Modulatory Effects of Acetylcholine and Dopamine on Evoked Synaptic Responses in the Entorhinal Cortex.

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ABSTRACT

Modulatory effects of Acetylcholine and Dopamine on Evoked Synaptic Responses in the Entorhinal Cortex.

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The entorhinal cortex connects neocortical areas with the hippocampal formation and other parahippocamal brain areas, and also receives cholinergic projections from the medial septum and dopaminergic projections from the ventral tegmental area. Dopamine and acetylcholine both may contribute to the processing of sensory information in the entorhinal cortex and in other areas of the brain. In Chapter 1, the application of the cholinergic agonist carbachol to entorhinal cortex slices suppressed synaptic transmission in vitro, and experiments determined that the effect was due primarily to activation of M_1 muscarinic receptors. Activation of cholinergic receptors also causes a relative *facilitation* of later responses during theta- and gamma-frequency trains, and because dopamine may modulate gamma and theta oscillations in the entorhinal cortex, Chapter 2 investigated the effect of amphetamine on the amplitudes of synaptic responses during trains of gamma- and theta-frequency stimulation in awake animals. A subset of animals that showed a facilitation of the response to the first pulse of theta-frequency trains due to amphetamine also expressed a synaptic suppression during mobility compared with immobility that was likely due to cholinergic receptor activation. These animals also showed a relative *suppression* of subsequent responses that was blocked by the D_1 receptor antagonist SCH23390 and the D_2 receptor antagonist eticlopride. Because previous work in our lab

has shown bidirectional effects of differing concentrations of dopamine, Chapter 3 investigated the role of both 10 and 50 μM dopamine in the entorhinal cortex during gamma- and theta-frequency stimulation in vitro. Ten μM dopamine facilitated responses during trains of both frequencies. In contrast, 50 μM dopamine induced a D_2 receptor-dependent *suppression* the first responses and induced a relative facilitation of later responses during the trains, an effect that was only significant for gamma-frequency trains. In general, then, low concentrations of dopamine may enhance repetitive synaptic transmission, while higher concentrations of dopamine may suppress repetitive synaptic transmission within the entorhinal cortex. Because dopamine may modulate learning-related synaptic strengthening, Chapter 4 investigated the effect of 10 μM dopamine on induction of long-term synaptic potentiation (LTP) in entorhinal cortex slices; although dopamine facilitated synaptic responses, it blocked the induction of LTP, suggesting that it may impede learning-related synaptic plasticity. Overall, results indicate that both dopamine and acetylcholine have strong modulatory influences on processes that may affect synaptic integration and plasticity within the entorhinal cortex.

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For all in vitro chapters, including Chapters 2, 4 and 5, Shawnna G. Barrett helped to design the experiments, performed the dissections, performed electrophysiological recordings, analysed data, created figures and wrote the manuscripts. Also for all in vitro chapters, C. Andrew Chapman designed the experiments and assisted with writing the manuscripts. For Chapter 4 regarding the role of dopamine on the amplitude of responses during trains of theta- and gamma-frequency stimulation in vitro, Marie Rose Popiel Jacobsen aided in conduting electrophysiological recordings and contributed to the writing of the manuscript as part of her thesis project in biology at Concordia University.

Research for Chapter 2 of this thesis regarding the receptors involved in the cholinergic suppression of fEPSPs in the medial entorhinal cortex began during my masters degree. My masters thesis included a 10uM carbachol group, the M_1 receptor antagonist pirenzepine and the M_2 receptor antagonist methoctramine. During my Ph.D., additional groups were added to the experiment to increase its value for publication, including groups investigating the effect of two different concentrations of M_1 receptor antagonist VU0255035 on the cholinergic suppression, as well as two groups investigating different concentrations of the M_4 receptor antagonist PD102806, a group investigating the cannabinoid 1 receptor antagonist AM281, additional slices for all conditions, as well multiple groups testing different concentrations of carbachol in order to determine the EC_{50} for carbachol. With the additional groups, this experiment was published in the journal Neuroscience Letters in 2013.

For Chapter 3 regarding the effect of injections of amphetamine on synaptic

responses in the entorhinal cortex durign trains of gamma- and theta-freqnency stimulation in freely moving animals, Shawnna G. Barrett helped with the experimental design, performed surgeries for the placement of electrodes, performed electrophysiological recordings, analysed data, created figures and wrote the manuscript. C. Andrew Chapman designed the experiments, also performed surgeries, and assisted with writing the manuscripts. Sandrine Cote also contributed to this experiment by conducting electrophysiological recorings, and Daniel Sparks contributed by performing surgeries.

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LIST OF ABBREVIATIONS

ACSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
CA1, CA3	*Cornu Ammois* fields of the hippocampus
Ca^{2+}	calcium
CaMKII	calcium/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CB1R	cannabinoid receptor type 1
CCh	carbachol
fEPSP	field excitatory postsynatic potential
GABA	gamma-aminobutyric acid
HFS	high-frequency stimulation
I_h	hyperpolarization-activated nonspecific cation current
LTD	long-term depression
LTP	long-term potentiation
NMDA	N-methyl-D-aspartate
6-OHDA	6-hydroxydopamine
PKA	protein kinase A

Chapter 1

General Introduction

General Introduction

The entorhinal cortex is part of the medial temporal lobe, and is thought to contribute to learning and memory (Witter et al., 1989; Lavenex and Amaral, 2000; Burwell, 2000) in part because it provides the hippocampus with its largest cortical sensory input (Burwell and Amaral, 1998). Cells in layer II of the entorhinal cortex receive sensory inputs from several major cortical areas, and the integration and modulation of synaptic strength within the entorhinal cortex is likely to alter how the entorhinal cortex contributes to sensory and mnemonic function. While the superficial layers of the entorhinal cortex provide inputs to the hippocampus, the deep layers of entorhinal cortex both receive outputs from the hippocampal formation, and send outputs back to neocortical areas (Eichenbaum and Lipton, 2008). Thus, the anatomical connections of the entorhinal cortex are interesting because they show that the entorhinal cortex is reciprocally connected both with cortical sensory and associational areas, and with the hippocampal formation, and it is therefore well positioned to contribute to processing of sensory information and mnemonic function (Witter et al., 1989; Burwell and Amaral, 1998; Lavenex and Amaral, 2000).

Synaptic strength can be altered over very short time-scales in response to release of neuromodulatory transmitters such as acetylcholine, serotonin, and dopamine. The synaptic effects induced by these neuromodulatory transmitters are likely to contribute to cognitive functions, but how is not known. Release of acetylcholine is associated with behavioural activation and exploration (Bland et al., 2003), and is also associated with the induction of theta (4- 12 Hz) and gamma-frequency (25 -80 Hz) EEG activities within the

entorhinal cortex (Golebiewski et al., 1994; van Der Linder et al., 1999) and hippocampus (van Der Linder et al., 1999). Interestingly, it has been found that acetylcholine can also *suppress* the strength of synaptic inputs to the entorhinal cortex (Hamam et al., 2007), and so it is not clear how acetylcholine might enhance synaptic processes contributing to cognitive function.

The modulatory transmitter dopamine also has strong effects on the strength of synaptic inputs to the entorhinal cortex, and can lead to a suppression or facilitation of synaptic responses depending upon the concentration applied (Caruana et al., 2006). Research has shown that dopamine contributes to mechanisms of appetitive motivation and reward (Berridge et al., 2009) and has also shown that memory performance is improved by administration of dopaminergic agonists in both rats (Lejeune et al., 2013) and humans (Macdonald et al., 2013). However, the cellular mechanisms by which dopamine contributes to memory are not yet known, and it is also not known how dopamine release during appetitively motivated states may modulate theta- and gamma-frequency synaptic responses within the entorhinal cortex.

This thesis has used a number of experimental techniques to investigate the cellular mechanisms through which acetylcholine and dopamine may alter the strength of cortical inputs to the superficial layers of the entorhinal cortex. In Chapter 2, recordings of synaptic responses in brain slices in vitro were used to investigate which muscarinic receptors are responsible for the suppression of synaptic transmission in the entorhinal cortex induced by acetylcholine. Chapter 3 used in vivo synaptic recordings in order to investigate how enhancing dopamine through injections of amphetamine alters repetitive

synaptic responses at both theta- and gamma-frequencies in behaving animals, and which dopamine receptors are involved in the changes caused by amphetamine. Chapter 4 used in vitro field recording techniques to determine the role of both high and low concentrations of dopamine on synaptic responses during trains of both theta- and gamma-frequency stimulation in slices, as well as which dopamine receptors are involved in dopamine-induced relative changes in the amplitude of responses. In addition, Chapter 5 reports experiments investigating how dopamine modulates the induction of lasting changes in synaptic strength that are thought to contribute to the mechanisms of memory formation. This Introduction will now provide an overview of the anatomy and functions of the medial and lateral entorhinal areas, and will also address what is already known in the literature about the role of dopamine and acetylcholine in the medial and lateral entorhinal areas.

Anatomy and Functions of the Medial and Lateral Entorhinal Areas

The entorhinal cortex can be broken up into the medial entorhinal cortex and the lateral entorhinal cortex based on the neocortical areas they receive inputs from, areas they project to, and the associated functions of these regions. The medial entorhinal cortex has been referred to as a major contributor to the 'where' pathway, as it receives information regarding spatial and contextual information from the postrhinal cortex, and sends projections to the hippocampus that contribute heavily to spatial processing (Burwell, 2000; Knierim et al., 2006; Eichenbaum et al., 2012). Neurons originating in the medial entorhinal cortex have also been shown to be involved in processing spatial information (Moser and Moser, 2008), with the existence of head direction cells (Sargolini et al.,

2006), border cells (Savelli et al., 2008) and grid cells (Moser et al., 2008; Moser and Moser, 2008) within this area. Conversely, the lateral entorhinal cortex receives information regarding objects from the perirhinal cortex (Burwell, 2000; Knierim et al., 2006; Eichenbaum et al., 2012), as well as the olfactory bulb and the piriform cortex (Burwell, 2000), and sends projections to the hippocampus. The lateral entorhinal cortex is therefore thought to be a key contributor to the 'what' pathway involved in object identification (Burwell, 2000; Knierim et al., 2006; Eichenbaum et al., 2012). These characteristics of the medial and lateral entorhinal regions provide a general perspective on their functions, but the literature dealing with these regions is complex in terms of the connections and functions of these regions. An understanding of the roles of the entorhinal cortex in learning and memory may therefore be aided by a more in depth review of the afferents and efferents of these regions (Figure 1.1) and behavioural evidence regarding their functions.

Inputs to the Medial Entorhinal Cortex. The superficial layers of the medial entorhinal cortex receive their major cortical projections from the posterior parietal cortex (Kerr et al., 2007) which receives somatosensory inputs (Lee et al., 2011), and it also receives inputs from the postrhinal (Burwell, 2000) and retrosplenial (Kerr et al., 2007) corticies; these are all areas of the brain that would suggest a major role of the entorhinal cortex in the "where" pathway that is thought to contribute to spatial processing (Eichenbaum et al., 2012). The lateral band of the medial entorhinal cortex has been show to receive projections from the posterior area of the parietal cortex (Kerr et al., 2007), an area heavily associated with the long-term memory of spatial information (Anderson,

1995; Calton and Taube, 2009; Kesner, 2009). The postrhinal cortex, part of the parahippocampal region along with the entorhinal cortex, also projects to the medial entorhinal cortex (Burwell and Amaral, 1998; Burwell, 2000; Witter et al., 2000). The synaptic link between the medial entorhinal area and the postrhinal cortex supports the role of the medial entorhinal cortex in processing information regarding the spatial environment of the animal because the postrhinal cortex is necessary in the retention of short- (Liu and Bilkey, 2002) and long-term spatial memory (Liu and Bilkey, 2002; Ramos, 2013). Excitotoxic NMDA lesions of the postrhinal cortex result in deficits in short-term memory on the radial arm maze task for working memory (Liu and Bilkey, 2002), and also result in a deficit in long-term memory on a delayed non-matching to place version of the radial arm maze task (Liu and Bilkey, 2002; Ramos, 2013), the T-maze, as well as the water maze task (Liu and Bilkey, 2002). The retrosplenial cortex also provides projections to the medial entorhinal cortex (Kerr et al., 2007), and it is possible that the retrosplenial cortex promotes spatial processing in the medial entorhinal cortex by providing mnemonic non-visual spatial information (Cooper et al., 2001; Cooper and Mizumori, 2001). Therefore, the posterior parietal, postrhinal, and retrosplenial corticies are all likely to provide inputs to the medial entorhinal cortex that aid in the role of the medial entorhinal cortex in the processing of spatial information.

The deep layers of the medial entorhinal cortex also receive projections from the dorsal CA1 region of the hippocampal formation, as well as from the presubiculum and the parasubiculum (Agster and Burwell, 2013). All of these brain regions contain cells that process information about the animals' spatial environment (Faust et al., 2013;

Robertson et al., 1999; Taube, 1995) that may contribute to spatial processing in the medial entorhinal cortex. Place cells use changes in spatial information to encode information about the location of an animal as well as the movement of objects, and they have been found in the CA1 region of the hippocampus (Faust et al., 2013), and are one of the most prominent cell types in the parasubiculum (Taube, 1995). The presubiculum, on the other hand, likely provides spatial information to the medial entorhinal cortex regarding the animals' environment through head direction cells (Robertson et al., 1999). Therefore, the medial entorhinal area receives inputs from areas of the hippocampal formation such as the CA1, parasubiculum and presubiculum that are consistent with a role of the medial entorhinal cortex in spatial processing.

Outputs from the Medial Entorhinal Cortex. The superficial layers of the medial entorhinal cortex also send multiple outputs to different components of the hippocampal formation including the dentate gyrus, CA3 and CA1 areas in both the dorsal and ventral hippocampus. The nature of these projections are dependent on the specific region of origin within the medial entorhinal cortex such that structures in the ventral hippocampus receive projections from the medial band of the medial entorhinal cortex, and structures in the dorsal hippocampus receive projections from the lateral band of the medial entorhinal cortex (Agster and Burwell, 2013). The ventral regions of the hippocampus have been most widely associated with mood-related behaviours such as stress, emotion, and affect (Bannerman et al., 2004; Engin and Treit, 2007; Fanselow and Dong, 2010), however, it has also been shown that the ventral hippocampal areas have some involvement in spatial memory processing (Loureiro et al., 2011) and response

learning (Fridalgo et al., 2012) while the dorsal hippocampal structures have been consistently associated with response learning and spatial memory (Potvin et al., 2007; Fanselow and Dong, 2010; Fridalgo et al., 2012), suggesting that the dorsal region of the hippocampus may be more involved in processing the spatial information provided by the inputs from the medial entorhinal cortex than the ventral hippocampal structure.

Role of the Medial Entorhinal Cortex in Cognitive Function. Differing methods investigating the role of the medial entorhinal cortex in cognitive function have shown that this area is involved in spatial information processing (Yasuda and Mayford, 2006; Brun et al., 2008). Brun et al. (2008) used neurotoxic lesions of layer III of medial entorhinal cortex, and found that the lesions resulted in larger and more broken up firing fields of place cells in the CA1, suggesting that inputs from this brain area are necessary for place cells in the CA1 to fire in a precise manner. Another study used transgenic mice with inducible overexpression of the calcium/calmodulin-dependent protein kinase II (CaMKII) in the entorhinal region to determine the role of the medial entorhinal cortex in the learning and memory of spatial information at a molecular level (Yasuda and Mayford, 2006). When CaMKII was overexpressed during training, the transgenic mice were impaired in the learning of a water maze task. Overexpression of CaMKII immediately after training disrupted spatial memory for the location of the hidden platform, but overexpression of CaMKII 3 weeks after training did not, suggesting that the entorhinal cortex plays a transient role in the formation of spatial memory but is not required for recall of consolidated spatial memory (Yasuda and Mayford, 2006).

In addition to behavioural experiments regarding the role of the medial entorhinal

cortex in spatial memory formation (Yasuda and Mayford, 2006; Brun et al., 2008), the discovery of "grid cells" (Moser and Moser, 2008) has led to an increase in interest in the entorhinal cortex in the scientific literature. Grid cells are cells that have been found in the medial part of the entorhinal cortex that fire when an animal is located in places within their environment aligned in a triangular grid-pattern (Moser and Moser, 2008), and grid cells fire consistently in the same grid pattern in all environments (Knierim et al., 2006). Grid cells are different from other cells that fire in a location-dependent manner, such as place cells or border cells, because they fire when the animal is in locations in their environment that correspond to the vertices of a grid of equilateral triangles (Moser and Moser, 2008). It has therefore been speculated that grid cells in the medial entorhinal cortex may serve to provide the hippocampus with inputs from multiple grid patterns in order to provide a coordinate system for the environment to support the spatial firing patterns of hippocampal place cells (Knierim et al., 2006).

Although grid cells are the most well known cells in the medial entorhinal cortex, there also exist boundary cells and head-direction cells in this brain area that may aid in different aspects of spatial processing (Moser and Moser, 2008; Sargolini et al., 2006; Bruce et al., 2006). Head-direction cells are cells that fire based on the direction that the animal's head is facing independent of other variables such as behavior, location in the environment, or body-position (Taube et al., 1990). Border, or boundary, cells fire when the animal is close to the borders of their respective environments, and these cells are found in all layers of the medial entorhinal cortex (Solstad et al., 2008). Zhang et al., (2013) used a combination of optogenetics and electrophysiology to record from only the

neurons in the medial entorhinal cortex with fibers reaching the hippocampus, and they found that although the majority of these cells were grid cells, there were also border cells and head direction cells. It has been suggested that the role of boundary cells is to aid in securing grid and place fields to a geometric reference frame (Solstad et al., 2008), and that the generation of place fields by the hippocampus is due to converging information from multiple cell types from the medial entorhinal cortex (Zhang et al., 2013).

A further look into spatially firing cells shows us that the nature of the 'equilateral triangle' that defines grid cell firing changes based on the location within the medial entorhinal cortex where the cell exists; grid cells in the ventral part of the dorsocaudal medial entorhinal cortex fire in larger grid patters, however, more dorsal locations in this brain region have grid patterns that are progressively smaller (Knierim et al., 2006; Hafting et al., 2005). This same pattern of grid-spacing or precision has been found for head direction cells, where there is narrower and more sharply tuned firing field for head-direction cells in the dorsal area of the medial entorhinal cortex and less sharply defined tuning in the ventral area of the medial entorhinal cortex (Giocomo et al., 2014; Jeffery, 2014). It has been suggested that the role of the varied precision of grid-spacing and tuning of head-direction cells as a function of location within the medial entorhinal cortex is to create various resolutions of spatial maps in order to help meet differing demands; when high levels of accuracy regarding the location and direction of the animal are required, head-direction cells that are more sharply tuned with narrower firing fields as well as smaller more specific grid patterns may be necessary, whereas in times when high levels of accuracy are not as critical, narrower firing fields for head direction cells and

smaller grid patterns are not as necessary (Jeffery, 2014). It is possible that these specific and non-specific grid cell firing patterns are controlled by theta-frequency activity in the medial entorhinal cortex, as theta-frequency oscillations dominate EEG activity while animals are navigating through their environment (Hasselmo and Stern, 2013).

Inputs to the Lateral Entorhinal Cortex. The lateral entorhinal cortex is associated with the 'what' pathway (Burwell, 2000; Eichenbaum et al., 2012; Knierim et al., 2006), due to its non-spatial, olfactory, and object recognition inputs via the perirhinal and piriform corticies (Agster and Burwell, 2013). The perirhinal cortex has large projections to the lateral entorhinal cortex (Witter et al., 2000) and receives inputs from brain regions that process non-spatial information about the identity of stimuli (Eichenbaum and Lipton, 2008) such as olfactory and auditory information (Burwell and Amaral, 1998). The lateral entorhinal cortex also receives inputs from the piriform cortex that is the primary olfactory cortex (Burwell, 2000; Kerr et al., 2007). The connections between the lateral entorhinal cortex and the perirhinal and piriform corticies suggest that the lateral entorhinal cortex has an important role in object identification and the olfactory characteristics of objects, that is consistent with a role of the lateral entorhinal cortex in the "what" pathway.

The ventral aspect of the hippocampal cornu ammonis areas CA1, CA2, and CA3, and of the subiculum provide inputs to the lateral entorhinal cortex (Agster and Burwell, 2013) that support the role of the lateral entorhinal cortex in non-spatial information processing (Jung et al., 1994; Rudy and Matus-Amat, 2005). The ventral hippocampus

and the ventral subiculum are both involved in contextual processing, where the ventral hippocampus is involved in memory of contexts alone (Rudy and Matus-Amat, 2005) as well as contextual fear conditioning (Rudy and Matus-Amat, 2005; Wang et al., 2013), while the ventral subiculum is involved in contextual fear conditioning (Burhams and Gabriel, 2007; Biedenkapp and Rudy, 2008) and latent inhibition (Quintero et al., 2011), all consistent with role of the lateral entorhinal cortex in processing of contextual, non-spatial, information about the environment. The pre- and para-subicular areas also provide projections to the lateral entorhinal cortex (Agster and Burwell, 2013).

Outputs from the Lateral Entorhinal Cortex. Layer II of the entorhinal cortex provides projections to the dentate gyrus (Dolorfo and Amaral, 1998[1]; Dolorfo and Amaral, 1998[2]). The dentate gyrus is a part of the hippocampal formation that has been associated with different functions both spatial (Kesner, 2013; Xavier and Costa, 2009) and non-spatial in nature (Eacott and Norman, 2004; Piterkin et al., 2008; Weeden et al., 2012; Kesner, 2013). Given the non-spatial information processed by the lateral entorhinal cortex, non-spatial functions of the dentate gyrus may be associated with information coming from lateral entorhinal inputs. These non-spatial functions include recognizing environments based on context cues such as colour (Eacott and Norman, 2004; Kesner, 2013), as well as object-recognition based on context (Piterkin et al., 2008; Kesner, 2013), and learning and memory for olfactory stimuli (Weeden et al., 2012; Kesner, 2013). All of these functions are based on information that is either processed by the lateral entorhinal cortex such as olfactory information (Bannerman et al., 2002; Petrulis et al., 2000; Staubli et al., 1984; Otto and Eichenbaum, 1992), or processed by

brain regions that provide inputs to the lateral entorhinal cortex such as object recognition information from the perirhinal cortex (Liu and Bilkey, 2001).

. **Roles of the Lateral Entorhinal Cortex in Cognitive Function.** While the role of the medial entorhinal cortex is more established in the literature than that of the lateral entorhinal cortex, there is a relatively clear understanding of what lateral entorhinal neurons are responsible for. The lateral entorhinal cortex has been heavily associated with processing olfactory information (Bannerman et al., 2002; Petrulis et al., 2000; Staubli et al., 1984; Otto and Eichenbaum, 1992). Thirty-five percent of neurons in the lateral entorhinal cortex fired selectively to different odors (Young et al., 1997), suggesting that a large function of the lateral entorhinal cortex is olfactory processing and the association of olfactory information with other stimulus dimensions. Lesions of the entorhinal cortex, both permanent (Staubli et al., 1984; Otto and Eichenbaum, 1992) and temporary (Chapuis et al., 2013), have led to deficits on odor-discrimination tasks, suggesting that the lateral entorhinal cortex is necessary for this type of processing. In vivo electrophysiological studies have also shown that neurons in the lateral entorhinal cortex display specific firing patterns to different odors presented in anesthetized animals (Xu and Wilson, 2012) and in awake animals (Young et al., 1997), indicating their responsiveness to olfactory stimuli. In addition, using temporary disruption of entorhinal function using the NMDA receptor antagonist APV, the lateral entorhinal cortex has also been shown to be involved in creating associations between tactile stimuli and olfactory stimuli (Boisselier et al., 2014)

In addition to olfactory processing, lesion studies have also suggested that the

lateral entorhinal cortex is involved in object-recognition (Wilson, Watanabe et al., 2013; Wilson, Langston et al., 2013; Vnek et al., 1995). Lesioning the angular bundle in rats in order to destroy connections between the entorhinal cortex and the hippocampus does not lead to impairments in object-discrimination, but does impair *retention* of that information (Vnek et al., 1995). Therefore, it has been suggested that the role of the lateral entorhinal cortex is not in object-discrimination alone, but that it also contributes to the ability to retain information regarding objects (Vnek et al., 1995). Similarly, it has been suggested that the entorhinal cortex may serve to maintain information about objects in short-term memory, and to provide this information to the hippocampus (Suzuki et al., 1997).

Research from Wilson et al. (2013[2]), showed that the lateral entorhinal cortex is necessary for the recognition of object-context associations, and for the long-term retention of that information. With lesions to the lateral entorhinal cortex, rats were able to perform object-recognition and context-recognition tasks, however, they were impaired on a combined object-context association task (Wilson et al., 2013[2]). A subsequent study that also used excitotoxic lesions to the lateral entorhinal cortex extended the last study by investigating other combinations such as objects in a certain context, objects in a certain place, and objects in both a place with a context (Wilson et al., 2013[1]). They found that the loss of the lateral entorhinal cortex led to a deficit in all of the combinations described, but as noted before, they were not impaired at recognizing objects, places, or contexts individually. Similar results have been shown in the entorhinal cortex of the rhesus monkey, where monkeys were impaired in learning new object-in-place

associations after ablations to the entorhinal cortex (Charles et al., 2004). These data suggest that the lateral entorhinal cortex plays a role in associative recognition memory regarding objects, contexts, and paces, but is not required for object-recognition alone (Wilson et al., 2013[1]).

Studies investigating object-recognition in humans have also shown that the entorhinal cortex is involved. A study using blood oxygenation level-dependent functional magnetic resonance imaging (fMRI) showed that the perirhinal and the entorhinal cortex in the left hemisphere were active while participants were performing an object-recognition task (Bellgowan et al., 2009). Although this study did not investigate the individual roles of the medial and lateral entorhinal cortices, it is interesting that they found hemispheric differences that have yet to be shown in animal models.

Acetylcholine and Dopamine in the Entorhinal Cortex

The foregoing section on the anatomical and functional characteristics of the medial and lateral entorhinal cortices indicates that the entorhinal region is an important region that integrates synaptic inputs from cortical and hippocampal structures, and which also provides the hippocampus with highly processed inputs that can contribute to sensory and mnemonic function. Perirhinal inputs to the lateral entorhinal region make an important contribution to object identification, and postrhinal inputs to the medial entorhinal region are thought to contribute to spatial and navigational functions (Ramos, 2013; Liu and Bilkey, 2002). Therefore, the information regarding stimuli characteristics from the lateral entorhinal area as well as spatial information from the medial entorhinal area converges within the hippocampal formation (Eichenbaum and Lipton, 2008). In

addition to these anatomical and functional considerations, the neuromodulatory transmitters acetylcholine (Gaykema et al., 1990) and dopamine (Bjorklund and Lindvall, 1984) send substantial inputs to both medial and lateral entorhinal regions, and they are likely to play a major role in modulating these functions. Because of this, to understand how the entorhinal cortex contributes to sensory and mnemonic processing, we must also consider how these areas are modulated by these important neurotransmitter systems, how these transmitters modulate cellular and synaptic functions within entorhinal neurons.

Acetylcholine

The entorhinal cortex receives strong cholinergic inputs from the medial septum (Gaykema et al., 1990). Acetylcholine in the medial entorhinal cortex has also been shown to modulate cognitive processes (McGaughy et al., 2005; Barak and Weiner, 2010). Barak and Weiner (2010) injected the muscarinic antagonist scopolamine through cannulae implanted in the medial entorhinal cortex of rats, and found that the rats failed to show latent inhibition, suggesting that acetylcholine neurons in this area of the brain are responsible for *inattention* to stimuli. Other work has shown that blocking cholinergic receptors in the medial entorhinal cortex results in an impairment in a delayed nonmatching to sample task involving novel odors, but not for familiar odors (McGaughy et al., 2005). It has also been shown that lesions to the medial septum, cutting off the supply of acetylcholine to the entorhinal cortex, results in an elimination or suppression of theta rhythm activity in the lateral entorhinal cortex and also results in a disruption in spatial learning on a radial arm maze task (Mitchell et al., 1982). The cholinergic system in the entorhinal cortex is also one of the most damaged parts of the brains of patients

who suffer from Alzheimer's disease, a disease that results in debilitating memory impairments, suggesting that cholinergic receptors in the entorhinal cortex are vital to memory processing (Kasa et al., 1997; Geula and Mesulam, 1989). All of this research combined suggests the role of cholinergic receptors in the entorhinal cortex on memory formation and retention.

Activation of cholinergic receptors primarily has excitatory effects on neuronal excitability and membrane potential (Klink and Alonso, 1997; Egorov et al., 2002; Brown, 2010), and activation of cholinergic receptors in the medial entorhinal cortex results in a both a depolarization of neurons and a *suppression* of excitatory synaptic transmission (Richter et al., 1999; Hamam et al. 2007; Hasselmo and McGaughy, 2004; Yun et al., 2000). The suppression of synaptic transmission has been found in layers III and V of the entorhinal cortex (Yun et al., 2000; Cheong et al., 2001) as well as in layer V inputs to layer II of medial entorhinal cortex (Richter et al., 1999), and the role of this cholinergic suppression may be to offset hyperexcitability of the region that could be caused by cholinergic depolarization of membrane potential (Friedman et al., 2007). The role of the cholinergic suppression could also be to enhance the selectivity of the way that that synaptic inputs contribute to learning-related synaptic plasticity by inducing a general suppression of synaptic strength that could raise the threshold for inductions of lasting synaptic plasticity (Hasselmo and McGaughy, 2004). In addition, it could reduce the level of background synaptic "noise", and thereby enhance the relative salience of the stronger synaptic inputs during sensory processing (Hasselmo and McGaughy, 2004).

It is not yet known which acetylcholine receptors are responsible for this reduction

in synaptic strength. Richter et al. (1999) showed that the application of a cholinergic agonist caused a reduction in the amplitude of EPSPs in layer II of the medial entorhinal cortex evoked by activation of inputs from layer V, and they also showed that application of the M_1 receptor antagonist pirenzepine blocked the cholinergic suppression. Richter et al. (1999) also showed that the cholinergic suppression was not associated with a change in paired-pulse facilitation ratio, suggesting that changes in postsynaptic responsivity, rather than in presynaptic transmitter release, mediated the suppression. In support of this general finding of the involvement of M_1 receptors, other studies have shown that M_1 receptors are responsible for the cholinergic suppression in the CA1 region of the hippocampus (Auerbach and Segal, 1996; Sheridan and Sutor, 1990). However, there are also several studies that suggested that the cholinergic suppression is due to activation of M_2-like receptors, including the M_4 receptor subtype (Dutar and Nicoll, 1988; Kremin et al., 2006; Dasari and Gulledge, 2011). The use of gallamine to block M_2 receptors in the CA1 resulted in a block of the cholinergic suppression (Dutar and Nicoll, 1988). Also, research using muscarinic receptor knock-out mice have shown that animals who lack M_1 receptors still show a reduction in EPSPs in the hippocampus, although the size of the reduction is somewhat smaller (Kremin et al., 2006), suggesting that M_1 receptors are not solely responsible for the cholinergic suppression effect (Kremin et al., 2006; Dasari and Gulledge, 2011). In addition, Dasari and Gulledge (2011) showed that application of a cholinergic agonist did not result in a significant suppression of EPSPs in M_4 knockout mice, suggesting that in the CA1 region, M_4 receptors likely play a larger role than M_1 receptors in the suppression effect. In addition to these inconsistencies in the hippocampal

literature, it is not known which muscarinic receptor is likely responsible for the cholinergic suppression in the medial entorhinal cortex.

Acetylcholine has been shown to promote both gamma frequency (25 - 80 Hz; van Der Linder et al., 1999) and slower theta-frequency oscillations (4-12 Hz) Golebiewski et al., 1994; Konopacki et al., 1992) in the entorhinal cortex (Mitchell and Ranck, 1980; Dickson et al. 2000; Glasgow and Chapman, 2007). Slow wave theta-frequency electroencephalographic (EEG) activity as well as fast wave gamma-frequency activity is generated in the entorhinal cortex and hippocampus during periods of behavioural mobility (Alonso and Garcia-Austt 1987; Dickson et al., 2000), and acts to synchronize neuronal firing in the entorhinal cortex (Brandon et al. 2011). The rhythms are thought to promote synaptic plasticity related to learning by enhancing postsynaptic depolarization (Huerta and Lisman, 1995; Chapman and Racine, 1997; Yun et al, 2000; Auerbach and Segal, 1996; Hasselmo, 2006). In vitro administration of carbachol onto guinea pig brain preparations results in gamma activity in the medial entorhinal cortex as well as the hippocampus (van Der Linden et al., 1999), however, not in the lateral entorhinal cortex. Although it has been suggested that gamma activity in the medial entorhinal cortex promotes gamma activity in the hippocampus, the activity seen in the hippocampus is visible at higher frequencies than in the entorhinal cortex, and is therefore likely part of a different aspect of signal-processing (van Der Linden et al., 1999).

Application of carbachol to slices of medial entorhinal cortex results in low-frequency theta oscillations that are blocked by an M_1 but not an M_2 receptor antagonist (Golebiewski et al., 1994). Sparks and Chapman (2013), showed that application of

carbachol, a cholinergic agonist, to entorhinal cortex slices resulted in an overall suppression of synaptic responses evoked during short trains of theta- and gamma-frequency stimulation. However, the amplitudes of responses to each of the pulses during the trains were facilitated relative to the amplitude of the first response, so that there was enhanced growth in responses during the trains in the presence of carbachol (Sparks and Chapman, 2013). Therefore, cholinergic receptor activation in the entorhinal cortex, which occurs as animals explore their environment during behavioural mobility, may be acting to both promote theta and gamma EEG activities, and to promote the transmission of representations carried by repetitive synaptic activation at these frequencies (Sparks and Chapman, 2013;Alonso and Garcia-Austt 1987; Dickson et al., 2000). Because cholinergic receptor activation is likely to help drive EEG activity during behavioural mobility, one of the goals of the present thesis was to determine if the relative synaptic facilitation effect observed by Sparks and Chapman (2013) in vitro might be observed in awake, behaving animals as they transition from immobility to mobility.

Dopamine

Dopamine is a neuromodulatory transmitter that is thought to play a role in the modulation of processes related to appetitive motivation and reward (Berridge et al., 2009) and learning and memory (El-Ghundi et al., 2007; Caruana et al., 2007). For example, it has been shown that blocking dopamine receptor activation results in a decrease in motivation to work for rewarding brain stimulation in the lateral hypothalamus (Ettenberg and Duvauchelle, 1988). Midbrain dopamine neurons of the mesocortical dopamine system in the ventral tegmental area and substantia nigra project

to the superficial layers of the lateral entorhinal cortex (Bjorklund and Lindvall, 1984; Oades and Halliday, 1987; Björklund and Dunnett, 2007), and are likely to have important modulatory functions on entorhinal neurons that contribute to sensory an mnemonic processing related to stimuli associated with motivation and reward. For example, pairing cues with desirable food rewards like chocolate results in reductions in synaptic responses that are dependent on dopamine receptor activation in the entorhinal cortex (Hutter and Chapman, 2013). It has also been shown that rewarding electrical brain stimulation administered in the lateral hypothalamus results in a reduction in synaptic responses in the lateral entorhinal cortex that is blocked by a D_2 receptor antagonist (Hutter et al., 2013). These data suggests that dopamine in the lateral entorhinal cortex plays a role in reducing synaptic activity in response to both natural rewards, and in response to electrical brain stimulation reward.

