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Hippocampal output to neocortex:
Examination of the electrophysiological and
plastic properties of CA1 projections to the
perirhinal cortex.

by

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Summary:

The hippocampal formation is an important structure in learning and memory that is required for the transfer of sensory information into long-term storage. This long-term storage is believed to occur in the neocortex and the physiological mechanism underpinning this transfer of information is believed to be long-term changes in synaptic plasticity, namely long-term potentiation (LTP) and depression (LTD). The aim of this thesis is to characterise synaptic plasticity in a particular hippocampal-neocortical projection. The CA1 to perirhinal cortex projection has been previously shown to sustain LTP; by stimulating the area CA1 and recording in the perirhinal cortex, we show that it can sustain short- and long-term changes in synaptic plasticity. Additionally we demonstrate that multiple frequencies of high-frequency stimulation can induce LTP in this projection and that LTP-induction may require AMPA/kainate receptor activation but not NMDA receptor activation; indicating that glutamatergic signalling underlies synaptic plasticity in this projection. We also determine the role of the CA1 to perirhinal cortex projection in a model of electrophysiologically excitatory and inhibitory hippocampal projections to the parahippocampal region of the neocortex. We propose that this projection forms part of an electrophysiologically excitatory circuit from the distal CA1 and proximal subiculum along with the lateral entorhinal cortex. Moreover, we investigate the roles of the hippocampus and perirhinal cortex in recognition and spatial memory. Utilising an object recognition task (a recognition memory task) and an object displacement task (a spatial memory task), we show that there are increased levels of hippocampal brain-derived neurotrophic factor (BDNF) following the spatial task. Furthermore, we demonstrate that AMPA/kainate glutamate receptors are necessary for performance in the object recognition task whereas both NMDA and AMPA/kainate receptors are required for the object displacement task. These findings suggest that glutamatergic signalling not only underlies synaptic plasticity in the CA1 to perirhinal cortex projection but that it is also required for learning and memory in recognition and spatial tasks.

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"There is no such thing as an empty space or an empty time. There is always something to see, something to hear. In fact, try as we may to make a silence, we cannot."

John Cage, *Silence*, p. 8.

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For their assistance in the lab, I would like to thank Dr. Sarah Craig and Dr. Deirdre Harvey for giving me their time and expertise. For their help outside the lab, I would also like to thank Dr. Richard Roche, Dr. Andrew Coogan and Mr. Derek Walsh for being a constant source of knowledge and advice. I wish to thank Dr. Bernard Mahon for access to his lab's facilities throughout my PhD. My thanks also go to

Chapter 1

Hippocampal output to neocortex: An overview of the anatomy, physiology and functions of the hippocampus and perirhinal cortex in the rat.

1.1 General introduction:

The hippocampal formation is an important structure for learning and memory (Scoville & Milner, 1957; O'Keefe & Nadel, 1978; Squire, 1992; Alvarez et al., 1995; Morris, 2007; Stark 2007). The hippocampal formation can be divided into a number of subregions, namely the dentate gyrus (DG), CA1, CA3 and the subiculum. Although the hippocampus is not considered to be the actual site of storage for long-term memory, it has been suggested that the hippocampus is the site of association of sensory information integrated to form long-term memories (Alvarez & Squire, 1994; Rolls, 1996). The hippocampus has been shown to be required in the formation of long-term memories (Scoville & Milner, 1957; Alvarez et al., 1994) but the actual storage of long-term memories is believed to occur in the neocortex and not in the hippocampus itself (Squire, 1992; McClelland et al., 1995; Squire & Alvarez, 1995; Qin et al., 1997).

Previous research has shown that the hippocampus projects to a number of areas with the area CA1 being the main output structure. Within the hippocampal formation it projects to the subiculum and it also projects to the entorhinal, perirhinal and postrhinal cortices (Swanson & Cowan, 1977; Witter et al., 1989; Van Groen & Wyss, 1990; Tamamaki & Nojyo, 1995; Naber at al., 2001a). Of these projections, those to the subiculum (Taube, 1993; Stewart, 1997; Commins et al., 1998) and the entorhinal (Craig & Commins, 2005; 2006; 2007) and perirhinal cortices (Cousens & Otto, 1998) have been shown to be electrophysiologically active in that they exhibit activity-dependent changes in synaptic plasticity. In addition, the area CA1 receives reciprocal projections from the entorhinal (Naber et al., 2001a) and perirhinal cortices (Kosel et al., 1983). These reciprocal projections from the entorhinal cortex (Remondes & Schuman, 2003) and perirhinal cortex (Naber et al., 1999) have also shown to be electrophysiologically functional and can undergo long-term potentiation (LTP).

The perirhinal cortex (cortical areas 35 and 36) forms part of the parahippocampal region of the temporal lobe (see section 1.2 for a review of the associated anatomy) and, as mentioned above, receives a direct input from the hippocampus. It is implicated in the long-term storage of object recognition

memory (Mumby & Pinel, 1994; Liu & Bilkey, 2001; Mumby et al., 2002b; Winters & Bussey, 2005a) and has also had roles suggested for it in spatial memory particularly spatial memory as it applies to objects (Bussey et al., 2000; 2001) and spatial reference memory (Abe et al., 2009). It has also been implicated in processing temporal order (Hannesson et al., 2004; Barker et al., 2007) and fear conditioning (Campeau et al., 1997; Herzog & Otto, 1998; Bucci et al., 2000; 2002; Schulz et al., 2004; Bang & Brown, 2009). The perirhinal cortex along with the entorhinal cortex, postrhinal cortex, presubiculum and parasubiculum forms the parahippocampal region of the neocortex in mammals including the rat, monkey and human (Witter, 2002). A circuit between the hippocampus and the parahippocampal region has been proposed to underpin various forms of learning and memory with parallel pathways between the hippocampus and the entorhinal, perirhinal and postrhinal cortices suggested as playing an important contribution to memory consolidation (Naber et al., 2000; Witter et al., 2000). Many of the invididual parts of this circuit have been shown to be electrophysiologically active (in that electrical stimulation in one area results in a measurable response at the terminus of the projection) including the projections running from CA1 to subiculum (Commins et al., 1998; Anderson et al., 2000), CA1 to the entorhinal cortex (Craig & Commins, 2005; 2007), CA1 to the perirhinal cortex (Cousens & Otto, 1998), subiculum to the entorhinal cortex (Craig & Commins, 2006; 2007), entorhinal cortex to area CA1 (Charpak et al., 1995), entorhinal cortex to the subiculum (Behr et al., 1998), entorhinal cortex to the dentate gyrus (Yaniv et al., 2003), perirhinal cortex to area CA1 (Liu & Bilkey, 1996a; 1996b; 1998b; Naber et al., 1999), perirhinal cortex to the subiculum (Naber et al., 1999), postrhinal cortex to the subiculum (Naber et al., 2001b), entorhinal to the perirhinal cortex (Biella et al., 2003; de Villers-Sidani et al., 2004; Pelletier et al., 2004) and perirhinal to the entorhinal cortex (de Villers-Sidani et al., 2004). Some parts of this circuit have received greater attention than others and the relationship between the perirhinal cortex and the hippocampus in terms of electrophysiology has undergone less scrutiny than the projections within the hippocampus and those involving the entorhinal cortex.

Therefore, the aim of this thesis is to investigate the electrophysiological (Chapters 2 and 4), molecular (Chapter 3) and pharmacological properties (Chapter 5) of the projection originating in the area CA1 of the hippocampus and terminating in the perirhinal cortex. In addition, analysis of the roles of the hippocampus and perirhinal cortex in the behaving animal using pharmacological and molecular techniques will complement the electrophysiological studies (Chapter 6). As such, this chapter will review the anatomy of the perirhinal cortex, detailing its boundaries and its cortical and subcortical connections. Following on from this, the physiology of the perirhinal cortex will be discussed concentrating on the electrophysiological and pharmacological characteristics of the perirhinal cortex. Finally, the functional roles of the perirhinal cortex and its relationship with the hippocampus will be described with a focus on their roles in recognition and spatial memory.

1.2 Anatomy:

1.2.1 Anatomical definition of the perirhinal cortex:

In the rat brain, the perirhinal cortex is located along the rhinal sulcus and it is composed of Brodmann's areas 35 and 36 (Brodmann, 1909), although earlier studies defined the perirhinal cortex as area 35 only (Krieg, 1946a). Area 36 occupies the dorsal bank of the rhinal sulcus and area 35 occupies the ventral bank, extending slightly more rostrally than area 36 (Burwell, 2001). It is bordered rostrally by the posterior agranular insular cortex (bordering with areas 35 and 36) and the visceral area (area 36 only), caudally by the postrhinal cortex, dorsally by the ventral temporal association cortex and ventrally by the lateral entorhinal cortex (Figure 1.1; Burwell et al., 1995; Burwell, 2001; Paxinos & Watson, 2005).
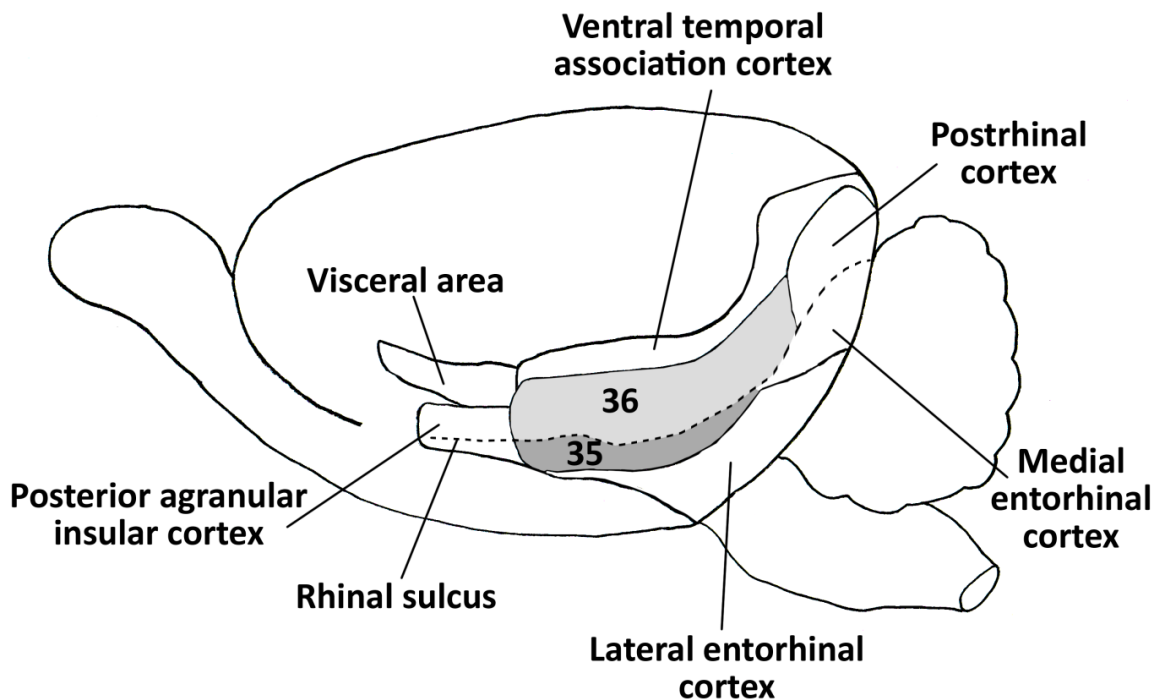
Figure 1.1: Location of the perirhinal cortex in the rat brain. The perirhinal cortex is found along the rhinal sulcus (dotted line) and is comprised of cortical areas 35 (dark grey) and 36 (light grey). Area 36 occupies the dorsal bank of rhinal sulcus whereas area 35 occupies the ventral bank. Neighbouring the perirhinal cortex are the posterior agranular insular cortex, the visceral area, the postrhinal cortex, the ventral temporal association cortex and the lateral entorhinal cortex. Diagram summarised from Burwell et al., 1995; Burwell, 2001; and Paxinos & Watson, 2005.

