the time points of; 1 minute, 26-30 minutes, and 86-90 minutes to control data, indicating TBS alone was ineffective in inducing LTP.

GABA Antagonist Picrotoxin paired with theta-burst stimulation unsuccessful in inducing long-term potentiation in lateral entorhinal cortex.

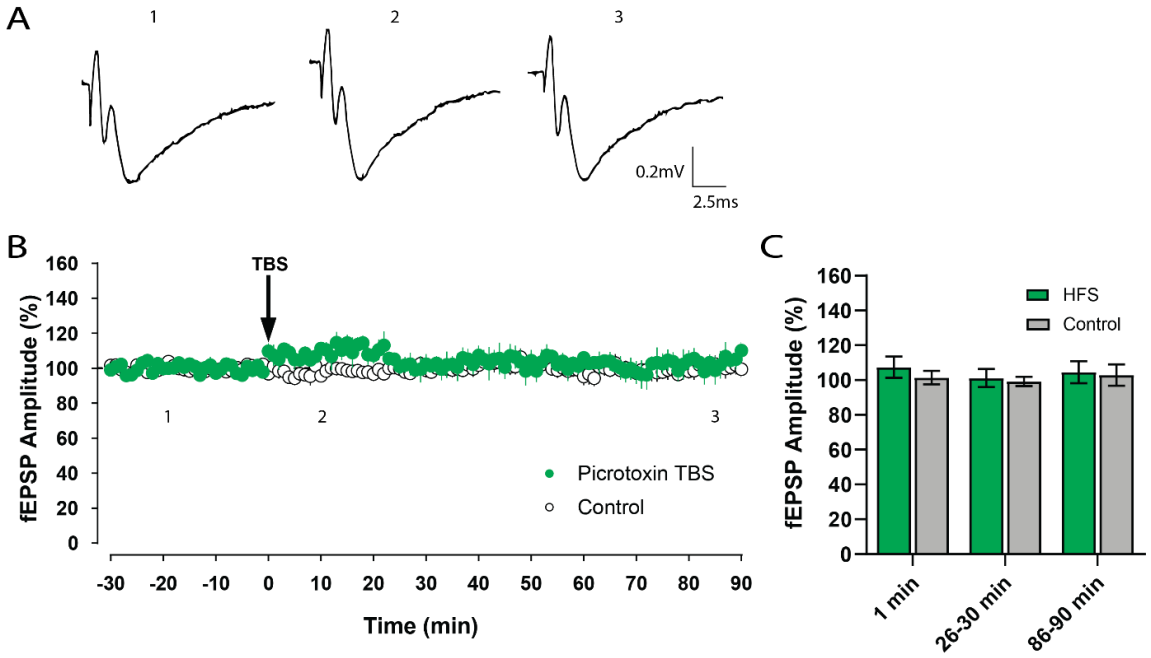

Figure 13. (A) Sample traces collected from the time points denoted in B. (B) Graphical presentation of data collected from TBS experiments in which the GABA inhibitor picrotoxin was present within the ACSF throughout, the HFS protocol was initiated at the point labelled. fEPSP amplitude data was then averaged and normalised to a percentage of the 30-minute baseline (n=3). (C) RM ANOVA comparison between the time points of; 1 minute, 26-30 minutes, and 86-90 minutes to control data. Picrotoxin application was unsuccessful in inducing LTP, however some STP is apparent during the 1-minute pot-tetanus period.

Experiments using the non-competitive GABA antagonist Picrotoxin were also carried out in conjunction with the TBS condition, the results of this experimental line are shown in figure 12 as well as the RM ANOVA statistical analysis for each time point (12C). Similarly to the non-picrotoxin condition no significant difference was found, when compared using an RM ANOVA statistical analysis no significant difference was found between either the HFS and control conditions ($F(1,2)=0.3605$, $p=0.6092$) or between time points ($F(1.1.72,2.34)=2.197$, $p=0.2670$). there was also no significant difference found between time points in each condition ($F(1,2)=0.1994$, $p=0.7$). Results of a Tukey's multiple comparisons test confirmed this result; at the 1 minut time point the mean difference between TBS and control data was 5.997 ± 4.940 ($p=0.8109$). Comparing the 26-30 minute period yielded similar results with the mean

difference of 1.811 ± 4.432 being insignificant (p=0.9961), as well as at 86 minutes in which the mean difference was found to be 1.695 ± 9.986 (p=>0.9999). Comparing between time points the closest result to statistical significance was the mean difference in TBS data at 1 minute and 26 minutes post tetanisation, here the difference was found to be 8.090 ± 5.269 (p=0.0696). Thus, concluding that picrotoxin and its resultant GABA antagonism was unable to cause theta burst stimulation to induce LTP in rat lateral entorhinal cortex slices.

10mM Calcium ACSF paired with theta-burst stimulation unsuccessful in inducing long-term potentiation

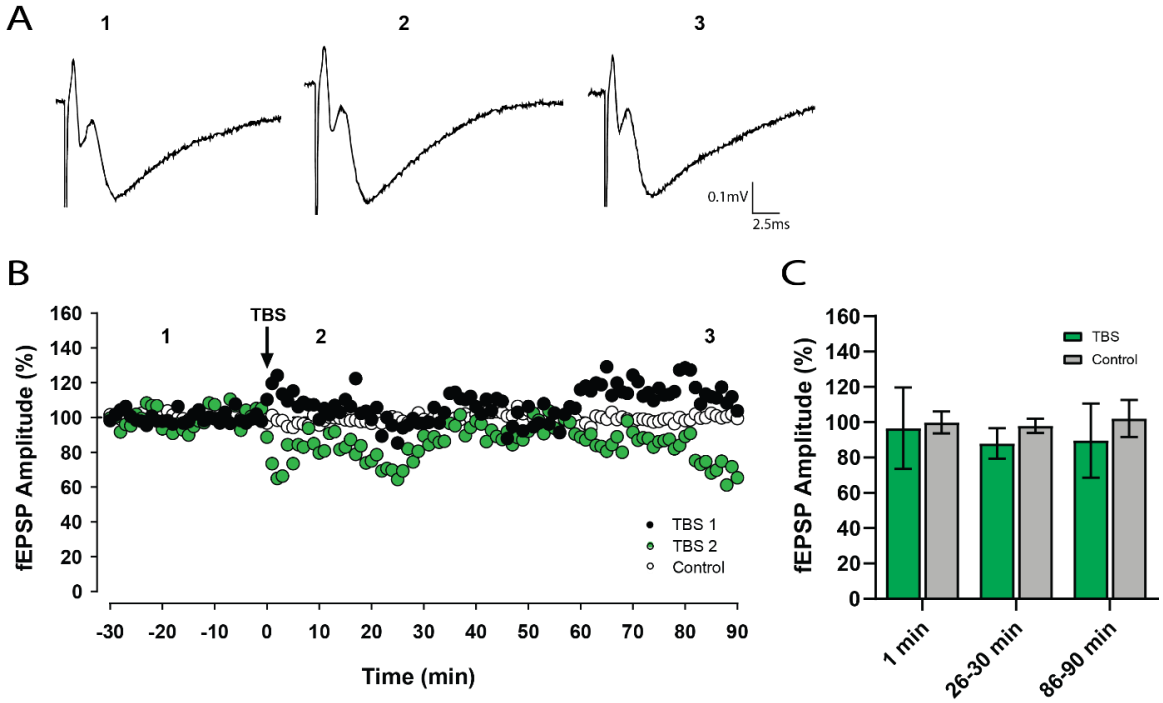

Figure 14. (A) Sample traces collected from the time points denoted in B. (B) Graphical presentation of data collected from high calcium ACSF TBS experiments in which all recording took place in high calcium ACSF (n=2). (C) Data was then compared to time matched control data using an RM ANOVA at the 1 minute, 26-30, and 86-90-minute time periods. High calcium ACSF also appears unsuccessful in inducing LTP with the TBS protocol, however more replicates are required to verify this conclusion.