There is a variety of evidence indicating roles for dopamine in memory formation. Administration of dopamine agonists has been shown to lead to an improvement in memory performance in both rats (Lejeune et al., 2013) and in humans (Macdonald et al., 2013). In humans, disorders that effect memory retrieval such as Parkinson's disease (Kish et al., 1988; Macdonald et al., 2013) have been associated with a depletion of dopamine in the brain. It has been shown that patients with Parkinson's disease have a depletion of dopamine in the striatum (Kish et al., 1988), and the use of dopaminergic replacement medications in patients with Parkinson's disease results in a significant improvement in tests of auditory memory as well as a significant improvement on an analogous test using symbols instead of words to produce a non-verbal memory task

(Macdonald et al., 2013). Similarly, elderly patients who suffered from dopamine receptor loss were tested on tasks assessing episodic memory with and without administration of the dopamine precursor L-dopa, and patients treated with L-dopa showed improvements in memory for images and scenes, while those not treated showed no improvements (Chowdhury et al., 2012). In contrast, when normal adults have *elevated* levels of dopamine due to administration of the same dose of L-dopa used in the previous experiment, they show impaired retrieval accuracy for recognition of old and new memories (Apitz and Bunzeck, 2013), suggesting a bidirectional effect of dopamine levels on memory. Research using animals has also provided a better definition of the role of dopamine in learning and memory (Gasbarri et al., 1996; Izquierdo et al., 1998; Barros et al., 2001; Balderas et al., 2013; Rossato et al., 2013).

Studies investigating the role of dopamine on learning and memory in the rat brain have found that dopamine is involved in long-term memory for fear (Rossato et al., 2009) and for the formation of spatial memory (Gasbarri et al., 1996; da Silva et al., 2012) in the hippocampus, while dopamine also contributes to object recognition memory in the perirhinal cortex (Balderas et al., 2013) and in the prefrontal cortex and amygdala (Rossato et al., 2013). However, results regarding the role of dopaminergic receptors in the entorhinal cortex have lead to inconsistent conclusions (Barros et al., 2001; Izquierdo et al., 1998; Gauthier and Soumireu-Mourat, 1981). Reducing dopamine projections to the hippocampus using 6-OHDA lesions results in spatial memory deficits on the Morris water maze task (Gasbarri et al., 1996), and administration of dopamine D_1 receptor agonists in the perirhinal (Balderas et al., 2013), and prefrontal cortex as well as the

amygdala (Rossato et al., 2013) results in an enhancement in object-recognition memory. Administration of a D_1 receptor antagonist in the entorhinal cortex results in a disruption of the memory that stepping onto a platform previously resulted in a foot-shock (Barros et al., 2001; Izquierdo et al., 1998), suggesting that dopamine is also involved in memory formation in the entorhinal cortex. However, Gauthier and Soumireu-Mourat (1981) showed that damaging dopamine receptors with 6-OHDA lesions in the lateral entorhinal cortex resulted in an *improvement* in performance on a continuously reinforced retention task, suggesting that dopaminergic receptor activation acts to *disrupt* memory formation. The role of dopamine on the formation of memory in the lateral entorhinal cortex is therefore unclear, and one of the chapters of this thesis will investigate the role of dopamine in the cellular mechanisms of memory in the lateral entorhinal cortex.

Dopamine in the prefrontal cortex has been shown to affect performance on working memory tasks differently, depending on the degree of dopaminergic activation (Dent and Neill, 2012; Goldman-Rakic et al., 2000). Moderate increases in dopamine in the prefrontal cortex have been shown to enhance performance on working memory tasks, while excessive amounts of dopamine result in impairments in performance on working memory tasks (Dent and Neill, 2012). Dent and Neill, (2012) injected a small dose of dopamine (5ug) in the medial prefrontal cortex of rats and found enhancements of working memory on a T-maze task, whereas higher doses of dopamine (10 and 30 μg) led to an impairment on the working memory T-maze task. Cai and Arnsten, (1997) showed similar results where rhesus monkeys showed improved performance on a spatial working memory task after injections of a low concentration of D_1 receptor agonists A77636 and

SKF81297, and showed impairments after high concentrations of both agonists. These studies suggest that different concentrations leading to different amounts of dopamine being released in the prefrontal cortex lead to an inverted U-shape in performance on working memory tasks (Dent and Neill, 2012; Goldman-Rakic et al., 2000; Cai and Arnsten, 1997), and this it likely because dopamine has differing effects on synaptic activity in the brain that is dependent on concentration (Caruana et al., 2006). However, it is not known how the sensory and mnemonic functions of the entorhinal cortex might be similarly affected by dopamine in a concentration-dependent manner.

Dopamine may modulate sensory and mnemonic functions in the entorhinal cortex by modulating the strength of excitatory synaptic transmission. Early work investigating the role of dopamine in modulating synaptic responses in the medial entorhinal cortex showed that dopamine causes a suppression of the amplitude of field EPSPs to about half the amplitude of baseline responses, and that this suppression was blocked by a D_2 receptor antagonist, suggesting the role of D_2 receptor activation on the dopaminergic suppression effect (Stenkamp et al., 1998; See also Pralong and Jones, 1993). More recently, dopamine in the lateral entorhinal cortex has been shown to have a bidirectional effect on synaptic transmission, where application of low doses of dopamine on slices result in a facilitation of synaptic responses via activation of D_1 receptors, and application of high concentrations of dopamine results in a suppression of synaptic responses via activation of D_2 receptors (Caruana et al., 2006; Caruana and Chapman, 2008; Glovaci et al., 2014). The D_1 receptor-mediated facilitation is not accompanied by a change in paired-pulse facilitation ratio and is mediated by protein kinase A (PKA) -mediated

suppression of protein phosphatase 1 that leads to increase in the AMPA-mediated component of the EPSP (Glovaci and Chapman, 2013). In contrast, the D_2 receptor-mediated suppression of responses is associated with an increase in paired-pulse facilitation ratio, indicating that a suppression of transmitter release contributes to the suppression. Dopamine D_1 receptors also provide a smaller contribution to the suppression effect through an increased K^+ conductance that suppresses synaptic responses by lowering cellular input resistance (Caruana and Chapman, 2008).

The bidirectional effect of dopamine on synaptic transmission suggests that low levels of dopamine that may be associated with tonic firing of dopamine neurons (Shultz, 2006) may contribute to an enhancement of synaptic responses in the entorhinal cortex that could increase the salience of stimuli, and that higher synaptic concentrations of dopamine might result in a suppression of synaptic responses. The functional effect of these changes is not known, but as noted for the suppressive effects of acetylcholine, the suppression induced by dopamine could serve to raise the threshold for the induction of long-term potentiation (LTP), or to silence background noise to give priority during sensory information processing to more active synaptic inputs (Hasselmo and McGaughy, 2004).

In addition to effects on discrete synaptic responses, dopamine may also have effects on the responsiveness of the superficial layers of the entorhinal cortex to repetitive synaptic inputs that could occur during rhythmic EEG activities. Rosenkranz and Johnston (2006) conducted a study in which they applied low concentrations of dopamine to entorhinal cortex slices and found that dopamine had no effect on the amplitude of

single intracellular synaptic responses, but that when 20 Hz trains of 10 pulses were delivered, there was a reduction in the temporal summation of synaptic responses. This reduction in summation was shown to be due to activation of D_1 receptors that can increase activation of the conductance I_h to result in a lowering of dendritic input resistance and poorer temporal summation (Rosenkranz and Johnston, 2006). In contrast, it has also been suggested that dopamine may serve as a high-pass filter to selectively *enhance* responses to higher-frequency synaptic inputs (Ito and Shuman, 2007). In synaptic inputs to the CA1 region, dopamine causes a suppression of responses to single stimulation pulses, but a stronger suppression of inhibitory synaptic inputs to CA1 neurons that are normally expressed at higher frequencies of stimulation, results in a disinhibition of excitatory responses during higher-frequencies of stimulation (Ito and Schuman, 2007). However, the manner in which amplitudes of responses during trains of stimulation pulses may be affected by high and low concentrations of dopamine, that induce suppression and facilitation effects on single EPSPs, has not yet been determined within the superficial layers of the entorhinal cortex.

Lasting Synaptic Plasticity

In addition to short-term effects on synaptic transmission, neuromodulatory neurotransmitters can also modify the extent to which long-term changes in the strength of synaptic responses are induced (Korchounov and Ziemann, 2011). Long-term potentiation is a long-lasting enhancement of synaptic responses that is induced by intense presynaptic stimulation, and it is an important experimental model for the mechanisms that contribute to long-term memory formation (Muller et al., 2002). Long-

term potentiation can be induced in the entorhinal cortex, and although there has been some research on the effects of acetylcholine on LTP induction in the entorhinal cortex, the information about how dopamine may modulate entorhinal LTP is incomplete.

Acetylcholine and Synaptic Plasticity

In the hippocampus, there has been a general finding that activation of muscarinic acetylcholine receptors results in a facilitation of long term potentiation, but there have been mixed reports of the contribution of M_1 vs M_2 receptors to this effect (Burgard and Sarvey, 1990; Boddeke et al., 1992; Auerbach and Segal, 1996; Seeger et al. 2004; Luo et al., 2008; Zheng et al., 2012; Anisuzzaman et al., 2013). Burgard and Sarvey (1990) investigated the role of muscarinic receptor activation on synaptic plasticity using muscarine application on dentate gyrus slices, and found that low concentrations (1 μM) of muscarine led to a facilitation of LTP induced by high-frequency stimulation (HFS) trains, while high concentrations (10 μM) led to a depression of synaptic responses, and they found that the facilitation of LTP was blocked by application of an M_1 receptor antagonist. Application of an M_1 receptor antagonist was also shown to suppress the amplitude of long-term synaptic plasticity in an additional study (Luo et al., 2008). Similarly activation of muscarinic M_1 receptors in the hippocampus can result in a facilitation of the amplitude of LTP (Boddeke et al., 1992; Anisuzzaman et al., 2013). There has also been evidence for a role of M_2 receptors. Auerbach and Segal (1996) showed that the induction of hippocampal LTP by HFS was blocked by M_2 receptor antagonists and not affected by M_1 receptor antagonists, and that long-term *depression* (LTD) was blocked by application of an M_3 receptor antagonist. Similarly, Seeger et al.,

(2004) were not able to induce long term potentiation in CA1 pyramidal cells in M_2 knockout mice, suggesting that M_2 receptors are necessary for the induction of LTP. Zheng et al., (2012) also found that that activation of M_2 receptors enhanced LTP induction at associational/commissural fibre inputs to the CA3 region, whereas activation of M_2 receptors at mossy-fibre to CA3 synapses reduced LTP. Different muscarinic receptors may therefore have pathway-specific effects on the induction of LTP.

The role of acetylcholine on LTP in the entorhinal cortex has also been investigated (Yun et al., 2000; Cheong et al., 2001). Stimulation of the medial septum to activate cholinergic neurons enhances LTP in the entorhinal cortex in vivo (Chapman and Racine, 1997). Yun et al. (2000) applied the muscarinic receptor blocker atropine to entorhinal slices and used theta-burst stimulation to induce LTP. They were able to consistently induce LTP in slices treated with atropine, but the amount of LTP was smaller than that in control slices. The same experiment was replicated in layer V of the medial entorhinal cortex by Cheong et al. (2001) and similar results were obtained in which long-term potentiation was induced in the medial entorhinal cortex, but the amplitude of LTP was smaller with atropine than in control slices. This suggests that the normal role of muscarinic receptor activation is to enhance LTP induction. Therefore, acetylcholine appears to play a similar role in enhancing LTP induction in both the entorhinal cortex and hippocampus.

Dopamine and Synaptic Plasticity

It is commonly believed that the role of dopamine in learning is to signal the salience of relevant events or stimuli (Berridge and Robinson, 1998; Smith et al., 2011).

In determining whether reward is relevant for learning, it has been shown that small rewards are associated with a longer time needed to learn, while larger rewards lead to faster learning (Rose et al., 2012). It has also been shown that dopamine activation occurs during rewarding events as well as in response to cues that have previously been associated with an award (Schultz, 2013). Others have shown that dopamine is affected by memory of both reward-paired experiences as well as punishment-paired experiences that lead to activation of the mesolimbic dopamine projections to nucleus accumbens (Howe et al., 2013; Oleson et al., 2012). It has been suggested that an interaction between the hippocampal and striatal dopamine networks contribute to determining whether to act or not act in times of uncertainty (Baudonnat et al., 2013), and this may mediated in part by motivational drive associated with nucleus accumbens dopamine.

The involvement of dopamine in LTP in the hippocampus has been extensively studied and the literature generally points to the necessity of dopamine in the induction and maintenance of long-term synaptic plasticity (Roggenhofer et al., 2013; Navakkode et al., 2012; Kwon et al., 2008; Stramiello and Wagner, 2008; Granado et al., 2008; Swant and Wagner, 2006; Mockett et al., 2004; Li et al., 2003; Kusuki et al., 1997; but see Wei et al., 2012). Further, most studies have indicated that dopamine acts through activation of D_1/D_5 receptors in order to either reduce the threshold for activation of LTP (Roggenhofer et al., 2013; Li et al., 2003; Kusuki et al., 1997) or to enhance the amplitude of synaptic responses during LTP (Stramiello and Wagner, 2008). Li et al. (2003) found that the threshold for LTP was lowered in the CA1 of awake animals while exposed to novel stimuli, and this lower threshold was blocked by D_1/D_5 antagonists. Application of a

D_1/D_5 agonist on slices of CA1 results in an increased potentiation that is modulated through NR2B-containing NMDA receptors (Stramiello and Wagner, 2008), and it has also been shown that this D_1/D_5-NMDA-dependent lasting potentiation is due to postsynaptic Ca^{2+} signalling and activation of PKA (Roggenhofer et al., 2013). While most studies have not been able to distinguish the contributions of D_1 vs D_5 receptors in LTP, Granado et al. (2008) showed that LTP was significantly reduced in the hippocampus of D_1 receptor knock-out mice, and that this reduction is not further suppressed by application of a D_1/D_5 antagonist, suggesting that the activation of D_1 receptors alone is necessary for the full induction and maintenance of LTP in the hippocampus.

Although many articles support the role of D_1/D_5 receptors in the induction and maintenance of LTP in the hippocampus, others point to the role of D_2 and D_3 receptors (Kwon et al., 2008; Swant and Wagner, 2006; Frey et al., 1990). Swant and Wagner (2006) applied a dopamine reuptake inhibiter to CA1 slices and found an enhancement of LTP that was blocked by a D_3 receptor antagonist but not by a D_1/D_5 antagonist. They also showed that application of a D_3 agonist results in the same degree of enhancement of LTP that was seen following application of a reuptake blocker, and that the D_3 agonist-induced enhancement was blocked by application of a D_3 antagonist. Frey et al., (1990), on the other hand, showed that application of the dopamine D_2/D_3 antagonist domperidone had no effect on the induction of LTP in the CA1, but that the maintenance of the potentiation was blocked after 4 hours, contrary to results seen in potentiated control slices. They also showed that this block of the maintenance of LTP was due to

activation of D_2 receptors (Frey et al., 1990). Thus, research has generally shown that dopamine receptor activation leads to an enhancement of the induction of LTP in the hippocampus, and that multiple receptor subtypes may contribute.

The enhancement of the induction of LTP caused by activation of dopaminergic receptors in the hippocampus (Kwon et al., 2008; Swant and Wagner, 2006; Frey et al., 1990) suggests that dopamine may contribute to learning and memory through synaptic mechanisms (Bliss and Collingridge, 1993; Morris et al., 1986), and the dopaminergic projections to the entorhinal cortex suggest that dopamine may also modulate lasting synaptic plasticity in the entorhinal cortex. However, the little research that has been conducted regarding the role of dopamine on LTP in the entorhinal cortex has not generated a consistent picture. For example, Chen et al. (1995) showed that dopamine activation leads to an enhancement of long-term *depression*, and not a potentiation of synaptic responses as that seen in the hippocampus (Swant and Wagner, 2006). Caruana et al., (2007) recorded synaptic responses in the entorhinal cortex of awake rats, and showed that injection of the dopamine reuptake inhibitor GBR12909 resulted in a *block* of the induction of both long term potentiation as well as long term depression seen in normal control animals. Because Caruana et al (2006) only used a single dose of GBR12909, it is not clear if dopamine may have a dose-dependent effect on the induction of synaptic plasticity in the entorhinal cortex that might parallel dose-dependent effects that have been observed for dopamine on basal synaptic transmission (Caruana and Chapman, 2007).

Overview of Experimental Chapters

The aim of this thesis is to explore the role of neuromodulatory transmitters on synaptic communication in cortical inputs to the superficial layers of the entorhinal cortex. The first chapter used electrophysiological field potential recordings in slices of entorhinal cortex tissue in order to assess which receptor subtypes are involved in the suppression of synaptic responses in inputs to layer II caused by activation of cholinergic receptors. Results showed that application of an M_1-family receptor antagonist prior to application of a cholinergic agonist resulted in almost a full block of the cholinergic suppression seen in slices treated with the cholinergic agonist alone. This was not the case in tests using either an M_2 or an M_4 receptor antagonist. However, when a more selective M_1 receptor blocker was applied, the cholinergic suppression was only partially blocked, suggesting that there are other mechanisms mediating the inhibition of fEPSPs caused by activation of cholinergic receptors. The role of cannabinoid-1-receptors was also assessed, and no significant role for these receptors was identified.

The second experimental chapter aimed to determine the role of dopamine activation on the amplitudes of responses during trains of high and low frequency stimulation to the entorhinal cortex. To do so, gamma- and theta-frequency trains were delivered to the piriform cortex of live rats before and after injection of a dopamine agonist, and field excitatory postsynaptic potentials were recorded in the entorhinal cortex. Dopamine receptor activation did not change the amplitude of synaptic responses during gamma-frequency trains. However, results showed that in a subgroup of animals, there was an increase in the amplitude of the first response during theta-frequency trains

following administration of the dopamine agonist, but that the rate of growth in later responses during the trains was reduced, resulting in a relative suppression of train-evoked responses. Both of these effects, the facilitation of the first response in the theta-frequency trains as well as the relative suppression of subsequent responses, were blocked by injection of a D_1 receptor antagonist prior to the dopamine agonist, as well as by a D_2 receptor antagonist. This suggests that, although activation of dopamine receptors can facilitate single synaptic responses in the lateral entorhinal cortex, that there may be a ceiling effect that may limit the growth of subsequent synaptic responses during repetitive synaptic activity occurring at theta-frequency.

The third experimental chapter was also aimed at determining the role of dopamine receptor activation on the amplitudes of field excitatory postsynaptic potentials in the entorhinal cortex during gamma- and theta-frequency trains, however this chapter was conducted using field potential recordings in acute brain slices and also tested the effects of two different concentrations of dopamine. Application of the *low* concentration of dopamine resulted in a facilitation of not only the first response in the trains, but also responses throughout both gamma- and theta-frequency trains. Results from slices therefore suggest that dopamine may have the capacity to enhance repetitive synaptic responses at both gamma and theta frequencies, and previous results suggest that this facilitation is mediated by D_1 receptors (Caruana et al., 2006; Glovaci et al., 2014),

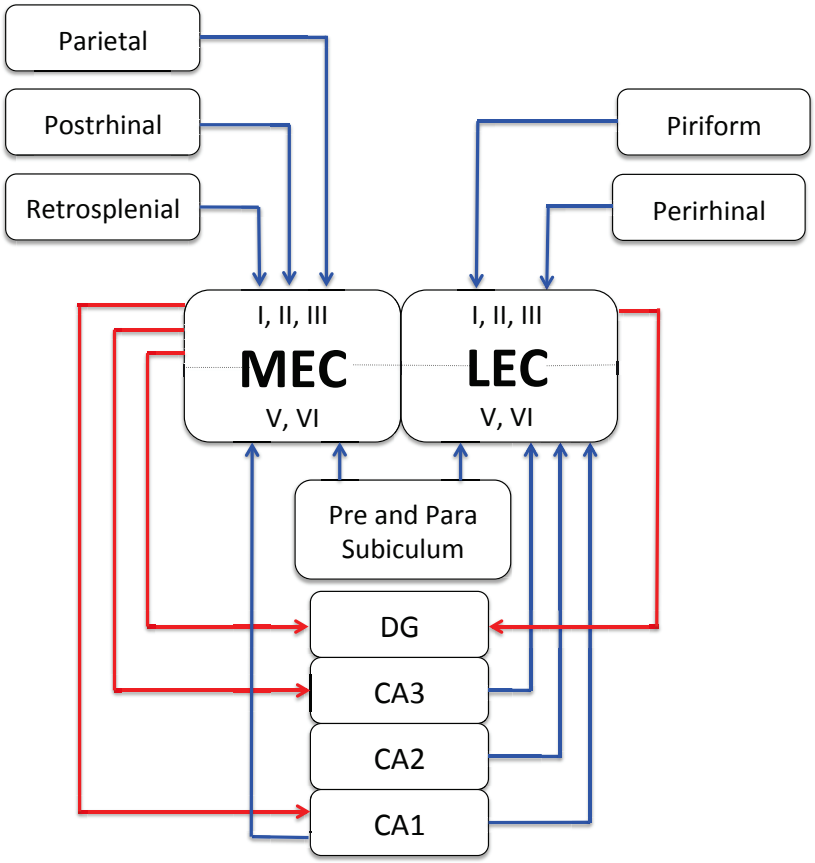
After application of the *high* concentration of dopamine, there was a *suppression* of the amplitude of the first response in the trains, and an associated increase in the relative amplitudes of later responses during the trains. This suggested that very strong

release of dopamine in the entorhinal cortex may result in a suppression of individual synaptic responses, but may also help maintain patterns of synaptic activation that occur with the frequencies of the theta and gamma rhythms. Both D_1 and D_2 receptors appear to be involved in changes in synaptic responses induced by 50 μM dopamine. Application of a D_2 receptor antagonist blocked the effects of 50 μM dopamine, including the suppression of the first response during trains as well as the relative facilitation effects. The D_1 receptor blocker, on the other hand, had no effect on the suppression of the response to the first pulse during both trains of stimulation, or the relative facilitation seen during the gamma trains, but it did block the suppression of later responses during theta-frequency trains. These results suggest that D2 receptors are involved in the suppression of single synaptic responses caused by high-concentrations of dopamine as well as the relative facilitation shown during trains of gamma frequency stimulation, and that D_1 receptors are involved in the suppression responses during trains of theta-frequency stimulation.

The last experimental chapter used in vitro field potential recordings to determine the role of dopamine on the induction of long-term synaptic potentiation in the entorhinal cortex. Application of a low concentration of dopamine alone to entorhinal cortex slices resulted in a facilitation of the amplitudes of synaptic responses, as has been observed before (Glovaci and Chapman, 2013). While administration of HFS in slices maintained in control artificial cerebral spinal fluid (ACSF) resulted in the induction of long-lasting potentiation of responses, the application of dopamine prior to HFS resulted in a block of LTP. Surprisingly, this suggests that dopamine in the entorhinal cortex may serve to

inhibit the induction of lasting increases in synaptic strength that may contribute to learning and memory.

Figure 1.1. The superficial layers of the medial entorhinal cortex (MEC; layers I, II and III) receive their cortical projections from the parietal (Kerr et al., 2007), postrhinal (Burwell, 2000) and retrosplenial (Kerr et al., 2007) corticies (Top left, blue lines), and project to the dentate gyrus, CA3 and CA1 areas of the dorsal and ventral hippocampus (Bottom left, red lines). Deep layers of the medial entorhinal cortex (layers V and VI) receive inputs from the CA1 region, and from the presubiculum and the parasubiculum (Agster and Burwell, 2013; Bottom left, blue lines). Superficial layers of the lateral entorhinal cortex (LEC; layers I, II, and III) receive inputs from the perirhinal and piriform corticies (Agster and Burwell, 2013; Top right, blue lines), and provide input to the dentate gyrus (Dolorfo and Amaral, 1998[1]; Dolorfo and Amaral, 1998[2]; Bottom right, red line). Deep layers of the lateral entorhinal cortex (layers V and VI) receive inputs from the CA1, CA2, and CA3, and from the presubiculum and parasubiculum (Agster and Burwell, 2013; Bottom right, blue lines).


Parietal
Postrhinal
Retrosplenial
Piriform
Perirhinal
I, II, III
MEC
V, VI
I, II, III
LEC
V, VI
Pre and Para Subiculum
DG
CA3
CA2
CA1

Chapter 2

CONTRIBUTION OF MUSCARINIC M_1 RECEPTORS TO THE CHOLINERGIC SUPPRESSION OF SYNAPTIC RESPONSES IN LAYER II OF THE ENTORHINAL CORTEX

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ABSTRACT

The entorhinal cortex is thought to play a role in mechanisms mediating sensory and mnemonic function, and the cholinergic suppression of the strength of synaptic inputs is likely to have important impacts on these processes. We have previously shown that field excitatory postsynaptic potentials (fEPSPs) in the medial entorhinal cortex evoked by stimulation of the piriform cortex are suppressed during theta EEG activity in behaving animals, and that cholinergic receptor activation suppresses synaptic responses both *in vivo*, and in layer II entorhinal neurons *in vitro*. Here, we have used *in vitro* field potential recordings to investigate the transmitter receptors that mediate the cholinergic suppression of synaptic responses in layer I inputs to layer II of the medial entorhinal cortex. Bath-application of the cholinergic agonist carbachol suppressed the amplitude of fEPSPs with an EC_{50} of 5.3 μM, and enhanced paired-pulse ratio consistent with a reduction in transmitter release. Application of the M_2-preferring receptor blocker methoctramine, or the M_4 receptor blocker PD102807 prior to addition of carbachol did not prevent the cholinergic suppression. However, the M_1- preferring receptor blocker pirenzepine and the M_1 receptor blocker VU0255035 markedly reduced the suppression, suggesting that the cholinergic suppression of excitatory synaptic responses in the entorhinal cortex is dependent in large part on activation of M_1 receptors. In addition to enhancements in neuronal excitability that follow cholinergic activation, therefore, cells in layer II of the entorhinal cortex also display a suppression of excitatory synaptic input that is mediated in part by M_1 muscarinic receptors. The role of cannabinoids on the cholinergic suppression of synaptic responses in the medial entorhinal cortex was

assessed because muscarinic receptor activation can increase cannabinoid synthesis, however, application of the CB1R antagonist AM281 did not block the cholinergic suppression suggesting cannabinoids are not involved.

INTRODUCTION

Neurons in layer II of the entorhinal cortex receive synaptic inputs from regions including the perirhinal and piriform cortices, and they also provide the hippocampal formation with its largest cortical sensory input (Burwell and Amaral, 1998). These strong interconnections suggest that the entorhinal cortex plays important roles in the sensory/integrative and mnemonic functions of the medial temporal lobe (Witter et al., 1989; Lavenex and Amaral 2000; Burwell, 2000). There has also been a growing interest in the role of the entorhinal cortex in spatial navigation because of the discovery of "grid cells" in the medial entorhinal cortex that fire in a manner that is dependent on the animal's spatial location as it moves through the environment (Derdikman and Moser, 2010). During periods of behavioural mobility, the entorhinal cortex and hippocampus generate theta-frequency (4-12Hz) electroencephalographic (EEG) activity that is promoted by cholinergic inputs from the medial septum (Alonso and Garcia-Austt 1987; Dickson et al., 2000). Theta activity helps to coordinate firing among entorhinal neurons (Brandon et al. 2011), modulates transmission through the circuitry of the hippocampal formation (Bland and Oddie, 2001; Schall and Dickson 2010), and is thought to promote learning-related synaptic plasticity by enhancing postsynaptic depolarization (Huerta and Lisman, 1995; Chapman and Racine, 1997; Yun et al, 2000; Auerbach and Segal, 1996; Hasselmo, 2006).

In contrast to the excitatory effects of acetylcholine on membrane potential and neuronal excitability (Klink and Alonso, 1997; Egorov et al., 2002; Brown, 2010), cholinergic input to the entorhinal cortex and hippocampus generally results in a

suppression of excitatory synaptic transmission (Richter et al., 1999; Hamam et al., 2007; Hasselmo and McGaughy, 2004; Yun et al., 2000). Synaptic responses in layer II of the entorhinal cortex are suppressed during theta activity in vivo, and cholinergic agonists suppress entorhinal EPSPs both in vivo and in vitro (Hamam et al. 2007). Similar suppression effects are observed in entorhinal layers III and V (Yun et al., 2000; Cheong et al., 2001) and in layer V inputs to layer II of medial entorhinal cortex (Richter et al., 1999). The cholinergic suppression of synaptic strength may serve to offset hyperexcitability associated with cholinergic depolarization (Friedman et al., 2007), enhance the salience of active synaptic inputs relative to a reduced level of background "noise", or may enhance the selectivity with which synaptic inputs contribute to learning-related synaptic plasticity (Hasselmo and McGaughy, 2004).

Although the cholinergic suppression of EPSPs has usually been attributed to M_1-preferring receptors (that include M_1, M_3 and M_5 receptors), there have been several reports that support the involvement of M_2-like receptors that include the M_4 subtype. The cholinergic suppression of EPSPs in layer V inputs to layer II of the medial entorhinal cortex is blocked by the M_1 receptor antagonist pirenzepine (Richter et al., 1999), and other reports have supported a role for M_1 receptors in the cholinergic suppression of EPSPs in the CA1 region (Auerbach and Segal, 1996; Sheridan and Sutor, 1990). However, the cholinergic suppression of CA1 EPSPs is also effectively blocked by gallamine which is an antagonist with a greater affinity for M_2 versus M_1 receptors (Dutar and Nicoll, 1988), and while the suppression of CA1 EPSPs is markedly reduced in M_1 receptor knockout mice, a residual suppression suggests that other receptor

subtypes also contribute (Kremin et al., 2006). Work with knock-out mice has also shown that, although there is an attenuation of the carbachol-induced suppression in M_1 knock-out mice, that the cholinergic suppression of EPSPs was completely blocked in M_4 knock-out mice (Dasari and Gulledge, 2011) indicating that the M_4 receptor plays a major role in the CA1 region.

In the present study, we used field potential recordings from acute brain slices to examine the muscarinic receptors that mediate the carbachol-induced suppression of fEPSPs in layer I inputs to layer II of the medial entorhinal cortex. The cholinergic suppression of synaptic responses in the entorhinal cortex is well established, and known to be associated with reduced transmitter release (Yun et al 2000; Cheong et al 2001; Hamam et al, 2007), but the muscarinic subtypes involved in the suppression of layer I inputs to layer II of the medial entorhinal cortex has not been determined. Muscarinic receptor blockers with differing affinities were used to assess the involvement of different muscarinic receptor subtypes in the cholinergic suppression of synaptic transmission (Caulfield and Birdsall, 1998). In addition, because muscarinic receptor activation can increase cannabinoid synthesis (Kano et al., 2009), and activation of CB1R receptors can suppress inhibitory synaptic transmission in the entorhinal cortex (Morgan et al., 2008) we also assessed the potential role of CB1 receptors using the CB1R antagonist AM281.

MATERIALS AND METHODS

In Vitro Slice Preparation

Acute brain slices were prepared in accordance with the guidelines of the Canadian Council on Animal Care. Slices were obtained from 5 to 7-week old rats that

were anesthetized with halothane and decapitated. The brain was extracted and submerged in ice-cold ACSF containing (in mM) 2 KCl, 1.4 NaH_2PO_4, 2.7 $MgSO_4$, 0.5 $CaCl_2$, 26 $NaHCO_3$, 10 dextrose, and 250 sucrose. Horizontal slices (400 μM thick) were cut using a vibratome (WPI, Vibroslice NVSL), and allowed to recover in room temperature ACSF (~22º C) containing (in mM) 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, 10 dextrose, L-ascorbic acid (0.4 mM), uric acid (0.35 mM) and indomethecine (40 μM) saturated with 95% O_2 and 5% CO_2 (pH ~7.3; 300-310 mOsm) for at least 1.5 h. Individual slices were transferred to a nylon net in a temperature-regulated gas-fluid interface chamber (Fine Science Tools). The upper surface of the slice was exposed to a humidified 95%/5% O_2/CO_2 atmosphere, and the chamber was perfused with oxygenated ACSF at a rate of 1.5 - 2.0 ml/min at 32 ± 0.5º C. There was a recovery period of at least 20 min before recordings.

Stimulation and Recording

Field potential recording electrodes were pulled from borosilicate glass (1.0 mm OD) using a horizontal puller (Sutter Instruments, P97), and were filled with ACSF (2-6 MΩ). Electrodes were positioned with the aid of a dissecting microscope (Leica, MS5) and the field potential recording electrode was placed in layer I near the border of layer II at a depth of roughly 200 μm below the surface. Synaptic responses were evoked with a concentric bipolar electrode (FHC) placed in layer I, 0.4 to .8 mm rostral to the recording electrode. Cathodal constant current pulses were delivered using a stimulus generator (WPI, Model A300) and stimulus isolation unit (Model A360). Evoked fEPSPs were filtered and amplified (DC-3 kHz, Axon Instr., Axoclamp 2B) and digitized (20 kHz,

Axon Inst., Digidata 1322A) using pClamp 8.2 software (Axon Instr.). Stimulation intensities were adjusted to evoke fEPSPs with amplitudes of ~65-75 % of the maximal response.

The cholinergic suppression of fEPSPs was characterized by evoking synaptic responses every 20 sec to establish a stable baseline of at least 10 min, followed by 10-min constant bath application of the cholinergic agonist carbachol (CCh, 1, 5, 10, 20, 50, or 100 μM), and a 20-min washout period in normal ACSF. Responses in a control group were recorded without CCh application. Nonlinear regression analysis using a four-parameter logistic equation was used to determine the EC_{50} of carbachol (SigmaPlot 11.0), and 5 μM CCh was used in subsequent tests in which the suppression of responses was challenged using receptor antagonists. To assess if the cholinergic suppression was due to pre- or post-synaptic mechanisms (Hamam et al., 2007; Richter et al., 1999), pairs of pulses were administered with an interpulse interval of 30 ms, and paired-pulse facilitation ratio was expressed as the amplitude of the response to the second pulse as a percentage of the amplitude of the response to the first pulse.

Following a stable baseline in normal ACSF, an antagonist was bath applied for 20 min prior to addition of carbachol for 10 min. The M_1-preferring receptor blocker pirenzepine dihydrochloride (1 μM, Ascent Scientific, Princeton, NJ) and the M_2-like receptor blocker methoctramine (5 μM, Sigma-Aldrich, St. Louis, MO) were used in initial tests, and concentrations were chosen based on previous research (Richter et al., 1999; Glasgow et al., 2012) and receptor subtype affinifies (Stoll et al., 2009). Pirenzepine blocks M_1 receptors, and can also block M_4 receptors at higher doses, and

methoctramine blocks both M_2 and M_4 receptors (Stoll et al., 2009; Caulfield and Birdsall, 1998; Dorje et al., 1991). The effects of the more selective M_1 receptor antagonist VU0255035 (5 and 10 μM; Eli Lilly and Company, Windlesham, UK; Sheffler et al., 2009; Xiang et al., 2012) and the M_4 receptor antagonist PD102807 (0.5 and 5 μM; Tocris Bioscience, Bristol, UK; Kitaichi et al., 1999) were also determined. Concentrated stock solutions were obtained by dissolving pirenzepine and methoctramine in distilled water, VU0255035 in DMSO (final concentration <0.1%), and PD102807 in 1% HCl (final concentration 0.003%). Changes in paired-pulse facilitation (30 ms interval) were also monitored in some tests (Hamam et al. 2007).

Because M_1 receptor-activation might lead to suppression of synaptic transmission via cannabinoid CB1 receptors on presynaptic terminals (Kano et al., 2009) the ability of the CB1R antagonist AM281 (1 μM; Leterrier et al., 2006) to block the cholinergic suppression of EPSPs was also tested. AM281 was obtained from Tocris Bioscience, prepared in a stock solution of 0.1% ethanol and 0.1% cremophor (final concentration 0.0003%).

Data Analysis

Peak amplitudes of synaptic potentials were measured relative to the prestimulus baseline, using pClamp 8.2 software, and expressed as the mean ± SEM. Changes in average fEPSP amplitudes measured during the 10 min baseline period prior to CCh application which occurred either during normal ACSF or during antagonist application, the last 5 min of CCh application, and the last 5 min of the follow-up period were analyzed to assess the effects of CCh and of co-application of receptor blockers. Effects

of varying concentrations of CCh on synaptic responses were assessed using a 1-way between-subjects ANOVA, and logistic regression was used to determine the EC_{50} of CCh (see above). The CCh-induced suppression was assessed relative to the control group using a mixed ANOVA of measures during baseline, CCh, and washout. Effects of adding receptor blockers to normal ACSF were assessed relative to controls using mixed 2 x 2 ANOVAs. Effects of receptor blockers on the CCh-induced suppression were assessed using mixed 2 x 3 ANOVAs and Neuman-Keuls tests. Paired-pulse facilitation was quantified by expressing the amplitude of responses evoked by the second pulse as a percentage of responses to the first pulses (Hamam et al., 2007), and ratios were compared between baseline, drug, and washout conditions. Averages of ten consecutive evoked responses were obtained for graphical display.