The borders between the areas 35 and 36 and their neighbouring areas can be determined by cytoarchitectonic means (Burwell, 2001; Witter, 2002). Brodmann's areas 35 and 36 are cytoarchitectonically different in that layer I of area 35 tends to be thicker than that of area 36, the cells in area 35 are organised radially, the presence of large, heart-shaped pyramidal cells in layer V of area 35 and finally layers II and III are distinctly separated in area 36 and not in area 35 (Krieg, 1946b; Burwell & Amaral, 1998a; 1998b; Burwell, 2001). Burwell subdivides areas 35 and 36 into two subregions (ventral and dorsal) and three subregions (dorsal, ventral and posterior) respectively (Figure 1.2). In the ventral subregion of area 35, there is a more pronounced radial organisation of neurons compared to the dorsal subregion and layers II and III have a more organised appearance. In addition, in the deeper parts of layer II/III of the dorsal subregion there is a lower cell density compared to the ventral subregion. Within area 36, the dorsal and ventral subregions make up the rostral region (with the ventral subregion sometimes protruding more rostrally than the dorsal subregion) and the posterior subregion is located caudally, bordering the postrhinal cortex. These three subregions are distinct from each other in that cells in layers II to V of the dorsal subregion are organised radially whereas cells in the same layers of the ventral subregion show no particular orientation and layers V and VI are narrower in the dorsal subregion (Burwell & Amaral, 1998b; Burwell, 2001). The posterior subregion differs from the rostral subregions of area 36 due to the presence of round, medium-sized cells throughout layers V and VI and the absence of a bilaminated layer VI (Burwell, 2001).
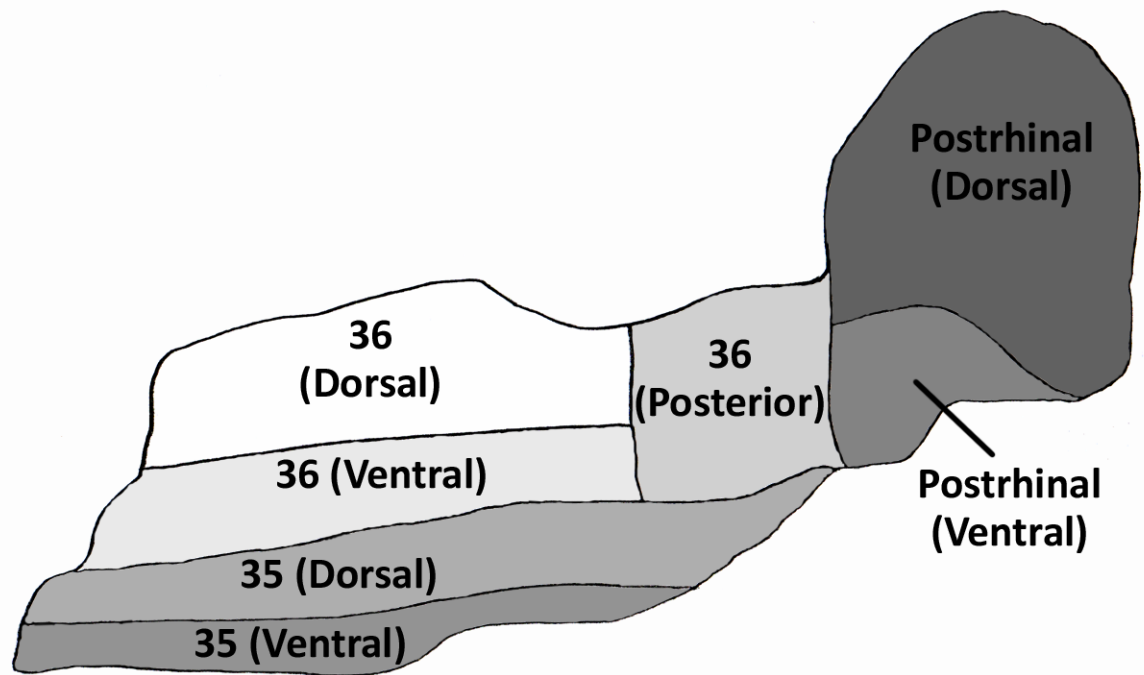
Figure 1.2: Subdivisions of the perirhinal and postrhinal cortices in the rat brain. Area 35 is composed of two subregions, the ventral and dorsal subregions. Area 36 is composed of three subregions, the ventral, dorsal and posterior subregions. Finally, the postrhinal cortex, like area 35, is also divisible into ventral and dorsal subregions. Adapted from Burwell, 2001.

The borders of the perirhinal cortex with the surrounding areas of neocortex can be determined in a similar way to the divisions within the perirhinal cortex. The border with posterior agranular insular cortex is located approximately 2.45 – 2.80 mm posterior to the Bregma line and the two areas of cortex differ in that the posterior agranular insular cortex has a trilaminar appearance and the presence of claustral cells but both these features are absent in the perirhinal cortex (Burwell, 2001). The visceral area has a granular layer IV making it distinct from the posterior agranular insular cortex but like the agranular insular cortex, the layers V and VI are distinct from each other. This is in contrast to the perirhinal cortex where the two layers are homogenous and is referred to as layer V (Burwell, 2001).

The border with the ventral temporal association cortex is determinable by observing cell types in layer II; this layer of area 36 of the perirhinal cortex is composed of round, medium-sized cells peppered with smaller pyramidal cells whereas a greater number of cells in the ventral temporal association cortex are

pyramidal in shape (Burwell, 2001). In addition, the ventral temporal association cortex sometimes features layers either side of layer V where cell density is lower but this feature is absent in the perirhinal cortex (Burwell, 2001). Layer VI in area 36 of the perirhinal cortex has a thick bilaminated appearance in contrast to both the ventral temporal association cortex and area 35 of the perirhinal cortex (Burwell, 2001). Finally, based on cortical input to either area, the perirhinal cortex receives markedly greater levels of input from various cortical areas compared to the ventral temporal association area, i.e. the ventral temporal association area receives no input from the piriform, more somatosensory and auditory input and lower levels of input from the insular and entorhinal cortices (Burwell & Amaral, 1998b).

The border with the entorhinal cortex is distinct in that the lamina dissecans layer (found in the entorhinal cortex) is absent in the perirhinal cortex (Burwell, 2001; Witter, 2002). The areas can also be defined based on the connections made with subcortical areas, the entorhinal cortex projects to the dentate gyrus (Wyss, 1981) whereas the perirhinal cortex does not (Insausti et al., 1997; Dolorfo & Amaral, 1998; Witter et al. 1999). In addition, the perirhinal cortex receives a much larger input from area CA1 of the hippocampus compared to the entorhinal cortex (Van Groen & Wyss, 1990). On a cellular level, the presence of large, "stellate" cells in layer I of the entorhinal cortex and differences in staining were found between the two areas for parvalbumin, calbindin and zinc (Timm's staining; Insausti et al, 1997; Burwell, 2001; Witter, 2002; Canto et al., 2008). Finally, a more subtle difference can be seen in layer IV of the perirhinal cortex which appears to be better defined in the entorhinal cortex (Witter et al., 2000b).

Although similar in terms of cell organisation and amounts of myelin present, the postrhinal and perirhinal cortices can be distinguished by the presence of ectopic layer II cells in the postrhinal cortex and the presence of smaller cells in postrhinal layer II compared to the perirhinal cortex (Burwell, 2001). It has a bilaminate appearance with layers II/III and V/VI forming homogenous bands (Burwell & Amaral, 1998a; 1998b). The two areas can also be distinguished based on their connections with the entorhinal cortex, the perirhinal cortex projects mainly to the lateral entorhinal cortex and the postrhinal cortex projects mainly to the medial entorhinal cortex (Naber et al., 1997). Despite these differences, there is

a debate in the literature as to whether to count the postrhinal cortex as a separate area to the perirhinal cortex (Deacon et al., 1983; Burwell et al., 1995) or whether to extend the definition of the perirhinal cortex to include the postrhinal cortex (Palomero-Gallagher & Zilles, 2004; Paxinos & Watson, 2005). There are a number of anatomical differences (Burwell et al., 1995; Burwell & Amaral, 1998a; 1998b; Pitkänen et al., 2000; Burwell, 2000; 2001) and functional differences (Norman & Eacott, 2005) existing between the two areas and it has been suggested that the rat postrhinal cortex is homologous to the monkey parahippocampal cortex (Burwell et al., 1995; Burwell, 2000). Therefore, we follow Burwell's classification (2000) and distinguish between the perirhinal and postrhinal cortices as two distinct anatomical regions in the rat brain.