The same high calcium assay used in figure 9 was also applied to the theta burst method of LTP induction. TBS had previously been unsuccessful in inducing any form of potentiation, either short or long term. At this stage, the aim was to assess whether a specific physiological

state, such as the levels of extracellular calcium, was required for LTP to occur in rat lateral entorhinal cortex. Figure 14 shows the results of the TBS protocol in the presence of high calcium ACSF, few replicates were collected from this assay as I found that the HFS protocol was more successful in producing a potentiative effect during these experiments. As few replicates were collected from this condition no statistical analysis was carried out, however general trends show no clear effect of high calcium in the ability of TBS to induce LTP. Although there is a high level of variation in the response amplitude, specifically, between 0 and 10 minutes, and 60-90 minutes, this effect is not due to the presence of picrotoxin, as the drug was present during the baseline period from -30 to 0 minutes. In fact, the baseline period shown in 13B is relatively flat and stable throughout, especially for only two replicates, it is therefore possible that theta burst stimulation is having some effect on the slice, affecting the stability of the recording. One would expect that, had there been no effect, then the data would be synonymous with that of control data. Further replicates would be required here to fully quantify the effect, if any, that picrotoxin is having during LTP induction by TBS, however these 2 replicates support the theory that picrotoxin has no effect on the efficacy of TBS in inducing LTP.

The methods used when aiming to induce LTP in rat lateral entorhinal cortex proved unsuccessful. Despite attempting two well documented LTP protocols in the form of TBS and HFS, these protocols unable to produce any potentiation within in vitro rat brain slices which could be categorised as long term. Successful methods came in the form of manipulating the conditions in which recording occurred, either by using high calcium ACSF or picrotoxin. In the case of HFS in the presence of high calcium ACSF 4 of the 9 total replicates successfully induced LTP which lasted 90 minutes. It is possible, therefore, that if further replicates are collected and with some refinement of the method, LTP can be induced in lateral entorhinal cortex, however not under physiological conditions. One factor which was not highly controlled

during these experiments was the age of rats used, although all brains were taken from rats aged between 14 and 21 days, further experiments may prove that the ability to induce LTP is age dependent. Notably the results of this chapter indicate that HFS was the more efficient method for LTP induction at least within this area. Experiments involving theta-burst stimulation showed no signs of potentiation, even under high calcium conditions. In the presence of picrotoxin (12B) TBS seemed to cause a 20-minute potentiation of synaptic response post-tetanus however this effect did not last, as verified by the results of the RM ANOVA (12C). It is apparent that an interaction between the lateral entorhinal cortex and LTP is occurring, and that changing external conditions can contribute to possibly inducing a long-term potentiation of a response, however further experimentation and more factors must be considered in order to fully explain the effect.

6. Discussion

6.1 Dopamine modulates activity dependent synaptic plasticity in lateral entorhinal cortex

The overall aim of this study was to analyse the dopaminergic modulation of activity dependent synaptic plasticity, with particular focus on dopamine's interaction with LTD. Early experiments concerned the direct effect that dopamine has on synaptic responses in rat lateral entorhinal cortex, particularly the apparent block of plasticity detailed in Caruana et. Al's 2006 paper. Dopamine was found to have bidirectional concentration dependent effects on entorhinal cortex field responses (Caruana et al., 2006). Here, the suppressive effect of dopamine was analysed as well as its interaction with LTD. The suppressive effect shown in figure 5B is similar to the effect depicted in Caruana et. Al's paper in which 100uM dopamine exhibited a peak suppression of fEPSP amplitude to 57.2 ± 6.1%, meaning that 100uM dopamine as able to suppress baseline fEPSP amplitudes by 42.8%. Compare this result to the

results collected in this study, the suppressive effect of dopamine at this concentration is consistent as results here indicate a 52.84 ± 6.1% suppression of synaptic responses. An apparent difference, however, is that within Caruana et. al's results the potentiation of synaptic response following dopamine wash-out occurs after a much shorter time frame (25 minutes post dopamine application period), 100uM dopamine caused an increase in fEPSP amplitude to 123.3 ± 6.1% at the end of the 65-minute recording period.

Caruana et. al (2008) found that co-application of the D1 receptor antagonist SCH23390 did not significantly reduce the dopamine-mediated suppression of fEPSP amplitude by 50μM dopamine, however the antagonist did block the potentiation rebound period in the last 10 minutes of recording. The D2 receptor antagonist Sulpride, however, caused a block of dopaminergic suppression of synaptic responses caused by 100uM dopamine. Continuation of this research should aim to apply the same D1 and D2 receptor antagonists to quantify this effect myself, as well as co-apply antagonists during experiments which concern the induction of LTD paired with dopamine application. One can infer from these results that the mechanism by which dopamine suppresses synaptic responses is D2 receptor mediated as only the D2 receptor antagonist Sulpride was able to block the dopaminergic effect, however these results only apply to the interaction of dopamine at high concentrations, and do not concern lower concentration applications which have been shown to cause a potentiation of fEPSPs. It is more likely that dopamine's interaction with either D1 or D2 receptors is dose dependent.

The experiment in Figure 5C involved inducing LTD using a Paired Pulse low frequency stimulation method (PP-LFS) and then applying 100uM dopamine as a follow up to LTD being induced. The aim here was to assess whether the mechanism underlying LTD induction would, in some way, modulate the suppressive ability of dopamine at a known concentration that

had previously been quantified. As the suppressive value of dopamine in 4B was tested one would then be able to compare the peak suppression of 100uM DA as a follow up to LTD. The notable result from this experiment is that the application of dopamine after prior activity-dependent LTD has been induced causes the dopamine-depressed responses to return to lower than the depressed level. This effect is more likely due to the reversible opening of potassium channels following LTD induction preventing the repolarization of the synapse during dopamine application.

To understand the interaction occurring it is important to first understand the mechanism underlying plasticity in neuronal circuits. LTD is induced by repeated activation of the synapse both postsynaptically and presynaptically through low frequency stimulation. It is widely accepted that it is the volume of calcium influx through NMDA receptors which causes LTD induction reaction by the postsynaptic cell (Yang, Tang and Zucker, 1999). Weak or low frequency activation presynaptically causes a low calcium influx which is interpreted postsynaptically by preferential activation of phosphatases which lead to the dephosphorylation of AMPA receptors and eventual endocytosis. It is this cellular signalling cascade which leads to an overall decrease in AMPA receptors postsynaptically and dampening of the synaptic response (Luscher and Malenka, 2012). The interaction between dopamine and plasticity comes in the form of dopamine's ability to inhibit glutamatergic and GABAergic transmission by D1 receptor mediation (Law-Tho et al., 1994), however research surrounding the interaction of the two dopamine receptors suggests that D2 receptors also play a role in LTD induction (Chen et al., 1996). Law-Tho et al. (1994) found that bath application of 50-100uM dopamine lead to a decrease in EPSP amplitude in layers I or VI of the rat prefrontal cortex, with a particularly strong effect on the EPSP caused by glutamate receptors. We can infer from this that high concentrations of dopamine are causing a

suppression of synaptic response by an attenuation of glutamate release. However, results from the study also indicate that the D2 receptor is capable of modulating LTD induction, further evidencing that the exact receptor via which LTD is induced is as yet unknown, however these results suggest NMDA is responsible for this mechanism.

The ability of dopamine to block the induction of long-term depression is a phenomenon that has been previously explored. Caruana et. al (2007) utilised the dopamine reuptake inhibitor GBR12909 in awake rats and found that they were unable to induce LTD or LTP in the presence of the reuptake inhibitor. The presence of a dopamine reuptake inhibitor such as GBR12909 would cause an excess of dopamine within the synaptic cleft. In the midbrain, LTD induction has been found to be blocked by activation of D2-like receptors (Thomas et al., 2000), these D2 receptors lead to an inhibition of calcium currents postsynaptically and, as described above, it is the constant low volume influx of calcium ions which causes the downstream cascade which eventually leads to a decrease in AMPA receptors and, therefore, LTD of synaptic response. Figure 6B shows LTD induction in lateral entorhinal cortex in vitro, leading to an overall depression of fEPSP amplitude which continued to the end of recording. Following from this, figure 6C shows the result of bath applying 30uM dopamine onto the slice preceding and during PP-LFS, the net result was a significant block of the depressive effect during the last 5 minutes of the experiment compared to PP-LFS alone, meaning that 30μM dopamine was sufficient in causing a block of LTD. Further experiments could be performed with a more refined series of dopamine concentrations below 30μM to examine the exact concentration at which LTD is blocked, as it is possible that 30μM is a higher concentration as is required to block LTD induction by PP-LFS.