RESULTS

Cholinergic suppression of evoked synaptic responses

Stimulation of layer I resulted in negative synaptic field potentials in layer II of the medial entorhinal cortex (e.g., Figure 2.1A). The effect of cholinergic receptor activation on evoked responses was assessed using 10-minute constant bath application of a range of concentrations of the cholinergic receptor agonist carbachol (1 to 100 μM, n = 4 to 7; Figure 2.1B, inset). Carbachol induced a dose-dependent, reversible suppression of the amplitude of synaptic field potentials ($F_{5,24} = 9.71$, $p < .001$). A concentration of 1 μM carbachol resulted in a suppression to 87.2 ± 4.9 % of baseline (-0.44 ± 0.02 versus -0.48 ± 0.03 mV), and 100 μM carbachol suppressed responses to 36.9 ±3.4 % of baseline values (-0.26 ± 0.02 versus -0.71 ± 0.08 mV). Nonlinear regression analysis indicated an

EC_{50} of CCh of 5.27 ± 3.03 μM ($F_{3,30} = 34.27$, $p < 0.0001$; $R^2 = 0.77$). Application of 5 μM CCh resulted in a suppression to 62.0 ± 5.4 % of baseline values (Figure 2.1A,B; $F_{2,22} = 8.91$, $p < 0.01$), and this concentration was used in subsequent tests of the effects of receptor blockers.

The contribution of muscarinic receptors to the suppression of fEPSPs was assessed initially by adding either the M_2-preferring receptor antagonist methoctramine (1 μM), or the M_1-preferring receptor antagonist pirenzepine (1 μM) to the bathing medium prior to addition of carbachol (Richter et al., 1999; Glasgow and Chapman, 2013; Glasgow et al., 2012). Amplitudes of synaptic responses tended to increase with time during addition of pirenzepine or methoctramine, and during application of other receptor blockers indicated below, but similar increases were observed in control recordings indicating that increases were not due to receptor antagonism (Figure 2.1B open circles, Figure 2.2A-C; drug by time interactions were non-significant: pirenzepine, $F_{1,11} = 1.39$, $p = 0.26$, methoctramine, $F_{1,9} = 1.46$, $p = 0.26$). Adding 1 μM methoctramine to the bath for 20 min prior to addition of carbachol failed to block the suppression of fEPSPs evoked by carbachol (58.9 ± 4.4%, n = 7, Figure 2.2A), and responses were reduced to 58.9 ± 4.4 % of baseline. A significant time by group interaction resulted from larger amplitudes in the methoctramine group during the washout period ($F_{2,20} = 3.77$, $p < 0.05$; n = 5) but there was no significant difference in the amount of depression induced by addition of CCh to methoctramine versus to normal ACSF (N-K, $p = 0.55$). Note also the significantly smaller responses at the end of the washout period in the presence of methoctramine vs normal ASCF (N-K, $p < .001$). Pirenzepine, however, blocked the CCh-induced

suppression of fEPSPs (n = 7, 94.8 ± 5.6 % of baseline levels; Figure 2.2B). This was reflected in a significant group by time interaction ($F_{2,24} = 11.7$, $p < 0.001$) with no significant CCh-induced suppression in the presence of pirenzepine (N-K, $p = 0.36$), and a significantly smaller CCh-induced suppression in pirenzepine versus normal ACSF (N-K, $p < 0.001$). The suppression is therefore dependent upon muscarinic receptors sensitive to pirenzepine.

An increase in paired-pulse facilitation ratio during CCh application, which is consistent with reduced transmitter release versus changes in postsynaptic mechanisms (Qian and Saggau, 1997; Zucker and Regehr, 2002), was been reported in entorhinal layer II by Hamam et al. (2007) but not by Richter et al., (1999). The cholinergic suppression of fEPSPs observed here was associated with a reversible increase in paired-pulse facilitation ratio from 110.1 ± 2.0% to 122.2 ± 4.0% ($F_{2,12} = 4.46$, $p = 0.036$; N-K, $p = 0.029$; 122.2 ± 4.0 %). Similarly, the suppression in fEPSP amplitude in slices exposed to methoctramine was also associated with an increase in paired-pulse ratio ($F_{2,12} = 4.67$, $p = 0.032$; N-K, $p = 0.025$; 117.6 ± 6.7 %), while paired-pulse ratios were stable in slices exposed to pirenzepine ($F_{2,12} = 3.37$, $p = 0.069$; 104.8 ± 2.5 %) (Figure 2.2D$_2$).

M_1 and M_4 Receptor Blockers

Pirenzepine can have effects on M_4 receptors as well as M_1 receptors (Caulfield and Birdsall, 1998; Dorje et al., 1991). We therefore compared effects of the more selective M_1 receptor antagonist VU0255035 (5 and 10 μM; Xiang et al., 2012; Sheffler et al., 2009) and the M_4 receptor antagonist PD102807 (0.5 and 5 μM; Bohme et al., 2002; Caulfield and Birdsall, 1998). Application of 5 μM VU0255035 did not block the

CCh-induced suppression ($F_{2,18} = 1.72$, $p = 0.21$; n = 4, 72.4 ± 8.9 %) but the suppression was significantly reduced by 10 μM VU0255035 ($F_{2,22} = 5.56$, $p = 0.011$; 82.1 ± 3.2 %; n = 6, Figure 2.3$A_{1,2}$). The reduction to 82.1 ± 3.2% of baseline levels in the presence of VU0255035 (N-K, $p < 0.001$) was significantly smaller than that induced by CCh in normal ACSF (62.0 ± 6.4, N-K, $p < 0.001$). The role of M_4 receptors was also tested using the antagonist PD102807. Application of 0.5 or 5 μM PD102807 did not prevent the CCh-induced suppression of synaptic responses (n=3 and 4 respectively, $F_{2,16} = 1.61$, $p = 0.230$, 54.3 ± 8.0 %; $F_{2,22} = 0.71$, $p = 0.503$, 54.0 ± 6.0%) suggesting that M_4 receptors are not required for the effect (Figure 2.3B).

CB1R Antagonism

Muscarinic receptor activation can increase synthesis of the endocannabinoid 2-AG (Kim et al., 2002; Kano et al., 2009), and activation of CB1 receptors suppresses synaptic transmission at entorhinal inhibitory synapses (Morgan et al., 2008). We therefore tested whether application of the CB1R antagonist AM281 (1 μM; Gifford et al., 1997) might attenuate the CCh-induced suppression of EPSPs. The size of the suppression of EPSPs induced by CCh in the presence of AM281 did not differ from that observed in normal ACSF, however, indicating that CB1 receptors are not required ($F_{2,24} = 1.01$, $p = 0.381$; n = 7, Figure 2.4).

DISCUSSION

The cholinergic suppression of synaptic responses within the hippocampal region (Benardo and Prince, 1982; McCormick and Prince, 1986; Glasgow and Chapman, 2011) and entorhinal cortex (Hamam et al., 2007; Richter et al., 1999, Cheong et al., 2001; Yun

et al., 2000; Sparks and Chapman 2013) is well established. Carbachol suppresses fEPSPs in layers III and V of the entorhinal cortex (Yun et al., 2000; Cheong et al., 2001), and in layer V inputs to layer II (Richter et al., 1999), and the present study has investigated the cholinergic suppression in layer I inputs to layer II of the medial entorhinal cortex. M_1 receptors are thought to contribute to cholinergic suppression effects in the hippocampus (Hasselmo and McGaughy, 2004; Dasari and Gulledge, 2011; Kremin et al., 2006). In the entorhinal cortex, the muscarinic antagonist atropine blocks the cholinergic suppression of layer II inputs to layer III (Yun et al., 2000) and layer V responses to local stimulation (Cheong et al., 2001), and the M_1- preferring receptor blocker pirenzepine also blocks the cholinergic suppression of layer V inputs to layer II of the medial entorhinal cortex (Richter et al., 1999). We have used muscarinic receptor blockers with differing affinities for muscarinic receptor subtypes, and have found that the M_1 receptor blockers pirenzepine and VU0255035 either block or attenuate the suppression of fEPSPs induced by carbachol, while M_2-like and M_4 receptor blockers do not. M_1 receptors are therefore likely to play a primary role in the cholinergic suppression of synaptic transmission in layer II of the entorhinal cortex.

The M_1-preferring receptor blocker pirenzepine effectively blocked the CCh-induced suppression of responses, but pirenzepine also has a partial affinity for M_4 receptors (Caulfield and Birdsall, 1998; Dorje et al., 1991). Experiments in knockout mice have also shown that, although the cholinergic suppression of hippocampal CA3-CA1 synapses is attenuated in mice lacking M_1 receptors, it is more completely blocked in mice lacking M_4 receptors (Dasari and Gulledge, 2011). The block of the cholinergic

suppression by pirenzepine observed here is most likely due to effects on M_1 receptors; higher doses of pirenzepine are required to obtain strong effects on M_4 receptors (Dorje et al., 1991), and there was no significant reduction in the cholinergic suppression in the presence of the M_2-like receptor blocker methoctramine which blocks both M_2 and M_4 receptors (Stoll et al., 2009; Caulfield and Birdsall, 1998). In addition, the selective M_4 receptor antagonist PD102807 (0.5 and 5 μM) did not block the cholinergic suppression, but bath application of the more selective M_1 receptor antagonist VU0255035 led to a strong reduction in the CCh-induced suppression. Interestingly, responses recorded during application of both methoctramine and PD102807 prior to carbachol resulted in a delayed return to baseline amplitudes that may be due to a block of M_4 receptors by both antagonists, however, mechansims of this delay are not yet known. Although muscarinic receptor blockers have overlapping affinities, and conclusions must therefore be tentative (Stoll et al., 2009; Caulfield and Birdsall, 1998; Dorje et al., 1991), the present results point to an important role for M_1 receptors in the cholinergic suppression of synaptic responses in layer II of the entorhinal cortex (Kremin et al., 2006).

The cholinergic suppression of synaptic responses is generally accompanied by a reversible increase in paired-pulse facilitation ratio (Auerbach and Segal, 1996; Hamam et al. 2007; Yun et al., 2000; Chong et al., 2001; but see Richter et al., 1999). This suggests that the suppression of synaptic responses is due to a reduction in transmitter release that can result in a larger readily releasable pool of transmitter available in response to the second stimulation pulse (Zucker and Regher, 2002). Muscarinic M_1 receptors on presynaptic terminals can inhibit release by inhibiting voltage-dependent

calcium channels (Sheridan and Suitor, 1990; Qian and Saggau, 1997; Kremin et al., 2006). In addition, M_1 receptor-mediated increases in diacylglycerol can enhance synthesis of the endocannabinoid 2-AG, and 2-AG can act as a diffusible retrograde signal that can activate presynaptic CB1Rs that modulate voltage-gated calcium channels (Ohno-Shosaku et al., 2003; Kano et al. 2009; Wilson and Nicoll, 2001; Twitchell et al., 1997). Activation of CB1Rs is known to suppress inhibitory synaptic transmission in the entorhinal cortex (Morgan et al., 2008), but the CB1R blocker AM281 did not block the cholinergic suppression of synaptic responses in the entorhinal cortex, indicating that CB1Rs are not required for this effect.

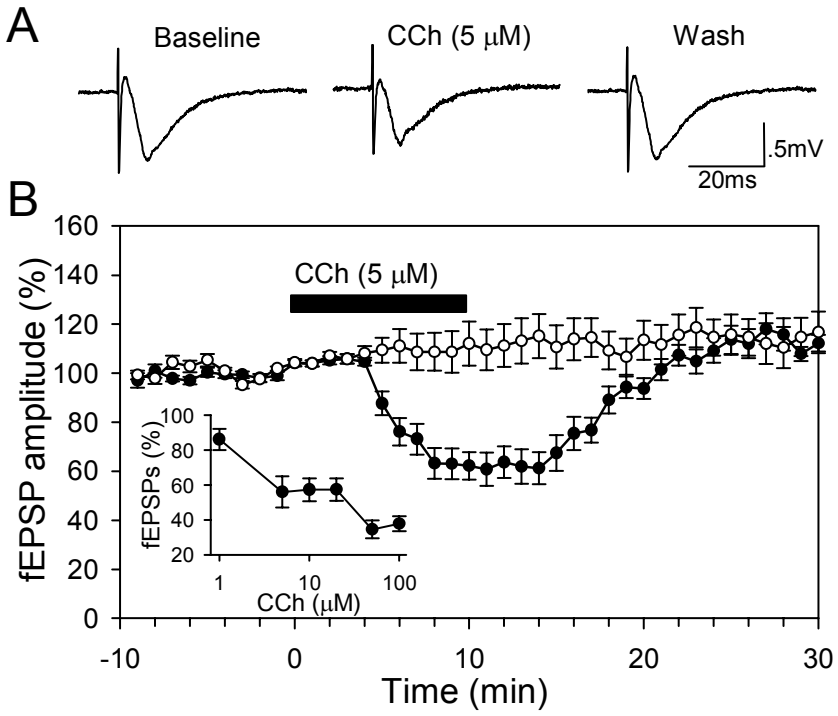
Periods of behavioural arousal and exploration are associated with release of acetylcholine in the entorhinal cortex that can enhance excitability of entorhinal neurons and promote neuronal synchrony by enhancing theta-frequency EEG activity (Alonso and Garcia-Austt, 1987; Bland and Oddie, 2001; Buzsaki, 2002). The cholinergic suppression of synaptic responses may serve to reduce excessive network activity that could lead to epileptogenesis (Friedman et al., 2007). However, there would be minimal therapeutic benefit of enhancing M_1 receptor activity in epilepsy because of the role of M_1 receptors in promoting development of seizures (Friedman et al., 2007). The cholinergic suppression may also serve to minimize potential interference between incoming sensory input and working memory representations, or may modulate which synaptic inputs may contribute to learning-related synaptic (Hasselmo and McGaughy, 2004). The cholinergic suppression may also make the relative timing of synaptic inputs with ongoing theta-frequency EEG activity particularly important, such that the strength of synaptic inputs

that repeat at theta or gamma frequencies may be better maintained (Sparks and Chapman, 2013) and may contribute more effectively to lasting synaptic potentiation effects (Huerta and Lisman, 1995; Chapman and Racine, 1997).

Conclusions

Both the M_1- preferring antagonist pirenzepine and the selective M_1 receptor blocker VU0255035 significantly attenuate the carbachol-induced suppression of synaptic responses in layer II of the entorhinal cortex, while the suppression of responses is not prevented be either the M_2-preferring antagonist methoctramine or the M_4 antagonist PD102807. This indicates that M_1 muscarinic receptors are likely to play a major role in the cholinergic suppression of synaptic transmission in layer I inputs to layer II of the medial entorhinal cortex.

Figure 2.1. Bath-application of the cholinergic agonist carbachol (CCh) reduces the amplitude of evoked fEPSPs in the entorhinal cortex *in vitro*. **A.** Averaged fEPSPs evoked in layer II by stimulation of layer I of the medial entorhinal cortex were attenuated by 10 min bath-application of 5 μM CCh. Responses returned to baseline levels during washout. **B.** Carbachol resulted in a dose-dependent reduction in fEPSP amplitude with an EC50 of 5.3 μM (inset). The time-course of changes in fEPSPs induced by 5 μM carbachol (black bar) is shown relative to the control group. Bars represent ± the SEM.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Wash
.5mV
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B
CCh (5 μM)
fEPSP amplitude (%)
Time (min)
fEPSPs (%)
CCh (μM)

Figure 2.2. The cholinergic suppression of the amplitude of fEPSPs in the entorhinal cortex is dependent on activation of M_1- preferring, but not M_2-like, muscarinic receptors. **A.** Traces in A_1 show averaged fEPSPs recorded during bath-application of ACSF, addition of the M_2-like receptor antagonist methoctramine (Methoc, 1 μM), addition of 5 μM carbachol (CCh), and during wash-off of carbachol. Application of the M_2 receptor antagonist methoctramine for 20 min prior to addition of carbachol failed to block the cholinergic suppression of fEPSPs (A_2). **B.** Pirenzepine, an M_1- preferring receptor antagonist, effectively blocked the suppression of fEPSPs induced by carbachol. Conventions are as in panel A. **C.** A summary histogram shows mean fEPSP amplitudes recorded in normal ACSF prior to addition of antagonists, during the 5 min period prior to addition of carbachol, the first 5 min after carbachol application, during the last 5 min of washout. Asterisks indicate a significant reduction of fEPSPs relative to the baseline period (*, $p < 0.05$; #, $p < 0.01$). **D.** The cholinergic suppression of fEPSPs is associated with enhanced paired-pulse facilitation ratio, suggesting that the suppression is expressed presynaptically. Representative responses to a pair of stimulation pulses (30 ms interpulse interval) recorded in control ACSF and in carbachol are shown, and have been scaled to the amplitude of the first response in control ACSF to illustrate changes in paired-pulse facilitation ratio (D_1). Note the larger scaled amplitude of the second response recorded in carbachol (arrow). Mean paired-pulse facilitation ratios were enhanced by addition of carbachol to normal ACSF or methoctramine (*, $p<0.05$) but were unaffected when the carbachol-induced suppression was

blocked by pirenzepine.

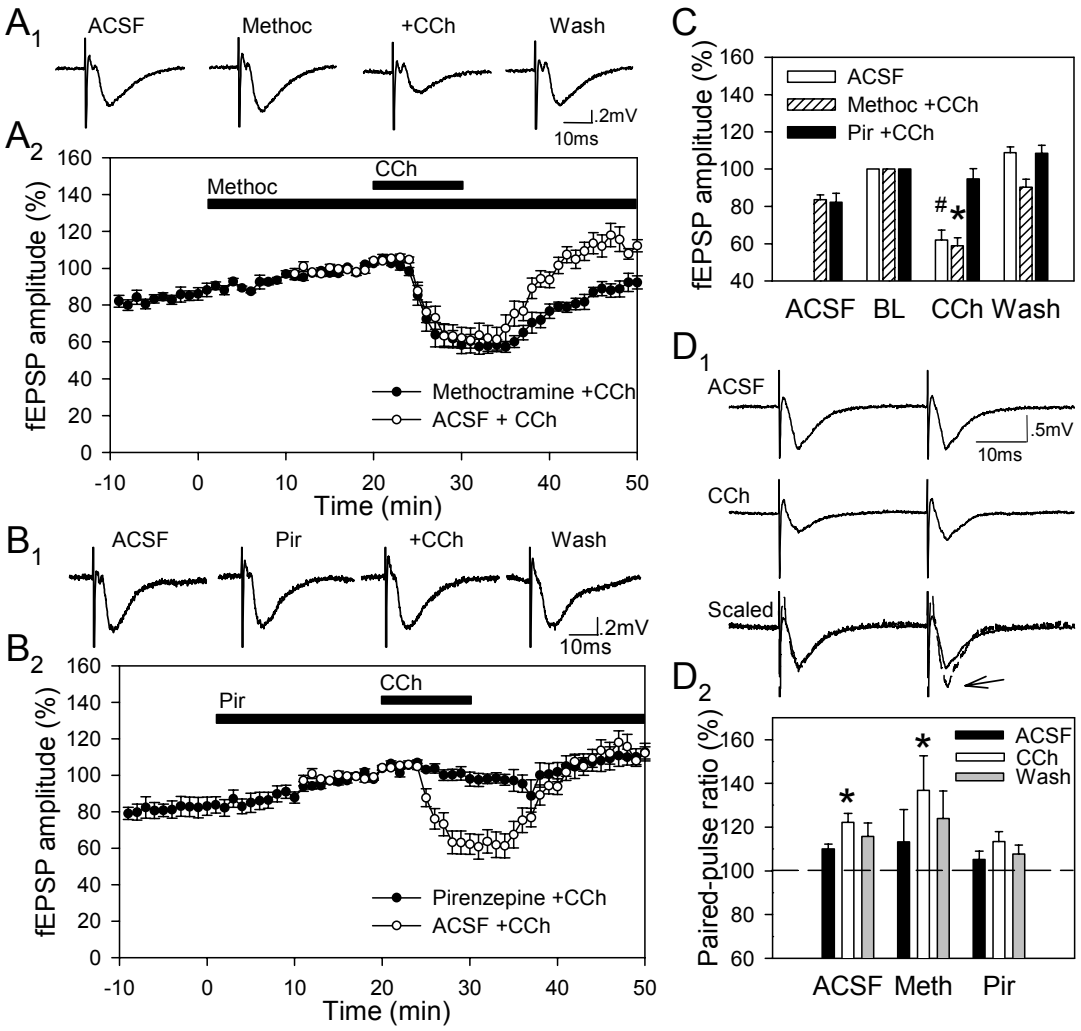
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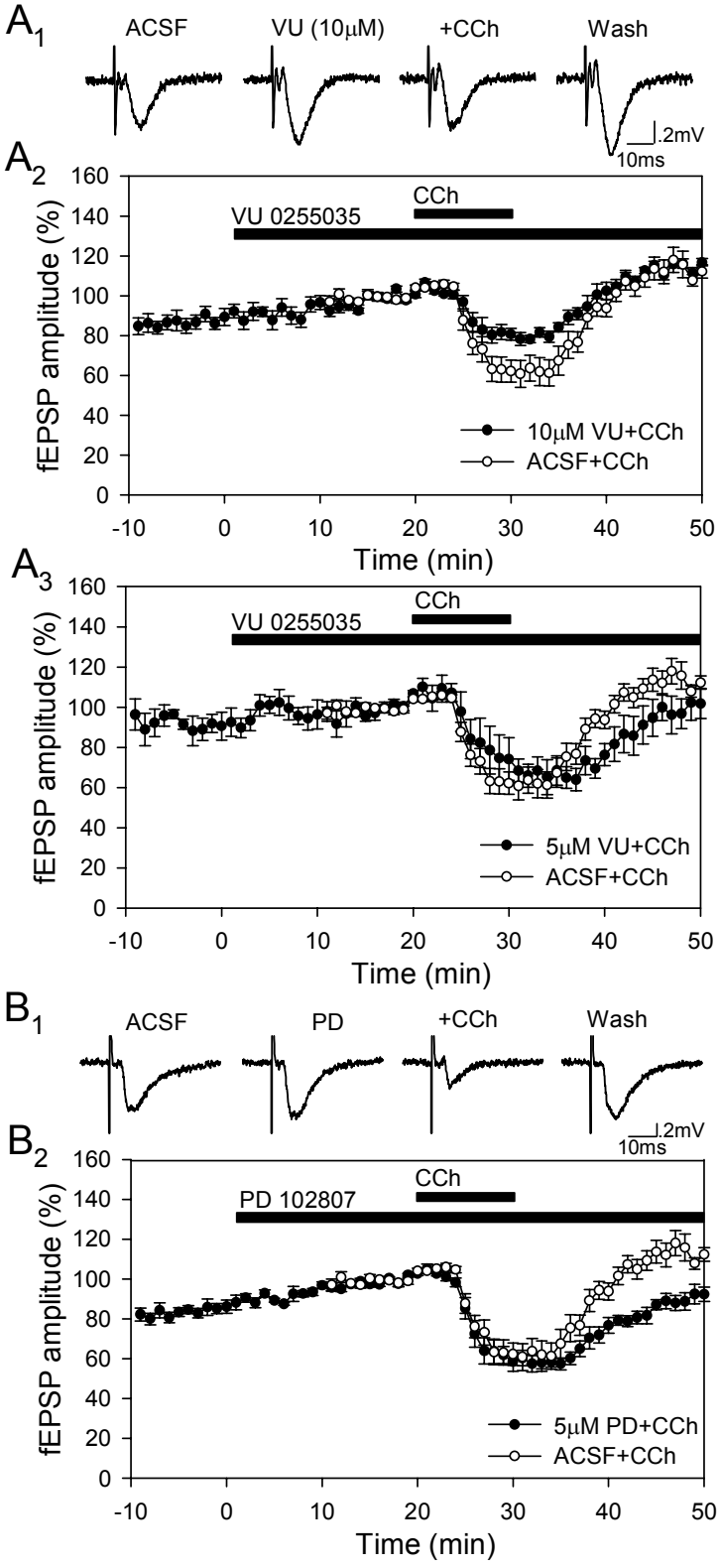
Figure 2.3. The cholinergic suppression of fEPSP amplitude is dependent in part on activation of M_1, but not M_4, muscarinic receptors. **A.** Application of the selective M_1 receptor antagonist VU0255035 (10 μM) for 20 min prior to addition of carbachol significantly reduced the suppression in fEPSPs induced by carbachol ($A_{1,2}$). A lower concentration of 5 μM VU0255035 did not block the carbachol-induced suppression (A_3). **B.** The selective M_4 receptor blocker PD102807 (5 μM) failed to block the suppression of fEPSPs induced by carbachol.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ACSF
VU (10μM)
+CCh
Wash
.2mV
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A2
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VU 0255035
fEPSP amplitude (%)
10μM VU+CCh
ACSF+CCh
Time (min)
A3
CCh
VU 0255035
fEPSP amplitude (%)
5μM VU+CCh
ACSF+CCh
Time (min)
B1
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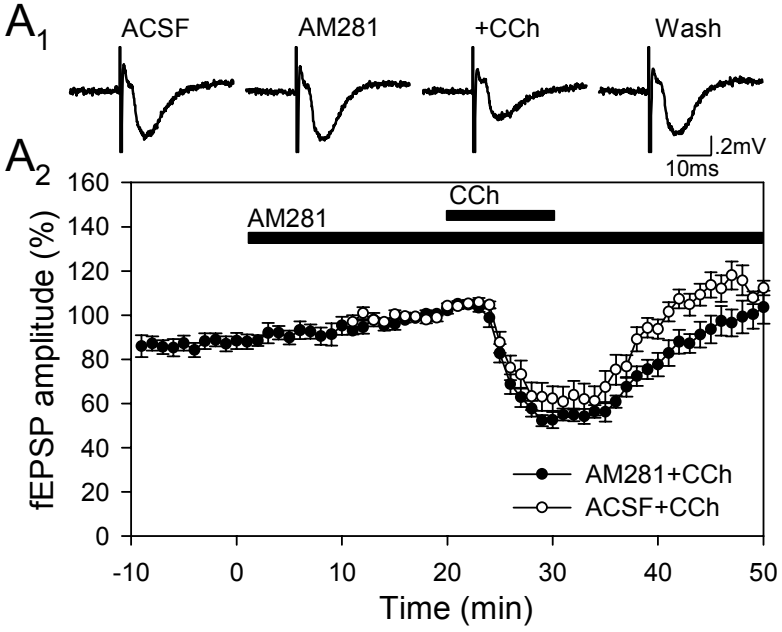
Wash
.2mV
10ms
B2
CCh
PD 102807
fEPSP amplitude (%)
5μM PD+CCh
ACSF+CCh
Time (min)

Figure 2.4. Activation of cannabinoid CB1R receptors is not required for the cholinergic suppression of fEPSPs in the medial entorhinal cortex. **A.** Averaged representative fEPSPs were recorded in normal ACSF, during addition of the CB1R receptor antagonist AM281 (1 μM), during addition of 5 μM carbachol (CCh), and during the washoff of carbachol (A_1). Application of AM281 failed to block the suppression suppression of fEPSPs induced by carbachol (A_2).


A1
ACSF
AM281
+CCh
Wash
.2mV
10ms
A2
CCh
AM281
fEPSP amplitude (%)
160
140
120
100
80
60
40
20
0
-10
0
10
20
30
40
50
Time (min)
AM281+CCh
ACSF+CCh

Chapter 3

EFFECTS OF AMPHETAMINE ON SYNAPTIC RESPONSES DURING SHORT TRAINS OF THETA- AND GAMMA-FREQUENCY STIMULATION IN THE LATERAL ENTORHINAL CORTEX IN VIVO

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ABSTRACT

The entorhinal cortex receives substantial dopaminergic inputs from midbrain neurons, which are thought to modulate the processing of sensory information. Past studies have shown that application of dopamine to brain slices results in a concentration-dependent bidirectional effect on amplitudes of synaptic responses from inputs to the entorhinal cortex such that high concentrations of dopamine suppress excitatory synaptic transmission, and low concentrations of dopamine can facilitate synaptic responses of the entorhinal cortex to inputs from sensory cortices. Gamma and theta EEG activities have been associated with processing of sensory information and they co-occur in the entorhinal cortex and in the hippocampal formation. In the current study, synaptic responses were recorded in the entorhinal cortex of freely moving animals during short trains of gamma- and theta-frequency stimulation to the piriform cortex before and after systemic administration of amphetamine (3 mg/kg, i.p.). The group of animals as a whole showed no reliable changes in the profile of responses to gamma-frequency stimulation due to dopaminergic receptor activation, but a subset of animals that showed an increase in the response to the first stimulation pulse of theta-frequency stimulation trains also showed reliable differences in responses associated with behavioral movement vs inactivity, and following amphetamine administration. In these animals, behavioral mobility was associated with a suppression in the amplitude of the response to the first pulse in the trains, and a relative facilitation of responses during the train, consistent with previous findings regarding the effects of acetylcholine on train-evoked responses. In addition, the amplitudes of synaptic responses were larger after these animals were

injected with amphetamine as compared to baseline responses recorded during behavioral mobility, and subsequent responses during the trains showed a relative *suppression.* Thus, the cholinergic suppression of single synaptic responses, and the dopaminergic facilitation of single synaptic responses, are associated with competing changes in the response evoked during theta-frequency stimulation. In additional tests, the D_1 receptor antagonist SCH23390 (1 mg/kg, i.p.) and the D_2 receptor antagonist eticlopride (0.1 mg/kg, i.p.) were injected prior to amphetamine injection in order to determine which dopaminergic receptors are responsible for the relative suppression caused by theta- and gamma-frequency trains. Both receptor antagonists blocked significant changes in the amplitude of the response to the first pulse in the trains, and also blocked the relative suppression of train-evoked responses, suggesting that both D_1 and D_2 receptor subtypes contribute to the effects of amphetamine on train-evoked responses.

INTRODUCTION

The entorhinal cortex receives large dopaminergic projections from midbrain dopamine neurons (Bjorklund and Lindvall, 1984; Akil and Lewis, 1993; Akil and Lewis, 1994; Arnsten, 1998; Erickson et al., 1998; Goldman-Rakic et al., 2000; Seamans and Yang, 2004; Björklund and Dunnett, 2007) but little is known about the behavioural role of dopamine in the entorhinal cortex. Activation of dopaminergic neurons has been linked to the motivation for obtaining rewards (Mogenson et al., 1980; Wise and Raptis, 1986), and midbrain dopamine neurons are activated in response to stimuli that predict the availability of rewards (Wise and Schwartz, 1981; Spyraki et al., 1982). Because the entorhinal cortex receives substantial dopaminergic inputs from the ventral tegmental area and substantia nigra (Björklund and Dunnett, 2007), it has been suggested that dopamine in the entorhinal cortex is likely to modulate processing of sensory stimuli associated with reward (Clark et al., 2012).

Activation of dopaminergic receptors has been shown to suppress glutamatergic synaptic transmission in layers II, III, and V of the medial entorhinal cortex (Pralong and Jones, 1993; Stenkamp, Heinemann and Schmitz, 1998). However, Caruana et al. (2006) discovered that application of dopamine to slices of entorhinal cortex tissue resulted either in a suppression or a facilitation of the strength of synaptic inputs to layer II of the lateral entorhinal cortex that was dependent on the concentration of dopamine; higher concentrations (50 μM) lead to a suppression of synaptic responses through activation of D_2-like receptors, as had been observed earlier, and lower concentrations (10 μM) lead to a facilitation of synaptic responses through activation of D_1-like receptors. Hutter et al.

(2013) have recently shown that rewarding electrical stimulation of the lateral hypothalamus leads to a suppression of synaptic responses in the entorhinal cortex evoked by stimulation of the piriform cortex, and that this suppression occurs due to activation of D_2-like receptors (Hutter et al, 2013). Research investigating the role of dopaminergic activation in the prefrontal cortex has lead to additional findings in which activation of D_1-like receptors suppresses glutamatergic responses in layer V neurons (Gao, Krimer and Goldman-Rakic, 2001; Seamans, Durstewitz, Christie, Stevens and Sejnowski, 2001) and enhances glutamatergic responses in layers II and III (Gonzalez-Islas and Hablitz, 2003). These findings indicate that dopamine can result in bidirectional changes in synaptic strength, and suggest that dopamine may serve to increase or decrease the salience of sensory stimuli associated with reward in the entorhinal cortex.

Theta- and gamma-frequency EEG activities occur endogenously within the superficial layers of the entorhinal cortex that contain neurons that have projections to the hippocampal formation (Alonso and Llinas, 1989; Dickson et al., 2000). Active exploration of the environment has been associated with the co-occurrence of gamma (30-80 Hz) and theta (4-12 Hz) frequency EEG rhythms (Mitchell and Ranck, 1980; Chrobak and Buzsaki, 1998) that can contribute to the synchronization of activity of neurons in the entorhinal cortex (Chrobak and Buzsaki, 1998; Colgin et al., 2009) and gamma-frequency activity in the olfactory system has been associated with odor sampling (Bressler, 1984; Freeman, 1978). Theta activity is found in multiple cortical structures including components of the hippocampal formation (Bland and Oddie 2001; Buzsaki 2002; Buzsaki et al. 1983; Green and Arduini 1954; Petsche et al. 1962), the entorhinal cortex

(Boeijinga and da Silva, 1988; Buzsaki, 2002), perirhinal cortex (Bilkey and Heinemann, 1999), parasubiculum (Glasgow and Chapman, 2007), cingulate cortex (Leung and Borst, 1987), and the amygdala (Pare and Collins, 2000). Prominent type I theta activity occurs both during REM sleep (Lerma and Garcia-Austt, 1985; Chrobak and Buzsaki, 1998), and during locomotor activities when the animal is exploring the environment (Vanderwolf, 1969; Mitchell and Ranck, 1980; Chrobak and Buzsaki, 1998). Type II theta, on the other hand, has been found to occur during immobility (Bland, 1986; Sainsbury, Heynen and Montoya, 1987) and during slow wave sleep (Chroback and Buzsaki, 1998). However, type II theta has been highly associated with immobility during a state of arousal (Sainsbury, Heynen and Montoya, 1987; Sainsbury and Montoya, 1984). Because of the strong relationship between mobility and oscillatory rhythms, it has been suggested that type I theta and gamma oscillations play a role in sensorimotor integration (Bland, 1986), and in information processing and memory formation (Winson, 1978; Ahissar et al., 1992; Lisman and Idiart, 1995). The synchronization of neuronal activity and firing in the entorhinal cortex induced during theta and gamma rhythms could help drive activation of neuronal targets in the hippocampal formation through the synchronization of synaptic inputs (Buzsaki and Wang, 2012; Fries, 2009).

Gamma activity in the entorhinal cortex can be observed superimposed on the slower theta-frequency activity (Chroback and Buzsaki, 1998), and the strength of gamma activity varies depending on theta oscillations (Bragin et al., 1995; Chroback and Buzsaki, 1998). Lesioning the entorhinal cortex leads to a decrease in or elimination of gamma activity in the hilus of the dentate gyrus (Bragin et al., 1995), suggesting that the

entorhinal cortex contributes to the generation of gamma activity in the dentate gyrus. Gamma rhythms in the neocortex have been proposed to contribute to the temporal binding of the activities of neurons so that they can better represent a coherent stimulus (Singer, 1993), and gamma activity has also been proposed to contribute to long-term auto-associative memory functions in the CA3 region of the hippocampus (de Almeida et al., 2007). However, the role of gamma activity within the entorhinal region is not known.