1.2.2 Cortical afferents and efferents of the perirhinal cortex:

The intrinsic projections of the perirhinal cortex follow a dorsal-ventral gradient within area 36 and between areas 35 and 36 (Burwell, 2000). Area 36 projects laterally into area 35 with the majority of connections originating in the ventral region of area 36 in layers II, V and VI and terminating in layers I and V of area 35 (Burwell & Amaral, 1998a). Burwell and Amaral (1998a) also show that area 35 of the perirhinal cortex projects into area 36 (to a lesser degree than the 36 into 35 projections) and forms a feedback pathway with most projections originating in layers II and III and terminating in all layers.

Projections to the perirhinal cortex from other cortical areas originate from the lateral entorhinal cortex, postrhinal cortex, precentral cortex, cingulate cortex, frontal pole of the frontal lobe, piriform cortex, insular cortex, periamygdaloid cortex and auditory cortex (Figure 1.3; Saper, 1982; Deacon et al., 1983; Wyss & Van Groen, 1992; Burwell & Amaral, 1998a; 1998b; Haberly, 2001; Naber et al., 2001a; Majak & Pitkänen, 2003). Postrhinal projections originate in layers II and V and terminate in both areas 35 and 36 but favouring area 36 (Burwell & Amaral, 1998a) forming a lateral pathway across all cortical layers in the perirhinal cortex (Deacon et al., 1983; Burwell & Amaral 1998a). Temporal and parietal projections terminate mainly in layer I of the perirhinal cortex, frontal terminate mainly in

deeper layers (V and VI; Deacon et al., 1983). The periamygdaloid cortex projection originates in layer III of the caudal region and terminates in layers II and III of area 35 (Majak & Pitkänen, 2003).

The most prominent of all afferent cortical projections to the perirhinal cortex is the one originating in the entorhinal cortex (Wyss, 1981; Kosel et al., 1982; Köhler, 1988; Insausti et al., 1997; Burwell & Amaral, 1998a). Lateral entorhinal projections project more to area 35 than 36 with rostral lateral entorhinal projections terminating in rostral regions of the perirhinal cortex and caudal lateral entorhinal projections terminating across the perirhinal cortex (Burwell & Amaral, 1998a). The lateral entorhinal cortex projection to the perirhinal cortex originates in all layers of the lateral entorhinal cortex but laminar distribution changes depending on location within the entorhinal cortex (Insausti et al., 1997) which supports later findings that suggest the projection originates in layers III and V of the lateral entorhinal cortex (Burwell & Amaral, 1998a). In any case, the lateral entorhinal cortex projects more heavily to area 35 than area 36 (Insausti et al., 1997; Burwell & Amaral, 1998a). These projections terminated across all layers of area 35 (albeit with heavier staining present in layers I and V comparatively) and a similar story is seen for area 36 (Burwell & Amaral, 1998a). There is only a minimal projection to area 35 from layer V of the medial entorhinal cortex (Burwell & Amaral, 1998a).

Projections originating in the perirhinal cortex and terminating in other cortical areas include the lateral entorhinal cortex, postrhinal cortex, ventral temporal association cortex, parietal cortex, piriform cortex, insular cortex and the frontal cortex (Figure 1.3; Saper, 1982; Kosel et al., 1983; Naber et al., 1997; Burwell & Amaral, 1998a; 1998b; Haberly, 2001; Agster & Burwell, 2009). Overall, projections from area 36 to the postrhinal cortex are heavier than those from area 35 and similarly projections from area 35 to the entorhinal cortex are heavier than those from area 36 (Burwell & Amaral, 1998a). Burwell and Amaral (1998a) show that both areas of the perirhinal cortex form a feedback pathway with the postrhinal cortex; projections originate from layers V and VI of the perirhinal cortex and terminate mainly in layers I/II and VI of the rostral part of the postrhinal cortex

with projections from the rostral region of area 36 terminating in the caudal postrhinal cortex.

The majority of projections from the perirhinal cortex to the entorhinal cortex terminate in the lateral entorhinal cortex (Naber et al., 1997). Cells from the rostral perirhinal cortex innervate rostral regions of the lateral entorhinal cortex and likewise those from the caudal perirhinal cortex go to caudal areas of the entorhinal cortex (Burwell & Amaral, 1998a). Area 36 efferents to the entorhinal cortex originate in layers II, V and VI and with more superficial cells projecting to lateral regions of the lateral entorhinal cortex and deeper cells projecting to more intermediate regions (Burwell & Amaral, 1998a). In area 35, more projections originate from superficial layers, i.e. II/III, with a smaller proportion originating from layers V and VI (Burwell & Amaral, 1998a). No matter which area of the perirhinal cortex that a projection originates from, the majority of projections terminate in layers II and III of the entorhinal cortex (Burwell & Amaral, 1998a).

Finally, tracing experiments have suggested that the perirhinal cortex is a "border area" where dorsal-ventral projections from neighbouring cortical areas are segregated (Deacon et al., 1983; Burwell & Amaral, 1998a). H^3-labelled amino acid autoradiographic and horse radish peroxidase tracing experiments show that projections from ventral areas do not traverse the perirhinal cortex dorsally and similarly projections from dorsal areas do not traverse ventrally (Deacon et al., 1983). Similarly, the situation described above with perirhinal-postrhinal connections focussing mainly in area 36 and perirhinal-entorhinal connections focussing mainly in area 35 (Burwell & Amaral, 1998a) thus supporting the idea of there being a segregation role for the perirhinal cortex along the dorsal-ventral axis.
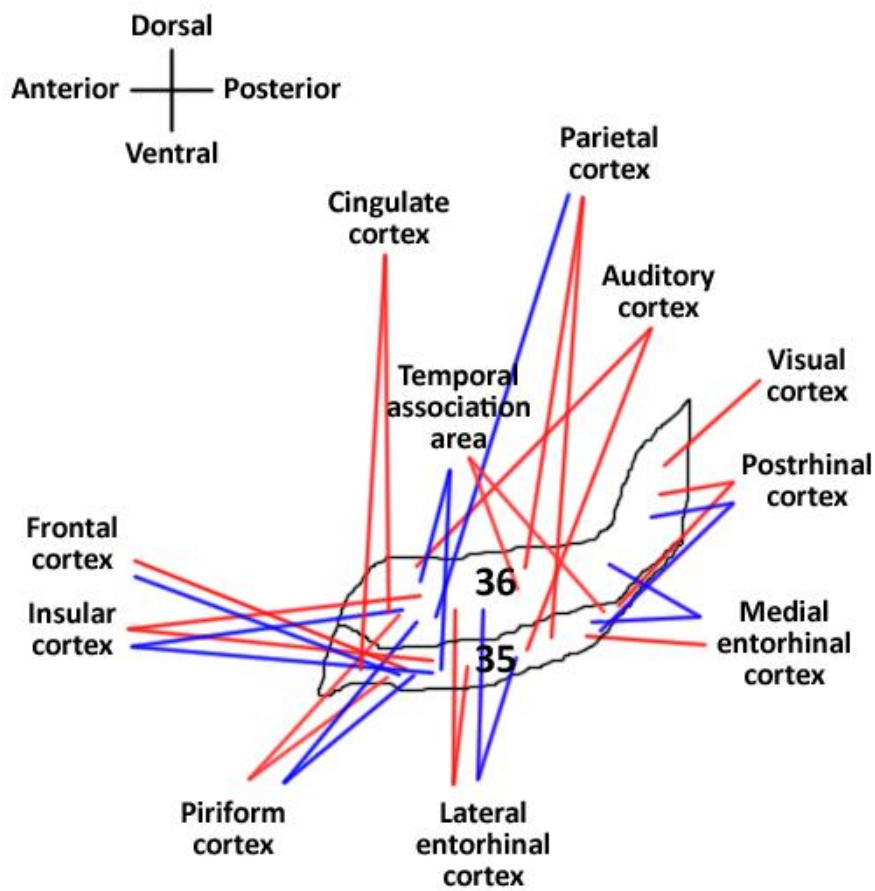
Figure 1.3: The main cortical afferent (red) and efferent (blue) projections of the perirhinal cortex (areas 35 and 35) summarised from Kosel et al. (1982); Saper (1982); Deacon et al. (1983); McIntyre et al. (1996); Insausti et al. (1997); Naber et al. (1997); Burwell & Amaral (1998a); Burwell & Amaral (1998b); Naber et al. (1999); and Agster & Burwell, 2009.

1.2.3 Subcortical afferents and efferents of the perirhinal cortex:

Subcortical projections to the perirhinal cortex originate from the hippocampal formation, amygdala, globus pallidus, thalamus and olfactory bulb (Figure 1.4; Swanson and Cowan, 1977; Deacon et al., 1983; Shammah-Lagnado et al., 1996; Van Groen et al., 1999; Pikkarainen & Pitkänen, 2001; Kloosterman et al., 2003b). From the amygdala, the perirhinal cortex receives projections from the accessory basal nucleus, basal nucleus and lateral nucleus (Pikkarainen & Pitkänen, 2001). These three regions of the amygdala project heavily to area 35 and form a weaker projection to area 36 (Pikkarainen & Pitkänen, 2001). Of the three regions, the heaviest projection comes from the lateral nucleus (McDonald & Jackson, 1987;

Pikkarainen & Pitkänen, 2001). The posterior cortical nucleus of the amygdala does not project to the perirhinal cortex at all (Kemppainen et al., 2002). The projection from the globus pallidus terminates across all layers in the perirhinal cortex (Shammah-Lagnado et al., 1996). Thalamic projections to the perirhinal cortex originate in the anteromedial nucleus and terminate in the layer V of the caudal perirhinal cortex (Van Groen et al., 1999). Finally, the input from the olfactory bulb terminates in across multiple layers of the perirhinal cortex (Santiago & Shammah-Lagnado, 2004).