LTD, as well as LTP, are known to contribute to the encoding of memory, acting at specific synapses to increase the strength of connections and thus affect the storage of sensory information in the form of memories (Takeuchi et. Al, 2014). Dopamine may act as a catalyst for learning during times of reward, for example eating, and this would assist in heightening the learning process to remember how this stimulus was received. To put it evolutionarily, stimuli which result in our survival, such as eating or finding a mate, would have higher priority within learning and memory encoding due to the release of dopamine, it is possible that dopamine blocks LTD within the entorhinal cortex in an effort to prevent the processing of important stimuli being dampened by LTD being induced as a result of repeat activation of the same synapse. Linking back to the anatomy of the entorhinal cortex and its position within the brain, the dopaminergic input to the entorhinal cortex as well its output and interface to the hippocampus mean that any dopaminergic modulation within the region would be beneficial to the storage and contextualisation of memory.

A key focus of the LTD experiments was not only the interaction between dopamine application and LTD induction but also the interaction between multiple dopamine applications, and also the interaction between dopamine-mediated and activity-dependent LTD, showing that the two forms can exist within the same synapses and only affect each other if they coincide temporarily. Experiments carried out in figure 7 included a double application of dopamine following the induction of LTD. Other research carried out alongside my own within the laboratory has shown that successive applications of the same dopamine concentration lead to a reduction in dopamine's suppressive ability during the second application, what appeared to be some form of dopaminergic plasticity. In figure 7B I attempted to examine the effect of a prior induction of LTD would have on this effect, in order to analyse whether the two effects were independent of each other. Initially results suggested

that induction of LTD by PP-LFS would block the observed interaction between successive dopamine applications, meaning both dopamine applications would cause the same peak suppression and the results of the peak suppression analysis show that there is no significant difference between the peak suppression of DA application 1 and 2. However, when analysing the area under the curve of the two application periods, it was shown that there was a significant difference between the two applications meaning that the initial dopamine application was affecting the second application. Figure 7C shows the dopamine application periods for both dopamine applications, from the data presented it would seem as though the second dopamine application has a much faster return to baseline compared to DA 1; DA 2 returns to baseline levels within 30 minutes of the end of application, whereas DA 1 returns to baseline within 60 minutes, it is possible that this difference causes the significant difference in the AUC analysis.

Looking at recent papers surrounding the potential role of dopamine in the entorhinal cortex gives some insight into the mechanism underlying dopamine's effects not only on LTD but also the lasting desensitisation of dopamine receptor function triggered by multiple exposures to dopamine. Theories have arisen that dopamine causes an increase in release of stored calcium by activation of D1 receptors (Glovaci and Chapman, 2019). A combination of electrophysiological recording and fluorescent imaging showed that bath application of either dopamine or the phosphatidylinositol linked D1-like receptor agonist SKF83959 caused an increase in fluorescence and postsynaptic activity in fan cells of the entorhinal cortex. Further evidence of the underlying mechanism of this effect comes from the fact that the classical D1-like receptor agonist SKF38939 did not cause any increase in fluorescence, similarly a block of the IP3 receptor or ryanodine receptor blocked the increase in calcium release as well as the facilitation of synaptic response typically caused by bath application of dopamine. One can

conclude from these results that it is the activation of IP3 and ryanodine receptors by dopamine which causes the release of calcium from internal stores and, therefore, the increase in synaptic current at low concentration. I have previously explained and given evidence to attest to the fact that dopamine's effect on synaptic response in lateral entorhinal cortex is bilateral, meaning that at high concentration dopamine causes a suppression of fEPSP amplitude, whereas at low concentrations a potentiation is present. Caruana et. Al (2006) concluded that dopamine exhibits bidirectional effects on synaptic response within layer II of the entorhinal cortex and that the activation of either D1 or D2 receptors was dose dependent. The experiments involving low dopamine concentration of 10μM were concluded to be D1 receptor dependent, whereas suppression of synaptic responses by 100μM dopamine were found to be D2 receptor dependent. The activation of D1 receptors by dopamine causes a G-protein mediated activation of adenylate cyclase, leading to the activation of PKA and eventual cAMP production and, therefore, calcium channel activation and calcium release into the synapse. D2 receptor activation by dopamine causes the inverse of this, inhibiting adenylate cyclase activity and cAMP production, leading to a block of calcium release into the synapse (Boyd and Mailman, 2012). The exact mechanism explaining the dose-dependent activation of either D1 or D2 receptors is unknown, however it is likely due to the varying affinity of either receptor for dopamine binding.

Present theories surrounding the mechanism of dopaminergic suppression of synaptic response in entorhinal cortex is that dopamine causes an inhibition of presynaptic glutamate release mediated by the D2 receptor (Caruana and Chapman, 2008). Other evidence suggests that the suppression of synaptic response is D1 mediated (Pralong and Jones, 1993), however it is more likely that dopamine is acting on both D1 and D2 receptors, dependent on concentration. The phenomenon of sequential DA applications causing exponential decrease

in fEPSP suppression is likely explained by the inhibition of glutamate release, the initial effect on D1 and D2 receptors appears to be dampened during the second application, serving as a form of dopaminergic plasticity or encoding. This could present evidence to the fact that LTD is controlled largely postsynaptically whereas dopaminergic effects are largely presynaptic (Caruana and Chapman, 2008) acting to inhibit glutamate transmission presynaptically whereas activity dependent synaptic plasticity would act postsynaptically to reduce the conductance and amount of AMPA receptors, further supporting the observation that dopamine-mediated LTD and activity-dependent LTD are independent of each other and are capable of coexisting within the same synapse.

The dampening down of responses following continued stimulation is often attributed to G-protein desensitisation, this phenomenon provides a possible explanation for the results shown, in which repeated dopamine applications of the same concentration lead to a dampening down of the dopaminergic suppression. The underlying mechanism of G-protein desensitisation occurs as a result of activation by a receptor agonist, such as dopamine. Upon activation G protein coupled receptors (GPCRs) are phosphorylated by GPCR kinases, it is the phosphorylation of the receptor by kinases which increases their affinity for arrestin, the eventual binding of arrestin leads to G protein deactivation as well as internalisation of the receptor itself (Gurevich et al., 2016). This would support the results shown here, as the second dopamine application yields a far lower decrease in fEPSP amplitude, likely due to the internalisation of the receptor mediating the dopamine-dependent depression.

This experiment could be improved by having greater control over which experiments were allowed to continue and be analysed, in many experiments the level of fEPSP depression in the 85-90 minute period was far greater than other replicates, and it is possible that the

degree to which the slice has undergone LTD may cause some variance in the effective block. By only accepting slices which depress a certain percentage, 15% for example, then any variation caused by a less effective LTD will be mitigated. The length of experiments, and therefore the stability of recordings, may also have affected results, one may look to replicate these experiments and aim to reduce the recording time with the intention of improving stability of recording for the entirety.

The blockage of LTD using AP5 (figure 8) was effective, reducing the level of fEPSP depression in the last 5 minutes of LTD induction prior to dopamine application. This experiment was carried out in order to analyse whether blocking the induction of LTD had any effect on the double application of 100uM dopamine. In the case of the AP5 experiments, there was found to be a significant difference in the peak suppression of dopamine, contrasting the results of figure 7. Based on these results alone it would appear as though blocking LTD returns the original phenomenon in which DA application 2 exhibits a lower fEPSP suppression (%) than DA application 1. The significant difference in this condition is the area under the curve analysis which, like figure 7, shows that there is a significant difference between the AUC of DA 1 and DA 2. Although the 30 minutes pre-dopamine application were re-normalized to baseline levels for the AUC analysis, DA 2 still has some run-up in its baseline period, unlike DA 1. Comparing the application periods between the AP5 and non-AP5 conditions it is possible that the run-up in the baseline of DA 2 is caused by the fact that the second dopamine application period occurs before the washout of DA 1 had managed to plateau. In figure 7 the fEPSP amplitude reaches a plateau point 30 minutes after application, whereas in Figure 7B the amplitude appears to be continuing to return to baseline levels when DA 2 is applied. There may be some differences in the onset of the application and desensitisation of dopamine occurring and this effect may be due to either AP5 or the block of LTD. This effect

could be analysed further by extending the washout period from 60 minutes to 90 minutes, so that the response is allowed to fully return to “baseline”. It is well understood that LTD is NMDAR mediated, the activation of NMDA receptors causes the activation of their AP2 clathrin adaptor complex responsible for the internalisation of AMPA receptors (Lee et al., 2002). This experiment supports this theory in that blockade of the NMDA receptor by AP5 causes a block of LTD, however results are inconclusive as to the effect that LTD, and the block of LTD, has on dopaminergic plasticity. It is possible that there is some form of metaplasticity occurring, meaning that the dopamine-mediated mechanism and activity-mediated mechanism are interacting however the experiments conducted here were unable to provide any evidence to support this. Perhaps, due to the fact that some replicates exhibited a clear block of dopaminergic plasticity, the effect is age-dependent or dependent on the suppression caused by LTD in the first instance.