Acetylcholine is thought to play a major role in the generation of theta- and gamma-frequency activities in the entorhinal cortex and hippocampus (Mitchell and Ranck, 1980; Dickson et al. 2000; Glasgow and Chapman, 2007). Both lesioning of the entorhinal cortex, or surgical isolation of the entorhinal cortex from its cortical and subcortical afferents leads to the elimination of theta rhythm oscillations generated in the CA1 region near the hippocampal fissure, and the remaining theta activity is sensitive to atropine and dependent on muscarinic receptor activation (Buzsaki et al., 1983). However, there is also evidence to suggest that activation of dopamine neurons enhances the acetylcholine release that drives theta activity (Rawlins et al., 1979).

In addition to the strong effects of acetylcholine on theta and gamma EEG activities, dopamine may also have modulatory effects on these rhythms that could affect processing of sensory information in the temporal lobe (Jay, 2003; Lisman and Grace, 2005; Orzel-Gryglewska et al., 2013). Midbrain dopamine neurons can contribute to the generation of theta activity in the medial temporal lobe, suggesting that dopamine may enhance theta activity (Jay, 2003; Lisman and Grace, 2005; Orzel-Gryglewska et al., 2013). It has been previously shown that dopamine activation leads to a modulation of

septal activity (e.g. Levin et al., 1990), which provides the entorhinal cortex with cholinergic innervation. A reduction of activity in the septum has been associated with reduced theta oscillations in the hippocampus (Rawlins et al., 1979), suggesting that dopamine may modulate theta oscillations in the hippocampal region by modulating cholinergic inputs. The findings of Wiess et al., (2003), however, suggest that activation of dopamine may lead to a *suppression* of gamma-frequency activity. They found that during carbachol-induced gamma frequency activity in brain slices containing the CA3 region, application of dopamine caused a reversible decrease in the power, but not frequency, of gamma activity in over half of cases that was due to activation of D_1 receptors. However, dopamine neurons project directly to the entorhinal cortex (Erickson et al., 1998), so that activation of dopamine receptors within the entorhinal cortex might modulate theta and gamma frequency activity more directly.

Although application of dopamine on slices of entorhinal cortex tissue has been found to result in a facilitation or suppression of single evoked synaptic responses depending upon the dose (Caruana et al., 2006; Glovaci et al, 2014), it is not known what effect dopamine may have on repetitive synaptic responses in the lateral entorhinal cortex in response to trains of stimulation pulses at theta- and gamma- frequencies. A modulatory effect on trains of synaptic responses could suggest how dopamine may normally affect synaptic transmission during periods when theta and gamma frequency activity dominates the EEG. For example, application of the cholinergic agonist carbachol results in an overall suppression of responses to trains of stimulation to layer II of the medial entorhinal cortex, but it also induces a relative facilitation effect in which the

amplitude of responses to each pulse during the trains was facilitated relative to the amplitude of the first response evoked during the train (Sparks and Chapman, 2013), suggesting that representations carried at theta and gamma frequencies may be well-maintained during theta and gamma frequency activity. Interestingly, dopamine in layer V of the lateral entorhinal cortex has been found to *reduce* the summation of synaptic responses recorded in response to 20 Hz trains of 10 pulses (Rosenkranz and Johnston, 2006). The effect was due to activation of D_1-like receptors and increases in the hyperpolarization-activated inward cationic conductance I_h (Rosenkranz and Johnston, 2006), and it suggests that dopamine may suppress repetitive synaptic transmission during rhythmic EEG activity. Dopamine is therefore likely to modulate synaptic function in the entorhinal cortex in a way that depends on the frequency of repetitive synaptic inputs (Ito and Schuman, 2008).

The present study involved determining the role of activation of dopamine receptors on the amplitude of field EPSPs in layer II of the entorhinal cortex in awake animals evoked by short 10-pulse trains of theta- and gamma-frequency stimulation of piriform cortex inputs to the superficial layers of the entorhinal cortex. Systemic administration of amphetamine was used to enhance dopaminergic transmission and the dependence of the effects on dopamine receptor activation was assessed in additional tests in which either the D_1 receptor blocker SCH23390, or the D_2 receptor antagonist eticlopride were administered prior to amphetamine. In addition, because previous work has shown that synaptic responses in vivo can be affected by release of acetylcholine associated with movement versus behavioral immobility, we also compared train-evoked

responses during movement versus immobility, and assessed responses evoked following administration of amphetamine relative to baseline responses recorded while animals were moving. Although results for effects on responses to gamma-frequency stimulation were not conclusive, results did show that an increase in the first response to trains of theta-frequency stimulation induced in most animals by amphetamine was associated with a relative suppression of later responses during the trains.

MATERIALS AND METHODS

Surgery

Male Long-Evans rats (8–10 weeks old; Charles-River) were housed individually and fed ad libitum. Animals were anesthetized with isoflurane (1.5–2% in O_2) and placed in a stereotaxic frame for chronic implantation of stimulating and recording electrodes. Electrodes were bipolar, twisted wire, electrodes constructed from Teflon-coated stainless-steel wire (125um exposed tips). The recording electrode was lowered into the superficial layers of the right lateral entorhinal cortex (P, 6.5 mm; L, 6.5 mm; V, 7.5–8.5 mm; tip separation 0.8 mm) that receive dopaminergic inputs from the ventral tegmental area and substantia nigra (Björklund and Lindvall, 1984; Oades and Halliday, 1987). The stimulating electrode was lowered into the right piriform cortex (P, 3.6 mm; L, 6.5 mm; V, 9.0 mm relative to bregma; tip separation 1.0 mm) that sends a strong projection to the superficial layers of the entorhinal cortex. Electrode positions were adjusted to minimize current thresholds and to maximize amplitude of evoked field excitatory postsynaptic potentials (fEPSPs). A jeweler's screw was used as a reference electrode in the contralateral frontal bone, and another screw was used as a ground electrode in the left

parietal bone. Two additional screws were used to secure the dental cement to the skull. Gold-plated Amphenol pins connected to electrode leads were mounted in a 9-pin plastic connector that was fixed to the skull by embedding the jeweler's screws, electrodes, and connector in dental cement. Buprenorphine (0.02 mg/kg, s.c.) was administered after surgery as an analgesic, and animals were given a 2–3 week recovery period before experimental testing.

Theta- and Gamma-Frequency Stimulation

During the lights-off phase of a 12-h light-dark cycle the animals were tested in a 40×60×40cm Plexiglas chamber surrounded by a Faraday cage. A computer digital-analog channel was used to deliver 0.1 ms biphasic constant-current square-wave pulses to the piriform cortex via a stimulus isolation unit (A-M Systems, Model 2200). Evoked field potentials were analog filtered (0.1–5 kHz pass-band) and amplified (A-M Systems, Model 1700). A piriform cortex stimulus intensity that evoked a synaptic response of 60 to 75% of the maximal response amplitude in the entorhinal cortex was determined for each animal, and this intensity was used during theta and gamma-frequency stimulation in subsequent tests. Ten responses to trains of 10 pulses at theta frequency (10 Hz) were delivered once every 30 sec, and this was followed by delivery of 10 trains of stimulation at gamma frequency (33 Hz) to avoid potential short-term plastic effects of high-frequency gamma stimulation interfering with the amplitude of responses during low theta-frequency stimulation. Responses were digitized at 10 kHz (12-bit) for storage on computer hard disk using the software package Sciworks (Datawave Tech.), and the train-evoked responses at each frequency were averaged. Because theta and gamma activities

occur naturally in the brain while an animals is exploring an environment (Vanderwolf, 1969; Mitchell and Ranck, 1980; Chrobak and Buzsaki, 1998), and because synaptic responses can be suppressed by enhanced cholinergic inputs to the entorhinal cortex as animals move through the environment (Hamam et al., 2007), responses to theta and gamma –frequency trains were recorded while the animals showed either active movements (ambulatory movements, rearing, sniffing), or were immobile (no trunk, neck, limb or facial movements). Responses were then also recorded after delivery of pharmacological agents.

Pharmacology

Baseline tests were conducted on each day in which responses to both theta- and gamma-frequency trains were recorded during both movement and immobility prior to administration of pharmacological agents. The effect of dopaminergic receptor activation on synaptic responses during theta and gamma frequency trains was assessed using systemic injection of amphetamine (3mg/kg, i.p.; Hankosky et al., 2013), and there was a 20 min period following injection prior to recordings.

Amphetamine enhances both dopaminergic and noradrenergic synaptic transmission (Berry, 2004), and tests on separate counterbalanced days were conducted to determine if the effects of amphetamine on synaptic responses could be affected by prior administration of either a D_1 or D_2 receptor antagonist. On each day following baseline tests during mobility and immobility, animals were injected with either the D_1 receptor antagonist SCH23390 (1mg/kg, i.p.; Ozaki et al., 1997) or the D_2 receptor antagonist eticlorpide (0.1mg/kg, i.p.; Hutter et al., 2013). For both antagonists, animals were given

20 minutes to rest before recording train-evoked synaptic responses. An additional set of recordings was then obtained 20 min following injection of amphetamine.

Histology

Animals were anesthetized with urethane (1.5 g/kg, i.p.) and perfused intracardially with 0.9% saline followed by 10% formalin. Brains were stored in 10% formalin and were flash-frozen with dry ice for 5 min prior to sectioning with a cryostat. Coronal sections (40 um thick) were obtained and stained with thionin in order to verify electrode placements histologically.

Data Analysis

The peak amplitudes of fEPSPs evoked by each pulse in the trains was measured relative to the level of the local field potential just prior to the stimulation pulse. Effects of movement versus immobility on averaged train evoked responses were assessed by normalizing the amplitudes of responses evoked during the trains to the amplitude of the response evoked by the first pulse in the train during movement. Because amphetamine induces locomotion, the effects of amphetamine on train-evoked responses were assessed by normalizing responses to the amplitude of the first response in the train during locomotion. The ability of dopamine receptor antagonists to block effects of amphetamine was assessed by assessing amphetamine-induced changes relative to the first response to trains in the presence of the receptor antagonist.

In all cases, repeated measures ANOVAs (with pulse number and testing condition as factors) were used to assess significant changes in train-evoked responses, and planned comparisons were used to compare responses to each individual pulse in the

trains recorded in each testing condition. In addition, data were re-normalized to better characterize the profile of changes in the response amplitudes during the course of the stimulation trains. The relative changes in fEPSP amplitudes during the 10-pulse trains in different testing conditions were compared by normalizing responses evoked during the trains to the amplitude of the first responses in each train. Repeated measures ANOVAs and planned comparisons were also used here for each pulse in the trains to test for differences in the relative change in the amplitudes of responses during the trains.

RESULTS

Amplitudes of synaptic responses evoked by each pulse in gamma-frequency trains were compared from tests conducted while animals were mobile vs after injection of amphetamine in order to control for the mobility shown by animals after injection of amphetamine (Figure 3.1A, n = 11). The amplitudes of synaptic responses increased during both conditions and peaked for the response to the third pulse (mobility, 163.7 ± 17.9% of the first response; amphetamine, 162.4 ± 21.1%), and then steadily decreased until the last pulse (mobility, 137.7 ± 28.3%; amphetamine, 133.7 ± 27.1 %). This was reflected in a significant main effect of pulse number ($F_{9,90} = 2.48$, $p = 0.014$) but the pattern of responses did not differ between mobility and amphetamine conditions (main effect of condition, $F_{1,10} = 0.13$, $p = 0.724$). Further, the amplitudes of the first pulses evoked during movement and amphetamine were similar (Figure 3.1A3, pulse 1), and so when the patterns of responses during the trains were compared by renormalizing the data to the first pulses in the movement and amphetamine conditions, there was also no difference in the relative change in responses during the trains (Figure 3.1A4; $p > 0.05$ for

responses two to ten). Amphetamine, therefore, did not affect responses to gamma-frequency stimulation.

Amplitudes of synaptic responses evoked by theta-frequency trains showed a somewhat different pattern in which the amplitudes of responses were strongly increased in response to the second pulse, and were maintained during the trains; amplitudes of synaptic responses in response to the tenth pulse reached 195.6 ± 19.2 % of the first response during mobility, and 189.6 ± 19.2 % after injection of amphetamine (Figure 3.1B). This was reflected in a main effect of pulse number in a 2-way repeated measures ANOVA ($F_{9,90} = 17.46$, $p < 0.001$). In addition, the amplitudes of responses recorded in response to theta-frequency stimulation were significantly greater than those recorded during gamma-frequency stimulation for later pulses in the trains (responses three and five: $p < 0.05$), indicating that synaptic responses are more robust when evoked by 10 Hz versus by 33 Hz stimulation (compare Figure 3.1A3 vs 1B3).

Relative Suppression of Train-Evoked Responses in a Subgroup of Animals

Although there was not a significant effect of amphetamine injection on responses to theta-frequency stimulation for the group of animals as a whole (n = 11; main effect of condition, $F_{1,10} = 0.34$, $p = 0.576$; interaction, $F_{9,90} = 1.36$, $p = 0.217$), there was a trend for responses to the first pulse to be enhanced by amphetamine (Figure 3.1B3, pulse 1; $p = 0.184$), and also a trend for a smaller relative increase in responses during amphetamine when the amplitudes of responses were expressed as a percentage of the amplitudes of the first responses in the trains (Figure 3.1B4; $p < 0.05$ for pulses 3 and 5). This trend was investigated (Figure 3.2A), and it was found that the magnitude of changes induced by

amphetamine in different animals was strongly related to the presence or absence of substantial changes in responses associated with movement versus immobility in baseline tests (Figure 3.2A). Animals were therefore separated into two sub-groups according to whether amphetamine induced a facilitation in the first response to theta-frequency stimulation trains, and it was found that that, while animals who did not show an amphetamine-induced facilitation of responses evoked by the first pulse also did not show a difference induced by mobility versus immobility (Figure 3.2A_2 ; n = 5, p = 0.599; train-evoked data not shown), animals that did show a significant facilitation due to amphetamine also showed significantly enhanced responses during immobility versus mobility for the theta-frequency stimulation trains (Figure 3.2A_3 and C; n = 6, $p < 0.001$).

In addition, although amphetamine increased the size of the response to the first pulse in the trains in these animals (Figure 3.2A3, 3.2C3), the responses to later pulses in the theta-frequency train during immobility were not similarly enhanced relative to mobility recordings (Figure 3.2C3; $F_{1,5} = 0.02$, p = 0.891), and this resulted in a significant *suppression* in the relative amplitudes of train-evoked responses with respect to the first pulses in the trains (Figure 3.2C4; pulse 2 to pulse 10, $p < 0.05$). This is consistent with less robust maintenance of repeated synaptic activation that was associated with the increased initial responses during immobility as compared to movement during theta-frequency stimulation in Figure 3.2. The facilitation of the first response in the trains may not have been maintained because of a limited capacity for growth in synaptic responses in this pathway during the trains of stimulation.

Release of acetylcholine during movement is known to suppress fEPSPs in the

entorhinal cortex in vivo (Hamam et al., 2007), and the absence of this effect in some animals is likely to be due to less robust movement in these animals due to the extensive testing and habituation to the recording chamber. It is therefore possible that facilitation of synaptic responses induced by amphetamine may have been masked in these animals because of generally larger synaptic responses in baseline tests driven by reduced cholinergic transmission associated with less behavioural mobility (Hamam et al., 2007). Because the focus of the present study was to assess the effects of dopaminergic transmission on synaptic responses, the subgroup of animals that showed a substantial facilitation of responses to the first pulse in theta-frequency trains of stimulation was used to characterize effects on train-evoked responses, and to assess the dopamine receptors involved.

A similar pattern of results for mobility versus immobility, including changes in the size of the response to the first pulse, was not observed for responses to gamma-frequency trains, suggesting that the repeated delivery of the more intense gamma-frequency trains may have suppressed the expression of the dopaminergic modulation of responses to first pulses (Figure 3.2B; $F_{1,5} = 0.12$, $p = 0.743$).

Effects of Amphetamine on Train Evoked Responses.

Animals in which amphetamine induced a facilitation of synaptic responses to the first pulse in theta-frequency trains ($n = 6$, $128.3 \pm 3.1\%$ of baseline; $n = 6$, $p < 0.001$) did not show a similar enhancement in responses evoked later in the train relative to responses recorded during movement (Figure $3.3B_3$) so that the relative growth in responses was significantly suppressed relative to those recorded during movement for

the third, fifth, and later pulses in the trains (Figure 3.3B_4). In this and later tests, however, similar effects of amphetamine were not observed for gamma-frequency trains either for the amplitude of responses during amphetamine compared to mobility (Figure 3.3A; $F_{1,5} = 2.42$, $p = 0.180$), or the main effect of pulse number ($F_{9,45} = 1.13$, $p = 0.364$). Thus, although there was no effect on the amplitude of responses due to amphetamine on gamma-frequency trains of stimulation, during theta-frequency stimulation amphetamine administration results in a facilitation of synaptic transmission evoked by single pulses that is not matched by continued facilitation of responses later in the train, suggestive of a ceiling effect.

Effect of* D_1 *and* D_2 *Receptor Antagonists

The facilitation of synaptic responses in response to low concentrations of dopamine in the entorhinal cortex in vitro is dependent on D_1 receptor activation (Caruana et al., 2007). To determine if the effects of amphetamine on train-evoked responses is mediated by actions on dopamine receptors, tests were conducted to determine if prior administration of D_1 or D_2 receptor antagonists could block the effects of amphetamine. Neither injection of the D_1 receptor antagonist SCH23390 or the D_2 receptor antagonist eticlopride alone resulted in significant effects on either the amplitudes of the first responses in the trains or on the pattern of train-evoked responses (SCH23390, $F_{9,36} = 1.33$, $p = 0.255$; eticlopride, $F_{9,45} = 1.64$, $p = 0.134$). However, the D_1 receptor antagonist SCH23390 did block both the amphetamine-induced facilitation of the first response in the trains (Figure 3.4B_3; 87.6 ± 11.8% of baseline values; $n = 5$, $F_{9,36} = 1.01$, $p = 0.454$), and the associated relative suppression of later responses in the trains (Figure 3.4B_4). The

D_2 receptor antagonist eticlopride had similar effects. Administration of amphetamine following eticlopride was associated with a non-significant reduction in the amplitude of the response to the first pulse in the trains to 94.9 ± 15.4% of baseline values ($F_{9,45} = 0.74$, $p = 0.673$), and there was also no significant change in the amplitudes of responses later in the train (Figure 3.5B_3; $n = 6$, $p = 0.752$) or in the relative amount of change in responses (Figure 3.5B_4; pulses 2 to 10, $n = 6$, $p > 0.05$). Activation of both D_1 and D_2 receptors is therefore involved in the effect of amphetamine on responses to theta-frequency trains of stimulation.

DISCUSSION

The entorhinal cortex provides the hippocampus with much of its cortical sensory input through its inputs from different cortical regions (van Groen and Wyss, 1990; Caballero-Bleda and Witter, 1993; Caballero-Bleda and Witter, 1994; Caruana and Chapman, 2004), including strong dopaminergic inputs from the midbrain (Bjorklund and Lindvall, 1984; Loughlin and Fallon, 1984; Erickson et al., 1998; Björklund and Dunnett, 2007). The entorhinal cortex expresses strong theta and gamma frequency EEG activities (Mitchell and Ranck, 1980; Chrobak and Buzsaki, 1998), and the aim of the current study was to investigate how dopamine modulates excitatory synaptic responses to short trains of theta- and gamma-frequency stimulation in the entorhinal cortex following injection of amphetamine in freely moving rats. Results showed that injection of amphetamine caused variable effects on synaptic responses in the group of animals tested but that, among animals in which amphetamine resulted in an enhancement of the first response during theta-frequency trains, there was a relative suppression of responses to later pulses during

the theta-frequency trains. Thus, while amphetamine caused an increase in the amplitude of single synaptic responses at the start of the trains, later responses in the train reached similar amplitudes as were observed during baseline tests (Figure 3.4B_3), such that amphetamine induced a suppression in the pattern of growth of responses during the trains (Figure 3.4B_4). This suggests that there may be mechanisms in the entorhinal cortex that limit the growth of repeated responses to stimulation and that these mechanisms may have limited the growth of train-evoked responses during amphetamine administration. It has previously been found that dopamine causes a significant suppression of synaptic responses in the entorhinal cortex (Caruana et al., 2006; Pralong and Jones, 1993), and it has been shown that this dopaminergic suppression is due to activation of D_2 receptors (Caruana et al., 2006). In investigating the individual role of D_1 versus D_2 receptor activation, it was found that injection of both receptor antagonists resulted in a block of significant facilitation of the first response, and also blocked significant relative suppression of subsequent responses during the trains. Thus, although dopamine may serve to enhance responses to discrete synaptic inputs, the current results suggest that it is not likely to enhance the responses of the entorhinal cortex to repeated synaptic activation at theta and gamma frequencies.

Animals were split into groups dependent on whether they showed an amphetamine-induced facilitation in the size of the first response or whether they did not show a facilitation. Initial analysis of responses of the entire group of animals indicated a trend towards a facilitation of the synaptic response to the first pulse in the theta-frequency trains, as well as a trend towards a relative suppression during the trains.

Investigation of this trend showed that separating animals based on the effect of amphetamine on the amplitude of the first response resulted in two groups of animals; animals that showed an amphetamine-induced facilitation of the first response that also showed a significant facilitation of the first response during immobility as compared to mobility, and animals that did not show a facilitation due to amphetamine and that also did not show a significant facilitation during immobility versus mobility. It has been demonstrated before that the amplitudes of EPSPs in the entorhinal cortex are reduced in animals that are mobile versus immobile due to the cholinergic suppression of synaptic responses during mobility (Hamam et al., 2007; Leung and Vanderwolf, 1980), and animals that did not show a significant facilitation during immobility compared to mobility in the present study are likely to have shown less robust behavioural activation during recordings throughout the mobility period (Hamam et al., 2007). The entire group of animals tested, then is likely to include animals that showed limited cholinergic suppression of synaptic responses in baseline testing during mobility, and this may have masked dopamine-dependent facilitation effects induced by subsequent amphetamine injection.

Because animals in the present study showed strong behavioral activating effects of amphetamine (Mabroak et al., 2014; Smith, 1965) that are likely associated with strong increases in cholinergic inputs associated with movement (Hamam et al., 2006; Kramis et al., 1975; Bland et al., 2006), the effect of amphetamine on train-evoked responses was assessed relative to the movement condition during baseline recordings. The procedure of dividing animals into two groups, and focussing analyses on animals that showed a

significant facilitation of responses in response to amphetamine (Glovaci et al., 2014) provided a method to better assess the effects of amphetamine on train-evoked responses in animals that likely displayed similar levels of movement-related cholinergic suppression of synaptic responses.

Gamma-Frequency Trains

A surprising finding was that, even among animals in which amphetamine caused a facilitation in the size of the first response to theta-frequency trains, there was no similar increase in the size of the first response to gamma-frequency trains: Injection of amphetamine did not change the amplitude of the first responses during gamma-frequency stimulation trains compared to the baseline mobility condition, nor did it result in a relative change in the amplitude of the subsequent responses (Figure 3.3A). The lack of an effect of amphetamine on single evoked responses at the beginnings of gamma-frequency trains was a consistent observation in this study (panel A of all Figures) that occurred even when responses to the first pulse in theta-frequency trains were enhanced (Figure 3.3B). This result may be due to the higher frequency of gamma-frequency stimulation that may have induced a transient facilitation effect that may have occluded further facilitation in response to amphetamine (Wang, 1999). Responses to gamma-frequency trains were recorded after response to theta-frequency trains in all recording conditions, and this may have limited possible effects of gamma-frequency stimulation on responses to theta-frequency trains. The results of the current study on the effects of amphetamine on gamma-frequency trains are therefore inconclusive, and will require further investigation in the future. It may be useful in the future to counterbalance the

order of gamma- and theta-frequecy stimulation, or to increase the interval between stimulation trials, to ensure that the high-frequecy gamma stimulation does not cause short-term plastic changes that may mask the effect of amphetamine on synaptic responses. Previous studies have shown that the medial and lateral entorhinal cortex are differentiated in terms of the role of the neurotransmitter acetylcholine on gamma-wave activity, such that administration of carbachol on brain slices resulted in an induction of gamma-frequency oscillations in the medial but not the lateral entorhinal cortex (van der Linden et al., 1999). Therefore, it could also be useful to test the effect of application of dopamine in the medial and lateral entorhinal corticies during gamma-frequency stimulation in order to determine whether the role of dopamine in these two brain areas may also differ.

A consistently observed effect was that the amplitudes of responses showed greater growth during theta-frequency trains as compared to gamma-frequency trains. In baseline recordings during mobility, mean responses to gamma-frequency trains were increased to their highest level after three pulses at 157 %, and then declined marginally to 146 % after the tenth pulse (Figure 3.1A3). In contrast, responses to theta-frequency stimulation increased to 191 % after three pulses, and then continued to increase to 223 % of the amplitude of the first pulse (Figure 3.1AB3). Chapman and Racine (1997) also found that the amplitude of responses to higher frequencies of stimulation in the entorhinal cortex were smaller than the amplitude of responses measured during lower frequency stimulation. Temporal summation of EPSPs should be greater for higher frequencies of stimulation, and the smaller growth during gamma frequency stimulation

therefore suggests that other factors such as recruitment of local synaptic inhibition may limit the growth of responses during gamma frequency stimulation, or that theta frequency stimulation may enhance facilitation through other postsynaptic mechanisms (Sparks and Chapman, 2013). This suggests that sensory inputs carried by repetitive synaptic inputs at theta-frequency may have greater synaptic effect in the entorhinal cortex as compared to patterns of activity that may be carried at gamma-frequency.

Theta-Frequency Trains

Among animals in which responses to the first pulses in theta-frequency trains were facilitated by injection of amphetamine, the subsequent responses in the trains grew to reach similar amplitudes as were observed during baseline recordings while the animal was mobile (Figure 3.3). The extremely similar amplitudes of responses late in the trains before and after amphetamine injection, even when the first response in the trains was significantly larger following amphetamine injections, suggests that there may be a ceiling effect that may limit growth in synaptic responses during trains. This ceiling effect may result from limited transmitter availability, or a growth in feedforward and feedback inhibitory processes that may limit the excitability within the entorhinal cortex (Finch et al., 1988). Thus, the significant relative suppression of synaptic responses during theta-frequency stimulation that is observed when data are re-normalized to the amplitudes of the first responses in the trains (Figure 3.3B4) is likely due to the facilitation of the first response after amphetamine injection combined with a ceiling effect that limits growth in the absolute size of responses.

Injection of amphetamine that was used in the present study is likely to have led to

effects on train-evoked responses by increasing activation of dopamine receptors. Caruana and Chapman (2008) found that a low concentration of dopamine (10 μM) led to a facilitation of evoked synaptic responses in recordings obtained from layer II in entorhinal cortex slices in vitro, and that higher concentrations lead to a suppression of synaptic responses. This is consistent with the idea that the dose of 3 mg/kg amphetamine used in the current study is appropriate for inducing moderate activation of dopaminergic inputs to the entorhinal cortex that can lead to a facilitation of synaptic responses. Caruana et al., (2006) showed that the facilitation of entorhinal synaptic responses induced by a low concentration of dopamine in vitro was blocked by a D_1 receptor antagonist. Other research has also shown that D_1 receptor activation in the prefrontal cortex enhances glutamatergic responses (Gonzalez-Islas and Hablitz, 2003), while others have found that it suppresses glutamatergic responses (Gao, Krimer and Goldman-Rakic, 2001; Seamans, Durstewitz, Christie, Stevens and Sejnowski, 2001). However, in the current study we have shown that both the D_1 receptor antagonist SCH23390 and the D_2 receptor antagonist eticlopride blocked both the facilitation of responses to the first pulse and the relative suppression effect (Figures 3.4 and 3.5). Other studies have found that D_2 receptor activation can induce a facilitation in the prefrontal cortex (Gorelova and Yang 2000). It is not clear why D_2 antagonist blocked the effects seen here, but the present results suggest that activation of both D_1 and D_2 receptors contribute to effects on train-evoked responses induced by amphetamine in vivo.

Amphetamine can enhance norepinephrine transmission as well as dopamine release (Spiller et al., 2013). However, norepinephrine leads to a *suppression* of synaptic

responses in the entorhinal cortex (Pralong and Magistretti, 1995) suggesting that norepinephrine did not mediate the facilitation of synaptic responses that was observed here. In addition, dopamine receptor blockers blocked the effects of amphetamine on train-evoked responses (Figure 3.4 and 3.5), suggesting that the effects of amphetamine were mediated by dopamine receptors rather than by norepinephrine receptors. Indeed, activation of noradrenergic inputs to the entorhinal cortex concurrently with dopaminergic inputs might partly counteract the facilitatory effects of dopamine, and could have lead to an underestimation of the facilitatory effect of dopamine in the present study.

The findings of the present study show that there is a dopamine-dependent facilitation of synaptic transmission in the entorhinal cortex in behaving animals, and these results extend previous findings that low concentrations of dopamine induce a facilitation of field EPSPs and intracellular EPSCs recorded in layer II of entorhinal cortex slices in vitro (Caruana et al., 2006; Glovaci et al., 2014). The enhanced response to the first pulses in theta-frequency trains of stimulation strongly suggests that activation of dopaminergic inputs to the entorhinal cortex in vivo can facilitate synaptic responses to discrete sensory inputs, and that this may enhance the impact or transmission of these inputs within the entorhinal cortex (Seamans and Yang 2004). The facilitatory effect of amphetamine on the first response in the trains was not maintained during theta-frequency stimulation due to an apparent ceiling effect, however, and this suggests that a facilitatory effect of dopamine on synaptic transmission of sensory inputs is not likely to be maintained during theta-frequency activity in vivo. An important caveat of this

conclusion is that the present experiment used stimulation pulses to synchronously activate large numbers of piriform cortex efferents to the entorhinal cortex, which cannot mimic the patterns of synaptic activation involved in real sensory processing (Carriero et al., 2010). In addition, it is possible that the use of weaker stimulation pulses in the present study could have reduced the possible contribution of a ceiling effect on large-amplitude synaptic responses, and might have permitted maintained facilitation of synaptic responses after amphetamine administration during theta-frequency trains.

The ceiling effect on growth of synaptic responses during theta-frequency stimulation, and the associated relative suppression effect observed here, may be related to findings in which dopamine can inhibit the induction of long-term synaptic potentiation (Caruana et al. 2007). Long-term potentiation is recognized as a cellular model of memory (Bliss and Collingridge, 1993) and theta-frequency activity has also been associated with mechanisms related to synaptic plasticity in the entorhinal cortex (Winson, 1978; Ahissar et al., 1992; Lisman and Idiart, 1995; Yun et al., 2002). Caruana et al. (2007) found that systemic administration of the dopamine receptor uptake inhibitor GBR12909 resulted in a block of the induction of both LTP and LTD in piriform cortex inputs to the entorhinal cortex in vivo. Although some research shows that dopamine activation is necessary for LTP induction (Matthies et al., 1997; Manahan-Vaughan and Kulia, 2003; Abe et al., 2009) or that dopamine activation can enhance LTP induction (Otmakhova and Lisman, 1996), other studies have also found that dopamine can suppress LTP induction (Caruana et al., 2007; Yanagihashi and Ishikawa, 1992; Wei et al., 2012; Law-Tho and Crepel, 1995). The present results suggest that a ceiling effect

limits growth of repetitive responses to synaptic input following amphetamine administration, and previous work has also shown that dopamine suppresses responses to repetitive stimulation in entorhinal layer V neurons (Rosenkranz and Johnston, 2006). These effects would not be expected to enhance postsynaptic depolarization contributing to the induction of LTP (Alonso, Curtis and Llinas, 1990), and the suppression of LTP induction by dopamine in the entorhinal cortex in vivo (Caruana et al, 2007) may be related to the relative suppression of growth responses to repetitive stimulation.

Future Directions

Additional experiments could be conducted to better determine how strong doses of dopamine that induce a *suppression* of single synaptic responses might alter repetitive responses to trains of theta- and gamma- frequency stimulation. A dose of 3 mg/kg amphetamine was sufficient in the present study to induce a facilitation of responses to the first pulses in stimulation trains, similar to the facilitation that is induced in vitro by 10 μM dopamine (Caruana et al., 2006; Glovaci et al., 2014). While this dose resulted in a facilitation of single synaptic responses and a relative suppression during theta-frequency trains, it may be interesting to determine the effect of a higher concentration of dopamine that can result in a suppression of single evoked responses rather than a facilitation (Caruana et al., 2006). Higher concentrations of dopamine result in a suppression of synaptic activity in response to single pulses in slices of the entorhinal cortex (Caruana et al., 2006), but it is not known how such a suppression may affect repetitive synaptic responses. This could be assessed by increasing the dose of amphetamine administered in vivo from 3 to 10 or 20 mg/kg (Proietti Onori et al., 2014; O'Neill and Gu, 2013). For this

purpose, a dose response curve for amphetamine may be useful to asses the levels of dopamine as well as other transmitters in the entorhinal cortex caused by different doses of amphetamine, and also to assess dose-depentent behavioural effects. However, higher doses of amphetamine, such as those sufficient to induce a suppression of single synaptic responses, might induce stronger changes in other neurotransmitter systems including norepinephrine (Maurizio et al., 1974), or indirect effects on acetylcholine (Guix et al., 1992). The in vitro slice preparation, however, has been used in the next chapter of this thesis in order to provide a method to investigate the effects of direct application of both low and high concentrations of dopamine on slices to see their effects on gamma- and theta-frequency trains induced in the lateral entorhinal cortex in vitro

Figure 3.1. Effects of systemic administration of amphetamine on synaptic responses in the entorhinal cortex during gamma- (**A**) and theta-frequency (**B**) trains of stimulation delivered to the piriform cortex in a group of 11 behaving animals. **A_1.** Sample traces show responses to 10-pulse trains of stimulation at gamma-frequency (33 Hz) during the mobility baseline condition and after amphetamine injection. **A_2.** Sample traces in response to the first pulse during gamma-frequency train stimulation (p1) are superimposed with the response to the last pulse in the train (p10) during mobility and after injection of amphetamine. Arrows point to the last response (p10, grey lines). **A_3.** Responses were normalized to the amplitude of the first field excitatory postsynaptic potential (fEPSP) during mobility, and show no significant difference induced by amphetamine in the amplitudes of responses evoked during the train at gamma-frequency stimulation. **A_4.** There was also no difference in the relative amplitudes of fEPSPs when responses evoked during each train were re-normalized to the amplitudes of the first fEPSPs evoked in each train. (Note the renormalized amplitudes are precisely 100% for the first responses in trains evoked both during mobility and after amphetamine injections. **B.** Similar effects were found for theta-frequency trains of stimulation after injection of amphetamine, but there was a trend for the first response in the train to be enhanced following amphetamine (B_3), and an associated significant suppression in the relative amplitudes of response to the 3rd and 5th pulses in the train expressed as a proportion of the amplitude of the first pulse (B_4; * = $p<0.05$)

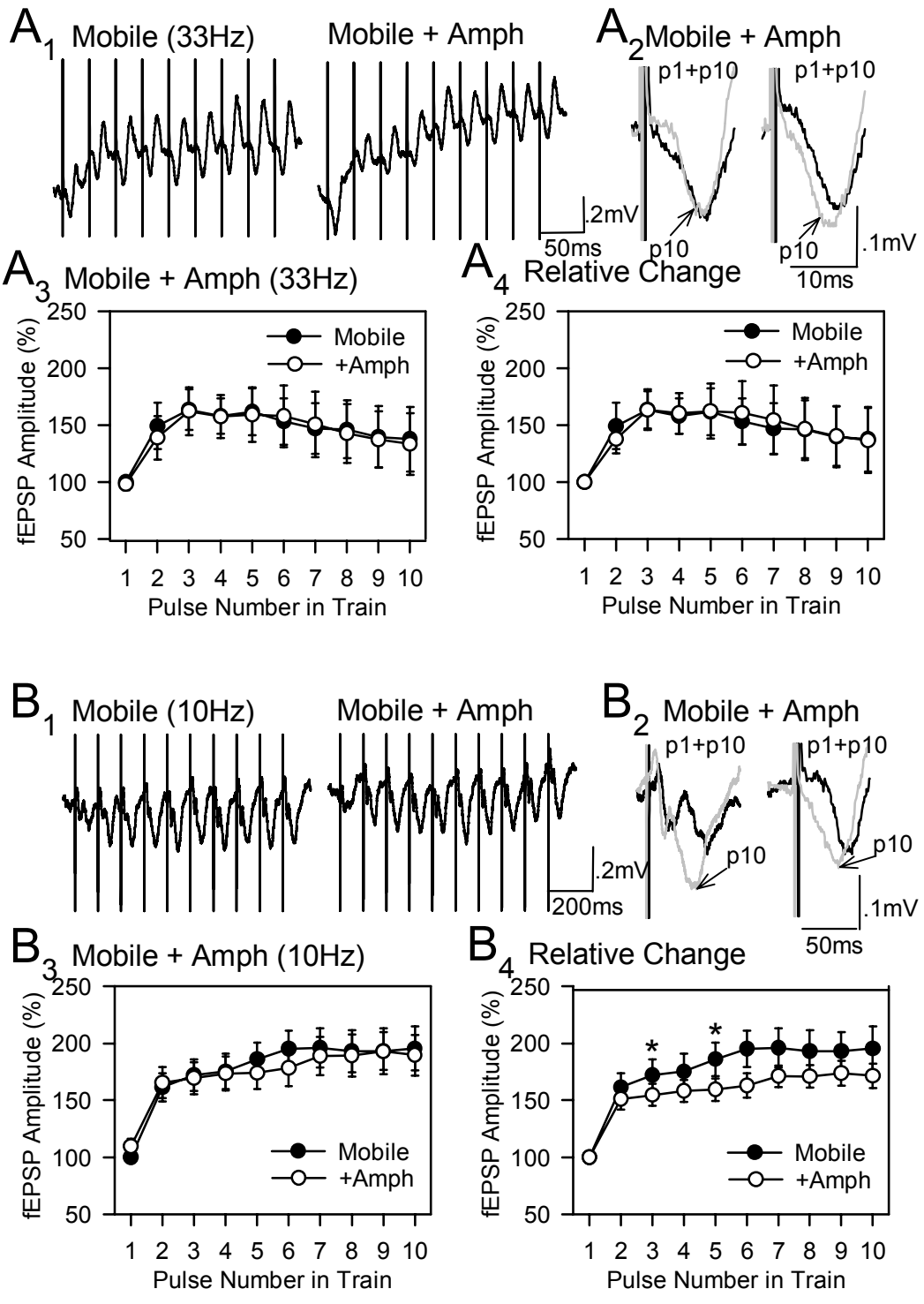

A1 Mobile (33Hz)
Mobile + Amph
A2 Mobile + Amph
p1+p10
p1+p10
.2mV
50ms
p10
p10
.1mV
10ms
A3 Mobile + Amph (33Hz)
A4 Relative Change
fEPSP Amplitude (%)
Mobile
+Amph
Pulse Number in Train
B1 Mobile (10Hz)
Mobile + Amph
B2 Mobile + Amph
p1+p10
p1+p10
p10
p10
.2mV
200ms
.1mV
50ms
B3 Mobile + Amph (10Hz)
B4 Relative Change
*
*

Figure 3.2. Animals were split into two groups depending on whether the amplitude of the first response during theta-frequency trains was facilitated after amphetamine injection; animals that showed an amphetamine-induced facilitation also showed differences in synaptic responses associated with behavioural state. **A_1**. Histograms show the amplitudes of fEPEPs evoked in response to the first pulses in the theta- (black bars) and gamma-frequency (grey bars) trains of pulses while the animals were mobile or immobile during baseline tests, and after injection of amphetamine (Amph). Note the trend towards larger responses during immobility, and larger responses after amphetamine, as compared to the mobility condition for theta-frequency trains. Histograms indicating the amplitudes of the first responses in the trains are also shown for animals that did not show a facilitation of the first responses during theta-frequency trains due to amphetamine (A_2), and that did show a facilitation of the first responses in theta-frequency trains following administration of amphetamine (A_3). Note the effects of movement versus immobility during theta-frequency trains in A_3, and also note the lack of effects of behavioural state or of amphetamine on responses to the first stimulation pulse in gamma-frequency trains of stimulation. **B.** Responses to gamma-frequency stimulation trains among animals that showed a facilitation of the first responses due to amphetamine (n = 6) were not affected by the behavioural states of movement versus immobility. Conventions are as in Figure 3.1. **C.** However, among the animals that showed a facilitation of the first responses following injection of amphetamine, there was also a facilitation of responses evoked by the first pulse of theta-frequency trains during immobility as compared to mobility during baseline testing (**C_3**), and this was associated

with a relative suppression of the amplitudes of later responses in the train expressed as a proportion of the amplitude of the first pulse (**C_4**;* = $p < 0.05$; # = $p < 0.01$).