The major subcortical input to the perirhinal cortex comes from hippocampal formation, principally from the septal region of the area CA1 and the dorsal region of the subiculum which both terminate in the deep layers of the perirhinal cortex (Swanson & Cowan, 1977; Deacon et al., 1983; Van Groen & Wyss, 1990). Projections from the septal proximal subiculum terminate ipsilaterally in layers V and VI of the perirhinal cortex (Kloosterman et al., 2003b). Kloosterman and colleagues (2003b) noted that there were some projections that terminated more superficially (layers I, II and III) and in addition, some of the subicular efferents projected contralaterally. Swanson and Cowan (1977) indicate that the CA1 projection may go through the subiculum and continue on to the perirhinal cortex by either passing straight through the subiculum or by terminating there and the direct connection to the perirhinal cortex actually originating in subicular neurons. However, retrograde tracing using horse radish peroxidase results in labelling of both the area CA1 and the subiculum (Deacon et al., 1983) which suggests that a direct connection from the area CA1 to the perirhinal cortex exists. In a later study using both anterograde and retrograde tracing, a projection originating the septal region of area CA1 was found to terminate in layer VI of the caudal perirhinal cortex (Van Groen & Wyss, 1990).

The projections from the perirhinal cortex terminate in the hippocampal formation, amygdala, striatum/caudate nucleus, nucleus accumbens, substantia nigra, thalamus (Figure 1.4; Ottersen, 1982; Kosel et al., 1983; McIntyre et al., 1996; Naber et al., 1999; McIntyre & Kelly, 2000) but there is no return projection to the olfactory bulb (Santiago & Shammah-Lagnado, 2004). The perirhinal cortex projects to the lateral, basolateral and basomedial nuclei in the amygdala; these projections

originating predominantly in the layers V and VI of the perirhinal cortex (McIntyre et al., 1996). McIntyre and colleagues (1996) also describe projections to the caudate nucleus, nucleus accumbens and substantia nigra originating in layer V of the perirhinal cortex, projections to the thalamus originating in layers V and VI of the perirhinal cortex and projections to the striatum originating in layers III and V of the perirhinal cortex.

The subcortical projections from the perirhinal cortex most pertinent to learning and memory are those to the hippocampus. The efferent projections to the hippocampal formation terminate in area CA1, subiculum, prosubiculum but not in the dentate gyrus, CA2, CA3 and CA4 (Kosel et al., 1983; McIntyre et al., 1996; Naber et al., 1999; Witter et al. 1999). A direct connection from area 35 to the molecular layer of the middle dorsoventral subiculum and adjacent parts of the prosubiculum and area CA1 has been described (Kosel et al., 1983). A later study found that the perirhinal cortex projected to the border area of CA1 and the subiculum but spared other regions of the hippocampus (Naber et al., 1999).
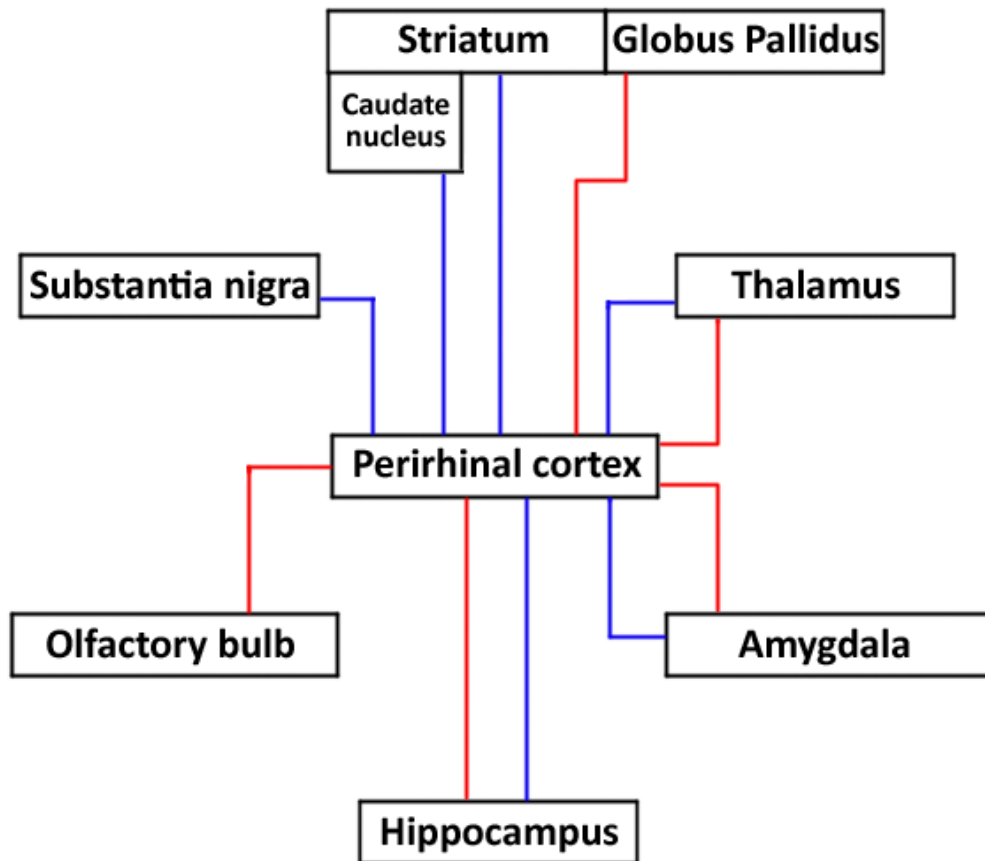
Figure 1.4: Schematic diagram showing main subcortical afferent (red) and efferent (blue) projections of the perirhinal cortex summarised from Swanson and Cowan (1977); Ottersen (1982); Deacon et al. (1983); Kosel et al. (1983); McIntyre et al. (1996); Shammah-Lagnado et al. (1996); Naber et al. (1999); Van Groen et al. (1999); McIntyre & Kelly (2000); Pikkarainen & Pitkänen (2001); and Kloosterman et al. (2003b).

1.3 Physiology:

1.3.1 Synaptic plasticity and its role in memory:

Before discussing perirhinal physiology and function, this section will provide a brief overview of the various forms of synaptic plasticity discussed in this thesis and the significance of synaptic plasticity in memory. Synaptic plasticity refers to changes, either positive or negative, in the strength of the synapses between two different populations of neurons. This plasticity can be transient in nature (short-term plasticity) or it can last for hours, days or weeks (long-term plasticity). These changes in synaptic strength are the currently accepted physiological models for memory formation.

Forms of short-term plasticity include paired-pulse facilitation (PPF), paired-pulse depression (PPD) and post-tetanic potentiation (PTP). PPF is a brief increase (in the realm of milliseconds) in synaptic strength first documented at the neuromuscular junction (Hutter & Loewenstein, 1955). When a pair of stimuli are delivered to the presynaptic neuron, one stimulus primes the synapse for firing so that the response to the second stimulus in the postsynaptic neuron is enhanced compared to the first response (Foster & McNaughton, 1991). This facilitation operates via a Ca^{2+}-dependent mechanism (Zucker, 1974; Thomson et al., 1993) whereby the first stimulus induces presynaptic increases in Ca^{2+} concentrations (Katz & Miledi, 1968; 1970; Thomson, 2000). This increase in Ca^{2+} concentrations may lead to an increase in the amount of neurotransmitter being released into the synapse (Foster & McNaughton, 1991) and thus a facilitation effect is seen following the second stimulus provided it is delivered within a number of milliseconds, i.e. before Ca^{2+} concentrations return to normal (Mallart & Martin, 1967; Thomson, 1997).

The second form of short-term plasticity of interest is PPD, a brief increase (in the realm of milliseconds) in synaptic strength (Thomson et al., 1993). As in the case of PPF, PPD is observed when a pair of stimuli are delivered to the presynaptic neuron but unlike PPF, the presynaptic neuron is not primed but enters a refractory state whereby the response to the second stimulus is attenuated compared to the

first (Thomson, 1997; 2000). This decrease in synaptic strength may be due to depletion of available presynaptic vesicles from the first stimulus, leaving less available neurotransmitter when the second stimulus is delivered (Debanne et al., 1996). The G protein RAB3 has been proposed as a mediator of this refractory state as it regulates the number of vesicles that can dock at any one time (Geppert et al., 1997; Geppert & Südhof, 1998). A postsynaptic mechanism involving alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor desensitisation has also been suggested but this appears to be dependent on what developmental stage the synapse is analysed at (Koike-Tani et al., 2008) as in the adult rat hippocampus there is no significant changes in PPD values following treatment with the AMPA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Debanne et al., 1996).

The final form of short-term plasticity that is of interest is PTP which usually lasts in the range of seconds to minutes (Liley, 1956; Hubbard, 1963; Zucker & Regehr, 2002). This increase in synaptic strength may be due to a presynaptic mechanism involving Ca^{2+}-dependent increase in the mobilisation of reserve neurotransmitter vesicles following high-frequency stimulation (HFS; Landau, 1969; Stanley, 1997; Kasai & Takahashi, 1999). There is also evidence to suggest that PTP may be the result of recruiting previously dormant active zones in the presynaptic neuron (Wojtowicz et al., 1994).

The two main forms of long-term plasticity are long-term potentiation (LTP) and long-term depression (LTD). LTP is a form of long-term synaptic plasticity first described in the rabbit hippocampus where a long-lasting increase in synaptic strength was observed following high-frequency stimulation (Bliss & Lømo, 1973). LTP can last anything from an hour up to a year (Abraham, 2003). LTP follows the requirements for a physiological mechanism for memory formation as set out by Hebb (1949): repeated activation of a neuron by another leads to a strengthening of the connection between the two neurons. LTP has been shown to rely on activity-dependent modifications of the presynaptic (Schultz et al., 1994) and postsynaptic nerve terminals (Isaac et al., 1995; Blundon & Zakharenko, 2008). The mechanisms underpinning LTP are different to those found in short-term plasticity (McNaughton, 1982) and two main forms of LTP have been identified; one that is

dependent on the N-methyl-D-aspartate (NMDA) glutamate receptor (Collingridge et al., 1983; 1988b; Bashir et al., 1994) and one that is independent of the NMDA receptor (Harris & Cotman, 1986; Bramham et al., 1991; Johnston et al., 1992). NMDA receptor-independent LTP has been found to be dependent on the kainate glutamate receptor (Castillo et al., 1997; Vignes & Collingridge, 1997; Bortolotto et al., 1999).