6.2 Modulation of activity dependent LTP

The data presented concerning LTP induction provides some points of interest, particularly in the apparent inability of the lateral entorhinal cortex to undergo LTP. This research utilised two methods of LTP induction which were well documented in inducing LTP in other brain regions, these being high frequency stimulation and theta burst stimulation (Grover et al., 2009; Volianskis et al., 2013). The intended aim of separating the methods this way and performing all conditions using two protocols allowed for better analyses of each protocol, as well as the ability to cover more possible induction methods. In future experimentation one should seek to further separate these methods into sub-groups, changing intervals for example in TBS protocols, would allow for a deeper analysis of LTP within lateral entorhinal cortex, it also decreases the likelihood that any observed inability to induce LTP is not due to using an incorrect, or poorly tuned, protocol. As described previously, other researchers have

induced LTP in brain regions with ease using either TBS or HFS (Yaniv et al., 2003), however it is notable that very little evidence exists of LTP induction in the lateral entorhinal cortex and, in the case of Yaniv et al's 2003 paper, entorhinal stimulation was used to induce LTP in either the amygdala or hippocampus. The methods used here attempted to induce and record long term potentiation of fEPSPs, using both HFS and TBS protocols, as well as altering bath conditions in order to successfully induce LTP.

Initial LTP experiments involved testing both protocols in typical, physiological conditions in 2-hour long experiments. This allowed for ample recording time post tetanisation, as well as for a 30-minute baseline period to ensure the stability of the response. Figure 9 shows the results of the HFS protocol in which no potentiation was induced. The TBS equivalent of this experiment, figure 12, shows the effect that theta burst stimulation had on fEPSP amplitude, and this method was less effective than the HFS method in causing any effect on fEPSP amplitude. HFS alone was able to induce a suppression of 15% in the 1-minute post-tetanisation which returned to baseline before the 10-minute time point. It was clear from this experiment alone that the HFS protocol was, at the very least, having some effect on synaptic responses although it did not cause potentiation.

These two protocols were then applied under different bath conditions, these conditions being the application of GABA antagonist picrotoxin as well as increasing the concentration of calcium in ACSF to 10mM; the effect of picrotoxin on HFS can be seen in figure 11. This experiment consists of a relatively low number of replicates (n=3) and it is likely that the high run down seen in this figure following HFS is due to an unstable recording, 2 of the 3 replicates showed no effect following HFS, not even the 15% suppression seen in the initial HFS experiment. The TBS equivalent experiment (Figure 12) exhibited a 5.9% increase in fEPSP

amplitude at the 1-minute time point compared to control data although this difference was found to be non-significant. Picrotoxin was used in these experiments as it was theorised that LTP induction may have been blocked due to feedback or feedforward inhibition within the slice (Finch et al., 1988), and this inhibition could be blocked through application of a GABA antagonist such as picrotoxin (Kleschevnikov, 2004). The mechanism underlying feedforward inhibition dictates that excitation of afferent excitatory neurons within the EC leads to the eventual excitation of inhibitory interneurons which, in turn, inhibit the activity of other excitatory neurons. Feedback inhibition involves the excitation of inhibitory interneurons by principal neurons, these inhibitory interneurons then synapse back to principal neurons and inhibit them. These experiments appear to show that the block of LTP induction is not solely due to the presence of feedforward or feedback inhibition, as block of inhibition by application of picrotoxin was insufficient in alleviating the apparent LTP block exhibited by the lateral entorhinal cortex in these experiments.

As previously explained when discussing the results of LTD experiments; the mediating factor in LTD or LTP induction is the volume of calcium influx. Therefore, the next group of experiments concerned the effect that an increase in calcium concentration in bath applied ACSF would have on the efficacy of each LTP induction protocol. From these data it seems as though increasing calcium concentration to 10mM has the greatest effect on the ability of HFS to induce LTP. Figure 10 shows that applying high calcium HFS to the slice throughout the experiment caused an increase in fEPSP amplitude at the 1-minute time period post tetanisation. Although results of the RM ANOVA suggest that this result is not statistically significant at the 0.05 level, this does display a positive result for potentiation of the fEPSP amplitude. Further evidence that high calcium ACSF contributes to an increase in efficacy of HFS's ability to induce LTP is that 4 of the 9 replicates collected displayed a potentiation of

synaptic response which continued until the end of the recording, meaning that LTP was successfully induced. No other experimental assay had induced potentiation to this degree of efficiency prior, and no other assay had successfully induced long-term potentiation. Although this may be the case, it is important to consider the entire data set when conduction analysis of this nature, a protocol cannot be considered truly successful at an efficiency of 44%. Interestingly, high calcium ACSF did not lead to a similar result when combined with the TBS protocol, however this condition included a very low number of replicates (n=2) which likely contribute to the high variance in these results, more replicates would need to be collected for this condition to fully analyse the effect, but from these experiments alone one would infer that HFS is likely to be the more efficient LTP induction protocol as HFS consistently delivered some effect on fEPSP amplitude.

These results suggest that LTP induction is not intrinsically possible within the lateral entorhinal cortex and that some modification of synaptic conditions is required. Yang et al. (1999) have conducted research which shows that LTP induction was possible by a brief, but high magnitude, increase in calcium. This mimics the calcium increase which would typically occur during electrical stimulation which causes LTP. When comparing the method used within this research to that of Yang et al. it is possible that applying high calcium ACSF throughout the experiment prevented any potentiation occurring as the physiological increase in calcium was masked by the high concentration of calcium in the recording bath. In future research the method could be modified such that high calcium ACSF would be applied in a short burst during tetanisation, although the application of calcium may mask the effect that tetanisation is having on the slice or make it difficult to differentiate between the calcium effect and tetanisation effect. It is surprising that LTD induction occurred with such ease within lateral entorhinal cortex, whereas LTP induction required particular conditions when

recording, it has been recorded previously that facilitation is possible within entorhinal cortex by application of low dopamine concentrations *in vivo* (Caruana et al., 2006) and perhaps the entorhinal cortex favours dopamine-dependent potentiation as a form of memory encoding, as opposed to activity dependence. The method of LTP induction used here are well documented as being favourable protocols in inducing LTP in other areas of the entorhinal cortex, such as the medial entorhinal cortex (Yun et al., 2002), however such research also suggest that TBS was most effective in inducing LTP in superficial layers of the entorhinal cortex, which contradicts the results collected. Further testing of methods would be required to fully understand the mechanism occurring, such as repeating the stimulation multiple times in order to cause potentiation.

Although the results of this study are inconclusive as to the optimal method for LTP induction in rat lateral entorhinal cortex, there are a few likely assays which could be performed in the event of successful LTP induction. These protocols would aim to assess the nature of the LTP mechanism within the lateral entorhinal cortex, as well as explore the metaplastic relationship between dopamine application and LTP. Firstly, bath application of an NMDA receptor antagonist such as AP5. As Morris showed in 1989, AP5 blocks the induction of LTP as well as reducing spatial learning in vivo, further research would aim to replicate such an experimental assay had LTP been successful, this would help to understand the mechanism of LTP induction within the lateral entorhinal cortex.

A method which may perhaps enhance the efficacy of both TBS and HFS would be to combine both high calcium ACSF and low concentration dopamine within the recording bath. Further research could apply picrotoxin throughout the experiment with a 15-minute dopamine application occurring during the tetanisation protocol. The reasoning behind this experiment

is that the work of Caruana et. al (2006) found that low dopamine concentrations, such as 10uM, caused a potentiation of the synaptic response of 119.3 ± 3.9% of baseline recording. Another possible experiment would aim to assess whether dopamine is capable of modifying the potentiative effect caused by a tetanisation protocol by pre-applying dopamine in low concentration to the slice. This work could be furthered by the use of dopamine transporter antagonists such as GBR 12935, D3 receptor antagonist U 99,194, and D1/D5 specific receptor antagonist SCH 23390 for example (Swant, 2006). In the case of Swant's findings, U 99194 was cable of blocking the DAT antagonist induced LTP, whereas SCH was not, future assays would not only aim to verify this effect but also combine the DAT antagonist induced LTP, as well as low concentration dopamine LTP with activity dependent LTP, analysing whether the induction of activity dependent LTP and drug-dependent LTP are independent of each other, or if some form of metaplasticity may occur.