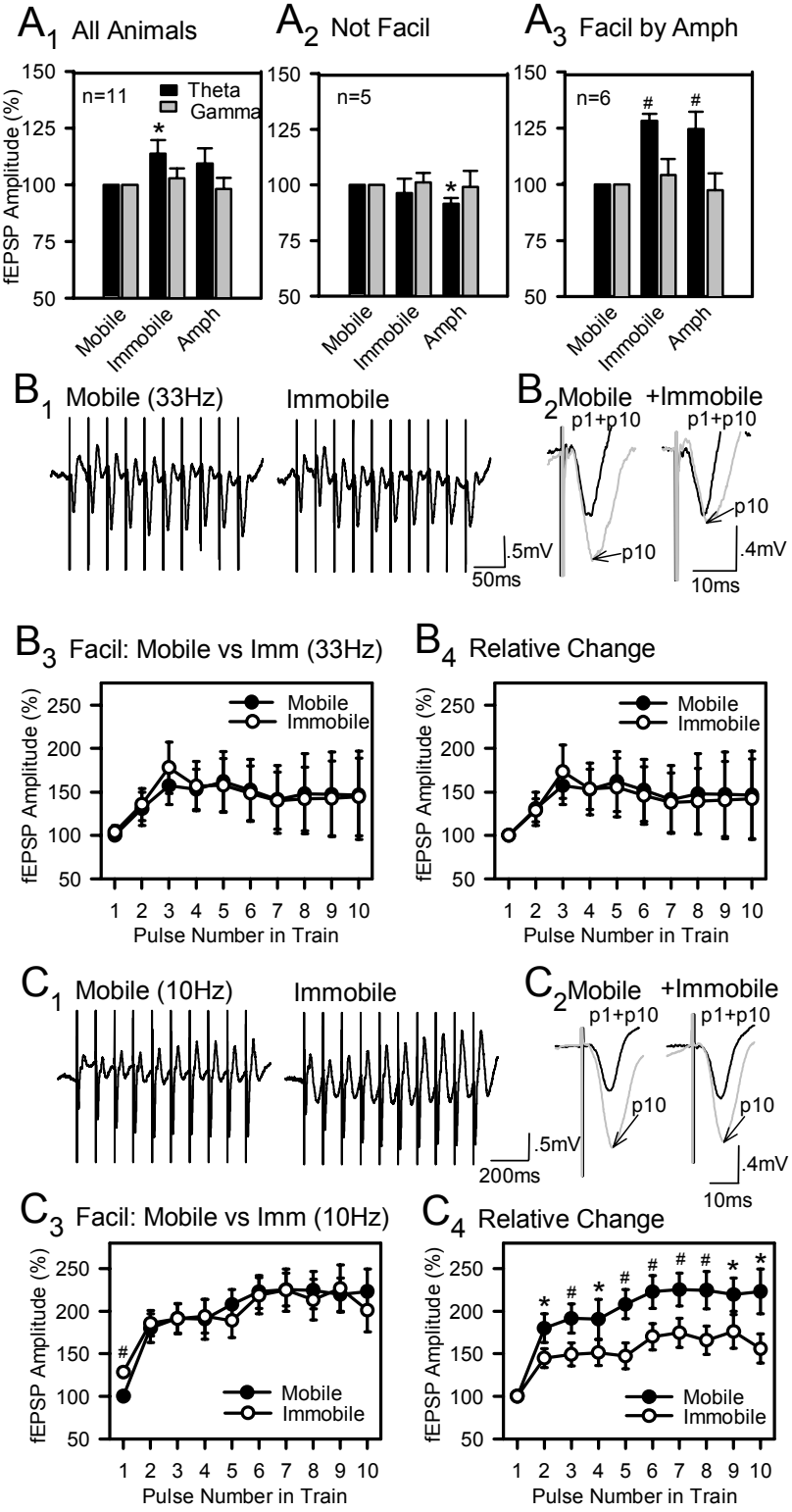
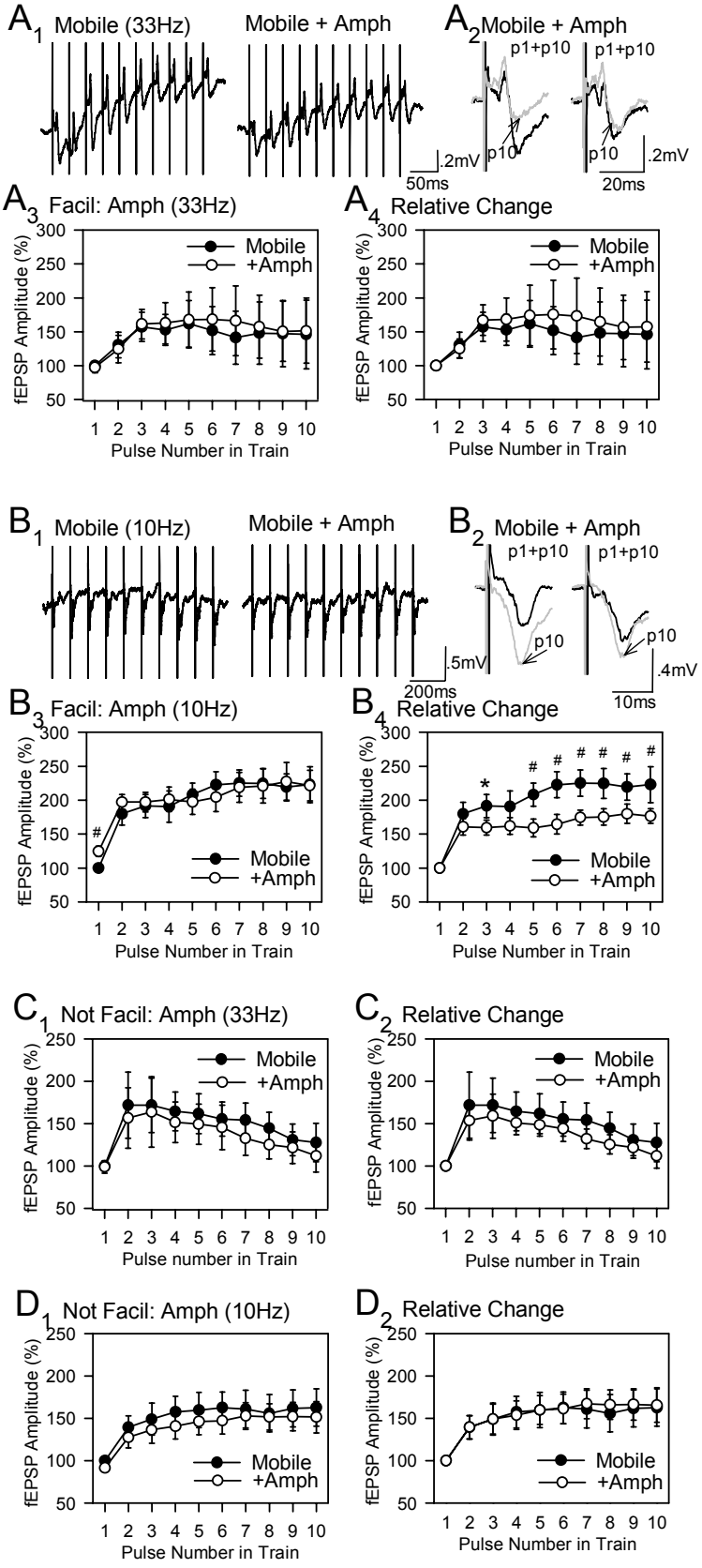

A1 All Animals
A2 Not Facil
A3 Facil by Amph
fEPSP Amplitude (%)
n=11
Theta
Gamma
n=5
n=6
Mobile
Immobile
Amph
B1 Mobile (33Hz)
Immobile
B2 Mobile +Immobile
p1+p10
p10
.5mV
50ms
.4mV
10ms
B3 Facil: Mobile vs Imm (33Hz)
B4 Relative Change
Pulse Number in Train
C1 Mobile (10Hz)
C2 Mobile +Immobile
200ms
C3 Facil: Mobile vs Imm (10Hz)
C4 Relative Change

Figure 3.3. Among animals that showed a facilitation caused by amphetamine during the first responses to theta-frequency stimulation, injection of amphetamine resulted in a significant relative suppression of responses during theta-frequency trains. **A.** Amplitudes of synaptic responses during gamma-frequency trains were not altered by amphetamine injection when responses were normalized to the first pulse during mobility (A_3), and when responses were normalized to the first pulse in the train (A_4). **B**. There was a significant facilitation of synaptic responses to the first pulse in the theta-frequency trains that does not persist throughout the train (B_3). When the amplitudes of responses were normalized to the first pulse during mobility, the majority of responses were *suppressed* due to amphetamine (B_4.; * = $p < 0.05$; # = $p < 0.01$). **C and D**. However, when animals were chosen based on amphetamine not causing a facilitation of the amplitude of the first response during theta-frequency trains, there were no changes in the amplitude of responses following injection of amphetamine for both gamma- (**C**) and theta-frequency (**D**) trains


A1 Mobile (33Hz)
Mobile + Amph
A2 Mobile + Amph
p1+p10
p10
.2mV
50ms
20ms
A3 Facil: Amph (33Hz)
A4 Relative Change
Mobile
+Amph
fEPSP Amplitude (%)
Pulse Number in Train
B1 Mobile (10Hz)
B2 Mobile + Amph
.5mV
.4mV
200ms
10ms
B3 Facil: Amph (10Hz)
B4 Relative Change
C1 Not Facil: Amph (33Hz)
C2 Relative Change
Pulse number in Train
D1 Not Facil: Amph (10Hz)
D2 Relative Change

F**igure 3.4.** Injection of the D_1 receptor antagonist SCH23390 prior to administration of amphetamine blocks the facilitation of synaptic responses to the first pulse in theta-frequency trains, as well as the relative suppression of responses. **A**. Similar to effects of amphetamine alone on responses to gamma-frequency stimulation, injection of amphetamine in animals that had previously received an injection of SCH23390 did not result in significant change in synaptic responses during gamma-frequency trains. **B**. The facilitation of the responses to the first pulse in theta-frequency trains induced by amphetamine was blocked by SCH23390 (B_3), as was the relative suppression when responses were normalized to the first pulse in their group (B_4).

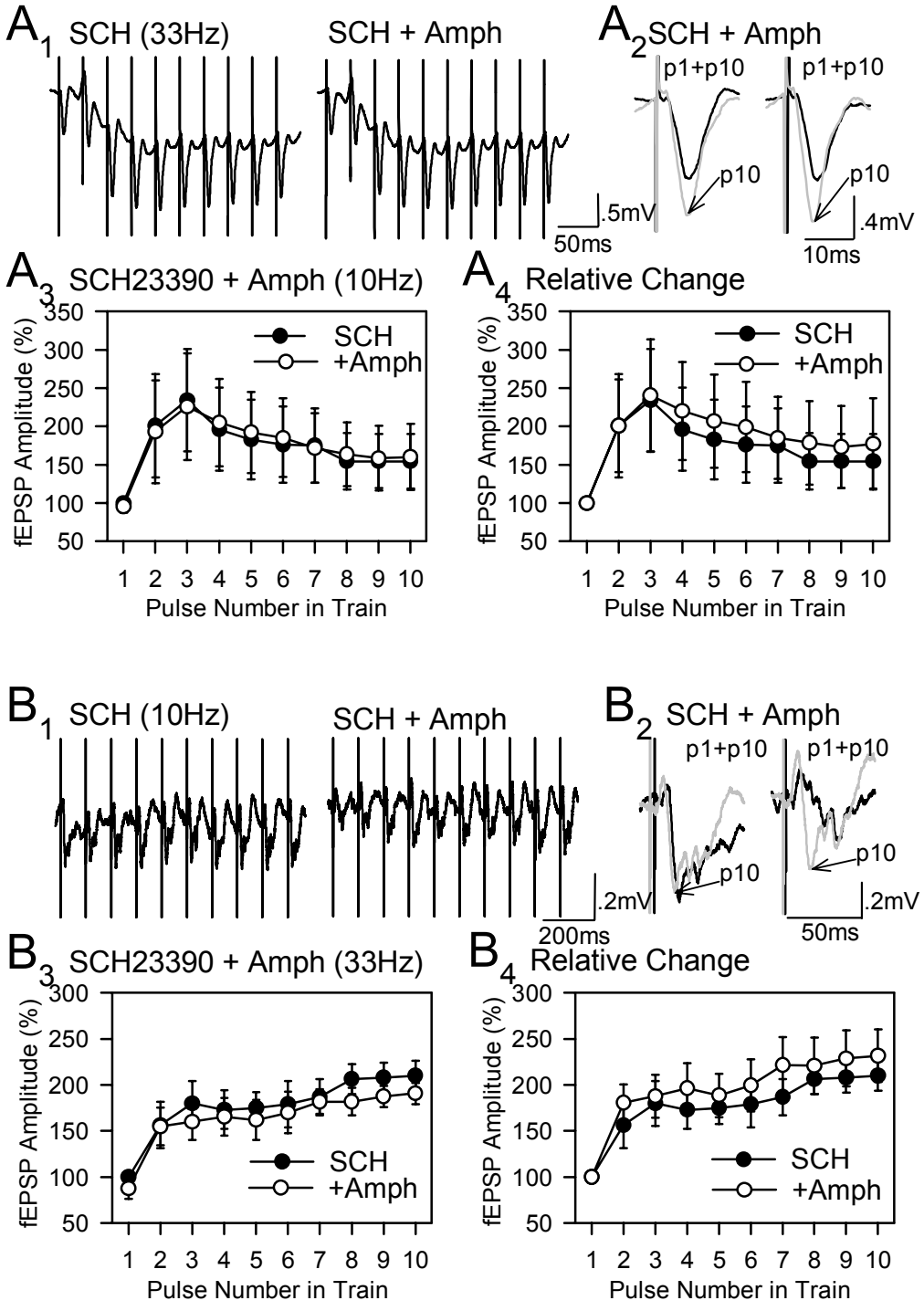
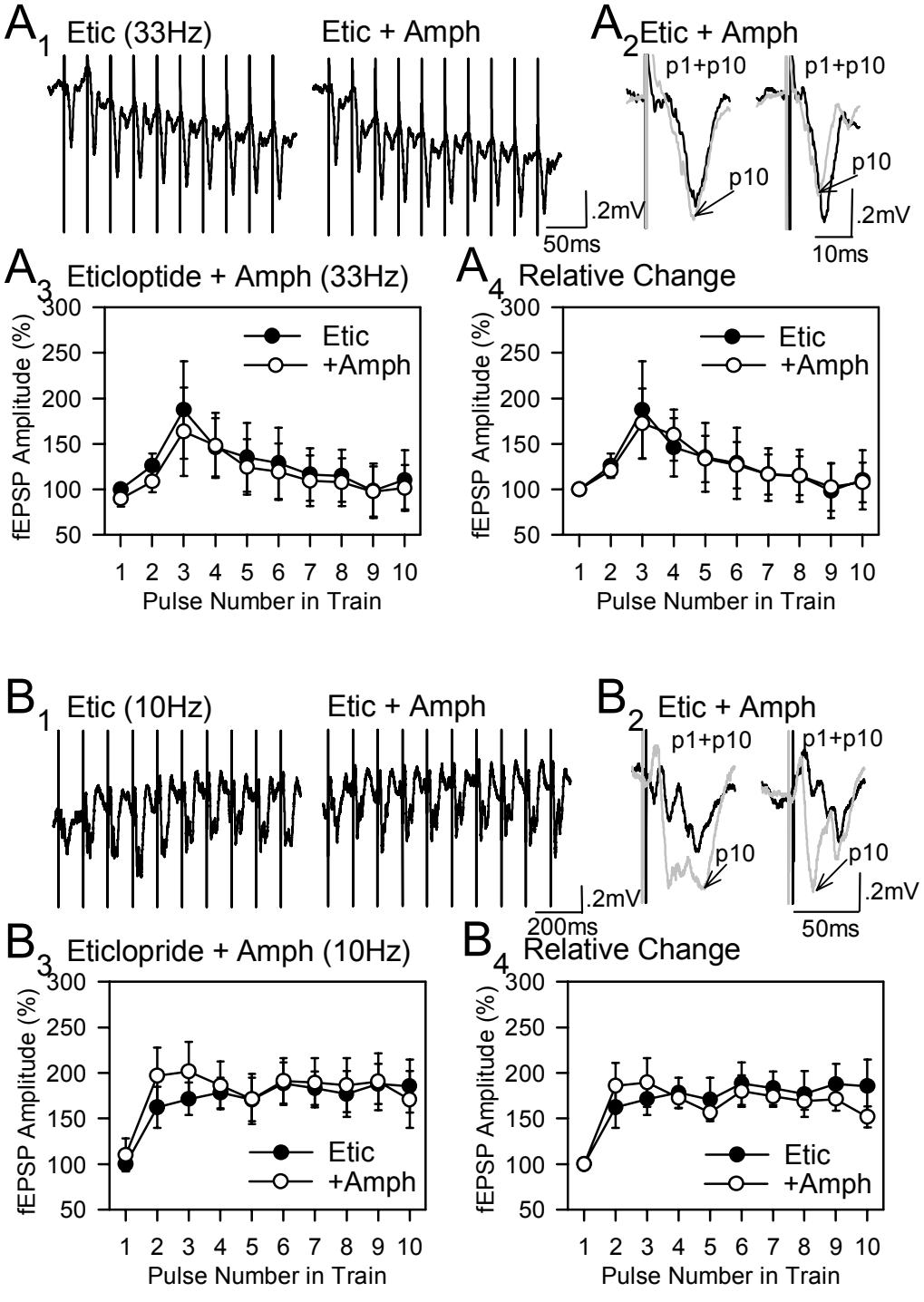

A1 SCH (33Hz)
SCH + Amph
A2 SCH + Amph
p1+p10
p10
.5mV
50ms
.4mV
10ms
A3 SCH23390 + Amph (10Hz)
A4 Relative Change
fEPSP Amplitude (%)
SCH
+Amph
Pulse Number in Train
B1 SCH (10Hz)
B2 SCH + Amph
.2mV
200ms
50ms
B3 SCH23390 + Amph (33Hz)
B4 Relative Change

Figure 3.5. Injection of the D_2 receptor antagonist eticlopride prior to administration of amphetamine blocked the facilitation and relative suppression caused by amphetamine during theta-frequency stimulation trains. **A.** While D_2 receptors were blocked by injection of eticlopride, administration of amphetamine did not result in significant changes in the amplitudes of responses during gamma-frequency stimulation. **B**. Eticlopride blocked the facilitation of the first response observed following injection of amphetamine alone (B_3), and also blocked relative suppression in response amplitudes when normalized to the first pulse (B_4).


A1 Etic (33Hz)
Etic + Amph
A2 Etic + Amph
p1+p10
p10
.2mV
50ms
10ms
A3 Eticloptide + Amph (33Hz)
A4 Relative Change
fEPSP Amplitude (%)
Etic
+Amph
Pulse Number in Train
B1 Etic (10Hz)
B2 Etic + Amph
200ms
B3 Eticlopride + Amph (10Hz)
B4 Relative Change

Chapter 4

EFFECTS OF DOPAMINE ON SYNAPTIC RESPONSES IN THE LATERAL ENTORHINAL CORTEX EVOKED BY SHORT TRAINS OF THETA- AND GAMMA-FREQUENCY STIMULATION: IN VITRO BRAIN SLICE RECORDINGS.

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ABSTRACT

The entorhinal cortex is involved in the processing of multimodal sensory information, and it receives substantial dopaminergic inputs from midbrain neurons. Past studies have shown that bath-application of dopamine on brain slices in vitro results in a concentration-dependent, bidirectional effect on amplitudes of synaptic responses from inputs to the entorhinal cortex, such that a low, 10 μM concentration of dopamine facilitates evoked synaptic responses in layer II of the entorhinal cortex in vitro, and a higher concentration of 50 μM dopamine results in a suppression of synaptic strength. Gamma- and theta-frequency EEG rhythms occur robustly within the entorhinal cortex, and are thought to contribute to neuronal mechanisms mediating sensory processing and learning and memory. To assess how dopamine may modulate repetitive synaptic responses during theta and gamma EEG rhythms, in the present study, the effect of application of dopamine on synaptic responses evoked in layer II of the lateral entorhinal cortex by short trains of gamma- and theta- frequency stimulation of layer I has been explored using an in vitro slice preparation. The application of 10 μM dopamine resulted in a facilitation of the amplitudes of synaptic responses evoked by the responses to the first stimulation pulse in the theta and gamma-frequency trains, as well as responses to later pulses in the trains. Application of 50 μM dopamine resulted in a significant suppression in the amplitudes of the responses to the first pulses in the trains of stimulation. This suppression of the response to the first pulse was associated with a relative facilitation of synaptic responses during gamma-frequency stimulation, and a similar trend during theta-frequency stimulation. In experiments in which either the D_1

receptor antagonist SCH23390 (50 μM) or the D_2 receptor antagonist sulpiride (50 μM) was applied prior to application of 50 μM dopamine, it was found that SCH23390 did not block the suppression of initial responses or the relative facilitation effect, and that sulpiride blocked the suppression of initial responses as well as the relative facilitation effect for gamma frequency stimulation. This suggests that the relative facilitation of synaptic responses induced by high concentrations of dopamine, in which smaller initial responses display greater relative growth during repetitive stimulation, is dependent mainly upon activation of D_2 receptors.

INTRODUCTION

Both the previous chapter of this thesis and other research reports have indicated that dopamine is likely to modulate synaptic function in the entorhinal cortex in a way that is dependent on repetitive synaptic inputs (Rosenkrantz and Johnston, 2007; Ito and Schuman, 2008; Hutter et al., 2013). Activation of cholinergic receptors is known to play a major role in the generation of theta- and gamma-frequency EEG activities in both the entorhinal cortex and hippocampus (Mitchell and Ranck, 1980; Dickson et al. 2000; Glasgow and Chapman, 2007), and dopamine is also likely to be released during these oscillatory activities, and to modulate synaptic communication during cholinergically-induced rhythmic states. Oscillatory activity is thought to promote sensory processing through the synchronization of population neuronal activity (Billock and Tsou, 2014; Munk et al., 1996) and gamma frequency activity has been thought to contribute to the temporal binding of representations across areas of neocortex (Dickson et al., 2000; Fries et al., 2007). Theta-frequency oscillations have also been linked to mechanisms thought to contribute to sensorimotor integration (Bland and Oddie, 2001) and mechanisms of learning and memory including induction of long-term synaptic potentiation (Buzsaki, 2002; Hasselmo, 2006). There has been no investigation, however, of how dopamine may modulate responses of layer II of the entorhinal cortex to repetitive stimulation in vitro, nor have the mechanisms of such a modulatory effect been investigated thoroughly.

In vitro field potential recordings in layer II of entorhinal cortex slices have shown that application of dopamine results in a bidirectional effect, in which high concentrations of dopamine lead to a facilitation of synaptic responses, while low concentrations result in

a suppression of synaptic responses (Caruana et al., 2006). Chapter 3 of this thesis investigated effects of systemic injection of amphetamine on train-evoked responses in the entorhinal cortex, but it was still unknown what effect different concentrations of dopamine in the entorhinal cortex may have on synaptic responses evoked during trains of stimulation pulses at the frequencies of the theta and gamma EEG rhythms. A relative facilitation in the amplitudes of synaptic responses recorded during theta- and gamma-frequency stimulation has been observed in the entorhinal cortex in vitro by Sparks and Chapman (2013); it was found that application of the cholinergic agonist carbachol resulted in an overall suppression of synaptic responses during trains, but that the amplitudes of responses to each pulse during the trains was facilitated relative to the amplitude of the first response (Sparks and Chapman, 2013). In a study of the effects of dopamine on synaptic responses in layer V entorhinal neurons, however, application of dopamine was not found to significantly affect the amplitudes of responses to single stimulation pulses, but activation of D_1 receptors did *reduce* the summation of intracellular synaptic responses recorded in response to 20 Hz trains of 10 pulses in acute brain slices (Rosenkranz and Johnston, 2006). Further, in the temporroammonic pathway that stretches from layer III of the entorhinal cortex to the hippocampal CA1 region, dopamine has been found to suppress responses to low-frequency trains of stimulation and to enhance responses to higher frequency trains, suggesting that dopamine may serve as a high-pass filter to selectively enhance responses to higher-frequency synaptic inputs (Ito and Schuman, 2007). The literature therefore shows variable effects of dopamine on temporal summation effects during trains of stimulation pulses.

The manner in dopamine may modulate responses to repetitive stimulation of synaptic inputs in layer II of the entorhinal cortex at theta- and gamma-frequencies is not yet known, and a full answer to this question will have to include consideration of the effects of both low concentrations of dopamine that facilitate single synaptic response, and higher concentrations of dopamine that cause a suppression of single synaptic responses (Caruana et al., 2006). The synaptic facilitation induced by lower concentrations of dopamine could be induced endogenously by tonic activation of dopaminergic input that could occur during appetitively motivated behaviours, and the synaptic suppression might be normally induced following phasic bursts of firing in dopamine neurons that can occur following exposure to reward-predictive cues (Shultz, 2007; Hutter and Chapman, 2013). Dopaminergic modulation of train-evoked responses in the entorhinal cortex, therefore, might reflect a mechanism through which changes in appetitive motivational state may have an effect upon the neuronal representations carried by rhythmic firing patterns at theta and gamma frequencies within the entorhinal cortex.

Chapter 3 of this thesis aimed to determine the role of dopamine receptor activation on synaptic responses in the entorhinal cortex during trains of stimulation pulses delivered at theta (10 Hz) and gamma (33 Hz) frequencies in freely moving rats. Results showed that injections of amphetamine (3 mg/kg) resulted in a facilitation of the first response within theta-frequency trains, and an apparent ceiling effect resulted in a concomitant relative suppression in the degree of growth in subsequent responses during the train. The facilitation and relative suppression effects induced by dopamine were blocked by prior application of either the D_1 receptor antagonist SCH23390 or the D_2

receptor antagonist eticlopride. These results indicated that activation of dopamine receptors by amphetamine results in a facilitation of the strength of individual evoked synaptic responses in the entorhinal cortex, but that this effect is associated with relatively less growth in the amplitude of synaptic responses during the train. Thus dopamine may enhance sensory inputs carried by a temporally discrete pattern of inputs to the entorhinal cortex, and is less likely to enhance the impact of inputs carried by repetitive synaptic activation at theta- and gamma- frequencies.

Experiments reported in Chapter 3 were conducted in live animals using systemic injections of amphetamine that can result in enhanced dopaminergic neurotransmission in multiple brain regions, and it is also not known to what extent amphetamine may have altered other neurotransmitter systems either directly or indirectly (Berry, 2004; Imperato et al., 1993). In addition, the results of Chapter 3 are likely to be most relevant to the question of the effects of a low concentration of dopamine on train-evoked responses, because the injection of 3 mg/kg amphetamine induced a dopamine receptor-dependent *facilitation* of synaptic responses. For that reason, it is not known how a higher concentration of dopamine that induces a suppression of single evoked responses might affect responses to trains of stimulation. The present study, therefore, has used in vitro slices of entorhinal cortex tissue using two different concentrations of dopamine (10 μM and 50 μM) to investigate the role of dopaminergic activation on synaptic responses during theta- and gamma-frequency stimulation. The low concentration of dopamine resulted in an overall facilitation of theta- and gamma-frequency train-evoked responses, and the higher concentration of dopamine resulted in a suppression of the synaptic

responses to the first pulse in the trains and a concomitant facilitation in the relative degree of growth in later synaptic responses evoked during the trains. This relative facilitation effect was investigated using the D_1 receptor antagonist SCH23390 and the D_2 receptor antagonist sulpiride to assess its dependence on D_1 and D_2 receptor activation.

MATERIALS AND METHODS

In Vitro Slice Preparation

Experiments were carried out observing the guidelines of the Canadian Council on Animal Care. Long-Evans rats between 4 and 7 weeks old were anaesthetized with halothane, decapitated and their brains rapidly dissected and placed in ice-cold, oxygenated (95% O_2 and 5% CO_2) high-sucrose artificial cerebrospinal fluid (ACSF) containing (in mM): 2 KCl, 1.25 NaH_2PO_4, 7 $MgCl_2$, 0.5 $CaCl_2$, 26 $NaHCO_3$, 10 dextrose and 250 sucrose. A quick dissection was then conducted to excise the frontal lobes, cerebellum and dorsal ~2 mm of the brain. The section of the brain containing the temporal lobe was then fixed on a pedestal using ethyl cyanoacrylate glue and submerged in ice-cold, oxygenated high-sucrose ACSF in order to make slices. Horizontal, 400 µm thick slices were taken using a vibrating microtome (Campden Instruments, Model HA752). The slices were placed on a nylon net submerged in oxygenated normal ACSF containing (in mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, 2 L-ascorbic acid, 10 dextrose. Slices were maintained at room temperature (22-24 ºC) and allowed to recover for at least 1 hour. All chemicals used were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Stimulation and Recording

In order to record synaptic responses, slices were transferred individually to a gas-fluid recording chamber (Fine Science Tools) and rested on a nylon net. Slices were perfused with oxygenated normal ACSF at 32 ºC at a flow rate of 1-1.5 ml/min throughout the experiments. A Leica MS5 microscope was used in order to view the slices and place the electrodes in the correct position and depth. A bipolar tungsten electrode (FHC, Bowdoin, ME, USA) was used to deliver cathodal constant square pulses (0.1 ms width, 40-180 μA amplitude) by means of a stimulus generator (WPI, Model A300) and a stimulus isolation unit (Model A360) to layer I of the lateral entorhinal cortex. Glass pipette recording electrodes were pulled using a horizontal micropipette puller (Sutter Instruments, Model P-97) and filled with normal ACSF in order to monitor field excitatory post-synaptic potentials (fEPSPs) in layer II of the lateral entorhinal cortex. Field potentials were low-pass filtered at 3 kHz, amplified (Axoclamp 2B; Molecular Devices, Sunnyvale, CA, USA) and digitized at 20 kHz. Field EPSPs were also displayed on a digital oscilloscope for monitoring during the experiments and stored on a computer hard disk using pClamp 8.2 software (Molecular Devices).

Effects of Dopamine on Evoked Responses

The effect of dopamine on synaptic responses evoked by short trains of theta- and gamma-frequency stimulation was evaluated by recording single pulses every 20 s, followed by short trains of theta and gamma stimulation during each of the following periods: normal ACSF, 10 μM dopamine, 50 μM dopamine (Sigma-Aldrich, St. Louis, MO, USA), and washout with normal ACSF. Ten and 50 μM dopamine solutions were

prepared by adding 1 mL or 200 µL of 3 mM dopamine solution to 60 mL of normal ACSF respectively. Sodium metabisulfite (Sigma-Aldrich, St. Louis, MO, USA) from a frozen stock solution (final concentration 50 µM) was added to minimize oxidization of dopamine.

During each period, ten 10-pulse trains at theta-(10 Hz) and gamma-(33 Hz) frequency were delivered 8 times with a 20 s interval between each train. Prior to baseline recordings of train-evoked responses, the stability of responses to single pulses of stimulation delivered every 20 s was evaluated for a period of 10 to 20 min, and slices were discarded if they showed constant drift of > 10 % in either an upward or downward direction. Following a stable baseline period of at least 10 min, train-evoked responses were recorded, and then 10 µM dopamine was applied to the slice. Train evoked responses were recorded once responses had increased by at least 10% in response to dopamine application, typically within 10 to 15 min of dopamine application. The concentration of dopamine applied was then increased to 50 µM dopamine, and responses to single pulses were monitored until responses had suppressed to less than 90% of the amplitude of baseline responses, typically within 15 min. Train evoked responses were then recorded in 50 µM dopamine prior to washout of dopamine and a final recording of train-evoked responses in normal ACSF.