The other main form of long-term plasticity is LTD which is a long-lasting reduction in synaptic strength following low-frequency stimulation (LFS; Lynch et al., 1977; Bramham & Srebro, 1987). Similar to the situation with LTP, there are both presynaptic (Stanton & Sejnowski, 1989; Xiao et al., 1995) and postsynaptic components underlying LTD (Bolshakov & Siegelbaum, 1994; Linden, 1994). Again, as in LTP, there are both NMDA receptor-dependent (Christie & Abraham, 1992; Thiels et al., 1996; Peng et al., 2009) and NMDA receptor-independent forms of LTD (Kemp & Bashir, 1997; Wang et al., 1996). There are a number of proposed subtypes of NMDA receptor-independent LTD; α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), kainate and metabotropic glutamate (mGlu) receptors have all been implicated in various forms of LTD (O'Mara et al., 1995; Park et al., 2006; Holman et al., 2007; Griffiths et al., 2008).

It has also been demonstrated that LTP and LTD may be opposing ends of the same process (Bear, 2003; Yu et al., 2008). LTP and LTD can be induced in the same synapses following a series of HFS and LFS protocols (Dudek & Bear, 1993; Heynen et al., 1996) and there are a number of molecular mechanisms found in both LTP and LTD including Ca^{2+} (Mulkey & Malenka, 1992; Cummings et al., 1996), protein phosphatases (Mulkey et al., 1993; 1994; Dickonson et al., 2009), brain-derived neurotrophic factor (BDNF; Aicardi et al., 2004), extracellular signal-regulated kinase (ERK; Norman et al., 2000; Thiels et al., 2002; Gallagher et al., 2004), NMDA receptors (Dudek & Bear, 1992; Kirkwood et al., 1993; Peng et al., 2009) and AMPA receptors (Lüscher et al., 1999; Wang & Linden, 2000). However, there has to be some control over when and where LTP or LTD is expressed if they serve some function in the intact brain. This control over which form of synaptic plasticity is expressed can be explained by metaplasticity; the changes that occur at

a given synapse that alter its potential for synaptic plasticity (Abraham & Bear, 1996).

In the visual cortex both LTP (Berry et al., 1989; Teyler et al., 1989; Tsanov & Manahan-Vaughan, 2009) and LTD (Kimura et al., 1990; Bröcher et al., 1992; McCoy & McMahon, 2007) can be expressed in a similar but less dramatic manner as is observed in the hippocampus (Teyler, 1989). It is within this cortical area that metaplastic effects have been studied in the most detail. The Bienenstock-Cooper-Munro (BCM) model of biphasic synaptic modification describes the modification threshold (θ_m) where a given stimulation frequency induces LTD instead of LTD (Bienenstock et al., 1982). The model predicts that changes in metaplasticity would be reflected by a shift in θ_m which would subsequently govern whether LTP or LTD is induced following a given stimulation frequency. A number of studies in the visual cortex have indentified that there are metaplastic effects that affect the induction of LTP and LTD (Artola et al., 1990; Aroniadou & Teyler, 1991). Moreover, a number of metaplastic mechanisms have been identified including silent synapses, the activity-dependent insertion of glutamate receptors into the postsynaptic membrane from internal stores (Wojtowicz et al., 1991; Isaac et al., 1995; Liao et al., 1995; Woo & Nguyen, 2002; Young & Nguyen, 2005; Cheyne & Montgomery, 2008), and alterations in NMDA receptor subtype expression; NMDA receptors expressing the NR2A subunit are associated with LTP and NR2B subunit-containing NMDA receptors are associated with LTD (Massey et al., 2004; Toyoda et al., 2005; Yashiro & Philpot, 2008). In the hippocampus, shifts in θ_m have been reported in the CA1 to entorhinal projection following various stimulation frequencies indicating that the BCM model is applicable to projections involved in learning and memory (Craig & Commins, 2007).

Evidence for the role of synaptic plasticity in learning and memory comes from a number of different experimental approaches. Coincidence detection was proposed by Hebb (1949) as a means by which two temporally aligned stimuli from two different projections could induce a larger effect in a common postsynaptic neuron even if neither presynaptic neuron could normally induce such a change on its own. Following studies in the sea slug, *Aplysia californica*, a neurophysiological model for conditioning supported Hebb's coincidence detection model; the gill and

siphon withdrawal reflex in *Aplysia californica* requires stimuli from two different sensory inputs (tactile stimulation of the mantle and a tail shock delivered at the same time) in order for the animal to demonstrate the conditioned response (Walters et al., 1979). An electrophysiological circuit was mapped out for this reflex (Castellucci et al., 1970) and molecular correlates between synaptic plasticity and learning and memory were also observed (Castellucci & Kandel, 1974; Brunelli et al., 1976; Carew et al., 1981).

Coincidence detection has been suggested to play a part in mammalian models of memory, the NMDA receptor in particular has been proposed to act as a coincidence detector during LTP and possibly also during memory formation (Nicoll & Malenka, 1999; Yuste et al., 1999; Tsien, 2000). In cortical neurons, spike timing-dependent plasticity which is reliant on NMDA receptor coincidence detection has been proposed as an *in vivo* mechanism by which LTP can be induced as experimental techniques like HFS are not naturally occurring phenomena (Dan & Poo, 2004; Bender et al, 2006; Billings & van Rossum, 2009). The NMDA receptor's role as a coincidence detector is reflected in its mode of action, in order to become active there must firstly be glutamate released into the synapse from the presynaptic neuron to bind to the NMDA receptor. Secondly, there must also be depolarisation within the postsynaptic neuron to remove the Mg^{2+} block in the channel of the NMDA receptor (Malenka & Nicoll, 1999). Mutations of the Grin1(N598R) allele lead to disruption of NMDA receptor coincidence detection with a concurrent impairment of LTP and performance in memory tasks (Chen et al., 2009). Overexpression of the NR2B NMDA subtype in the mouse forebrain led to an enhancement of LTP and performance in a range of memory tasks but had no effect on LTD (Tang et al., 1999). Similar findings have since been reported in the rat hippocampus (Wang et al., 2009) and roles for the NMDA receptor acting as a coincidence detector during LTD have also been found (Sjöström et al., 2003). NMDA-dependent coincidence detection appears to be controlled by postsynaptic $GABA_B$ receptors (Bradley et al., 2004). In the chick, Bradley and colleagues posit that where both postsynaptic NMDA and $GABA_B$ receptors are expressed in a neural circuit, the circuit will act as a coincidence detector when the balance of firing is weighted towards the NMDA receptors but when the balance is shifted towards the

GABA$_B$ receptors the circuit will cease to act as a coincidence detector (i.e. after learning has occurred). Recently, both presynaptic forms of NMDA receptor-dependent (Duguid & Sjöström, 2006) and non-NMDA receptor-dependent forms of coincidence detection underlying LTP and LTD have been found (Egger et al., 1999; Schrader et al., 2004).

Further evidence for synaptic plasticity being a suitable physiological model for learning and memory comes from the pharmacological and molecular analysis of electrophysiological and behavioural data. Early experiments in LTP came at a time when a number of novel glutamate receptor antagonists were being developed (Collingridge, 2003) and there are a number of studies that show that both LTP (Collingridge et al., 1983; Blake et al., 1988; Collingridge et al., 1988a; 1988b; Blake et al., 1989) and learning (Morris et al., 1986; Jerusalinsky et al., 1992; Nilsson et al., 1997) could be disrupted by the same compounds. AMPA glutamate receptor trafficking has been found to underpin long-term changes in synaptic plasticity as well as learning and memory (Griffiths et al., 2008; Kessels & Malinow, 2009).

A number of second messenger and signalling molecules required for synaptic plasticity have also been shown to be necessary for learning and memory (Alberini, 2009). Alterations in BDNF signalling (see Section 1.3.2) are observed following induction of LTP (Gooney & Lynch, 2001; Gooney et al, 2002) and learning (Mizuno et al., 2000; 2003). Additionally, BDNF has shown to be required for LTP induction (Korte et al., 1995) and learning (Linnarsson et al., 1997). Downstream BDNF signalling has also been shown to occur with synaptic plasticity and learning memory; there is increased phosphorylation of ERK observed after LTP induction (Wu et al., 1999; Gooney et al., 2002) and learning (Crow et al., 1998; Maguire et al., 1999). Moreover, inhibition of ERK signalling leads to deficits in LTD induction in the visual cortex (McCoy & McMahon, 2007) and impaired learning (Atkins et al., 1998; Bozon et al., 2003). There is also increased levels of cAMP response element binding protein (CREB) following LTP induction (Impey et al., 1996) and learning (Mizuno et al., 2002). Addition of active CREB using a viral vector has been shown to enhance LTP (Marie et al., 2005) and contextual fear memory (Restivo et al., 2009). Furthermore, the expression of the immediate early gene c-Fos has been shown to

increase following LTP induction (Dragunow et al., 1989; Jeffery et al., 1990). It has also been shown to be necessary for learning to occur (Countryman et al., 2005; Labrousse et al., 2009) and knockout mice for c-Fos show impairments in LTP induction and learning (Fleischmann et al., 2003).

In addition to these molecular correlates between synaptic plasticity and learning and memory, there are a number of behavioural interventions that can affect synaptic plasticity and learning and memory. Environmental enrichment (Gardner et al., 1975; Tees, 1999; Leggio et al., 2005) and exercise (Vaynman et al., 2004; Asl et al., 2008; Griesbach et al., 2009) have been shown to enhance performance in learning and memory tasks and they have also been shown to enhance changes in synaptic plasticity (Christie et al., 2005; Huang et al., 2006; O'Callaghan et al., 2007). Similarly, behavioural interventions that impair learning and memory such as environmental deprivation (Gardner et al., 1975; Schrijver et al., 2004) and stress (de Quervain et al., 1998; Grace et al., 2009) also impair synaptic plasticity (Foy et al., 1987; Marrone, 2005; Yamauchi et al., 2005). These correlations between changes in synaptic plasticity and learning and memory has lead to the development of the synaptic plasticity and memory (SPM) theory which identifies activity-dependent synaptic plasticity as the as the process by which memories are encoded and consolidated (Martin et al., 2000).