Throughout research, the age of rats used was limited to between 14 and 21 days, for future research rats would be grouped based on age more discriminately with the aim of assessing whether the ability to induce LTP within the entorhinal cortex by HFS and TBS is age dependent. This would be tested by not only categorising p14-21 rats by age, but also expanding research to much older rats. Experiments could then be carried out across a variety of age groups and the efficacy of LTP induction methods could be compared. The use of a positive control would also be advantageous in this situation, for example successfully inducing LTP within the hippocampus utilising the same protocols as were used here would verify that it is the entorhinal cortex which is resistant to LTP induction, and the results depicted here are not due to an ineffective protocol or other conditions of the experiment. A positive control would, therefore, verify the conclusion that the entorhinal cortex is resistant to LTP induction by HFS or TBS.

The final point of expansion for this research in the future would be to utilise various rat models of disease, such as depression, and Alzheimer's. This would improve the ability of the research to be applied to pharmacological research surrounding these conditions, as well as give some insight into the role of dopamine deficiency within such conditions and activity dependent synaptic plasticity. The Flinders Sensitive Line (FSL) genetic rat model of depression has shown cognitive impairment similar to depressed humans, this model could be used to examine the effect of depression on activity dependent synaptic plasticity. Experiments using this genetic rat line could be pivotal in explaining the cognitive learning deficits witnessed by past researchers (Overstreet, 1993), and how synaptic plasticity in the entorhinal cortex plays a role in this cognitive deficiency.

7. Implications

A large and overbearing issue within modern society is the prevalence of depression, around 300 million people suffer from depression globally according to the world health organisation. It is, therefore, vital that novel treatments are studied and tested, as well as current treatments refined. A common symptom of those with major depressive disorder (MDD) is anhedonia, a loss of sensation of pleasure for activities which would previously be enjoyable to the person (Rizvi et al., 2016). Anhedonia is not only a symptom of depression, but also anxiety, stress, schizophrenia, and substance abuse, all of which are pressing issues and conditions which require precise and effective treatment, and anhedonia is believed to affect the appetitive reward system, specifically the anticipation and consumption of said reward (Craske et al., 2016). It is also believed that there is a disconnect in MDD between the anticipation of reward and the motivation to expend effort to receive reward (Sherdell et al., 2012). It is possible that within MDD the entorhinal cortex is contributing to the anhedonia

witnessed, particularly the link between memory and reward anticipation. In Sherdell et al.'s 2012 study, participants were asked to look at cartoons which were either humorous or non-humorous, representing a reward and non-reward respectively, participants would then have to exert some degree of effort in order to receive the reward stimuli, and those in depressed condition did not favour performing a task to receive the reward. This finding concluded that the level of preference towards a reward is not reduced, but rather the anticipation of the reward. The findings of this study suggest that there is a possible link between the entorhinal cortex and its dopamine-dependent plasticity and the lack of reward-seeking behaviour in depressed individuals. The anatomy of the entorhinal cortex and the large dopaminergic input to the region suggest that a dopaminergic deficiency, as seen in depressed individuals, would lead to a change in memory encoding which would make previously rewarding stimuli unfavourable, as is the case in anhedonia.

This links heavily to the findings presented in this study, in which dopaminergic release within the lateral entorhinal cortex mediates a block of LTD, and further evidences the bidirectional effects of dopamine within the region (Caruana et al., 2006). It is possible that the anhedonia symptom of depression is caused by a lack of dopaminergic regulation of the entorhinal cortex, that the lack of dopamine means that high concentrations of dopamine are not available to block LTD induction, meaning that no sensory input may be prioritised in terms of memory formation. As anhedonia is categorised as a sensory deficit, leading to a lack of desire to fulfil activities which were previously rewarding to the individual. As the entorhinal cortex serves as a point of convergence for sensory information, as well as an interface region between said sensory information and the hippocampus an inference can be made that the dopamine-dependent modulation of synaptic plasticity within the entorhinal cortex is dampening the appetitive value of memories formed and stored within the hippocampus. The

findings presented suggest that the dopaminergic deficiency exhibited in MDD sufferers leads to an inability to modulate LTD within the entorhinal cortex, causing an eventual dampening of sensory information by LTD within the region.

Research suggests that the dopaminergic modulation of activity dependent synaptic plasticity is an innate encoding mechanism employed by the entorhinal cortex, a method of “tagging” inputs as high priority, preventing any LTD occurring. Referring to MDD sufferers, it is likely that this tag is deficient or not present at all, meaning reward-based learning is lacking, particularly with regard to appetitive reward. It is also likely that a lack of dopamine within the entorhinal cortex would mean that the low concentration effects of dopamine would not occur, lacking a potentiation of synaptic response.

8. Conclusion

The data collected not only provides evidence to support the previous research of Caruana et. al, but also provides new insights into the ability of dopamine to block LTD induction at high concentration. These results show that prior exposure to activity dependent plasticity, in the form of LTD, does not have any significant effect on the desensitisation of dopamine in multiple concentrations. Not only this, but these results support the evidence that dopamine is capable of blocking LTD in rat lateral entorhinal cortex, thus showing that two forms of plasticity within the region are independent and do not influence one another. The experiments into long-term potentiation within the entorhinal cortex yielded an apparent inability for the EC to support LTP, although application of high calcium ACSF in the case of high frequency stimulation appeared to alleviate this inability to some degree. It is clear from these findings that the relationship between dopamine and activity dependent plasticity requires further investigation, in order to fully understand the mechanism underlying their

interaction in the hopes of developing novel treatments for dopaminergic conditions such as Alzheimer's disease, and depression. These findings also allude to the role of dopamine within the entorhinal cortex as a mediator of memory encoding.

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11. Appendix

Number of families	1							
Number of comparisons per family	3							
Alpha	0.05							
Bonferroni's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
Control vs. LTD	-18.70	-22.46 to -14.94	Yes	****	<0.0001	A-B		
Control vs. LTD+DA	-9.085	-12.84 to -5.325	Yes	****	<0.0001	A-C		
LTD vs. LTD+DA	9.614	5.854 to 13.37	Yes	****	<0.0001	B-C		
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
Control vs. LTD	-0.6357	18.06	-18.70	1.353	5	5	13.82	12
Control vs. LTD+DA	-0.6357	8.449	-9.085	1.353	5	5	6.716	12
LTD vs. LTD+DA	18.06	8.449	9.614	1.353	5	5	7.107	12

Table 1. results of Bonferroni's multiple comparisons test as part of one-way ANOVA from data collected from the average depression (%) from the last 5 minutes of control, LTD, and LTD + 30uM DA data.

Column B	DA Application 2
Column A	DA Application 1
Unpaired t test	
P value	<0.0001
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=7.764, df=10
How big is the difference?	
Mean of column A	2587
Mean of column B	1629
Difference between means (B - A) ± SEM	-958.0 ± 123.4
95% confidence interval	-1233 to -683.1
R squared (eta squared)	0.8577
F test to compare variances	
F, DFn, Dfd	2.625, 5, 5
vP value	0.3131
P value summary	ns
Significantly different (P < 0.05)?	No
Data analyzed	
Sample size, column A	6
Sample size, column B	6

Table 2. Unpaired T test of area under the curve data from DA application period 1 and 2. Presented in figure 7.

Table Analyzed	Area under Curve
Column B	DA 2
vs.	vs.
Column A	DA 1
Unpaired t test	
P value	<0.0001
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=8.449, df=8
How big is the difference?	
Mean of column A	2663
Mean of column B	1804
Difference between means (B - A) ± SEM	-859.0 ± 101.7
95% confidence interval	-1093 to -624.6
R squared (eta squared)	0.8992
F test to compare variances	
F, DFn, Dfd	1.683, 4, 4
P value	0.6265
P value summary	ns
Significantly different (P < 0.05)?	No
Data analyzed	
Sample size, column A	5
Sample size, column B	5

Table 3. Results from unpaired T test of area under the curve analysis comparing DA application 1 and 2 in the AP5 condition. Presented in figure 8.

Table 4. Two-way Repeated measures ANOVA results from HFS data, shown in figure 9.