Because 50 µM dopamine was found to result in a suppression of responses to the first pulses, and a relative facilitation of later responses during gamma frequency trains, the dopamine receptors underlying this phenomenon were investigated by applying 50 µM dopamine to slices previously perfused with either the D_2 receptor antagonist

sulpiride (50 μM, Sigma-Aldrich, St. Louis, MO, USA; Caruana et al., 2006) or the D_1 receptor antagonist SCH23390 (50 μM, Tocris Bioscience; Caruana et al., 2006). The protocol for the antagonist conditions was as follows: normal ACSF (10-15 min), 50 μM sulpiride or SCH23390 (10-15 min), 50 μM dopamine (10-15 min), and a washout of dopamine in the presence of the antagonist (10-15 min). The 10-pulse trains at theta- and gamma-frequencies were again delivered at the end of each of these periods.

Data Analysis

The peak amplitudes of fEPSPs evoked by each pulse in the trains were measured relative to the level of the local field potential just prior to each stimulation pulse. Effects of dopamine on amplitudes of responses versus baseline recordings in normal ACSF were assessed by normalizing the amplitudes of responses evoked during the trains to the amplitudes of the response evoked by the first pulse in the trains delivered in normal ACSF. The ability of dopamine receptor antagonists SCH23390 or sulpiride to block effects of dopamine was determined by normalizing the amplitudes of responses evoked in the presence of dopamine relative to the first response to trains recorded in the presence of the respective antagonist. Repeated measures ANOVAs (with pulse number and testing condition as factors) were used to assess significant changes in train-evoked responses, and planned comparisons were also used to compare responses to each individual pulse number between each of the testing conditions.

Data were also re-normalized to characterize the profile of changes in the amplitudes of responses during the course of the stimulation trains. The relative changes in fEPSP amplitudes during the 10-pulse trains in different testing conditions were

compared by normalizing responses evoked during the trains to the amplitudes of the first responses within each train. Planned comparisons were used to compare the renormalized responses obtained at each pulse number across the testing conditions to test for differences in relative changes in the amplitudes of responses during the trains.

RESULTS

In normal ACSF during gamma-frequency stimulation using 33 Hz trains, there was a slight facilitation of the amplitude of the response to the second pulse in the train to 106.2±6.6 % of the amplitude of the first fEPSP, but subsequent pulses in the train evoked progressively smaller fEPSPs such that the last fEPSP was 69.6±7.8 % of the amplitude of the first fEPSP (Figure 4.1A_3, closed symbols; $t = 4.56$, $p = 0.003$). During theta-frequency stimulation using 10 Hz trains in normal ACSF, the amplitude of the second pulse was facilitated to 106.6 ± 4.9 %, but there was no significant suppression of the tenth pulse relative to the first pulse (Figure 4.1B_3; 92.9 ± 6.8; $t = 1.29$, $p = 0.237$). Therefore, responses showed a larger and more reliable decline during gamma-frequency stimulation than during theta-frequency stimulation ($p < 0.05$ for pulses 4 to 10), and this general pattern was maintained in subsequent tests.

Effects of 10 μM Dopamine on Train-Evoked Responses

The effect of either 10 or 50 μM dopamine on responses to trains of stimulation pulses delivered at theta- and gamma-frequencies was tested by delivering short 10-pulse trains at either 10 or 33 Hz respectively (n = 8 for 10 μM, and n = 9 for 50 μM).

Following application of 10 μM dopamine, the amplitude of the response to the first pulse in the gamma-frequency trains was increased to 118.9 ± 4.9 % of baseline (t =

3.86, p = 0.006), reflecting the basic facilitatory effect of 10μM dopamine (Caruana et al., 2006). In addition, an ANOVA showed both a significant main effect of drug ($F_{1,7}$ = 12.52, p = 0.009) and a significant drug by pulse-number interaction ($F_{9,63}$=3.98, p < 0.001), and planned comparisons of response amplitudes for each pulse number showed that there was a significant effect of dopamine for all pulses (p < 0.05) except for the last pulse in the train (78.2 ± 9.4 % versus 69.3 ± 7.8 %; t = 2.21, p = 0.063; Figure 4.1A_3). However, the *relative* amplitudes of responses evoked during the trains, compared to the first response in the trains under each recording condition, was similar in normal ACSF and in 10 μM dopamine for all pulse numbers (p > 0.05 for all pulses; Figure 4.1, A_4). Thus, 10 μM dopamine resulted in an overall facilitation of responses evoked during gamma-frequency stimulation trains, but did not alter the relative pattern amplitudes of responses during the train.

The effects of 10 μM dopamine on responses evoked by theta-frequency trains were similar to the effects of 10 μM dopamine on responses to gamma-frequency trains. During theta-frequency stimulation, 10 μM dopamine increased the amplitude of the response to the first pulse in the train (116.4 ± 4.7 %; t = 3.49, p = 0.010), similar to its effects on the response to the first pulse in gamma-frequency trains (Figure 4.1B). Application of dopamine also resulted in a significant main effect of drug ($F_{1,7}$ = 6.60, p = 0.037) but the drug by pulse-number interaction did not reach significance ($F_{9,63}$ = 2.03, p = 0.051). Planned comparisons of response amplitudes to each pulse number showed that there were significant effects of dopamine for all pulses (p < 0.05) except for the 5^{th}, 7^{th}, and 10^{th} pulses in the train (p > 0.05; Figure 4.1B_3). Similar to effects observed for

gamma-frequency trains, the facilitation of responses to theta-frequency trains was not associated with a significant change in the relative amplitudes of responses evoked during theta-frequency trains relative to the first pulse in the train (Figure 4.1B4; $p > 0.05$ for all pulse-numbers). Thus, for both theta- and gamma- frequency stimulation, application of 10 μM dopamine lead to an overall facilitation of train-evoked responses (Figure $4.1A_3$, B_3), but did not lead to a significant change in the relative pattern of growth in response during the trains (Figure $4.1A_4$, B_4).

The reversibility of the effects of dopamine were assessed using ANOVAs comparing responses obtained before dopamine application and after washoff of dopamine, and there was no significant differences found for gamma-frequency stimulation ($F_{1,5} = 0.10$, $p = 0.765$) as well as for theta-frequency stimulation ($F_{1,5} = 3.15$, $p = 0.136$), suggesting that dopamine had a transient effect on gamma-frequency responses.

Effects of 50 μM Dopamine on Train-Evoked Responses

In contrast to 10 μM dopamine, which caused a facilitation of responses to the first pulse in the trains, application of 50 μM dopamine caused a *suppression* in the amplitudes of the first responses in the trains, and an accompanying relative facilitation effect similar to that induced by muscarinic receptor activation (Sparks and Chapman, 2013). After application of 50 μM dopamine, the response to the first pulse in gamma-frequency trains was suppressed to 79.7 ± 5.1 % of baseline ($p = 0.004$) an effect that reversed during washout ($p = 0.583$). In addition, there was no significant effect of dopamine on all later responses evoked during the trains ($p > 0.05$), which was reflected

in a significant drug by pulse-number interaction ($F_{9,72} = 4.82$, $p < 0.001$; Figure 4.2A). When these data were re-normalized to the amplitudes of the first responses in the trains to highlight changes in the amplitudes of responses during trains, there was a trend for responses evoked in the presence of dopamine to show greater relative growth during the trains, and this effect was significant for the 3rd and 6th response in the train (97.5 ± 8.0 versus 86.2 ± 7.4 % for the third pulse, and 71.1 ± 6.0 versus 61.7 ± 5.7 % for the sixth pulse; $p<0.05$; Figure 4.2A$_4$). Therefore, the suppression in the amplitude of the first pulse during gamma-frequency trains due to 50 μM dopamine was accompanied by a trend for an increase in the relative magnitude of responses later in the train

A somewhat different pattern of effects of 50 μM dopamine was observed on responses to theta-frequency stimulation trains. After the application of 50 μM dopamine, the response to the first stimulation pulse in the trains was significantly decreased to 76.1 ± 3.8 % of baseline levels ($t = 6.28$, $p < 0.001$), and most of the subsequent responses during the train were also suppressed, resulting in a significant main effect of drug ($F_{1,8} = 11.48$, $p = 0.010$; Figure 4.2B). The response to the last pulse in the train in the presence of dopamine was 73.5 ± 6.4 % of baseline, as compared to 89.0 ± 6.2 % of baseline in normal ACSF). Re-normalizing these data to the amplitudes of the first responses in the respective trains showed that the pattern of change in amplitudes during the train were similar in normal ACSF and in the presence of 50 μM dopamine ($p > 0.05$ for all pulses).

The results shown in Figures 4.1 and 4.2 replicated the finding reported by Caruana et al. (2006) in which application of 50 μM dopamine resulted in a significant suppression of single evoked fEPSPs in layer II of entorhinal cortex slices, and 10 μM

resulted in a significant facilitation of synaptic responses. The overall facilitation induced by 10 μM dopamine in the amplitudes of responses to gamma- and theta-frequency stimulation trains is therefore likely due to D_1 receptor, activation, and the suppression of initial responses in trains induced by 50 μM dopamine is likely due to D_2 receptor activation. In addition, however, it is not clear which dopamine receptors might contribute to the relative facilitation effect induced by 50 μM dopamine, in which there was relatively greater growth in responses evoked by later pulses in the trains (Figure 4.2A4). For this reason, the effects of D_1 and D_2 receptor antagonists on the changes in responses induced by application of 50 μM dopamine were assessed.

Dopaminergic Effects on Train-Evoked Responses in the Presence of D_1 Antagonist SCH23390

The role of activation of D_1 receptors on the suppression and relative facilitation of synaptic responses due to application of 50 μM dopamine was assessed using the D_1 receptor antagonist SCH23390. Application of SCH23990 alone did not have an effect on the amplitudes of fEPSPs during trains of gamma-frequency stimulation (n = 5; main effect of group: $F_{1,4} = 0.82$, $p = 0.383$), or during trains of theta-frequency stimulation (n = 5; main effect of group: $F_{1,4} = 1.31$, $p = 0.311$).

During gamma-frequency stimulation, dopamine had similar effects on responses in the presence of SCH23390 as it did in normal ACSF; dopamine resulted in a suppression in the amplitude of the first response evoked in the train (78.0 ± 4.0%; $t = 5.46$, $p = 0.005$) and did not significantly alter the amplitudes of subsequent responses during the train (Figure 4.3$A_{1,2,3}$; interaction: $F_{9,36} = 6.89$, $p < 0.001$, main effect of group:

$F_{1,4} = 1.39$, $p = 0.303$). SCH23390 also did not block the significant relative facilitation of synaptic responses induced by 50 μM dopamine (Figure $4.3A_4$; $p < 0.05$ for pulses two to five).

During theta-frequency stimulation, SCH23390 also did not block the dopamine-induced suppression of the amplitude of the first response in the trains (Figure $4.3B_{1,2,3,}$; 79.5 ± 7.9; $t = 2.95$, $p = 0.042$), but interestingly, SCH23390 did prevent the suppression of responses to later pulses in the trains that was observed with dopamine alone (compare Figures 4.2B and 4.4B; main effect of group: $F_{1,4} = 1.82$, $p = 0.249$; interaction: $F_{9,36} = 1.93$, $p = 0.079$). The suppression of the response to the first pulse was also associated with a significant relative facilitation effect for the fourth and fifth pulses in the theta-frequency trains (Figure $4.3B_4$; $p = 0.008$, $p = 0.032$ respectively). Thus, D_1 receptor blockade did not block the initial suppression and relative facilitation of responses to both gamma and theta-frequency trains.

Dopaminergic Effects on Train-Evoked Responses in the Presence of D_2 Antagonist Sulpiride.

It has been shown previously that the suppression of single synaptic responses caused by 50 μM dopamine is mediated by D_2-like receptors, and that the D_2 receptor antagonist sulpiride inhibits the suppression (Caruana et al., 2006). I therefore investigated whether the relative facilitation evoked during trains of stimulation induced by 50 μM dopamine is also blocked by activation of D_2 receptors using sulpiride (Figure 4.4).

While sulpiride itself had no significant effect on responses evoked during

gamma-frequency stimulation (main effect group: $F_{1,7} = 0.12$, $p = 0.745$), it did block both the dopaminergic suppression of responses evoked by the fist pulse in trains (106.1 ± 3.8%, $t = 0.16$, $p = 0.154$; interaction: $F_{9,63} = 1.08$, $p = 0.390$; Figure 4.4$A_{1,2,3}$), as well the relative facilitation of responses (Figure 4.4A_4; $p > 0.05$ for all pulses).

Sulpiride alone also had no effect on responses to theta-frequency stimulation (main effect group: $F_{1,7} = 0.10$, $p = 0.757$), but it did block the suppression of the first response induced by 50 μM dopamine that was observed in normal ACSF (100.2 ± 6.5 %; $t = 0.03$, $p = 0.977$; main effect $F_{1,7} = 3.51$, $p = 0.103$; interaction $F_{9,63} = 2.29$, $p = 0.027$; Figure 4.4B). However, application of dopamine in the presence of sulpiride was associated with a significant relative facilitation of responses evoked by theta-frequency trains, and this effect was significant for pulses two through five ($p = 0.015$, $p = 0.003$, $p = 0.009$, $p = 0.014$, respectively; Figure 4.4B4). Thus, block of D_2 receptors was effective at blocking the suppression of single synaptic responses evoked at the beginning of trains of stimulation, as well as the associated relative facilitation evoked during gamma-frequency stimulation. For theta-frequency stimulation, however, sulpiride appeared to *enhance* the reliability of a trend towards a relative facilitation effect that was observed with dopamine alone

DISCUSSION

The previous Chapter in this thesis investigated the role of dopamine receptor activation on the amplitudes of responses evoked during gamma-frequency trains (33Hz) and theta-frequency trains (10Hz) of stimulation in awake animals. It was found that, while injection of amphetamine had no effect on responses to gamma-frequency

stimulation trains, animals that showed a significant facilitation in fEPSPs due to amphetamine injection after the first pulse in the theta-frequency trains also showed less growth in responses during the trains. In addition, this relative suppression effect was found to be partially dependent upon activation of both D_1 and D_2 receptors. The present Chapter has aimed to investigate the effects of both a dopaminergic facilitation of synaptic strength by 10 μM dopamine, and a dopaminergic suppression of synaptic strength induced by 50 μM dopamine on responses evoked by trains of gamma- and theta-frequency stimulation in slices of entorhinal cortex tissue.

Results from this Chapter have shown that the pattern of responses evoked during trains of stimulation differed as compared to that observed in in vivo recordings. In in vitro recordings, the general pattern was a decline in amplitudes of responses as the pulse number increased, similar to what has been observed for parasubicular inputs to the entorhinal cortex in vitro (Sparks and Chapman, 2013). This differs from the previous chapter, where synaptic responses during trains of stimulation in vivo showed a facilitation, eventually reaching an asymptote. The preparation of in vitro slices can reduce the amount of synaptic inhibition because of reductions in the axonal arbour of inhibitory interneurons (Buckmaster and Schwartzkroin, 1995). Reduced inhibition in slices, however, would be expected to *enhance* train-evoked responses in slices, but this effect was not observed. The difference between the patterns of baseline responses in in vivo vs. in vitro recordings is not known, but it may relate to a reduced capacity of slices to maintain stores of neurotransmitter available for release.

Differences between baseline responses to gamma and theta-frequency stimulation

were also observed in in vitro recordings. During trains of gamma stimulation in normal ACSF in all tests in vitro, the amplitudes of synaptic responses decreased steadily after the first several pulses to amplitudes about half of the first response, while responses to theta-frequency stimulation showed lesser declines. The greater declines observed during gamma-frequency stimulation in vitro may be due to stronger recruitment of inhibitory mechanisms, or greater reduction in transmitter availability during these higher-frequency trains.

When investigating the effects of both high and low concentrations of dopamine on the amplitudes of single pulses as compared to measures in normal ACSF, results from the current study are consistent with that of previous literature; during both gamma- and theta-frequency stimulation, application of 10 μM dopamine resulted in a facilitation of the first response, while application of 50 μM resulted in a suppression of the first response (Caruana et al., 2006). However, it is unclear what the function of the dopaminergic suppression and facilitation of single synaptic responses due to high and low concentrations of dopamine are. The facilitation of single synaptic responses may serve to enhance neuronal communication between the entorhinal cortex and the hippocampus, while the suppression may serve to enhance the signal to noise ratio for salient events (Kroener et al., 2009). Differences in responses to repetitive stimulation were also found, and the current study has characterized how both concentrations of dopamine affect the amplitude of responses evoked by gamma- and theta-frequency stimulation. Ten μM dopamine caused an enhancement of synaptic responses throughout trains of both theta and gamma frequency stimulation without inducing a relative

facilitation of responses, and although 50 μM dopamine suppressed the initial responses, it was associated with a relative facilitation that was non-significant for gamma-frequency stimulation, but significant for the 3rd and 6th pulses during theta-frequency stimulation. The effects on synaptic strength caused by dopamine may play a role in enhancing synaptic communication during EEG rhythms in the entorhinal cortex, and adds to previous research that has shown that dopamine may serve to selectively enhance responses to repetitive stimulation (Kroener et al., 2009).

Effects of 10 μM Dopamine

Low concentrations of dopamine applied to slices of entorhinal cortex tissue resulted in an expected facilitation of the first pulse in both the gamma- and theta-frequency trains of stimulation (Caruana et al., 2006). Interestingly, this concentration also led to a facilitation of all pulses during both frequencies of trains, and no relative change when responses were normalized to the amplitudes of the first responses during each testing condition (Figure 4.1). It has been previously hypothesized that this synaptic facilitation by low concentrations of dopamine may serve to increase the impact of reward-relevant sensory inputs within the hippocampus (Caruana et al., 2006). However, we have found here that although application of 10 μM dopamine results in a maintained overall facilitation of responses during gamma-frequency trains, dopamine does not result in a further relative facilitation of train-evoked responses relative to the first pulse in the baseline trains. This facilitation of synaptic responses is known to be due to D_1 receptor activation (Caruana et al., 2006) and, in contrast to findings reported in vivo in Chapter 3, this D_1 receptor activation by low concentrations of dopamine therefore appears to be

maintained during repetitive stimulation in vitro.

Effects of 50 µM Dopamine

Application of the high concentration of dopamine (50 µM) during both gamma- and theta-frequency stimulation resulted in a suppression of the first pulse relative to measures in normal ACSF. This affect is consistent with the D_2 receptor-mediated suppression seen in the entorhinal cortex in previous research (Pralong and Jones, 1993; Stenkamp, Heinemann and Schmitz, 1998; Caruana et al., 2006). Application of 50 µM dopamine during *gamma*-frequency stimulation resulted in a suppression of the amplitude of the first synaptic response, while subsequent responses had similar amplitudes as in normal ACSF. This resulted in a relative facilitation effect (Figure 4.2A4), in which there was greater growth of responses during the train expressed relative to the amplitude of the first pulse. A similar relative facilitation effect was observed in parasubicular inputs to the entorhinal cortex by Sparks and Chapman (2013) during application of a cholinergic agonist. Fifty µM dopamine also had marked effects on responses during *theta*-frequency stimulation, but there was a greater suppression of responses throughout the train (Figure 4.2B3), and a correspondingly weaker and non-significant relative facilitation effect (Figure 4.2B4). This relative facilitation effect was only significant during gamma stimulation, suggesting that during periods of dopaminergic activation, patterns of synaptic activity exhibiting gamma-frequencies may be less susceptible to suppression than patterns that are active at theta-frequencies. This frequency-dependent effect on the amount of relative facilitation, in which there is greater relative facilitation for the higher frequency trains, could be due to greater potential for temporal summation among fEPSPs

during the higher frequency trains (Ito and Schuman, 2008).

Effects of Antagonists on Gamma and Theta-Frequency Trains

Application of D_1 antagonist SCH23390 as well as the D_2 receptor antagonist sulpiride on slices of lateral entorhinal cortex tissue did not result in a significant change in the amplitude of responses throughout both theta- and gamma-frequency trains compared to the amplitude of reponses recorded prior to application of the antagonist in normal ACSF. However, when the amplitudes of responses are compared to those recorded during normal ACSF in the slices that received dopamine alone, the responses during antagonist application are significantly suppressed relative to those recorded in normal ASF (compare baseline values in Figure 4.2 with antagonist values in Figure 4.3 and 4.4). This may be due to spontaneous changes in th amplitude of baseline recordngs on the different testing days.

Because the suppression of synaptic responses induced by high concentrations of dopamine has been shown to be mediated by activation of D_2 receptors (Caruana et al., 2006), experiments were also conducted in the presence of the D_2 blocker sulpiride in order to determine whether D_2 receptors also mediate the increased relative facilitation effect in response to application of 50 μM dopamine. Application of sulpiride prior to wash-on of 50 μM dopamine resulted in a block of the suppression of the first pulse during gamma-frequency trains that was observed in normal ACSF, and it also blocked the suppression of responses throughout the theta-frequency trains that was observed in normal ACSF (Compare Figures 4.2A3,B3 and 4.4A3, B3). Sulpiride also blocked the relative facilitation of responses during gamma-frequency stimulation, suggesting that

activation of D_2 receptors is involved in the relative facilitation of responses during gamma-frequency stimulation (Compare Figures 4.2A4 and 4.4A4). Interestingly, application of dopamine in the presence of sulpiride did not block the trend for a relative facilitation effect during *theta*-frequency trains; the larger responses during the train in the presence of the D_2 receptor blocker could be due to a D_1 receptor-mediated synaptic facilitation.

The D_1 receptor antagonist SCH23390 was applied to slices prior to application of 50 μM dopamine in order to determine the role of D_1 receptors on synaptic responses to trains of gamma- and theta-frequency stimulation. The D_1 receptor antagonist did not block the suppression of synaptic responses due to the first pulse of the gamma- or theta-frequency trains (Caruana et al., 2006), and in addition, it did not block associated relative facilitation effects that were observed for pulse numbers early in the trains for both gamma- and theta-frequency stimulation (Figure 4.3A4, B4). This suggests that the suppression of synaptic responses and associated relative facilitation effects are not dependent upon D_1 receptor activation.

During theta-frequency stimulation, the maintained suppression of responses to later pulses in the trains that was induced by 50μM dopamine was blocked by SCH23390 (see Figure 4.3B3). This suggests that, although D_1 receptor activation is not involved in the initial suppression of single responses, it may contribute to the sustained suppression during theta trains. Caruana and Chapman (2008) found that the suppression of synaptic responses induced by high concentrations of dopamine was due in part to a D_1-receptor-mediated reduction in cellular input resistance caused by an increase in a K+

conductance, and it is possible that a D1 receptor-mediated reduction in input resistance is might contribute to the suppression of train-evoked responses by 50 μM dopamine.

Future Directions

Dopamine could result a relative facilitation during low- and high-frequency stimulation in part by suppressing inhibitory GABAergic transmission. Dopamine has been shown to inhibit GABAergic inhibitory synaptic transmission in the rat neostriatum, which is mediated by activation of D_2 receptors (Harsing and Zigmond, 1997; Delgado et al., 1999). In the entorhinal cortex, although Glovaci et al (2013) found that 1 μM dopamine did not significantly affect GABA-mediated IPSCs in layer II of the entorhinal cortex, Caruana and Chapman (2007) found that 50 μM dopamine did suppress synaptic inhibition in these cells. Therefore, future studies could assess the possibility that the D_2 mediated relative facilitation may arise due to the dopamine-induced reduction of inhibitory GABAergic transmission during repetitive stimulation in the entorhinal cortex. This could be investigated by examining train-evoked synaptic responses during application of the GABA receptor blocker bicuculine that would remove the influence of inhibition, and by examining how the amplitude of pharmacologically isolated inhibitory synaptic potentials is altered during trains of stimulation.

It has been shown that the relative facilitation caused by application of the cholinergic agonist carbachol in the entorhinal cortex occurs due to the enhancement of NMDA glutamate receptor-mediated responses along with a reduction in the hyperpolarization activated current I_h that results in increased summation of EPSPs

(Sparks and Chapman, 2013). Because dopamine facilitates NMDA receptor currents (Harvey and Lacey, 1997; Caruana and Chapman, 2008), future experiments could investigate the contribution of NMDA receptors to the relative facilitation during low and high-frequency trains of stimulation through the use of the NMDA receptor blocker APV (Sparks and Chapman, 2013). Sparks and Chapman (2013) found that the relative facilitation of responses during theta and gamma-frequency trains caused by carbachol was also in part due to a suppression of

The role of the hyperpolarization activated inward cationic current I_h, could also be investigated; while activation of cholinergic receptors is known to facilitate summation of EPSPs by increasing input resistance due to a *suppression* of the current I_h (Heys et al., 2010; Magee, 2000; Chevaleyre and Castillo, 2002), application of dopamine in layer V neurons of the lateral entorhinal cortex leads to an *increase* in I_h to result in the opposite effect of reducing the extent of temporal summation of fEPSPs during stimulation with 20 Hz trains (Rosenkranz and Johnston, 2006). Nevertheless, the effects of dopamine on Ih in layer II entorhinal neurons is unknown, and this could be investigated using the I_h blockers Cs^+ or Zd7288 to determine whether reductions in I_h might contribute to the relative facilitation due to application of high concentrations of dopamine observed here.

Conclusions

Recordings of entorhinal cortex fEPSPs were used in the current study to investigate the role of low and high concentrations of dopamine on the amplitudes of responses evoked by trains of gamma- and theta-frequency stimulation. While 10 μM dopamine resulted in a significant facilitation of all pulses during both the gamma- and

theta-frequency trains, 50 μM dopamine resulted in a suppression of the first response in both trains relative to baseline values, and a relative facilitation of synaptic responses that was statistically significant for gamma-frequency stimulation. Application of the D_1 receptor antagonist SCH23390 prior to application of 50 μM dopamine did not block the suppression of the response to the first pulse or the relative facilitation of responses during gamma- or theta-frequency stimulation. However, application of the D_2 receptor antagonist sulpiride prior to 50 μM dopamine blocked the suppression of the initial responses to the trains, and also blocked the relative facilitation of responses during gamma-frequency stimulation. Results are therefore consistent with a role for D_2 receptors in the suppression and relative facilitation of responses in inputs to layer II of the entorhinal cortex in vitro.

Figure 4.1. Application of 10 μM dopamine facilitates responses to single pulses and does not result in a relative facilitation in response to gamma- and theta-frequency trains of stimulation. **A_1.** Representative sample traces in response to 10-pulse trains at gamma-frequency before and after application of 10 μM dopamine. **A_2.** Sample response to the first pulse of the ten-pulse gamma-frequency train (p1) superimposed with the response to the last pulse in the train (p10) for both the baseline condition during normal ACSF and during application of 10 μM dopamine. Arrows indicate the response to the tenth stimulation. **A_3.** Responses for both groups were normalized to the amplitude of the first fEPSP in normal ACSF showing the facilitatory effect of 10 μM dopamine throughout the train at gamma-frequency stimulation. **A_4.** All responses were normalized to the amplitude of the first fEPSP in the respective trains, where application of 10 μM dopamine shows no relative facilitation at gamma frequencies. **B_1.** Representative sample traces during normal ACSF and in response application of 10 μM dopamine during theta-frequency trains. **B_2.** Sample response to the first pulse of the theta-frequency train (p1) superimposed with the response to the last pulse in the train (p10). **B_3.** Theta-frequency stimulation shows similar effects during application of 10 μM dopamine, where dopamine application results in a facilitation of responses, with no relative facilitation (B_4).

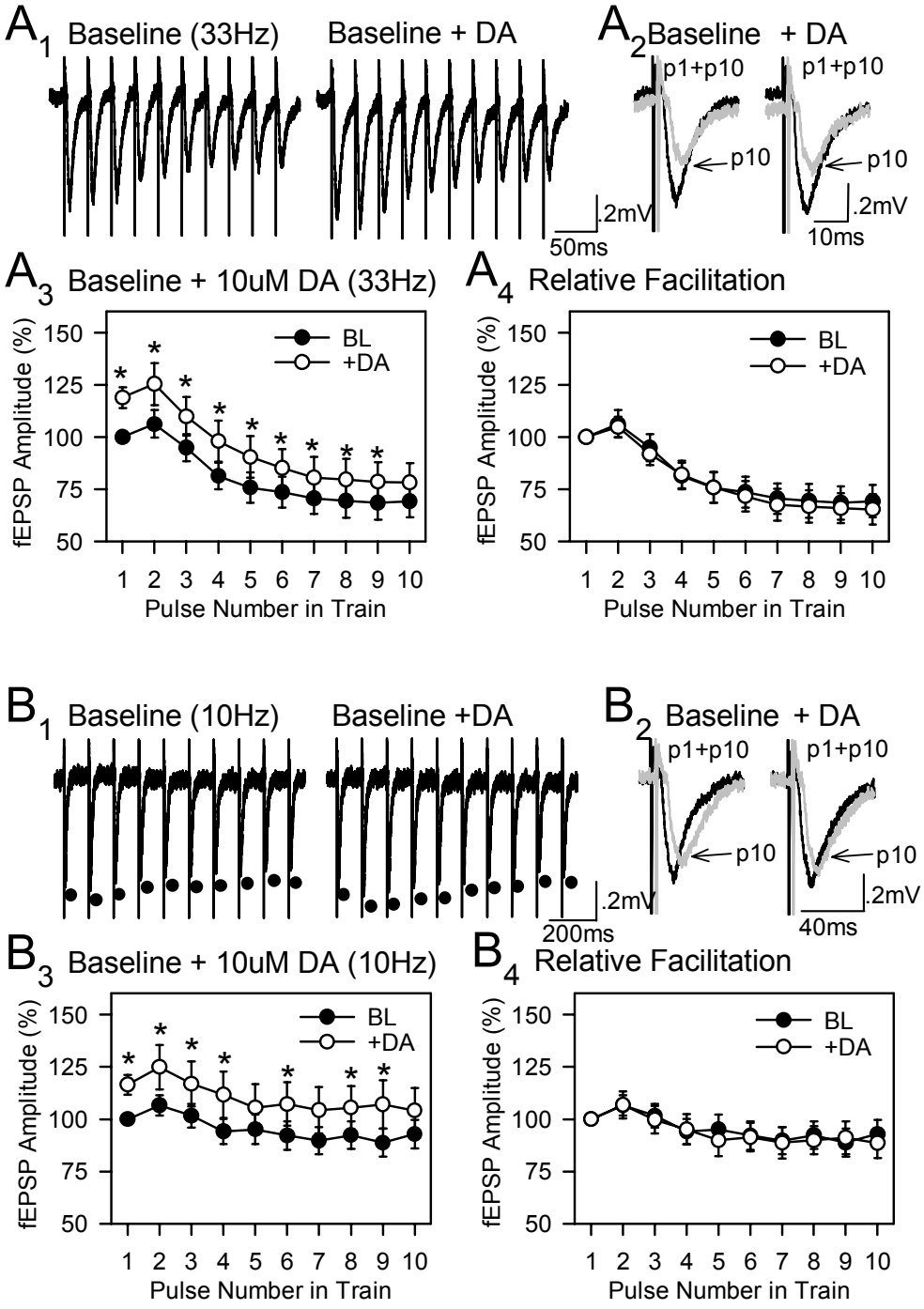
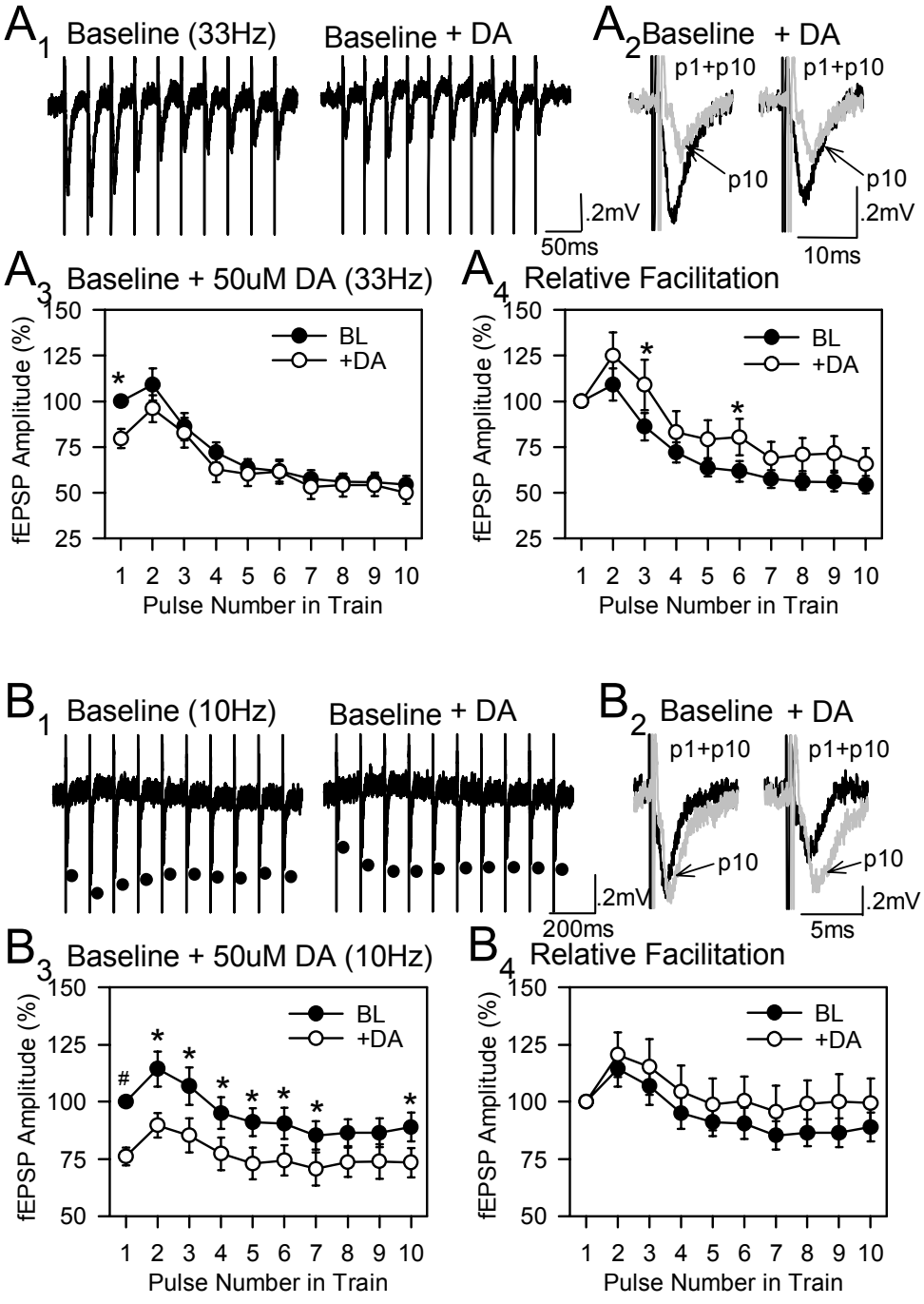

A1 Baseline (33Hz)
Baseline + DA
A2 Baseline + DA
p1+p10
p10
.2mV
50ms
10ms
A3 Baseline + 10uM DA (33Hz)
A4 Relative Facilitation
fEPSP Amplitude (%)
BL
+DA
Pulse Number in Train
B1 Baseline (10Hz)
Baseline +DA
B2 Baseline + DA
200ms
40ms
B3 Baseline + 10uM DA (10Hz)
B4 Relative Facilitation

Figure 4.2. Dopaminergic activation of receptors with 50 μM dopamine suppresses responses to the first pulse in a trains of pulses, and shows a trend towards a relative facilitation of responses when subjected to gamma- and theta-frequency trains of stimulation. **A.** Responses during ACSF and 50 μM dopamine normalized to the amplitude of the first fEPSP during ACSF shows a suppressive effect of 50 μM dopamine of the first pulse, and no change throughout the train at gamma-frequency stimulation (A_3). When responses were normalized to the amplitude of the first fEPSP in the respective trains, 50 μM DA shows a trend towards a relative facilitation at gamma frequencies (A_4; * = $p<0.05$). **B.** Theta-frequency stimulation shows a suppression of the amplitude of the majority of the responses due to 50 μM dopamine (B_3; # = $p<0.01$), with no significant relative facilitation (B_4).