Out of the above list of signalling molecules involved in both synaptic plasticity and learning and memory, a large proportion of them are transcription factors. As transcription factors act in the nucleus of the neuron, away from the synapses being acted upon (whether experimentally via electrophysiological stimulation such as HFS or from upstream signalling in a circuit involved in learning and memory), a problem of synapse specificity emerges: how does the cell body know which synapses to target with newly transcribed genes? Synaptic tagging has proposed as a mechanism by which specific genes induced during changes in synaptic plasticity are delivered to the appropriate synapses (Frey & Morris, 1998b; Morris et al., 2003; Pittenger & Kandel, 2003). These synaptic tags are believed to play a role in memory as well as in experimentally-induced changes in synaptic plasticity (Moris & Frey, 1997).

LTP can be split into two mechanistically different forms: early LTP (E-LTP) and late LTP (L-LTP; Frey et al., 1988). During E-LTP in area CA1 of the rat, there is an increase in synaptic strength which lasts for only a few hours and it can be induced in the absence of protein synthesis (Frey & Morris, 1997). On the other hand, Frey and Morris (1997) show that L-LTP lasts longer than E-LTP and requires protein synthesis in order to be induced. They also show that E-LTP involves the creation of a synaptic tag which can capture newly synthesised proteins at the appropriate synapses during L-LTP (Frey & Morris, 1998a). Morris and colleagues (2003) propose that the relevant synapses for forming a particular long-term memory is primed with an E-LTP type process which tags the respective synapses before an L-LTP type process completes the memory formation. Evidence for this can be found with their experiment which shows that the D1/D5 dopamine receptor antagonist SCH23390 blocks both L-LTP and impairs long-term memory (Morris et al., 2003). A molecular candidate for synaptic tagging is CREB which has been shown to be necessary for long- but not short-term spatial memory (Guzowski & McGaugh, 1997). Additionally, the immediate early gene *Arc* has been shown to be necessary for both the consolidation of long-term memory in a Morris water maze task and for the induction of L-LTP (Guzowski et al., 2000).

Based on the above evidence, the case for synaptic plasticity being the physiological underpinning for learning and memory in the brain is a strong one. The shared molecular mechanisms between synaptic plasticity and memory are many but the exact roles of short- and long-term synaptic plasticity in memory formation are still to be fully elucidated.

1.3.2 Brain-derived neurotrophic factor in synaptic plasticity, learning and memory:

Brain-derived neurotrophic factor (BDNF) is part of a family of neuropeptides called the neurotrophins (Barde et al., 1982); the other members of this neuropeptide family include nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5; Barde, 1990; Maisonpierre et al., 1990; Hallböök et al., 1991). The neurotrophins all bind to one of two types of neurotrophin receptor: the three tropomyosin-receptor-kinase (Trk) receptors (Barbacid, 1994) and the p75

neurotrophin receptor (Chao, 1994). The p75 neurotrophin receptor binds all of the neurotrophins but the Trk receptors show specificity for the various neurotrophins (Rodríguez-Tébar et al., 1991): NGF binds to the TrkA receptor (Kaplan et al., 1991); BDNF (Klein et al., 1991; Squinto et al., 1991) and NT-4/5 (Klein et al., 1992) bind to the TrkB receptor; and NT-3 binds to the TrkC receptor (Lamballe et al., 1991). However, both the TrkA (Cordon-Cardo et al., 1991) and TrkB receptors (Klein et al., 1991; Squinto et al., 1991) also show some specificity for NT-3.

Neurotrophins are stored in neurons as immature precursors (pro-neurotrophins; Carvalho et al., 2008); in the case of BDNF it is stored as proBDNF (Mowla et al., 1999; 2001). In the presynaptic neuron, proBDNF is stored in large dense-core vesicles (Fawcett et al., 1997) and, in the postsynaptic neuron, proBDNF is stored in secretory granules (Haubensak et al., 1998). proBDNF is released in an activity- (Goodman et al., 1996; Hartmann et al., 2001; Kohara et al., 2001) and Ca^{2+}-dependent manner (Sadakata et al., 2007) into the synapse (Figure 1.5). Here it is cleaved by plasmin to form an activated BDNF dimer (Pang et al., 2004). These activated BDNF dimers bind to two postsynaptic TrkB receptor monomers, causing the TrkB receptor to dimersise and become activated (Jing et al., 1992; Ibáñez et al., 1993). BDNF-induced activation of the postsynaptic TrkB receptor results in downstream signalling via a number of intracellular pathways including phospholipase C_γ (PLC$_\gamma$; Widmer et al., 1993; Middlemas et al., 1994), mitogen-activated protein kinase (MAPK)/ERK (Marsh et al., 1993; Takei et al., 2001), and phosphoinositide 3-kinase (PI3K). These signal transduction pathways lead to increases in transcription factors such as c-Fos (Ip et al., 1993; Roback et al., 1995) and Arc (Yin et al., 2002) and phosphorylation of proteins such as synapsin I (Jovanovic et al., 1996). BDNF can then be recycled from the synapse and reused in an activity-dependent manner (Santi et al., 2006).
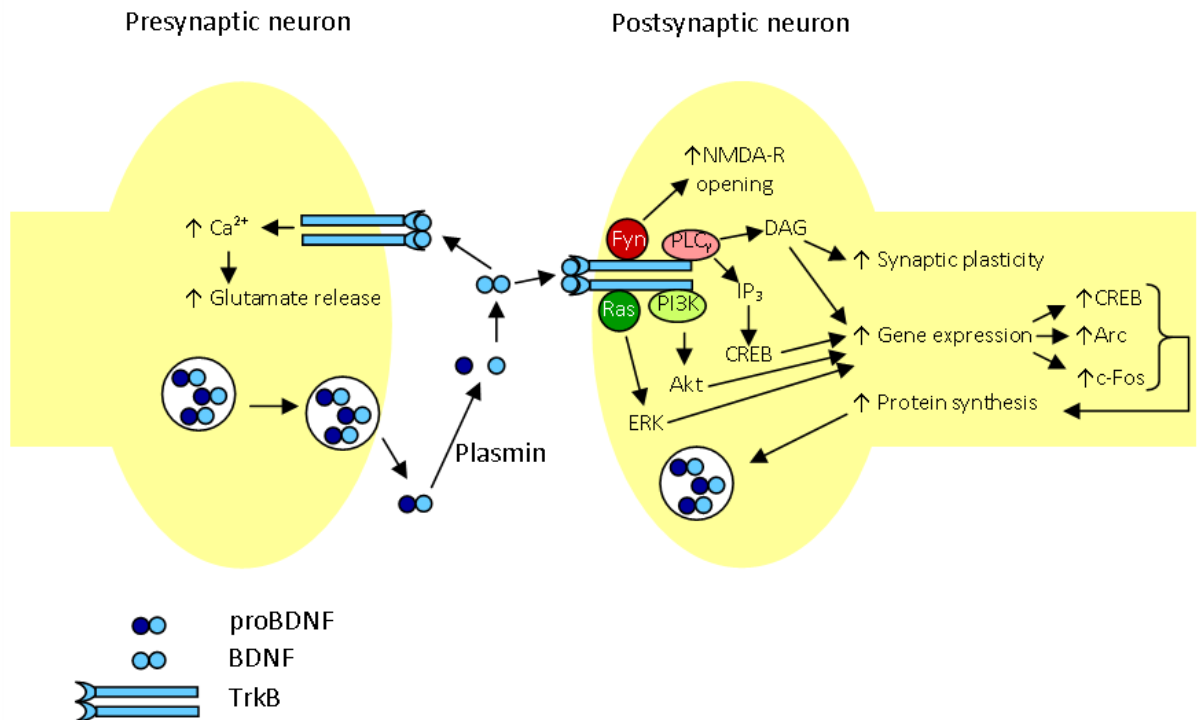
Figure 1.5: Interactions between BDNF and the TrkB receptor. Following release of proBDNF into the synapse, it is cleaved by plasmin to form mature BDNF which can then activate pre- and postsynaptic TrkB receptors. Presynaptic TrkB receptor activation induces a Ca2+-dependent increase in glutamate release. Postsynaptic TrkB receptor activation results in changes in gene expression, protein synthesis and synaptic plasticity via a number of different signalling pathways. See Section 1.3.2 for details. Diagram adapted from Carvalho et al. (2008) and Minichiello (2009).

BDNF can also bind with low affinity to the p75 neurotrophin receptor but although p75 dimers have been found (Jing et al., 1992), recent evidence from NGF-p75 binding studies suggest that neurotrophin dimers bind to a single p75 neurotrophin receptor (He & Garcia, 2004). Activated p75 neurotrophin receptors signal intracellularly via a number of second messenger pathways distinct from those seen following TrkB receptor activation; p75 activation can be followed by increases in nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB; Carter et al., 1996) and c-Jun amino-terminal kinase (JNK; Casaccia-Bonnefil et al., 1996). In general, the TrkB receptor and the p75 neurotrophin receptor have been shown to be involved in separate functions with TrkB receptor activation leading to promotion of cell survival (Frade et al., 1997) and increases in dendritic spine

density (Shimada et al., 1998; Tyler & Pozzo-Miller, 2003). On the other hand, activation of the p75 neurotrophin receptor often has an opposite effect including cell death (Frade et al., 1996) and reductions in dendritic spine density (Zagrebelsky et al., 2005). These conflicting affects suggest that there is a feedback system between Trk and p75 signalling (Yoon et al., 1998). However, there is increasing evidence that the interactions between Trk and p75 receptors; it has been shown that the TrkB receptor and the p75 neurotrophin receptor seem to be involved in similar functions such as upregulating neurotransmitter release (Tyler & Pozzo-Miller, 2001; Numakawa et al., 2001; 2003). As mentioned above, neurotrophin dimers appear to bind to a single p75 neurotrophin receptor (He & Garcia, 2004). This form of binding may allow the p75 neurotrophic receptor to act as a co-receptor (Carter & Lewin, 1997; Zampieri & Chao, 2006).