Two-way RM ANOVA	Matching: Both factors				
Assume sphericity?	Yes				
Alpha	0.05				
Source of Variation	**% of total variation**	**P value**	**P value summary**	**Significant?**	
Time	1.284	0.7484	ns	No	
HFS vs Control	0.02220	0.9339	ns	No	
Time x HFS vs Control	11.30	0.0524	ns	No	
Replicate x Time	21.51				
Replicate x HFS vs Control	14.59				
Replicate	37.23				
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Time	93.46	2	46.73	F (2, 10) = 0.2984	P=0.7484
HFS vs Control	1.616	1	1.616	F (1, 5) = 0.007607	P=0.9339
Time x HFS vs Control	822.9	2	411.5	F (2, 10) = 4.018	P=0.0524
Replicate x Time	1566	10	156.6		
Replicate x HFS vs Control	1062	5	212.5		
Replicate	2711	5	542.3		
Residual	1024	10	102.4		
Difference between column means					
Mean of HFS	97.77				
Mean of Control	97.35				
Difference between means	0.4238				
SE of difference	4.859				
95% CI of difference	-12.07 to 12.91				
Data summary					
Number of columns (HFS vs Control)	2				
Number of rows (Time)	3				
Number of subjects (Replicate)	6				
Number of missing values	0				

Table 5. Tukey's multiple comparisons test performed on HFS data in figure 9.

Number of families	1
Number of comparisons per family	15
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
1 Minute:HFS vs. 1 Minute:Control	-12.65	-32.95 to 7.642	No	ns	0.3300
1 Minute:HFS vs. 30 Minute:HFS	-11.87	-32.17 to 8.420	No	ns	0.3889
1 Minute:HFS vs. 30 Minute:Control	-7.899	-28.19 to 12.39	No	ns	0.7525
1 Minute:HFS vs. 90 Minute:HFS	-14.56	-34.85 to 5.738	No	ns	0.2138
1 Minute:HFS vs. 90 Minute:Control	-4.607	-24.90 to 15.69	No	ns	0.9633
1 Minute:Control vs. 30 Minute:HFS	0.7782	-19.52 to 21.07	No	ns	>0.9999
1 Minute:Control vs. 30 Minute:Control	4.753	-15.54 to 25.05	No	ns	0.9584
1 Minute:Control vs. 90 Minute:HFS	-1.903	-22.20 to 18.39	No	ns	0.9993
1 Minute:Control vs. 90 Minute:Control	8.045	-12.25 to 28.34	No	ns	0.7393
30 Minute:HFS vs. 30 Minute:Control	3.975	-16.32 to 24.27	No	ns	0.9803
30 Minute:HFS vs. 90 Minute:HFS	-2.682	-22.98 to 17.61	No	ns	0.9966
30 Minute:HFS vs. 90 Minute:Control	7.267	-13.03 to 27.56	No	ns	0.8070
30 Minute:Control vs. 90 Minute:HFS	-6.657	-26.95 to 13.64	No	ns	0.8545
30 Minute:Control vs. 90 Minute:Control	3.292	-17.00 to 23.59	No	ns	0.9914
90 Minute:HFS vs. 90 Minute:Control	9.948	-10.35 to 30.24	No	ns	0.5588

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
1 Minute:HFS vs. 1 Minute:Control	88.96	101.6	-12.65	5.843	6	6	3.062	10.00
1 Minute:HFS vs. 30 Minute:HFS	88.96	100.8	-11.87	5.843	6	6	2.874	10.00
1 Minute:HFS vs. 30 Minute:Control	88.96	96.86	-7.899	5.843	6	6	1.912	10.00
1 Minute:HFS vs. 90 Minute:HFS	88.96	103.5	-14.56	5.843	6	6	3.523	10.00
1 Minute:HFS vs. 90 Minute:Control	88.96	93.57	-4.607	5.843	6	6	1.115	10.00
1 Minute:Control vs. 30 Minute:HFS	101.6	100.8	0.7782	5.843	6	6	0.1884	10.00
1 Minute:Control vs. 30 Minute:Control	101.6	96.86	4.753	5.843	6	6	1.150	10.00
1 Minute:Control vs. 90 Minute:HFS	101.6	103.5	-1.903	5.843	6	6	0.4607	10.00
1 Minute:Control vs. 90 Minute:Control	101.6	93.57	8.045	5.843	6	6	1.947	10.00
30 Minute:HFS vs. 30 Minute:Control	100.8	96.86	3.975	5.843	6	6	0.9621	10.00
30 Minute:HFS vs. 90 Minute:HFS	100.8	103.5	-2.682	5.843	6	6	0.6491	10.00
30 Minute:HFS vs. 90 Minute:Control	100.8	93.57	7.267	5.843	6	6	1.759	10.00
30 Minute:Control vs. 90 Minute:HFS	96.86	103.5	-6.657	5.843	6	6	1.611	10.00
30 Minute:Control vs. 90 Minute:Control	96.86	93.57	3.292	5.843	6	6	0.7967	10.00
90 Minute:HFS vs. 90 Minute:Control	103.5	93.57	9.948	5.843	6	6	2.408	10.00

Table 6. Results of two-way Repeated measures ANOVA of 10mM Calcium ACSF data shown in figure 10.

Table Analyzed	Group x Time RM ANOVA Example				
Two-way RM ANOVA	Matching: Both factors				
Assume sphericity?	No				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	Geisser-Greenhouse's epsilon
Time	10.59	<0.0001	****	Yes	0.7295
Group	3.053	0.4859	ns	No	1.000
Time x Group	6.143	0.0484	*	Yes	0.7651
Slice x Time	2.449				
Slice x Group	45.76				
Slice	20.40				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Time	1128	2	563.8	F (1.459, 11.67) = 34.60	P<0.0001
Group	325.0	1	325.0	F (1.000, 8.000) = 0.5338	P=0.4859
Time x Group	654.0	2	327.0	F (1.530, 12.24) = 4.234	P=0.0484
Slice x Time	260.7	16	16.30		
Slice x Group	4872	8	608.9		
Slice	2172	8	271.5		
Residual	1236	16	77.24		
Difference between column means					
Mean of HFS	106.9				
Mean of Control	102.0				
Difference between means	4.907				
SE of difference	6.716				
95% CI of difference	-10.58 to 20.39				
Data summary					
Number of columns (Group)	2				
Number of rows (Time)	3				
Number of subjects (Slice)	9				
Number of missing values	0				

Table 7. results of a Tukey's multiple comparisons test performed on 10mM calcium ACSF HFS data.

Number of families	1							
Number of comparisons per family	15							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
1 min:HFS vs. 1 min:Control	13.88	-4.909 to 32.66	No	ns	0.1774			
1 min:HFS vs. 26-30 min:HFS	12.42	1.499 to 23.35	Yes	*	0.0260			
1 min:HFS vs. 26-30 min:Control	16.36	-2.948 to 35.66	No	ns	0.1051			
1 min:HFS vs. 86-90 min:HFS	19.44	6.947 to 31.94	Yes	**	0.0041			
1 min:HFS vs. 86-90 min:Control	16.35	-13.59 to 46.30	No	ns	0.4184			
1 min:Control vs. 26-30 min:HFS	-1.453	-20.47 to 17.56	No	ns	0.9997			
1 min:Control vs. 26-30 min:Control	2.482	-5.276 to 10.24	No	ns	0.8397			
1 min:Control vs. 86-90 min:HFS	5.566	-20.23 to 31.36	No	ns	0.9620			
1 min:Control vs. 86-90 min:Control	2.475	-12.94 to 17.89	No	ns	0.9892			
26-30 min:HFS vs. 26-30 min:Control	3.935	-16.80 to 24.67	No	ns	0.9776			
26-30 min:HFS vs. 86-90 min:HFS	7.019	-3.405 to 17.44	No	ns	0.2408			
26-30 min:HFS vs. 86-90 min:Control	3.928	-26.68 to 34.54	No	ns	0.9961			
26-30 min:Control vs. 86-90 min:HFS	3.084	-24.37 to 30.54	No	ns	0.9979			
26-30 min:Control vs. 86-90 min:Control	-0.006461	-12.28 to 12.27	No	ns	>0.9999			
86-90 min:HFS vs. 86-90 min:Control	-3.090	-41.59 to 35.40	No	ns	0.9996			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
1 min:HFS vs. 1 min:Control	117.5	103.7	13.88	5.141	9	9	3.817	8.000
1 min:HFS vs. 26-30 min:HFS	117.5	105.1	12.42	2.990	9	9	5.876	8.000
1 min:HFS vs. 26-30 min:Control	117.5	101.2	16.36	5.284	9	9	4.378	8.000
1 min:HFS vs. 86-90 min:HFS	117.5	98.09	19.44	3.420	9	9	8.040	8.000
1 min:HFS vs. 86-90 min:Control	117.5	101.2	16.35	8.195	9	9	2.822	8.000
1 min:Control vs. 26-30 min:HFS	103.7	105.1	-1.453	5.205	9	9	0.3948	8.000
1 min:Control vs. 26-30 min:Control	103.7	101.2	2.482	2.123	9	9	1.653	8.000
1 min:Control vs. 86-90 min:HFS	103.7	98.09	5.566	7.061	9	9	1.115	8.000
1 min:Control vs. 86-90 min:Control	103.7	101.2	2.475	4.219	9	9	0.8298	8.000
26-30 min:HFS vs. 26-30 min:Control	105.1	101.2	3.935	5.676	9	9	0.9804	8.000