A1 Baseline (33Hz)
Baseline + DA
A2 Baseline + DA
p1+p10
p1+p10
p10
p10
.2mV
.2mV
50ms
10ms
A3 Baseline + 50uM DA (33Hz)
A4 Relative Facilitation
fEPSP Amplitude (%)
BL
+DA
Pulse Number in Train
B1 Baseline (10Hz)
Baseline + DA
B2 Baseline + DA
p1+p10
p1+p10
p10
p10
.2mV
.2mV
200ms
5ms
B3 Baseline + 50uM DA (10Hz)
B4 Relative Facilitation
fEPSP Amplitude (%)
BL
+DA
Pulse Number in Train

F**igure 4.3.** Application of 50 μM dopamine during application of the D_2-like receptor antagonist sulpiride resulted in a block of the relative facilitation effect in gamma-frequency trains of stimulations, and a new significant relative facilitation during theta-frequency trains. **A**. Normalized responses to the first fEPSP in normal ACSF-sulpiride showed no difference in the amplitude of any of the responses during 50 μM dopamine and sulpiride versus sulpiride alone (A_3). There was also no relative facilitation when all responses were normalized to the amplitude of the first fEPSP in the respective trains (A_4). **B**. Application of sulpiride prior to dopamine resulted in a block of the overall suppression of responses during the theta-frequency train (B_3). Sulpiride application along with dopamine caused a relative facilitation when responses were normalized to the amplitude of the first response in the respective trains that was not evident during application of 50 μM dopamine alone (B_4).

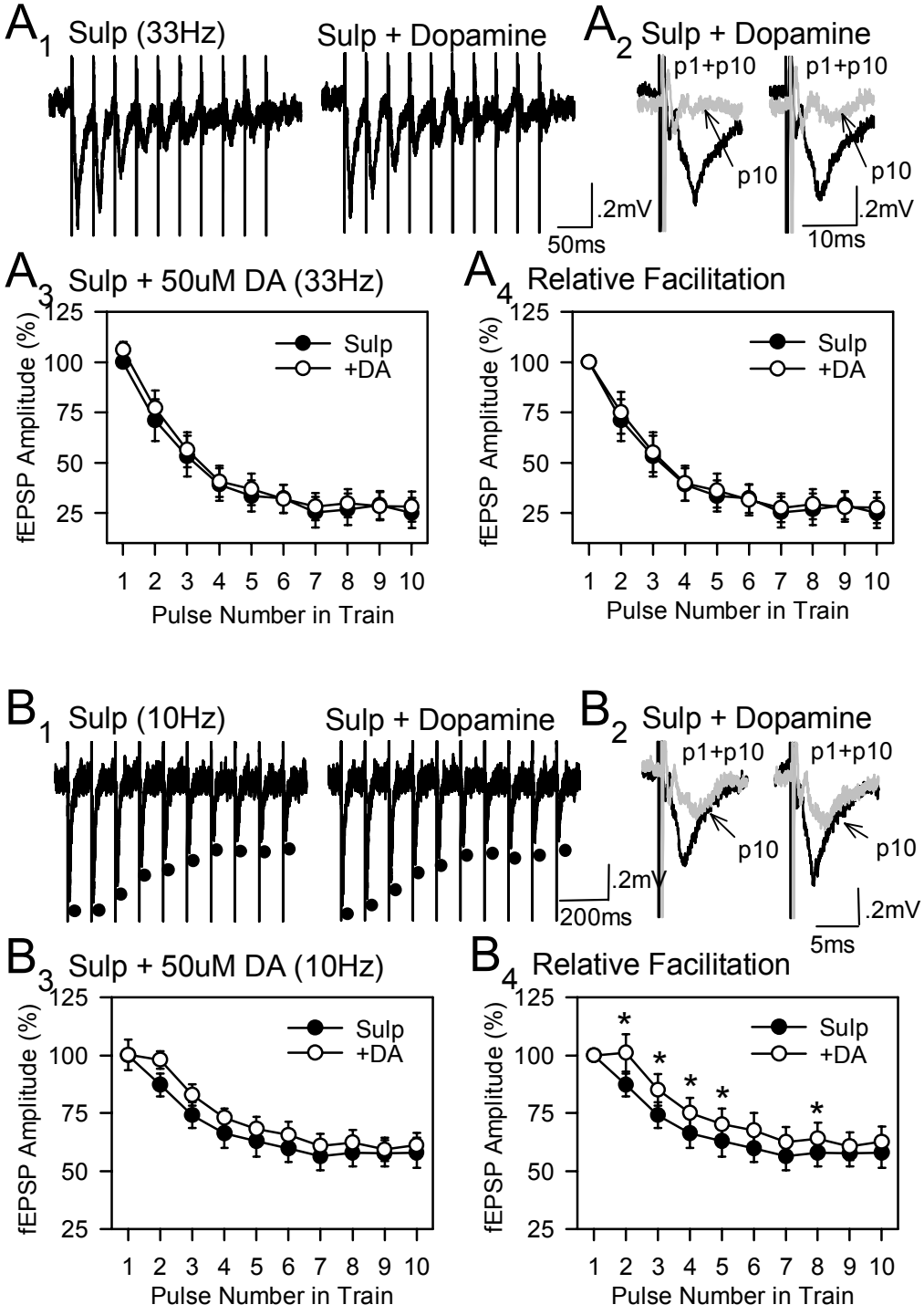
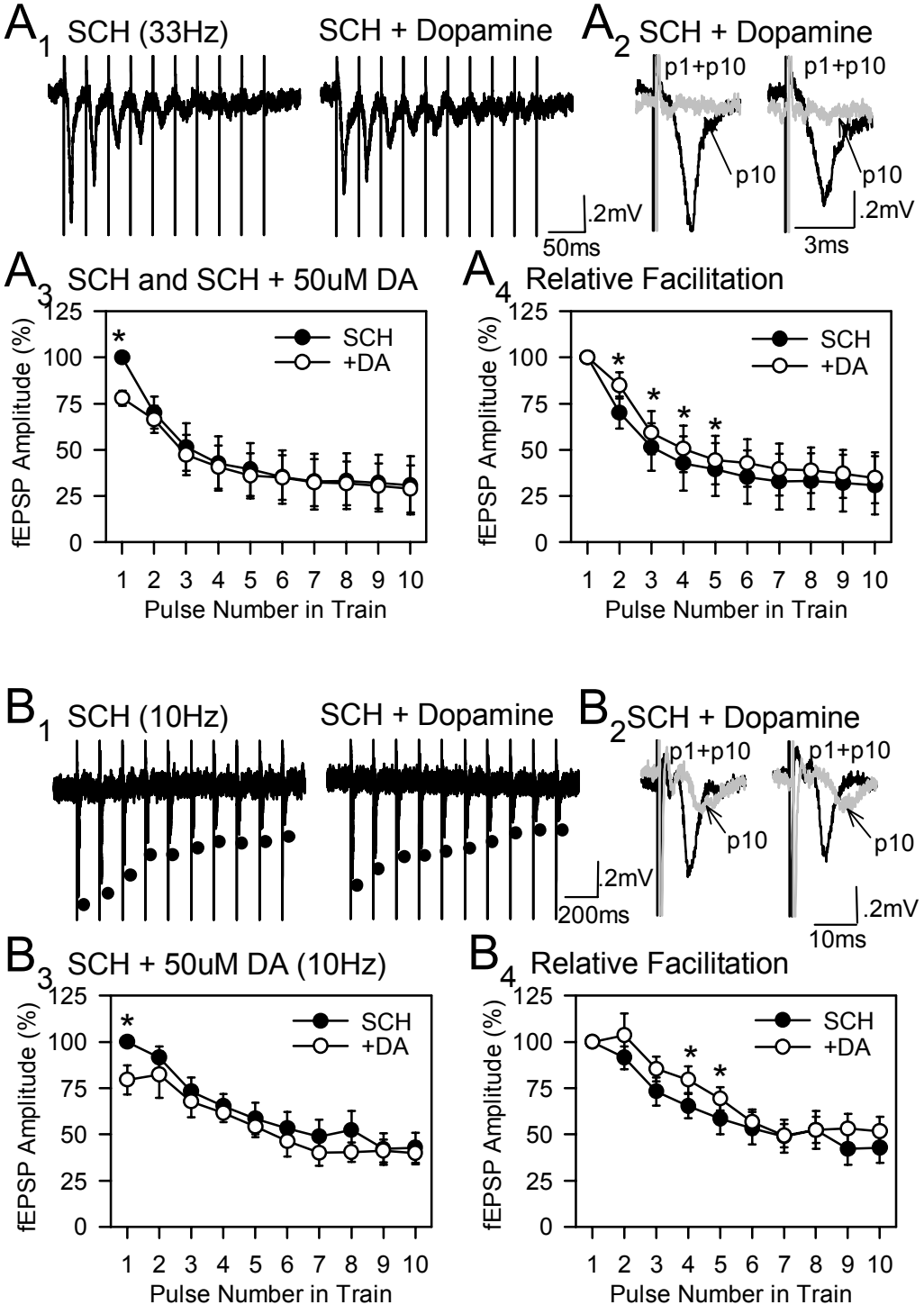

A1 Sulp (33Hz)
Sulp + Dopamine
A2 Sulp + Dopamine
p1+p10
p10
.2mV
50ms
10ms
A3 Sulp + 50uM DA (33Hz)
A4 Relative Facilitation
fEPSP Amplitude (%)
Sulp
+DA
Pulse Number in Train
B1 Sulp (10Hz)
B2 Sulp + Dopamine
200ms
5ms
B3 Sulp + 50uM DA (10Hz)
B4 Relative Facilitation

Figure 4.4. Application of 50 μM dopamine during application of the D_1-like receptor antagonist SCH23390 resulted in no change in the amplitude of responses during gamma-frequency stimulation, however, SCH23390 blocked the suppression of responses 2 to 10 during theta-frequency stimulation and induced a significant relative facilitation. **A**. SCH23390 did not block the suppression of the first fEPSP in the gamma-frequency group caused by 50 μM dopamine (A_3), nor did it block the relative suppression due to dopamine application (A_4). **B**. SCH23390 application did not block the suppression of the first pulse due to dopamine during the theta-frequency trains, but it did block the suppression of all subsequent pulses (B_3). Similar to results during application of sulpiride, SCH23390 resulted in a significant relative facilitation for two pulses during the theta-frequency trains that did not exist during application of 50 μM dopamine alone (B_4).


A1 SCH (33Hz)
SCH + Dopamine
A2 SCH + Dopamine
p1+p10
p10
.2mV
50ms
3ms
A3 SCH and SCH + 50uM DA
A4 Relative Facilitation
fEPSP Amplitude (%)
SCH
+DA
Pulse Number in Train
B1 SCH (10Hz)
B2 SCH + Dopamine
200ms
10ms
B3 SCH + 50uM DA (10Hz)
B4 Relative Facilitation

CHAPTER 5:
INDUCTION OF LONG-TERM SYNAPTIC POTENTIATION IS BLOCKED BY DOPAMINE IN LAYER II OF THE LATERAL ENTORHINAL CORTEX IN VITRO

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ABSTRACT

The entorhinal cortex is a major structure linking neocortical areas with the hippocampal formation, and it is thought to contribute to the integration and encoding of sensory information. The superficial layers of the entorhinal cortex receive a large projection from the piriform (primary olfactory) cortex, and synaptic plasticity within this input pathway may affect olfactory information processing. In addition, the mesocortical dopamine system provides a substantial input to layer II of the lateral entorhinal cortex and dopamine may therefore play an important role in modulating neuronal processing related to learning and memory in the entorhinal cortex. In the present study, to assess the effects of dopamine on a cellular model of learning, we have used field potential recordings from slices of entorhinal cortex maintained in vitro to assess the effects of dopamine on the induction of LTP in synaptic inputs to layer II. Stimulation pulses were delivered to layer I using a concentric bipolar electrode, and field excitatory postsynaptic potentials were obtained in layer I adjacent to layer II. High-frequency stimulation (HFS) was delivered to induce LTP in slices exposed either to normal ACSF or to 15 min bath application of dopamine. Changes in synaptic responses were compared to those obtained in control groups in which responses were monitored with no HFS in either normal ACSF or in response to dopamine alone. Similar to previous observations in vivo, application of dopamine alone resulted in a facilitation in the amplitude of synaptic responses that was partially reversed during wash in normal ACSF. In addition, while delivery of HFS in normal ACSF resulted in significant LTP of synaptic responses, the presence of dopamine

blocked the induction of LTP. These findings are consistent with previous observations in vivo, and suggest that release of dopamine associated with reward-relevant behaviours may inhibit the induction of long-term changes in synaptic strength that may contribute to learning and memory.

INTRODUCTION

The entorhinal cortex is thought to contribute to the integration and encoding of sensory information because it received inputs from multiple areas including the piriform cortex, perirhinal cortex and the hippocampus (Burwell, 2000). While neurons in the medial entorhinal cortex are involved in processing spatial information (Moser and Moser, 2008), the lateral entorhinal cortex receives monosynaptic projections from the olfactory bulb as well as the piriform cortex (Burwell, 2000) suggesting that the lateral part of the entorhinal cortex is more involved in processing olfactory information. Lesions in the parahippocampal cortex have indicated that this region plays a role in olfactory memory; lesions to the perirhinal cortex (Feinberg et al., 2012), entorhinal cortex (Bannerman et al., 2002; Petrulis et al., 2000) and subiculum (Bannerman et al., 2002) result in deficits in social odor recognition, while lesions in the lateral entorhinal cortex (Staubli et al., 1984; Otto and Eichenbaum, 1992), perirhinal cortex (Otto and Eichenbaum, 1992), and ventral hippocampus result in deficits in odor discrimination (Kesner et al., 2011; Kesner et al., 2002). Other studies have indicated that the lateral entorhinal cortex is necessary for object-recognition (Wilson, Watanabe et al., 2013; Wilson, Langston et al., 2013). Collectively, these data point to the importance of the medial and lateral entorhinal cortices in memory processes.

The superficial layers of the lateral entorhinal cortex receive projections from mesocortical dopamine neurons in the ventral tegmental area and the substantia nigra (Bjorklund and Lindvall, 1984; Oades and Halliday, 1987; Björklund and Dunnett, 2007), and dopamine may therefore play an important role in modulating sensory processing in

the entorhinal cortex. Dopamine is a neuromodulatory transmitter that can strongly affect cortical neurophysiology (Schultz, 2007) and which is thought to modulate processes of learning and memory related to appetitive motivation and reward (Caruana et al., 2007; Berridge et al., 2009). Although the lateral entorhinal cortex has been associated with olfactory memory in the past (Staubli et al., 1984; Otto and Eichenbaum, 1992; Petrulis et al., 2000; Bannerman et al., 2002), the specific role of dopamine on memory in the entorhinal cortex is not yet clear. Gauthier and Soumireu-Mourat (1981) showed that damaging dopamine cells in the lateral entorhinal cortex with 6-hyroxydopamine resulted in an *improvement* in performance on a continuously reinforced retention task. Contrary to the findings of Gauthier and Soumireu-Mourat (1981), other studies have shown that long-term inhibitory avoidance memory due to foot shock is *impaired* in rats infused with a dopamine D_1 receptor antagonist in the entorhinal cortex (Barros et al., 2001; Izquierdo et al., 1998). Therefore, although it is clear that dopamine in the entorhinal cortex is somehow involved in regulating learning and memory for motivated tasks, it is unclear what the role is and what the cellular mechanisms for this involvement may be.

Previous work in our lab has shown that application of dopamine to slices containing the lateral entorhinal cortex has a bidirectional effect on synaptic responses, in which low doses of dopamine facilitate synaptic responses via D_1 receptors, and higher concentrations cause a suppression of synaptic responses mainly via D_2 receptors (Caruana et al., 2006; Caruana and Chapman, 2008; Glovaci et al., 2014). It is therefore possible that a facilitation or suppression of synaptic responses in the entorhinal cortex

due to activation of dopamine receptors could result in a transient enhancement or inhibition of the salience of sensory inputs to the entorhinal cortex. In addition, these transient effects of dopamine on synaptic responses may also contribute to the regulation of the induction of longer-lasting changes in synaptic responses that could contribute to the lasting synaptic changes required for learning and memory. Because dopamine has been found previously to contribute to learning and memory (Lejeune et al., 2013; Macdonald et al., 2013; da Silva et al., 2012; Rossato et al., 2009), we were interested in determining how LTP, a cellular model of memory, is affected by dopamine in the entorhinal cortex in vitro.

In both the hippocampus and prefrontal cortex, studies have found that dopamine can result in an enhancement of the induction of LTP (Manahan-Vaughan and Kulia, 2003; Matthies et al., 1997; Otmakhova and Lisman, 1996), suggesting that it may contribute to synaptic mechanisms of learning and memory (Bliss and Collingridge, 1993; Morris et al., 1986). Similarly, inactivation of the ventral tegmental area leads to a suppression of LTP in the hippocampus (Ghanbarain and Motamedi, 2013). However, other studies have found that activation of dopamine receptors can inhibit LTP induction (Wei et al., 2012; Blond et al., 2002; Law-Tho and Crepel, 1995). For example, Wei et al (2012) showed that in the CA1 region of the hippocampus, dopamine inhibits the induction of LTP by HFS, and that this inhibition is due to activation of D_1 receptors. Research investigating the role of dopamine activation on synaptic plasticity has also shown that activation of dopamine enhances long-term *depression* (Chen et al., 1995). In addition, previous research in our lab has shown that dopamine in the entorhinal cortex

also has powerful effects on induction of synaptic plasticity (Caruana et al., 2007). Caruana et al. (2007) found that systemic injections of the dopamine reuptake inhibiter GBR12909 in the entorhinal cortex blocked the induction of both long-term synaptic potentiation and LTD in vivo. However, the effects of GBR12909 on basal synaptic transmission were not clear in the study by Caruana et al. (2007) and it is unknown whether LTP and LTD were blocked under levels of dopamine that would induce either a D_1 receptor-mediated facilitation, or a D_2 receptor-mediated suppression, of synaptic transmission. The suppression of synaptic plasticity might have occurred if dopamine suppressed synaptic transmission via D_2 receptors (Caruana et al., 2006) to result in reduced postsynaptic depolarization and reduced NMDA glutamate receptor activation during trains of stimulation. Alternatively, lower effective concentrations of dopamine may have concurrently facilitated basal synaptic transmission (Caruana et al., 2006) and inhibited synaptic plasticity via D_1 receptor-mediated mechanisms (Wei et al., 2012; Matthies et al., 1997; Otmakhova and Lisman, 1996).

The purpose of the current study was to investigate the role of dopamine receptor activation on the induction of LTP in slices of the lateral entorhinal cortex using field potential recordings in vitro. A low concentration of 10 μM dopamine that has previously been found to cause a facilitation of fEPSPs in the entorhinal cortex (Caruana et al., 2006) was used in order to determine whether the transient synaptic facilitation induced by lower, more physiological levels of dopamine might enhance the induction of lasting synaptic strengthening. High-frequency stimulation of synaptic inputs to layer II of the entorhinal cortex was delivered in either normal ACSF, or following 15 min application

of dopamine, and results were compared to slices that were exposed only to normal ACSF or to dopamine alone. Results obtained were consistent with those found by Caruana et al. (2007) in vivo, and indicate that a low concentration of dopamine in the entorhinal cortex blocks induction of LTP, suggesting that synaptic plasticity in this region is inhibited during periods in which animals are exposed to reward-related stimuli.

MATERIALS AND METHODS

In Vitro Slice Preparation

The guidelines of the Canadian Council on Animal Care were used in order to prepare acute brain slices of the lateral entorhinal cortex. Brains were obtained from 4 to 7-week old rats anesthetized with halothane, and were submerged in ice-cold ACSF containing (in mM) 124 NaCl, 5 KCl, 1.25 NaH2PO4, 2 MgSO4, 2 CaCl2, 26 NaHCO3, 10 dextrose, L-ascorbic acid (0.4 mM), uric acid (0.35 mM) and indomethecine (40 μM), saturated with 95% O_2 and 5% CO_2. Horizontal slices (400 μM) were cut using a vibratome (WPI, Vibroslice NVSL), and recovered in room temperature ACSF (~22ºC) for ≥1.5 h. Slices were transferred to a nylon net in a gas-fluid interface chamber (Fine Science Tools) containing a humidified 95%/5% O_2/CO_2 atmosphere, and perfused with oxygenated ACSF (1.5-2.0 ml/min; 32 ± 0.5ºC).

Stimulation and Recording

Field potential recording electrodes were pulled using borosilicate glass (1.0 mm OD) with a horizontal puller (Sutter Instruments, P97), and filled with ACSF (2-6 MΩ). The concentric bipolar stimulating electrode (Frederick Hauer Co.) was positioned with the aid of a dissecting microscope (Leica, MS5) in layer I of the lateral entorhinal cortex,

and the recording electrode was placed 0.3 to 0.5 mm caudal to the stimulating electrode in layer I close to the border of layer II. Cathodal constant current pulses were delivered using a stimulus generator (WPI, Model A300) and isolation unit (Model A360). Evoked field excitatory postsynaptic potentials (fEPSPs) were amplified (DC-3 kHz, Axon Instr., Axoclamp 2B) and digitized using pClamp 8.2 software (20 kHz, Digidata 1322A, Molecular Devices). Stimulation intensities were adjusted to evoke fEPSPs with amplitudes of ~65-75 % of the maximal, and responses to test pulses were recorded once every 20 sec throughout testing.

In order to assess the role of dopamine on the induction of LTP in the lateral entorhinal cortex, slices received high-frequency stimulation (HFS; three 1-sec, 100 Hz trains of pulses, delivered once every 30 sec) to induce LTP either after a 10-min baseline period in normal ACSF, or following an additional 15-min bath application of 10 μM dopamine (with 50 μM sodium metabisulphite, Sigma-Aldrich), the concentration of dopamine shown previously to facilitate synaptic responses in the lateral entorhinal cortex (Caruana et al., 2006). The protocol for the induction of LTP was chosen based on previous research on induction of LTP in the entorhinal cortex (Yun et al., 2002; Ma et al., 2008). Results obtained in LTP tests in normal ACSF were compared to responses observed in a control group in which responses to test pulses were recorded during constant exposure to normal ACSF without HFS. Results obtained in LTP tests following application of dopamine were compared to a group in which responses were obtained during a baseline period of 10 min followed by 15-min constant bath application of dopamine, and a 40-min washout period in normal ACSF.

Data analysis

Peak amplitudes of synaptic potentials were measured using pClamp 8.2 software (Molecular Devices). Averages of five consecutive evoked responses were obtained for graphical display. For each slice, the amplitudes of synaptic responses were normalized to the mean amplitude of synaptic responses recorded during the baseline period, and amplitudes of each set of three consecutive synaptic responses (obtained every one minute) were calculated and then averaged across the group for graphical display. Data are presented as means ± SEM.

All measures of synaptic responses used for statistical analyses were based on averaged fEPSPs obtained during the last 5 min of each recording period. The amplitude of responses recorded 20 sec following HFS were also assessed. Planned comparisons were also used to compare amplitudes of synaptic responses during baseline versus 20 sec post HFS, baseline versus 5 min after application of dopamine where applicable, baseline versus 40 min post HFS, and analogous time periods in the normal control and dopamine alone groups. To assess the amount of short- and LTP induced by HFS versus the respective control groups, mixed 2x2 ANOVAs were conducted focusing on baseline versus immediately after HFS, and baseline versus 40 min post HFS.

In addition, the level of LTP was compared between the dopamine and no-dopamine HFS conditions using a mixed 2x2 ANOVA on time points previously described to see the effect of application of dopamine on the induction of LTP.

RESULTS

Stimulation of layer I resulted in negative synaptic field potentials in layers I-II of the lateral entorhinal cortex (e.g., Figure 5.1A) similar to responses that have been observed previously (Caruana et al., 2007). In slices maintained in normal ACSF, delivery of HFS to layer I resulted in significant increases in the amplitude of synaptic responses recorded in layer II of the entorhinal cortex. The amplitudes of responses were increased to 122.4 ± 8.2% of baseline values 20 seconds after delivery of HFS (n = 13, from -0.44 ± 0.04 to -0.54 ± 0.07 mV; $t = 2.74$, $p = 0.018$). There was a transient reduction in the amplitude of responses from 20 sec to roughly 15 min following HFS, but responses were significantly increased to 123.9 ± 7.4% of baseline values (-0.53 ± 0.05 mV) at the end of the 40 min follow up period ($t = 3.24$, $p = 0.007$; Figure 5.1A). Responses in control slices were stable, with no significant change in the amplitudes of response from baseline to periods corresponding to 20 sec after HFS (from -1.26 ± 0.20 to -1.25 ± 0.20 mV, or 99.1 ± 2.4% of baseline; n=8, t=0.37, p=0.722) or 40 min after HFS (from -1.26 ± 0.20 to -1.27 ± 0.23 mV, or 98.6 ± 4.3% of baseline; $t = 0.32$, $p = 0.759$; Figure 5.1B). In addition, the potentiation of responses immediately and 40 min after delivery of HFS was reflected in significant planned interaction comparisons of responses obtained in the HFS group versus the control group both at 20 sec post HFS ($F_{1,19} = 4.74$, $p = 0.042$) and at 40 min post-HFS ($F_{1,19} = 6.29$, $p = 0.021$). This indicates that HFS resulted in significant increases in synaptic responses.

Effects of HFS were also tested in slices following 15 min pre-exposure to 10 μM dopamine (Figure 5.2A), and results were compared to control slices in which dopamine

was applied without subsequent HFS. Application of dopamine alone resulted in a significant increase in the amplitude of synaptic responses both in slices that received dopamine alone (from -0.55 ± 0.05 to -0.62 ± 0.06 mV or 113.3 ± 5.2% of baseline; n = 10, t = 2.57, p = 0.030) or with subsequent HFS (from -0.58 ± 0.09 to -0.67 ± 0.10 mV or 115.5 ± 5.1% of baseline; n = 14, t = 3.05, p = 0.009). Dopamine did not result in a significant lasting increase in responses, and responses were 111.2 ± 8.9 % of baseline 40 min after wash off of dopamine (-0.55 ± 0.05 to -0.61 ± 0.08 mV of baseline; t = 1.29, p = 0.230). This increase in the amplitude of evoked responses in consistent with the findings of Caruana et al (2006), and provided a means to test for evaluating how facilitation of synaptic responses by dopamine may modulate induction of LTP.

Administration of HFS immediately following 15 min dopamine application resulted in a significant short-term facilitation of responses, but no lasting potentiation of responses. Responses recorded immediately after HFS increased significantly to 120.2 ± 4.8% of values at the end of dopamine application (from -0.67 ± 0.09 to -0.81 ± 0.12 mV, baseline; t = 4.09, p = 0.001). However, this facilitation returned to baseline values by the end of the recording period, and there was no significant difference between the amplitudes of synaptic responses recorded 40 min after HFS versus during the baseline period (-0.66 ± 0.13 versus -0.58 ± 0.09 mV, or 115.6 ± 12.3 % of baseline; t = 1.28, p = 0.224). Similarly, the amplitudes of response at the end of the recording period were 100.0 ± 10.2% of the amplitudes of responses recorded in the presence of dopamine. Statistical comparisons of response obtained following HFS in the presence of dopamine, versus in in the presence of dopamine alone also suggest that significant LTP was not

observed following HFS in the presence of dopamine. Interaction comparisons showed that HFS did result in a significantly larger response 20 sec after HFS ($F_{1,22}$ = 7.95, p = 0.010) but that synaptic responses were not significantly greater following HFS in dopamine versus cells exposed to dopamine alone ($F_{1,22}$ = 0.07, p = 0.788). Dopamine therefore appears to block the LTP induced by HFS.

DISCUSSION

In vitro field potential recordings have been used here to assess the effect of dopamine on the induction of long-term synaptic potentiation in layer II of the lateral entorhinal cortex. Previous results showed that LTP and LTD were blocked by systemic injection of the dopamine reuptake blocker GBR12909 in vivo, but it was not clear to what extent dopamine levels were enhanced by that treatment, and it was possible that synaptic plasticity might have been blocked by a dopamine-mediated suppression of synaptic strength that could reduce postsynaptic excitability (Caruana et al., 2007; Nicoll and Malenka, 1999). The present study used in vitro recordings to determine if LTP might be enhanced by a low concentration of dopamine that has been found to cause an acute facilitation of synaptic responses (Glovaci et al., 2014; Caruana et al., 2006; Caruana et al., 2008). Slices of entorhinal cortex in normal ACSF showed long-term synaptic potentiation as evidenced by a significant facilitation of synaptic responses following HFS that persisted over time. Application of dopamine alone caused an increased the amplitude of fEPSPs as seen in previous literature (Glovaci et al., 2014; Caruana et al., 2006; Caruana et al., 2008), and this facilitation returned towards baseline levels after dopamine was washed off. In slices that received HFS in the presence of dopamine, there

was no significant lasting potentiation induced by HFS, indicating that relatively low concentrations of dopamine can inhibit the induction of long term synaptic potentiation in the lateral entorhinal cortex. This suggests that release of dopamine in the entorhinal cortex during appetitive motivation and reward may *impede* the induction of learning-related synaptic plasticity in the entorhinal cortex.

Effects of Dopamine Alone on Synaptic Responses

Application of dopamine alone facilitated synaptic responses. This result is consistent with previous findings that 10 μM dopamine results in a D_1 receptor-dependent facilitation of synaptic responses in layer II of the lateral entorhinal cortex in vitro (Caruana et al., 2006). In slices bathed in dopamine that did not receive HFS, the degree of facilitation of synaptic responses observed here was about 113.3 %, which is similar to that observed previously (Caruana et al., 2006). However the time course of changes in responses during the wash-off of dopamine observed here was slower than in the study of Caruana et al. (2006) in which responses returned to baseline within about 30 min. Data obtained here showed a non-significant elevation of responses at 111.2 % of baseline levels after 40 min of washoff of dopamine. The maintained mean amplitudes of responses during the washoff period were found to be due to two slices that showed a slow drift up in EPSP amplitudes (data not shown), but the reasons for the drift in these slices is not known.

Work conducted in our lab has shown that the facilitation of synaptic responses caused by application of a low concentration of dopamine is mediated by activation of D_1 receptors, and increased protein kinase A levels that result in an inhibition of PP1-

dependent dephosphorylation of AMPA receptors (Glovaci et al., 2014). The reduction in dephosphorylation by PP1 leads to an increase in AMPA-receptor-mediated synaptic currents (Yan et al., 1999). Glovaci et al. (2014) also found that the size of the increase, and the consistency of facilitation caused by dopamine were dependent on the type of cell that was recorded from in the lateral entorhinal cortex; where fan cells consistently showed moderate synaptic facilitation effects, facilitation effects in pyramidal cells were less reliable, and were also more variable in size, and there was no change in synaptic responses induced in a small group of multiform cells (Glovaci et al. 2014; Tahvildari and Alonso, 2005). In the current study, recordings of the effect of dopamine application on synaptic responses have been obtained through use of synaptic field potentials that represent the combined activation of many entorhinal neurons, and is likely to represent changes mainly in fan and pyramidal neurons.

It is not clear how the low, 10 μM concentration of dopamine that was used here may relate to concentrations of dopamine that result from endogenous release of dopamine in behaving animals. This uncertainty is partly due to the degradation of dopamine that takes place over time as dopamine is oxidized in ACSF. The 10 μM concentration used here, which results in a synaptic facilitation, is much lower than concentrations of 50 and 100 μM dopamine which result in a suppression of synaptic strength (Caruana et al., 2006). The lower dose of dopamine is likely to be closer to physiological levels of dopamine, and this suggests that the most common physiological effect of endogenous dopamine might be to result in a facilitation that could enhance entorhinal responses to sensory inputs. However, endogenous dopamine can be released

either tonically, during slow regular firing of dopamine neurons, or phasically following burst firing in dopamine neurons (Shultz, 2007). It is possible that the low concentration of dopamine used here may correspond best to low levels of dopamine induced during tonic firing, and that the associated facilitation may enhance the salience of reward-relevant stimuli during task performance (Glovaci et al., 2014). Similarly, higher concentrations of dopamine that result in a suppression of synaptic strength may reflect higher concentrations of dopamine resulting from intense periods of burst-firing of dopamine neurons. Consistent with this idea is that rewarding electrical stimulation of the lateral hypothalamus results in a transient reduction in the amplitude of evoked synaptic responses in the entorhinal cortex that is dependent on activation of D_2 receptors (Hutter et al., 2013). This suggests that bursting in dopamine neurons, that can be associated with reward-related cues (Shultz 2007), may suppress synaptic transmission. In conclusion, it cannot be known with certainty to what extent the 10 μM concentration of dopamine used here may correspond to dopamine concentrations induced by tonic vs phasic release in behaving animals, but it may most closely relate to states of tonic release that may occur as the animal engages in reward-relevant behaviors.

Effect of Dopamine on Induction of LTP

The effect of large concentrations of dopamine in the entorhinal cortex is to suppress glutamatergic synaptic transmission via activation of D_2 receptors (Pralong and Jones, 1993; Stenkamp et al., 1998; Caruana, 2006) but lower concentrations facilitate synaptic transmission via activation of D_1 receptors (Caruana et al., 2006; Glovaci et al., 2014). This led to the hypothesis that lower, more physiologically realistic dopamine

concentrations might both enhance the salience or impact of reward-relevant stimuli processed while the animal is in a motivated state, and might also contribute to lasting memory of these stimuli by contributing to the induction of learning-related synaptic plasticity in the entorhinal cortex (Caruana et al., 2006). Dopamine was therefore predicted to enhance the induction of LTP in the entorhinal cortex. Synaptic facilitation by dopamine could enhance NMDA receptor-mediated components of EPSPs during trains and contribute to calcium influx required for LTP induction (Yang, 2000; Nicoll and Malenka, 1999). However, experiments in awake animals in which entorhinal dopamine was increased using systemic injections of the drug GBR12909 showed that, although groups of control animals showed robust LTP and LTD, administration of the dopamine reuptake inhibitor GBR12909 caused a block of both LTP and LTD (Caruana et al., 2007). The findings of that study were not definite, however, because the extent of transient synaptic facilitation or suppression induced by GBR12909 during stimulation to induce LTP and LTD was not clear. This led to the current study in which in vitro recordings were conducted in which the concentration of dopamine could be controlled more precisely to determine if lower concentrations of dopamine might enhance or inhibit LTP induction.

The current finding that dopamine results in an inhibition of the induction of LTP is consistent with research in other areas of the brain, where application of dopamine in acute brain slices containing the hippocampal CA1 region (Wei et al., 2012), the dentate gyrus (Yanagihashi and Ishikawa, 1992) or the prefrontal cortex (Law-Tho and Crepel, 1995; Blond et al., 2002) resulted in a block of the LTP induced either by HFS or by

tetanization. However, activation of dopamine has also been observed in other brains regions to result in a facilitation of LTP induction (Manahan-Vaughan and Kulia, 2003; Matthies et al., 1997; Otmakhova and Lisman, 1996). The inhibitory effects of the application of dopamine on LTP induction in the lateral entorhinal cortex suggest that dopamine in this area of the brain plays a different role in regulating synaptic plasticity than it may in the hippocampus, such that learning-related plasticity may be suppressed rather than enhanced during behaviours associated with activation of dopamine neurons.