Although the neurotrophins have been traditionally viewed as primarily being involved in neuronal development and survival (Levi-Montalcini et al., 1968; Barde et al., 1978; Edgar et al., 1981; Barde, 1994; Davies, 1994), more recently research has shown that they are also involved in synaptic plasticity (Kang and Schuman, 1995a; 1995b; Korte et al., 1995; Gooney & Lynch, 2001; Aicardi et al., 2004) and learning and memory (Falkenberg et al., 1992a; 1992b; Mizuno et al., 2000; Gooney et al., 2002; Harvey et al., 2008). Both BDNF (Ernfors et al., 1995) and TrkB knockouts (Klein et al., 1993) are lethal (with most animals only surviving a few weeks after birth) but even so, they have revealed prominent roles for both proteins in synaptic plasticity. BDNF-knockout mice showed impaired induction of E-LTP (Korte et al., 1995) and L-LTP (Korte et al., 1998) in the area CA1. However, viral transfer of BDNF (Korte et al., 1996a; 1996b) or application of recombinant BDNF to area CA1 (Patterson et al., 1996) restores the plastic capabilities of these neurons in these mice. It has also been noted that stimulus strength or stimulation pattern may play a factor in inducing E-LTP in BDNF knockout mice (Kang et al., 1997). In addition to impairments in LTP, BDNF knockout mice show a number of presynaptic deficits in the hippocampus such as impaired high-frequency transmission (higher levels of synaptic fatigue compared to wild type mice), lower levels of vesicle docking and reduced neurotransmitter release (Pozzo-Miller et al., 1999). Pozzo-Miller and colleagues (1999) also showed that these deficits could be

reversed by treating BDNF knockout hippocampal slices with recombinant BDNF. Knocking out TrkB in area CA1 also points to a presynaptic mechanism for BDNF modulation of LTP (Xu et al., 2000). Finally, although knockouts for the p75 neurotrophin receptor show no impairments in LTP compared to wild type controls (Xu et al., 2000), more recent evidence suggests that the p75 neurotrophin receptor may play an inhibitory role in synaptic plasticity (Sandoval et al., 2007).

Evidence for BDNF's role in synaptic plasticity and learning also comes from studies which show that BDNF is released in an activity-dependent manner following electrophysiological stimulation (Goodman et al., 1996; Hartmann et al., 2001; Kohara et al., 2001). Exogenous application of BDNF to hippocampal slices promoted the induction of LTP and removal of BDNF using a TrkB-IgG conjugate inhibited the induction of LTP (Figurov et al., 1996). The induction of L-LTP (Korte et al., 1998) and maintenance of LTP are both impaired following treatment of hippocampal slices with TrkB-IgG or TrkB antiserum (Kang et al., 1997). Significantly, direct application of BDNF in the absence of HFS to Schaffer collateral neurons is enough to lead to a long-lasting enhancement of synaptic strength (Kang & Schuman, 1995a; 1995b). Kang and Schuman (1995a; 1995b) also report a decrease in PPF which suggests that BDNF-induced enhancement of synaptic strength is presynaptic in nature. Moreover, in CA1 pyramidal neurons a spike-timing protocol (repetitive pairing of postsynaptic spikes and glutamate release) lead to a BDNF-dependent enlargement of dendritic spines (Tanaka et al., 2008) and BDNF-induced changes in dendritic spine morphology occur through ERK1/2 (Alonso et al., 2004).

Taken together, these findings indicate that BDNF and the TrkB receptor play important roles in synaptic plasticity. Furthermore, the case for synaptic plasticity being a physiological model for learning and memory is strengthened by BDNF's pivotal role in synaptic plasticity and in learning and memory (Pang & Lu, 2004). Hippocampal levels of BDNF have been shown to increase following spatial learning in the Morris water maze (and these increases are distinct from exercise-induced increases in BDNF; Harvey et al., 2008), following spatial learning in a radial arm maze (Mizuno et al., 2000) and following learning in an inhibitory avoidance task (Alonso et al., 2002a). Application of an antisense BDNF disrupts acquisition, maintenance and recall of spatial memory in a radial arm maze (Mizuno et al.,

2003). Mizuno and colleagues (2003) also show that this impairment of spatial memory also affects downstream signalling of the TrkB receptor as learning-induced increases in phosphorylation of the TrkB receptor, PI3K and Akt are reduced following application of antisense BDNF. BDNF$^{+/-}$ mice express significantly lower levels of BDNF and are significantly impaired in the Morris water maze compared to wild type controls (Linnarsson et al., 1997). Additionally, hippocampus-specific lentiviral knockouts of BDNF impair object recognition and spatial memory in mice (Heldt et al., 2007). Overexpression of the truncated form of the TrkB receptor results in spatial learning impairments in the Morris water maze (Saarelainen et al., 2000).

In a hippocampal-dependent inhibitory avoidance task, BDNF synthesis was shown to be required in order to consolidate learning to long-term memory (Bekinschtein et al., 2007). Other studies have shown that downstream signalling of BDNF is required for learning this inhibitory avoidance task (Alonso et al., 2002a; 2002b) and that protein synthesis blockade-induced impairment in this task could be reversed by application of BDNF protein to the hippocampus (Bekinschtein et al., 2008). In contextual fear conditioning, hippocampal BDNF has been shown to be required for consolidation of memories into long-term memory but not for reconsolidation (Lee et al., 2004).

A role for cortical BDNF in learning and memory has also been proposed; application of anti-BDNF antibody to the rat parietal cortex produced impairments in short- and long-term memory in a fear-motivating learning task (Alonso et al., 2005). Alonso and colleagues (2005) also showed that these impairments can be reversed by application of exogenous BDNF to the parietal cortex. Overexpression of the truncated form of the TrkB receptor in cortical neurons has also been reported to impair spatial memory in the Morris water maze but this finding has a caveat in that the hippocampus was also expressing the truncated form of the TrkB receptor so hippocampal and cortical roles for the TrkB receptor could not be distinguished from each other (Saarelainen et al., 2000). In the perirhinal cortex, application of antisense BDNF resulted in impairments in long-term recognition memory (Seoane et al., 2010). Furthermore, exercise-induced enhancement of

recognition memory in rats is accompanied by increases in perirhinal BDNF levels (Griffin et al., 2009).

From the above evidence, it can be seen that BDNF and the TrkB receptor may underlie several important processes in synaptic plasticity and learning and memory. As several aspects of BDNF signalling are conserved between changes in synaptic plasticity and in a number different types of task and in multiple brain areas during learning and memory formation, the argument for synaptic plasticity as a physiological model of learning and memory is strengthened considerably.

1.3.3 Physiological properties of the perirhinal cortex:

The perirhinal cortex, as defined anatomically as areas 35 and 36 in the rat brain, It can sustain glutamatergic transmission as it expresses mGlu7 receptors (Ohishi et al., 1995), NMDA glutamate receptors (Monaghan & Buller, 1994), AMPA glutamate receptors (Palomero-Gallagher & Zilles, 2004) and kainate glutamate receptors (Nagahara et al., 1993). Ionotropic glutamate receptors (which are the main receptors involved in synaptic plasticity, see above) appear in lower densities in the perirhinal cortex compared to surrounding cortical areas and perirhinal ionotropic glutamate receptors show a layer-specific distribution; NMDA and AMPA glutamate receptors are found in greater densities in layers I-III whereas kainate receptors around found in greater densities in the layers V-VI in the perirhinal cortex (Palomero-Gallagher & Zilles, 2004).

Cholinergic transmission can be sustained through muscarinic acetylcholine (Massey et al., 2001) and nicotinic acetylcholine receptors (Palomero-Gallagher & Zilles, 2004). Along with glutamate, acetylcholine has been proposed to play a role in memory formation in the hippocampal formation (Hasselmo, 2006). However, although a number of studies have shown the physiological and behavioural importance of perirhinal cholinergic transmission (Massey et al., 2001; Abe & Iwasaki, 2001; Bang & Brown, 2009) there is little anatomical and histological information on the distribution and density of perirhinal cholinergic receptors available.

The perirhinal cortex can also sustain dopaminergic transmission (Pum et al., 2007); it expresses D1 and D2 dopamine receptors (Richfield et al., 1989; Goldsmith & Joyce, 1994), D4 receptors (Rivera et al., 2008) and dopamine transporters (Belcher et al., 2005). Based on several tracing studies, there are low levels of GABAergic input to the perirhinal cortex (Christie et al., 1987; Kosaka et al., 1987; Beart et al., 1990) but GABAergic inputs from the temporal and entorhinal cortices have been identified (Garden et al., 2002). Additionally, $GABA_B$ receptors have been shown to play a role in local perirhinal circuits (Ziakopoulos et al., 2000). Adrenergic transmission can be sustained through α_2-adrenergic receptors (King et al., 1995) and noradrenaline transporters (Hébert et al., 2001). Finally, the perirhinal cortex receives serotonergic input (Pum et al., 2007) with a projection from the raphe nucleus terminating in superficial layers of the perirhinal cortex (Hermann et al., 1997; Vertes et al., 1999; Harding et al., 2004). The perirhinal cortex expresses $5\text{-}HT_{1A}$ receptors (Nyakas et al., 1997), $5\text{-}HT_2$ receptors (Altar et al., 1985; Osterlund et al., 1999) and 5-HT transporters (Hébert et al., 2001; Belcher et al., 2005).

In addition to the classic neurotransmitters, the perirhinal cortex is also capable of signalling via the neurotrophins and their receptors (see Section 1.3.2; Sobreviela et al., 1996). Although there are low levels of NGF and TrkA present in the neocortex (Altar et al., 1991; Merlio et al., 1992), the other neurotrophins and their receptors are more widely expressed throughout the neocortex (Klein et al., 1990; Merlio et al., 1992; Altar et al., 1994). However, the parahippocampal region shows higher levels of neurotrophins compared to rest of the neocortex: NGF (Lin et al., 1996; Conti et al., 2009), BDNF (Sato et al., 1996; Vezzani et al., 1999; Engler-Chiurazzi et al., 2009), NT-3 (Eagleson et al., 2001) and all three Trk receptors (Bengzon et al., 1993) have been shown to be strongly expressed in the perirhinal cortex. It must be noted that so far, no study has looked at NT-4/5 expression in the perirhinal cortex. Evidence for a physiological role for BDNF in the perirhinal cortex comes from infusion studies where recombinant human BDNF (rhBDNF) is applied to the occipital and entorhinal cortices and subsequently transported retrogradely to the perirhinal cortex (Sobreviela et al., 1996).