26-30 min:HFS vs. 86-90 min:HFS	105.1	98.09	7.019	2.853	9	9	3.479	8.000
26-30 min:HFS vs. 86-90 min:Control	105.1	101.2	3.928	8.377	9	9	0.6632	8.000
26-30 min:Control vs. 86-90 min:HFS	101.2	98.09	3.084	7.515	9	9	0.5803	8.000
26-30 min:Control vs. 86-90 min:Control	101.2	101.2	-0.006461	3.359	9	9	0.002720	8.000
86-90 min:HFS vs. 86-90 min:Control	98.09	101.2	-3.090	10.54	9	9	0.4148	8.000

Table 8. Two-way repeated measures ANOVA results from Picrotoxin HFS data, shown in figure 11.

Assume sphericity?	No				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	Geisser-Greenhouse's epsilon
Row Factor	18.45	0.0846	ns	No	0.5040
Group	20.55	0.3276	ns	No	1.000
Row Factor x Group	19.49	0.0601	ns	No	0.6974
Slice x Row Factor	3.606				
Slice x Group	24.90				
Slice	8.794				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Row Factor	455.8	2	227.9	F (1.008, 2.016) = 10.23	P=0.0846
Group	507.9	1	507.9	F (1.000, 2.000) = 1.651	P=0.3276
Row Factor x Group	481.5	2	240.8	F (1.395, 2.790) = 9.256	P=0.0601
Slice x Row Factor	89.10	4	22.27		
Slice x Group	615.4	2	307.7		
Slice	217.3	2	108.6		
Residual	104.0	4	26.01		
Difference between column means					
Mean of HFS	90.62				
Mean of Control	101.2				
Difference between means	-10.62				
SE of difference	8.269				
95% CI of difference	-46.20 to 24.95				
Data summary					
Number of columns (Group)	2				
Number of rows (Row Factor)	3				
Number of subjects (Slice)	3				
Number of missing values	0				

Table 9. Tukey's multiple comparisons test results from Picrotoxin HFS data set.

Number of families	1							
Number of comparisons per family	15							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
1 min:HFS vs. 1 min:Control	0.3749	-41.00 to 41.75	No	ns	>0.9999			
1 min:HFS vs. 26-30 min:HFS	11.40	-20.99 to 43.79	No	ns	0.3193			
1 min:HFS vs. 26-30 min:Control	3.632	-39.72 to 46.99	No	ns	0.9656			
1 min:HFS vs. 86-90 min:HFS	24.67	-20.78 to 70.12	No	ns	0.1560			
1 min:HFS vs. 86-90 min:Control	0.1970	-60.21 to 60.60	No	ns	>0.9999			
1 min:Control vs. 26-30 min:HFS	11.03	-62.68 to 84.73	No	ns	0.8006			
1 min:Control vs. 26-30 min:Control	3.257	-36.00 to 42.51	No	ns	0.9668			
1 min:Control vs. 86-90 min:HFS	24.30	-62.47 to 111.1	No	ns	0.4411			
1 min:Control vs. 86-90 min:Control	-0.1779	-29.69 to 29.33	No	ns	>0.9999			
26-30 min:HFS vs. 26-30 min:Control	-7.770	-73.67 to 58.13	No	ns	0.8936			
26-30 min:HFS vs. 86-90 min:HFS	13.27	0.2071 to 26.34	Yes	*	0.0485			
26-30 min:HFS vs. 86-90 min:Control	-11.21	-101.0 to 78.59	No	ns	0.8749			
26-30 min:Control vs. 86-90 min:HFS	21.04	-56.10 to 98.18	No	ns	0.4564			
26-30 min:Control vs. 86-90 min:Control	-3.435	-34.27 to 27.40	No	ns	0.9102			
86-90 min:HFS vs. 86-90 min:Control	-24.48	-126.7 to 77.76	No	ns	0.5343			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
1 min:HFS vs. 1 min:Control	102.6	102.3	0.3749	4.986	3	3	0.1063	2.000
1 min:HFS vs. 26-30 min:HFS	102.6	91.24	11.40	3.904	3	3	4.131	2.000
1 min:HFS vs. 26-30 min:Control	102.6	99.01	3.632	5.225	3	3	0.9830	2.000
1 min:HFS vs. 86-90 min:HFS	102.6	77.97	24.67	5.478	3	3	6.370	2.000
1 min:HFS vs. 86-90 min:Control	102.6	102.4	0.1970	7.280	3	3	0.03826	2.000
1 min:Control vs. 26-30 min:HFS	102.3	91.24	11.03	8.883	3	3	1.756	2.000
1 min:Control vs. 26-30 min:Control	102.3	99.01	3.257	4.731	3	3	0.9736	2.000
1 min:Control vs. 86-90 min:HFS	102.3	77.97	24.30	10.46	3	3	3.286	2.000
1 min:Control vs. 86-90 min:Control	102.3	102.4	-0.1779	3.556	3	3	0.07074	2.000
26-30 min:HFS vs. 26-30 min:Control	91.24	99.01	-7.770	7.943	3	3	1.383	2.000

26-30 min:HFS vs. 86-90 min:HFS	91.24	77.97	13.27	1.575	3	3	11.92	2.000
26-30 min:HFS vs. 86-90 min:Control	91.24	102.4	-11.21	10.82	3	3	1.464	2.000
26-30 min:Control vs. 86-90 min:HFS	99.01	77.97	21.04	9.298	3	3	3.201	2.000
26-30 min:Control vs. 86-90 min:Control	99.01	102.4	-3.435	3.716	3	3	1.307	2.000
86-90 min:HFS vs. 86-90 min:Control	77.97	102.4	-24.48	12.32	3	3	2.809	2.000

Table 10. Two-way Repeated measures ANOVA results from Theta-burst stimulation data shown in figure 12.

Two-way RM ANOVA	Matching: Both factors				
Assume sphericity?	No				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	Geisser-Greenhouse's epsilon
Row Factor	10.15	0.1901	ns	No	0.5787
Group	1.152	0.6425	ns	No	1.000
Row Factor x Group	1.280	0.4207	ns	No	0.9643
Slice x Row Factor	28.63				
Slice x Group	28.96				
Slice	21.52				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Row Factor	271.7	2	135.9	F (1.157, 6.945) = 2.127	P=0.1901
Group	30.85	1	30.85	F (1.000, 6.000) = 0.2387	P=0.6425
Row Factor x Group	34.26	2	17.13	F (1.929, 11.57) = 0.9248	P=0.4207
Slice x Row Factor	766.5	12	63.88		
Slice x Group	775.4	6	129.2		
Slice	576.1	6	96.02		
Residual	222.3	12	18.52		
Difference between column means					
Mean of HFS	96.19				
Mean of Control	97.90				
Difference between means	-1.714				
SE of difference	3.508				
95% CI of difference	-10.30 to 6.870				
Data summary					
Number of columns (Group)	2				
Number of rows (Row Factor)	3				
Number of subjects (Slice)	7				
Number of missing values	0				

Table 11. Tukey's multiple comparisons test results from Theta-burst stimulation data.