The cellular mechanisms that mediate the dopamine-dependent block of LTP in the entorhinal cortex need to be determined. Previous research in our lab has shown that the facilitation of synaptic responses caused by 10 μM dopamine is blocked by application of the D_1 receptor antagonist SCH23390, and that the suppression caused by 50 μM dopamine is blocked by the D_2 receptor blocker sulpiride (Caruana et al., 2007; Glovaci et al., 2014). Research in the CA1 has shown that blocking D_1 receptors via the D_1 receptor antagonist SCH23390 results in a block of the inhibition of LTP induction caused by activation of dopamine receptors (Wei et al., 2012). It is therefore likely that the block of LTP observed in this study is dependent upon D_1 receptor activation that also results in the facilitation of synaptic responses. In a future study, the D_1 receptor antagonist SCH23390 could be used to determine if blocking D1 receptors would both block the dopaminergic facilitation of synaptic responses, and also permit the induction of LTP in the lateral entorhinal cortex in the presence of dopamine. Glovaci and Chapman (2014) have found that the facilitation of EPSPs induced by D_1 receptor activation is dependent upon activation of PKA and protein-phosphatase 1. Dopamine-dependent

activation of PKA is one mechanism that can contribute to lasting potentiation of AMPA glutamate receptors (Wolf et al., 2003), and it is possible that effects of dopamine on PKA signaling in entorhinal neurons may also interfere with the induction of LTP. Activation of PKA is the result of activation of cAMP, which occurs due to D_1 receptor-mediated increases in adenylate cyclase (Jay et al., 1996). Therefore, it may therefore be worthwhile to investigate the role of PKA signaling on the dopaminergic block of LTP induction in the entorhinal cortex using whole cell patch clamp recordings in order to manipulate intracellular signaling pathways. Glovaci et al. (2014) also found that the facilitation of EPSCs by dopamine was dependent on increases in intracellular calcium, likely by increases in activation of L-Type Ca^{2+} channels or by release of Ca^{2+} from internal stores. Dopamine-dependent increases in intracellular calcium would be expected to facilitate induction of Ca-dependent LTP (Nicoll and Malenka, 1999), and so enhanced Ca^{2+} entry per se induced by dopamine is unlikely to explain the block of LTP induction observed here.

Changes in susceptibility to LTP induction induced by dopamine may also be due to hyperpolarization of membrane potential induced by dopamine. Dopamine can hyperpolarize membrane potential by several mV (Caruana et al., 2006) and this could reduce depolarization-dependent activation of NMDA receptors during HFS that mediates LTP. Long-term potentiation and depression in the entorhinal cortex have been shown to be dependent on activation of NMDA receptors (Alonso et al., 1990; Kourrich and Chapman, 2003), and application of dopamine in this study may impede plasticity through the reduction of postsynaptic depolarization required for NMDA receptor

activation (Nicoll and Malenka, 1999). A suppression of NMDA receptor currents in the prefrontal cortex can occur due to D_4 receptor activation (Wang et al., 2003), and it has been shown that D_4 receptors are also present in the entorhinal cortex (Defagot et al., 1997; Primus et al., 1997). Therefore, D_4 receptor activation might suppress NMDA receptor-mediated currents in the entorhinal cortex (Caruana et al., 2007). However, this idea still remains to be explored.

In the present study, the role of dopamine receptors in the lateral entorhinal cortex on *induction* of LTP was assessed. It may also be helpful in the future to also assess the role of dopamine on the *persistence* of the lasting strengthening of synapses; dopamine may have effects on the persistence of LTP induced in the absence of dopamine. Similar to the variability in effects of dopamine on synaptic responses and LTP induction, results from other studies have also shown variable effects of dopamine on the persistence of LTP (Lemon and Manahan-Laughan, 2006; Swanson-Park et al., 1999). Some studies have shown that while blocking dopamine D_1 receptors has no effect on the induction of LTP in the CA1, it does block its persistence, so that LTP only lasts for an hour or so (Swanson-Park et al., 1999; Lemon and Manahan-Laughan, 2006). Other studies that have shown, however, that activation of D_1 receptors have no effect on the persistence or induction of LTP in the dentate gyrus (Swanson-Park et al., 1999). Other work has shown that D_2 receptors are necessary for both the full induction of LTP as well as for the persistence of LTP in the dentate gyrus (Manahan-Laughan and Kulia, 2003; Abe et al., 2009). While it may be true that dopamine D_1 receptors are necessary for the persistence but not induction of LTP in the CA1 (Lemon and Manahan-Laughan, 2006; Swanson-

Park et al., 1999), and D_2 receptor activation is necessary for the full induction of LTP as well as the persistence in the dentate gyrus (Abe et al., 2009; Manahan-Vaughan and Kulia, 2003), it is unclear what the role dopamine in the entorhinal cortex plays on persistence of LTP. Because dopamine blocks LTP induction in the entorhinal cortex, the role of dopamine in the persistence of LTP may be examined by determining how dopamine affect the maintenance or decay of previously initiated changes in synaptic strength.

Additional experiments could be performed to assess the effects of endogenously released dopamine on induction of LTP in the entorhinal cortex, because it is not known exactly how levels of dopamine induced by GBR12909 or by 10 μM dopamine relate to the concentrations of dopamine released at the synapse in vivo. The concentrations of dopamine measured in the entorhinal cortex in vivo using microdialysis following administration of GBR12909 by Caruana et al. (2007) were similar to those observed in the prefrontal cortex using the same equipment, but it is not clear how these levels may relate to endogenous tonic or phasic dopamine release (Schultz, 2007). Further, dopamine is known to degrade with oxidization over time and it is not known to what extent the concentration of 10 μM dopamine that was applied to slices was reduced by oxidation (Ogawa et al., 1993). It would therefore be interesting to assess the effect of endogenously released dopamine on both basal synaptic responses and on the induction of LTP. This might be accomplished using selective optogenetic activation of dopaminergic cells in vivo (Bass et al., 2010), the induction of LTP shortly after rewarding hypothalamic stimulation (Hutter et al., 2013), or might also be assessed by comparing

the LTP induced during a control task versus during a motivated behavior or appetitive state (Hutter and Chapman, 2013; Krawczyk et al., 2013). Interestingly, both rewarding hypothalamic stimulation, and anticipation and consumption of chocolate is associated with a suppression of synaptic responses in the entorhinal cortex (Hutter et al., 2013; Hutter and Chapman, 2013), suggesting that these latter two approaches would most likely help to assess the effect of dopaminergic *suppression* of synaptic responses on induction of LTP.

Functional Implications

Results of the present study indicate that a relatively low concentration of dopamine prevents LTP induction, and suggests that the block of LTP observed in vivo by Caruana et al. (2007) may have taken place during exposure to relatively low concentrations of dopamine induced by systemic injection of the dopamine re-uptake inhibitor GBR12909 in vivo. The finding that dopamine prevents the induction of LTP suggests that the role of dopamine in the lateral entorhinal cortex may be to aid in the stable and reliable processing of physiologically relevant stimuli from the olfactory and associational areas by preventing changes in synaptic strength (Bouras and Chapman, 2003; Chapman and Racine, 1997). The role of dopamine may, therefore, be to restrict activity-dependent synaptic changes in the entorhinal cortex in order to aid in processing of olfactory information in both the entorhinal cortex and in targets of the entorhinal cortex including the hippocampus. It has been found that dopamine is released in the hippocampus in response to novel stimuli, and that this may enhance plasticity and encoding of new information in hippocampal CA3 projections to the CA1 (Lisman and

Grace, 2005). Therefore, it is possible that the dopaminergic facilitation of synaptic strength in the entorhinal cortex may serve to enhance the transmission of reward-relevant information from the entorhinal cortex to the hippocampus, and to enhance the storage of related representations in the hippocampus at the same time (Caruana et al., 2006; Lisman and Grace, 2005). This scenario could provide for enhanced processing of reward-relevant information in both the entorhinal cortex and hippocampus during appetitive states, and to also preferentially support a greater role for reward-related lasting synaptic plasticity in the hippocampus (Abe et al., 2009; Manahan-Vaughan and Kulia, 2003; Matthies et al., 1997; Otmakhova and Lisman, 1996)

Figure 5.1. The HFS results in the induction of long-term synaptic potentiation of fEPSPs recorded in layer II of the lateral entorhinal cortex in vitro. **A_1**. Representative traces in show responses recorded during the baseline period (1), immediately following HFS (2), and at the end of the recording period (3) for the HFS in normal ACSF group. **A_2**. Changes in the mean fEPSP amplitudes (n = 12) were reflected in a significant potentiation of synaptic responses ($F_{2,35} = 9.51$, $p < 0.05$). Points represent the mean ±1 standard error of the mean. **B_1**. Representative traces show responses recorded during periods corresponding to significant events in the control group. **B_2**. The amplitude of synaptic responses remained stable in control slices (n = 5) that did not receive conditioning stimulation.

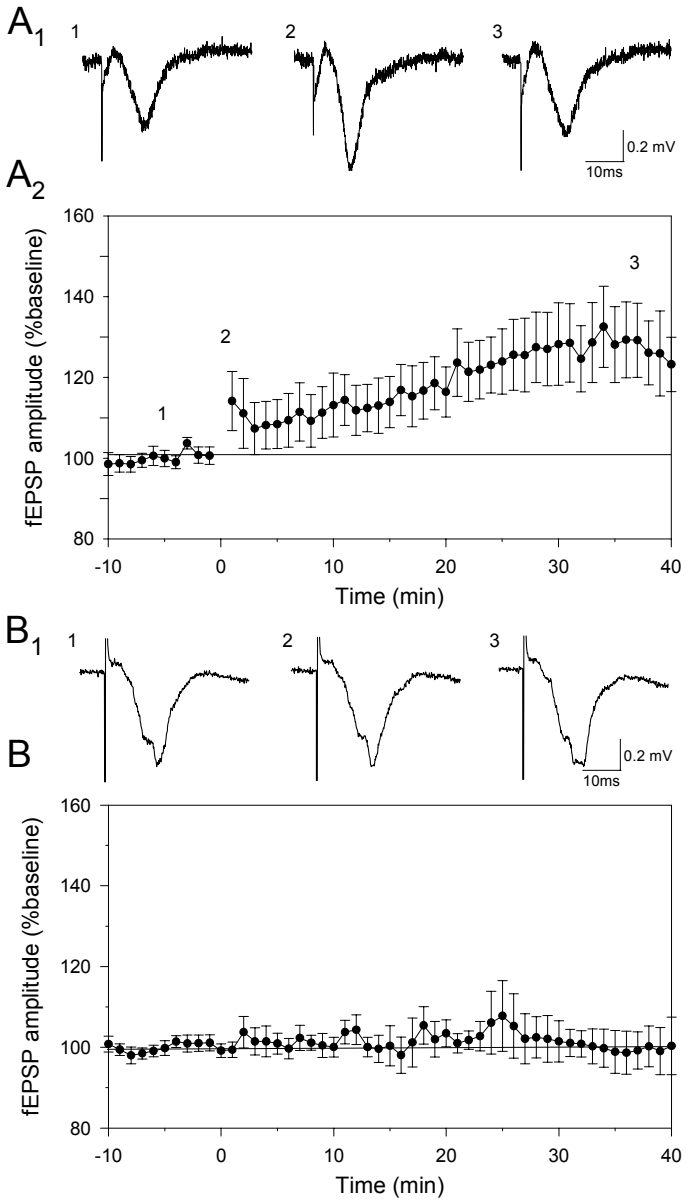
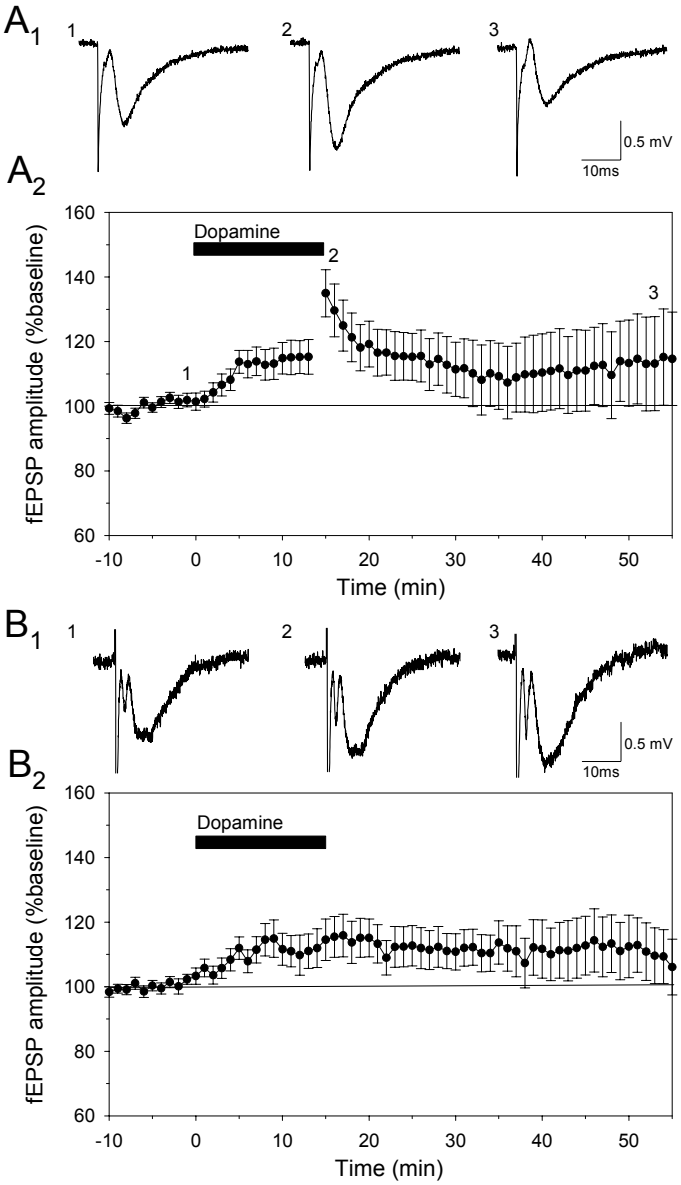

A1
1
2
3
0.2 mV
10ms
A2
fEPSP amplitude (%baseline)
160
140
120
100
80
1
2
3
-10
0
10
20
30
40
Time (min)
B1
B
1
2
3
0.2 mV
10ms
fEPSP amplitude (%baseline)
160
140
120
100
80
-10
0
10
20
30
40
Time (min)

Figure 5.2. Dopamine application results in a significant facilitation of responses in both groups, however, HFS delivered following bath application of dopamine does not result in significant potentiation of fEPSPs in layer II of the lateral entorhinal cortex. **A_1**. HFS to induce LTP was delivered at the end of a 15-min bath application of 10 μM dopamine. Traces show averaged responses recorded during the baseline period (1), after 15 min dopamine application (2), immediately after the HFS (3), and at the end of the recording period (4). **A_2**. Changes in response amplitudes at the end of the recording period were variable (n = 6), and there was no significant potentiation of synaptic responses ($F_{3,23} = 4.69$, $p < 0.05$) due to HFS. **B_1**. Sample traces represent averaged responses recorded during baseline, application of dopamine, and follow up period. **B_2**. The amplitude of synaptic responses increased following application of dopamine with no clear reversal of the effect (n = 4).


A1
1
2
3
0.5 mV
10ms
A2
Dopamine
fEPSP amplitude (%baseline)
Time (min)
B1
B2

Chapter 6

General Discussion

GENERAL DISCUSSION

This thesis used electrophysiological techniques in order to investigate the role of two modulatory neurotransmitters in altering synaptic communication in the entorhinal cortex. Experiments have demonstrated that the suppression of synaptic activity in the medial entorhinal cortex caused by activation of cholinergic receptors heavily involves activation of M_1, but not M_2 or M_4 receptors (Chapter 2). Injections of amphetamine in awake animals, that were used to enhance dopaminergic transmission, resulted in a facilitation of the strength of single synaptic responses, as well as an associated relative *suppression* of synaptic responses during short trains of theta-frequency stimulation that could affect synaptic processing during theta-frequency EEG activity (Chapter 3). Further experiments in slices of lateral entorhinal cortex tissue showed that a low concentration of dopamine resulted in an overall facilitation of train-evoked responses, with no *relative* change in the amplitudes of responses during trains of gamma- and theta-frequency stimulation. In addition, a *high* concentration of dopamine that induces a suppression of responses to single stimulation pulses was associated with a relative *facilitation* of responses later in the trains (Chapter 4). Dopaminergic effects on a cellular model of learning and memory was also assessed, and LTP in the lateral entorhinal cortex was found to be blocked by dopamine (Chapter 5).

Taken together, these findings indicate that both acetylcholine and dopamine have strong actions on synaptic activity in the entorhinal cortex that are likely to modulate how sensory inputs to the entorhinal cortex are processed and passed on to other structures within the hippocampal formation. These results are consistent with the idea that

modulatory neurotransmitters can regulate the proposed role of the entorhinal cortex as a "gatekeeper" for sensory and mnemonic functions that filters information that is transmitted to the hippocampal formation for further processing (Takehara-Nishiuchi, 2014).

Cholinergic Effects on Synaptic Transmission

The first experimental chapter in this thesis investigated the suppression of evoked synaptic responses in the medial entorhinal cortex that results from activation of cholinergic receptors (Richter et al., 1999; Hamam et al., 2007). Through application of the muscarinic M_1 receptor antagonists pirenzepine and VU0255035 on slices of medial entorhinal cortex prior to application of the cholinergic agonist carbachol, it was shown that the cholinergic suppression is due mainly to activation of M_1 receptors. The application of methoctramine, which blocks M_2 and M_4 receptors, or the application of PD102807 which blocks M_4 receptors, however, did not significantly reduce the suppression of responses induced by carbachol. Increases in paired-pulse facilitation indicated that the suppression of EPSPs was mediated by reduced neurotransmitter release, and it was hypothesized that postsynaptic M_1 receptors could be acting through retrograde cannabinoid signaling to cause the suppression via activation of CB1R receptors (Kano et al. 2009). The CB1R receptor antagonist AM281, however, had no effect on the synaptic suppression induced by carbachol, suggesting that the synaptic suppression is mediated by presynaptic M_1 receptors (Hamam et al., 2007) rather than through postsynaptic M1 receptors combined with retrograde cannabinoid signalling.

It has been shown in the past that activation of cholinergic receptors results in a

suppression of synaptic responses in the entorhinal cortex and hippocampus (Richter et al., 1999; Hamam et al., 2007; Hasselmo and McGaughy, 2004; Yun et al., 2000). However, pharmacological agents targeting muscarinic receptors can have effects on multiple muscarinic receptor subtypes (Caulfield and Birdsall, 1998; Dorje et al., 1991) and previous work identifying the muscarinic receptors mediating synaptic suppression effects has been inconsistent (M_1: Richter et al., 1999; Auerbach and Segal, 1996; Sheridan and Sutor, 1990; M_4: Dasari and Gulledge, 2011). This study has used muscarinic receptor blockers that target M_1 and M_4 receptors more specifically, and has provided an important contribution to the literature by showing that the suppression of synaptic responses in the medial entorhinal cortex is mainly due to activation of M_1 receptors (Barrett and Chapman, 2013). Application of the $M_2/_4$ receptor blocker methoctramine, or the highly selective M4 receptor blocker PD102807 had no effect on the cholinergic suppression of synaptic responses, but the highly selective M_1 receptor antagonist VU0255035 significantly reduced the suppression of synaptic transmission induced by carbachol, and verified that M_1 receptors play a major role in the cholinergic suppression. There was a residual suppression of synaptic strength observed in the presence of VU0255035, and it is not clear why a complete block of the suppression effect was not obtained. The lack of effects of the $M_2/_4$ receptor blocker methoctramine, and the M_4 receptor blocker PD102807, however, suggests that these receptor subtypes are not required for the suppression. The residual suppression may therefore have been due to incomplete block of M_1 receptors by VU0255035.

The cholinergic suppression of synaptic responses may reduce excessive network

activity caused by cholinergic depolarization of membrane potential that could lead to epileptogenesis (Friedman et al., 2007), and may also contribute to sensory and mnemonic functions by minimizing interference between incoming sensory inputs and existing memory representations (Hasselmo and McGaughy, 2004). In addition, activation of cholinergic inputs to the entorhinal cortex is associated with theta- and gamma-frequency EEG activities (Golebiewski et al., 1994; Konopacki et al., 1992; van Der Linder et al., 1999), and the strength of synaptic input to the entorhinal cortex may depend strongly on whether inputs arrive during the peak or trough of these rhythms (Huerta and Lisman, 1995), and might affect repetitive synaptic inputs at these frequencies.

Results obtained in experiments described in Chapter 3 provided evidence indicating how enhanced cholinergic input to the entorhinal cortex during mobility may modulate synaptic responses of the entorhinal cortex to theta-frequency stimulation. The changes in the amplitudes of train-evoked responses during mobility versus immobility were examined, and were in agreement with the findings obtained by Sparks and Chapman (2013) in vitro. Sparks and Chapman (2013) showed that application of the cholinergic agonist carbachol in slices of entorhinal cortex resulted in a suppression of the first response during trains of gamma- and theta-frequency stimulation, and led to a relative facilitation in the growth of subsequent responses during the trains. Cholinergic receptors are activated during mobility (Hamam et al., 2007) and it was of interest to determine if the changes in synaptic responses induced by carbachol by Sparks and Chapman (2013) might be replicated in vivo by more normal release of acetylcholine

during mobility as compared to immobility. The observed suppression of the first response during theta-frequency trains, and the associated relative facilitation of responses observed during mobility versus immobility (Figure 5.3.2.C_4) were consistent with the effects observed by Sparks and Chapman in vitro (2013). The dependence of this effect on cholinergic receptor activation was not explicitly tested in vivo, by determining if the relative facilitation effect during mobility would be blocked by a cholinergic receptor blocker, but the results are consistent with the known effects of mobility on single synaptic responses (Hamam et al., 2007), and suggest that, although single synaptic responses are likely to be suppressed during cholinergically induced theta activity, mechanisms exist to help maintain repetitive, theta-frequency synaptic inputs within the entorhinal cortex in vivo.

Dopaminergic Effects on Synaptic Transmission

The entorhinal cortex is one of the main cortical targets of midbrain dopamine neurons (Bjorklund and Dunnett, 2007), but in comparison to the prefrontal cortex (Goldman-Rakic et al., 2000), relatively little is known about how dopamine modulates neuronal activity in the entorhinal cortex. Dopamine neurons are thought to play an important role in appetitive motivation and reward (Berridge et al., 2009), and dopamine release in the entorhinal cortex is likely to play an important role in shaping the synaptic responsiveness of the entorhinal cortex to reward-relevant stimuli. A major previous finding specific to the entorhinal area is that dopaminergic activation leads to either a suppression or facilitation of single evoked synaptic responses, depending on the concentration of dopamine being used; low concentrations induce a facilitation of

synaptic responses via activation of D_1 receptors, and higher concentrations induce a suppression of synaptic responses via D_2 receptors (Carauna et al., 2006; Caruana and Chapman, 2008; Glovaci and Chapman, 2013). The different responses to low and high concentrations of dopamine might reflect differences in the manner in which slow, tonic activation of dopamine neurons, versus transient burst firing may modulate synaptic activity in the entorhinal cortex (Shultz, 2006). Thus, during periods of in which the animal is mobile and engaged in exploring reward relevant stimuli, both acetylcholine and dopamine are likely to modulate the responsiveness of the entorhinal cortex to sensory inputs. This thesis has explored, using both in vivo field potential recording techniques (Chapter 3) and in vitro brain slice recordings (Chapter 4) how dopamine may modulate the strength of synaptic transmission during repetitive trains of pulses delivered at the frequencies of the theta and gamma EEG rhythms.

In Vivo Recordings

Sparks and Chapman (2013) previously showed that the cholinergic agonist carbachol applied in the medial entorhinal cortex in vitro results in a suppression of single synaptic responses, and a relative facilitation of synaptic responses to later pulses in trains of theta- and gamma-frequency stimulation. In contrast, in layer V entorhinal neurons, dopamine has been found to result in a *reduction* in temporal summation of synaptic responses during 20 Hz trains of stimulation (Rosenkranz and Johnston, 2006). Experiments in Chapter 3 were therefore used to investigate the role of dopamine in awake animals on the amplitudes of responses during gamma- and theta-frequency trains of stimulation in layer II of the entorhinal cortex. Results for responses to gamma-

frequency stimulation were inconclusive because amphetamine did not affect the amplitudes of initial synaptic responses in gamma-frequency trains; the effect of amphetamine may have been masked by transient increases in initial synaptic responses induced by the repeated delivery of the relatively intense gamma-frequency trains. However, among the entire group of animals tested, findings for theta-frequency trains showed that injection of amphetamine resulted in a trend towards a facilitation of the first response in the train, and reduced growth in responses to later pulses in the trains.

Further, when animals were split into two groups based on the presence or absence of an amphetamine-induced increase in the amplitude of the response to the first pulse during the theta-frequency stimulation, animals that showed a facilitation also showed a significant behavior-dependent facilitation of responses during baseline tests in which responses to the first pulses were suppressed during mobility as compared to immobility. Because amplitudes of fEPSPs are known to be suppressed during mobility due to cholinergic receptor activation (Hamam et al., 2007; Leung and Vanderwolf, 1980) the animals in the group that did not show a suppression during mobility versus immobility likely expressed weaker behavioural activation during recordings in the mobility-period (Hamam et al., 2007). Therefore, following amphetamine injection, cholinergic suppression of synaptic responses associated with increased movement in these animals is likely to have masked synaptic facilitation effects associated with increased dopamine release.

Subsequent analysis of the effects of amphetamine therefore focused on animals that showed an amphetamine-induced facilitation of initial responses to theta-frequency

stimulation. These animals demonstrated reduced relative growth in responses to later pulses in the trains, suggesting that the facilitation of synaptic responses is associated with a ceiling effect on growth in responses to theta-frequency stimulation. An important caveat of these results is that the ceiling effect may have resulted largely from the artificial nature of the strong, synchronous evoked synaptic stimulation that was used here. However, these results imply that, during periods in which animals express theta-frequency EEG activity, the release of dopamine within the entorhinal cortex is unlikely to enhance the strength of repetitive synaptic activation.

Further tests showed that pretreatment with either the D_1 receptor antagonist SCH23390 or the D_2 receptor antagonist eticlopride blocked both the facilitation of the response to the first pulse and the relative suppression during the rest of the train, suggesting that both receptor subtypes are required for this effect. This finding contrasts with previous findings showing a role for only D1 receptors in the facilitation of single evoked synaptic responses in the entorhinal cortex (Glovaci and Chapman, 2013). However, there was an increase in the mean amplitude of the first response when dopamine was applied during block of D2 receptors, as would be expected on the basis of results of Glovaci and Chapman (2013). Systemic injections can have effects in other brain areas that project to the entorhinal cortex, however, and it is possible that such unknown effects may have contributed to the block of the effect of dopamine on theta-frequency trains following injection of eticlopride.

Injection of amphetamine resulted in a synaptic facilitation, suggesting that it resulted in concentrations of dopamine in the entorhinal cortex that correspond to the

lower concentration of dopamine applied in vitro which also results in a facilitation of single synaptic responses (Caruana et al., 2006; Chapter 4). Although higher concentrations of dopamine result in a suppression of synaptic responses in vitro (Caruana et al., 2006; Chapter 4), a higher dose of amphetamine was not tested in vivo because it could introduce other effects such as immobility and increased changes in other neurotransmitter systems (Sudilovsky, 1975; O'Neill and Gu, 2013). Higher concentrations of dopamine are likely to occur at synapses during burst firing of dopamine neurons (Shultz, 2006) however, and dopamine-dependent suppression of synaptic responses has been shown to occur in the entorhinal cortex following rewarding brain stimulation in the lateral hypothalamus (Hutter et al., 2013) and during exposure to cues associated with chocolate reward (Hutter and Chapman, 2013). This suggests that higher concentrations of dopamine, and associated synaptic suppression effects, are likely to be associated with natural rewards in vivo. Experiments conducted in Chapter 4 therefore assessed the effects of both low and high concentrations of dopamine on responses to repetitive stimulation in entorhinal cortex slices.

Comparison of Baseline Responses In Vitro vs. In Vivo

Baseline responses showed a different profile of changes in responses during trains of stimulation in vivo versus in vitro. In vivo, the amplitudes of responses became larger during the trains of stimulation, eventually reaching an asymptote reflecting an apparent ceiling effect of the amplitudes of responses. In vitro, the amplitudes of responses were generally increased in response to the second pulse, but became smaller later in the trains. Previous in vivo recordings have also shown facilitation effects during

trains of stimulation in the in entorhinal cortex, hippocampal region and prefrontal cortex (Chapman and Racine, 1997; Caruana and Chapman, 2004; Takita et al., 2007; Stepan et al., 2012; Jones 1993), and in vitro studies can show both synaptic facilitation and suppression effects (Day et al., 2005; Carr and Surmeier, 2007; Sparks and Chapman, 2013). It is not clear why train-evoked responses recorded here show greater suppression effects in vitro as compared to the in vivo experiments, but this is likely largely due to reduced capacity of axons and synaptic terminals in slices to rapidly maintain a pool of readily releasable neurotransmitter.

In both in vivo and in vitro recordings, the amplitudes of responses at the end of the trains were larger for theta-frequency stimulation in comparison to responses to gamma-frequency trains. The growth in responses was greater during theta- than during gamma-frequency trains, suggesting that the higher frequency of gamma-frequency trains may either result in a depletion of available neurotransmitter or recruit inhibitory mechanisms that reduce the growth of responses during trains (Sparks and Chapman, 2013). In addition, theta-frequency stimulation may result in greater facilitation through reductions in inhibitory transmission (Arai and Lynch, 1992; Staubli and Otaky, 1994; Ito and Schuman, 2007). Thus, although greater summation is expected to occur for higher frequencies of stimulation due to temporal overlap of synaptic potentials, other neuronal mechanisms appear to promote greater strength of repetitive synaptic inputs that occur for theta-frequency versus gamma-frequency stimulation (Jones 1993; Pernía-Andrade and Jonas, 2014; Takita et al., 2007).

Effects of 10 µM Dopamine In Vitro

The low concentration of 10 µM dopamine used in vitro resulted in a facilitation of initial responses to the trains that was similar to the facilitation of single synaptic responses induced by amphetamine in vivo, but there were differences in the way that increased dopamine affected responses evoked later in the trains in vivo and in vitro (compare Figures 5.3.1 and 5.4.1). During theta-frequency stimulation in vivo, amphetamine increased the amplitude of the first response in the train, and an apparent ceiling effect on the growth in responses during the train resulted in a relative facilitation effect. In slice recordings, however, 10 µM dopamine increased the absolute amplitude of *all* responses during both gamma and theta-frequency trains (without affecting the profile of relative changes in the sizes of the responses expressed as a function of the first responses).

This difference in the effects of dopamine on the pattern of train-evoked responses may be due to 1), mechanisms in vivo that lead to a ceiling effect in the growth of baseline responses, so that addition of dopamine cannot result in further increases in responses, and 2), the response decrements that are observed during baseline train-evoked responses in slices, that may better allow increases in responses following application of dopamine to be expressed. The facilitation of individual synaptic responses that is induced by D1 receptor activation in vitro is known to be mediated by postsynaptic factors that enhance AMPA receptor responses (Glovaci and Chapman, 2013). Thus, even though reduced presynaptic transmitter availability is likely to mediate much of the decrements in baseline responses to trains of stimulation in vitro, the postsynaptic

mechanisms that enhance single AMPA mediated responses (Glovaci and Chapman, 2013) are likely to mediate much of the dopamine-induced facilitation of synaptic responses during theta-frequency trains observed here in vitro.

The differences in the effects of dopamine on train-evoked responses that have been observed here in vivo versus in vitro make it difficult to infer how low concentrations of endogenous dopamine may affect repetitive synaptic responses during theta- and gamma-frequency EEG activities. The results of in vitro experiments suggest that dopamine has the capacity to increase synaptic strength during gamma- and theta-frequency activities, and this would be consistent with a possible role of dopamine in enhancing the salience or impact of representations of reward-relevant stimuli carried by repetitive synaptic inputs to the entorhinal cortex (Berridge and Robinson, 1998). However, the results of experiments conducted in vivo indicate that, although activation of dopamine receptors can enhance synaptic responses, there may be mechanisms that limit the dopaminergic facilitation of synaptic responses during repetitive synaptic activation. Future experiments could test the effects of dopamine on repetitive synaptic responses evoked by less-intense stimuli, to reduce the possible contribution of a ceiling effect on growth in synaptic responses. However, the data presented here suggest that, in vivo, dopamine at low concentrations may have a limited effect on repetitive synaptic responses during periods of theta activity.

Effects of 50 µM Dopamine In Vitro

The use of the in vitro preparation also allowed a direct test of the effects of a high concentration of dopamine, that is known to suppress single synaptic responses (Caruana

et al., 2006), on responses to repetitive synaptic activation. Similar to the results obtained by Sparks and Chapman (2013), who used a cholinergic agonist that suppresses single evoked responses, the suppression of initial evoked responses by 50 μM dopamine was associated with greater relative growth in responses later in the trains. The increased relative growth during trains is important, because it suggests that mechanisms exist to maintain the strength of representations carried by repetitive synaptic responses during exposure to dopamine. However, it is also important to recognize that the absolute amplitudes of train-evoked synaptic responses were still reduced overall by dopamine, particularly for theta-frequency stimulation. Thus, the overall effect of high concentrations of dopamine is most likely to reduce the salience or impact of representations reaching the entorhinal cortex, while the relative increase in growth of responses during the trains may help limit reductions in these train-evoked responses.

Dopaminergic Effects on Induction of LTP

Dopamine is known to contribute to mechanisms of memory formation (El-Ghundi et al., 2007; Caruana et al., 2007), and has also been shown to modulate the induction of long-term synaptic potentiation (LTP) in the hippocampus and prefrontal cortex(Yanagihashi and Ishikawa, 1992; Law-Tho and Crepel, 1995; Blond et al., 2002; Abe et al., 2006; Manahan-Vaughan and Kulia, 2003; Abe et al., 2009). The last experimental chapter of this thesis was therefore designed to provide the first investigation of the role of dopamine on the induction of LTP in entorhinal cortex slices. Previous literature regarding the role of dopamine on long-term synaptic plasticity in hippocampal and parahippocampal areas has resulted in a variety of findings indicating that dopamine is

necessary for, or can enhance induction of LTP (Abe et al., 2006; Manahan-Vaughan and Kulia, 2003; Abe et al., 2009; Ghanbarain and Motamedi, 2013; Otmakhova and Lisman, 1996), or that dopamine can inhibit LTP (Yanagihashi and Ishikawa, 1992; Law-Tho and Crepel, 1995; Wei et al., 2012; Caruana et al., 2007). Results presented here show that application of dopamine to slices of entorhinal cortex prior to delivery of high-frequency stimulation results in a block of the induction of LTP. Law-Tho and Crepel (1995) also observed a dopaminergic block of LTP induction in the prefrontal cortex following high-frequency stimulation. The present results, indicating that dopamine blocks induction of LTP in the entorhinal cortex in vitro, therefore add support to the findings of Caruana et al. (2007) who found that administration of a dopamine reuptake blocker that enhances the concentration of dopamine in the entorhinal cortex also resulted in a block of the induction of both LTP and LTD in vivo.

The initial findings of Caruana et al. (2007) showing a dopaminergic block of entorhinal LTP in vivo were not associated with clear effects of dopamine alone on single synaptic responses; it was therefore not known if the dopamine reuptake inhibitor used resulted in a consistent facilitation of synaptic strength. The results shown here verify that a transient synaptic facilitation induced by 10 μM dopamine is associated with a block of LTP induction. Because the synaptic facilitation is mediated by D1 receptor activation (Glovaci and Chapman, 2013), this suggests that D1 receptor activation leads to an inhibition of processes contributing to LTP induction in entorhinal cortex neurons. Similarly, Wei et al., (2012) found that the inhibition of LTP in the CA1 pyramidal neurons that was induced by dopamine was blocked by a D1 receptor antagonist.

Findings in this thesis have indicated that low concentrations of dopamine that are likely to be released during reward-relevant behaviours (Shultz, 2006) can have multiple effects on mechanisms of synaptic transmission. Responses to individual evoked responses are enhanced by 10 μM dopamine, but synaptic enhancements induced by systemic injection of amphetamine are not maintained during short trains of theta-patterned stimulation in vivo. Thus, although dopamine release in the lateral entorhinal cortex during reward-relevant behaviours may lead to a facilitation of sensory inputs mediated by discrete synaptic inputs, it is not clear that this facilitation is maintained during repetitive synaptic activation at gamma and theta-frequencies. In addition, 10 μM dopamine also results in an inhibition of the induction of LTP induced by high-frequency stimulation, and dopamine also therefore appears to inhibit neuronal mechanisms that mediate long-term changes in synaptic strength that are thought to contribute to memory formation.

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