Number of families	1							
Number of comparisons per family	15							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
1 min:HFS vs. 1 min:Control	-2.904	-20.29 to 14.49	No	ns	0.9800			
1 min:HFS vs. 26-30 min:HFS	-1.397	-9.075 to 6.281	No	ns	0.9714			
1 min:HFS vs. 26-30 min:Control	-0.5585	-17.25 to 16.13	No	ns	>0.9999			
1 min:HFS vs. 86-90 min:HFS	5.703	-10.09 to 21.50	No	ns	0.7093			
1 min:HFS vs. 86-90 min:Control	2.627	-24.66 to 29.91	No	ns	0.9983			
1 min:Control vs. 26-30 min:HFS	1.507	-11.57 to 14.58	No	ns	0.9961			
1 min:Control vs. 26-30 min:Control	2.346	-8.415 to 13.11	No	ns	0.9417			
1 min:Control vs. 86-90 min:HFS	8.608	-9.577 to 26.79	No	ns	0.4864			
1 min:Control vs. 86-90 min:Control	5.531	-13.66 to 24.73	No	ns	0.8465			
26-30 min:HFS vs. 26-30 min:Control	0.8385	-9.550 to 11.23	No	ns	0.9993			
26-30 min:HFS vs. 86-90 min:HFS	7.100	-5.582 to 19.78	No	ns	0.3435			
26-30 min:HFS vs. 86-90 min:Control	4.024	-18.14 to 26.19	No	ns	0.9717			
26-30 min:Control vs. 86-90 min:HFS	6.262	-3.977 to 16.50	No	ns	0.2753			
26-30 min:Control vs. 86-90 min:Control	3.185	-9.668 to 16.04	No	ns	0.9070			
86-90 min:HFS vs. 86-90 min:Control	-3.076	-21.57 to 15.42	No	ns	0.9803			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
1 min:HFS vs. 1 min:Control	97.63	100.5	-2.904	4.369	7	7	0.9400	6.000
1 min:HFS vs. 26-30 min:HFS	97.63	99.02	-1.397	1.929	7	7	1.024	6.000
1 min:HFS vs. 26-30 min:Control	97.63	98.18	-0.5585	4.193	7	7	0.1884	6.000
1 min:HFS vs. 86-90 min:HFS	97.63	91.92	5.703	3.968	7	7	2.033	6.000
1 min:HFS vs. 86-90 min:Control	97.63	95.00	2.627	6.855	7	7	0.5419	6.000
1 min:Control vs. 26-30 min:HFS	100.5	99.02	1.507	3.285	7	7	0.6488	6.000
1 min:Control vs. 26-30 min:Control	100.5	98.18	2.346	2.704	7	7	1.227	6.000
1 min:Control vs. 86-90 min:HFS	100.5	91.92	8.608	4.569	7	7	2.664	6.000
1 min:Control vs. 86-90 min:Control	100.5	95.00	5.531	4.823	7	7	1.622	6.000
26-30 min:HFS vs. 26-30 min:Control	99.02	98.18	0.8385	2.610	7	7	0.4543	6.000

26-30 min:HFS vs. 86-90 min:HFS	99.02	91.92	7.100	3.187	7	7	3.151	6.000
26-30 min:HFS vs. 86-90 min:Control	99.02	95.00	4.024	5.570	7	7	1.022	6.000
26-30 min:Control vs. 86-90 min:HFS	98.18	91.92	6.262	2.573	7	7	3.442	6.000
26-30 min:Control vs. 86-90 min:Control	98.18	95.00	3.185	3.230	7	7	1.395	6.000
86-90 min:HFS vs. 86-90 min:Control	91.92	95.00	-3.076	4.648	7	7	0.9361	6.000

Table 12. Two-way Repeated measures ANOVA results from Theta-burst stimulation data in the presence of GABA antagonist Picrotoxin, shown in figure 13.

Two-way RM ANOVA	Matching: Both factors				
Assume sphericity?	No				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	Geisser-Greenhouse's epsilon
Row Factor	5.483	0.2670	ns	No	0.5859
Group	4.082	0.6092	ns	No	1.000
Row Factor x Group	1.629	0.7000	ns	No	0.5030
Slice x Row Factor	4.992				
Slice x Group	22.65				
Slice	44.83				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Row Factor	60.66	2	30.33	F (1.172, 2.344) = 2.197	P=0.2670
Group	45.16	1	45.16	F (1.000, 2.000) = 0.3605	P=0.6092
Row Factor x Group	18.02	2	9.012	F (1.006, 2.012) = 0.1994	P=0.7000
Slice x Row Factor	55.22	4	13.81		
Slice x Group	250.5	2	125.3		
Slice	495.9	2	247.9		
Residual	180.8	4	45.20		
Difference between column means					
Mean of HFS	104.3				
Mean of Control	101.2				
Difference between means	3.168				
SE of difference	5.276				
95% CI of difference	-19.53 to 25.87				
Data summary					
Number of columns (Group)	2				
Number of rows (Row Factor)	3				
Number of subjects (Slice)	3				
Number of missing values	0				

Table 13. Results of Tukey's multiple comparisons test performed on Picrotoxin TBS data.

Number of families	1							
Number of comparisons per family	15							
Alpha	0.05							
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
1 min:HFS vs. 1 min:Control	5.997	-34.99 to 46.99	No	ns	0.8109			
1 min:HFS vs. 26-30 min:HFS	6.279	-1.186 to 13.74	No	ns	0.0696			
1 min:HFS vs. 26-30 min:Control	8.090	-35.63 to 51.81	No	ns	0.6889			
1 min:HFS vs. 86-90 min:HFS	2.822	-55.80 to 61.44	No	ns	0.9965			
1 min:HFS vs. 86-90 min:Control	4.517	-28.18 to 37.22	No	ns	0.8360			
1 min:Control vs. 26-30 min:HFS	0.2818	-34.65 to 35.22	No	ns	>0.9999			
1 min:Control vs. 26-30 min:Control	2.093	-7.922 to 12.11	No	ns	0.6170			
1 min:Control vs. 86-90 min:HFS	-3.175	-78.20 to 71.85	No	ns	0.9980			
1 min:Control vs. 86-90 min:Control	-1.480	-23.22 to 20.26	No	ns	0.9845			
26-30 min:HFS vs. 26-30 min:Control	1.811	-34.96 to 38.58	No	ns	0.9961			
26-30 min:HFS vs. 86-90 min:HFS	-3.457	-59.15 to 52.24	No	ns	0.9894			
26-30 min:HFS vs. 86-90 min:Control	-1.762	-32.00 to 28.48	No	ns	0.9919			
26-30 min:Control vs. 86-90 min:HFS	-5.268	-74.03 to 63.49	No	ns	0.9754			
26-30 min:Control vs. 86-90 min:Control	-3.573	-34.50 to 27.35	No	ns	0.8998			
86-90 min:HFS vs. 86-90 min:Control	1.695	-81.16 to 84.55	No	ns	>0.9999			
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
1 min:HFS vs. 1 min:Control	107.4	101.4	5.997	4.940	3	3	1.717	2.000
1 min:HFS vs. 26-30 min:HFS	107.4	101.1	6.279	0.8997	3	3	9.870	2.000
1 min:HFS vs. 26-30 min:Control	107.4	99.29	8.090	5.269	3	3	2.171	2.000
1 min:HFS vs. 86-90 min:HFS	107.4	104.6	2.822	7.066	3	3	0.5649	2.000
1 min:HFS vs. 86-90 min:Control	107.4	102.9	4.517	3.941	3	3	1.621	2.000
1 min:Control vs. 26-30 min:HFS	101.4	101.1	0.2818	4.211	3	3	0.09465	2.000
1 min:Control vs. 26-30 min:Control	101.4	99.29	2.093	1.207	3	3	2.452	2.000
1 min:Control vs. 86-90 min:HFS	101.4	104.6	-3.175	9.043	3	3	0.4965	2.000
1 min:Control vs. 86-90 min:Control	101.4	102.9	-1.480	2.620	3	3	0.7989	2.000
26-30 min:HFS vs. 26-30 min:Control	101.1	99.29	1.811	4.432	3	3	0.5780	2.000

26-30 min:HFS vs. 86-90 min:HFS	101.1	104.6	-3.457	6.713	3	3	0.7283	2.000
26-30 min:HFS vs. 86-90 min:Control	101.1	102.9	-1.762	3.645	3	3	0.6837	2.000
26-30 min:Control vs. 86-90 min:HFS	99.29	104.6	-5.268	8.287	3	3	0.8990	2.000
26-30 min:Control vs. 86-90 min:Control	99.29	102.9	-3.573	3.728	3	3	1.356	2.000
86-90 min:HFS vs. 86-90 min:Control	104.6	102.9	1.695	9.986	3	3	0.2400	2.000