Morphological analysis of the perirhinal cortex in the adult rat reveal that pyramidal neurons are the most common type of perirhinal neuron and are

morphologically divisible into five subtypes horizontal, upright, inverted, bifurcating and oblique (with upright pyramidal neurons being the most numerous; Furtak et al., 2007a). Although the most common neuron type, Furtak and colleagues (2007a) note that the numbers of pyramidal neurons in the perirhinal cortex are comparatively lower than other neocortical areas. Electrophysiological examination of perirhinal cortex has revealed a number of different neuron types based on spiking patterns; a range of neurons falling into the categories of fast spiking, regular spiking, burst spiking and late spiking have been described by Faulkner and Brown (1999). These neurons were all found in layers II/III, V and VI of the perirhinal cortex (Faulkner & Brown, 1999). The various cell layers in the perirhinal cortex show differential distribution of these neurons compared to each other; in layers II/III later spiking neurons account for 54 % of the total of pyramidal neurons followed by regular spiking pyramidal neurons (46 %; Beggs et al., 2000). Whereas in layer V regular spiking pyramidal neurons are the most common of the three types (76 % of total) followed by late spiking (14 %) and burst spiking pyramidal neurons (9 %; Moyer et al., 2002). Lastly, layer VI has been shown to be unique amongst cortical regions in having late spiking neurons account for 86 % with of the total number of pyramidal neurons followed by single spiking (7 %), fast spiking (5 %) and regular spiking pyramidal neurons (<1 %; McGann et al., 2001). The baseline field excitatory postsynaptic potential (fEPSP) in the perirhinal cortex is dependent on the voltage-gated Ca^{2+} and Na^+ ion channels (D'Antuono et al., 2001), the AMPA glutamate receptor (Cho et al., 2000b) but less reliant on NMDA or mGlu receptors (Ziakopoulos et al., 1999) or extracellular Ca^{2+} (McCaffery et al., 1999). A long-lasting, positive $GABA_B$ component has also been described (Ziakopoulos et al., 2000).

Importantly, these neurons can sustain long-term changes in synaptic plasticity *in vitro*; 100 Hz HFS in layers II/III neurons of the perirhinal cortex produces NMDA receptor-dependent LTP in the same layers (Bilkey, 1996) and tetanic and theta-burst stimulation at 100 Hz in layers II/III can also induce NMDA receptor-dependent LTP in layer I neurons (Ziakopoulos et al., 1999). LTP in the perirhinal cortex is associated with an increase in BDNF secretion in the first 5-12 post-stimulation and this LTP can be blocked by inhibiting the BDNF receptor TrkB

(Aicardi et al., 2004). Perirhinal LTP can be blocked by inhibiting CREB as shown by adenoviral transduction of a CREB-inhibitor onto perirhinal slices (Warburton et al., 2005) but it appears to be independent of L-type voltage-dependent Ca^{2+} channel activation (Seoane et al., 2009). The induction of LTP in the perirhinal cortex has also been demonstrated to be $GABA_A$-dependent as enhancement of $GABA_A$ receptor function by lorazepam disrupts the induction of LTP in neurons within layers II/III (Wan et al., 2004).

The bulk of research on synaptic plasticity within the perirhinal cortex has focussed on LTD due to its possible role in recognition memory (Jerusalinsky et al., 1997; Warburton et al., 2003; Wan et al., 2004; Barker et al., 2006; Griffiths et al., 2008; Seoane et al., 2009). Ziakopoulos and colleagues (1999) demonstrated that the perirhinal cortex could also undergo depressive synaptic plasticity; short-term depression in the form of paired-pulse depression (PPD) could be induced with a 200 ms IPI and this PPD was $GABA_B$-dependent (Ziakopoulos et al., 2000). Perirhinal long-term depression was also demonstrated; 1 Hz and 5 Hz LFS can induce perirhinal LTD lasting approximately 40 min and 180 min respectively and this LTD is associated with transient decreases in BDNF secretion following LFS (Aicardi et al., 2004). Induction of perirhinal LTD has been found to be reliant on L-type voltage-dependent Ca^{2+} channels (Seoane et al., 2009). Like LTP, LTD in the perirhinal cortex seems to be largely glutamatergic in nature with the ionotropic glutamate receptors being of particular importance (Ziakopoulos et al., 1999).

However, even though Ziakopoulos and colleagues (1999) concluded that both LTP and LTD in the perirhinal cortex were mGlu receptor-indpendent, it has been shown that a range of mGlu receptor agonists (specific for group I, II and III mGlu receptors) can induce perirhinal LTD in the absence of LFS (McCaffery et al., 1999). These conflicting results were reconciled with the finding that NMDA and mGlu receptors may be both required for LTD induction and that the contribution of NMDA and mGlu receptors to LTD require different electrophysiological conditions induced; group II mGlu receptor-dependent LTD can only be induced at resting membrane potentials whereas NMDA receptor-dependent LTD can be induced even when depolarisation has occurred (Cho et al., 2000b; Cho & Bashir, 2002). Furthermore, Ca^{2+} signalling in mGlu receptor-dependent LTD relies on

neuronal Ca^{2+} sensor protein interacting with protein C kinase whereas NMDA receptor-dependent LTD relies on calmodulin (Jo et al., 2008). It also appears that interaction between group I and group II mGlu receptors is required for LTD to be induced at resting membrane potentials and this synergy requires protein kinase A and protein phosphatase 2B (Cho et al., 2002). This interaction between group I and group II mGlu receptors is explained by the cAMP-dependent enhancement of mGluR5 (group I mGlu receptor) by mGluR2 (group II mGlu receptor); this enhancement of mGluR5 in turn contributes to the induction of perirhinal LTD (Harris et al., 2004a). NMDA receptor-dependent LTD involves the internalisation of AMPA receptors (Griffiths et al., 2008) but mGlu receptor-dependent LTD appears to modulate AMPA receptors via a different molecular mechanism to that found with NMDA receptors (Harris et al., 2004b). Kainate receptor-dependent LTD has been described which appears to be independent of NMDA receptor activation and also appears to be regulated by mGluR5 (Park et al., 2006). These findings indicate that glutamatergic LTD in the perirhinal cortex is highly organised and regulated across multiple glutamate receptor systems, perhaps reflecting the complexity of the perirhinal cortex's functional role in the behaving animal (see section 1.4).

In addition to LTD due to glutamatergic neurotransmission, there appears to other forms of LTD in the perirhinal cortex. Muscarinic M1 cholinergic receptor-dependent LTD can be induced without electrical stimulation or NMDA receptor activation by the application of carbachol (Massey et al., 2001). Additionally, Massey and colleagues (2001) found that blockade of intracellular Ca^{2+} release and protein synthesis could impair this carbachol-induced LTD. Moreover, muscarinic receptor antagonism by scopolamine can block perirhinal LTD while sparing LTP (Warburton et al., 2003). It is worth noting that in the developing rat brain, there is a visual experience-dependent switch from mGlu receptor-dependent to muscarinic receptor-dependent LTD (Jo et al., 2006) which indicates not only that LTD might play a functional role in the perirhinal cortex but also that the forms of LTD expressed in the area are not set in stone and could be further modified with behavioural experience. Yet other forms of LTD have been identified in the literature such as $GABA_A$ and D2 dopamine receptor-dependent LTD; enhancement of $GABA_A$ receptor function using lorazepam disrupts induction of LTD in the

perirhinal cortex (Wan et al., 2004) and the D2 dopamine receptor has been implicated in synaptic plasticity abnormalities found in the perirhinal cortex in a mouse model of Huntington's disease (Cummings et al., 2006; 2007). Again, this complex array of processes underlying different forms of LTD may be functionally significant in terms of the perirhinal cortex's role in cognitive processes.

The relationship between LTP and LTD in the perirhinal cortex appears to follow the predictions outlined in the BCM model of synaptic plasticity (Bienenstock et al., 1982) as the θ_m threshold appears to be dependent on intracellular Ca^{2+} concentrations (Cho et al., 2001). Cho and colleagues (2001) have also shown that the magnitude of perirhinal synaptic plasticity is also Ca^{2+}-dependent but they also propose that even though synaptic plasticity in the perirhinal cortex obeys the predictions of the BCM model, this LTP and LTD may be two separate co-existing processes instead of one biphasic process. Learning has also been shown to affect the perirhinal cortex's tendency to exhibit LTP or LTD; perirhinal cortex slices taken from rats that had previously been exposed to multiple trials in a visual recognition learning task showed impaired LTD induction but no changes in LTP were observed (Massey et al., 2008). Massey and colleagues (2008) also demonstrated that scopolamine administration during learning prevented the learning-induced LTD impairment, indicating that muscarinic receptors are needed for learning to modify synaptic plasticity in the perirhinal cortex. These findings reviewed in this section suggest that although synaptic plasticity in the perirhinal cortex may appear to follow the predictions set out in the BCM model, due to the varying types of LTP and LTD and the complex variations in LTD observed in several experiments, it may be that perirhinal LTP and LTD are separate but related processes which would allow for finer control of physiological functions compared to a biphasic LTP/LTD process.

1.3.4 The hippocampal-parahippocampal network:

The perirhinal cortex does not exist on its own, along with the rest of the parahippocampal region and the hippocampus it forms a part of hippocampal-parahippocampal network (Figure 1.6); the hippocampus is reciprocally connected

to all the regions of the parahippocampal neocortex to form a network of parallel pathways that are proposed to work in concert during learning and memory formation (Witter et al., 2000a; 2000b; Witter et al., 2002). Not only do these areas form anatomical connections with each other but many of them have been shown to sustain changes in synaptic plasticity. Within the parahippocampal region, the perirhinal cortex forms reciprocal connections with the entorhinal (Wyss, 1981; Kosel et al., 1982; Köhler, 1988; Insausti et al., 1997; Naber et al., 1997; Burwell & Amaral, 1998a) and postrhinal cortices (Burwell & Amaral, 1998b; Burwell, 2000). These three cortical areas have been shown to communicate with each other (de Curtis & Biella, 2002); stimulation of the entorhinal cortex elicits an electrophysiological response in the perirhinal cortex and this projection from the entorhinal to the perirhinal cortex can sustain short-term synaptic plasticity in the form of PPD (Garden et al., 2002). Stimulation of the perirhinal cortex can also generate fEPSPs in the entorhinal cortex and this projection is capable of sustaining LTP (Ivanco & Racine, 2000).
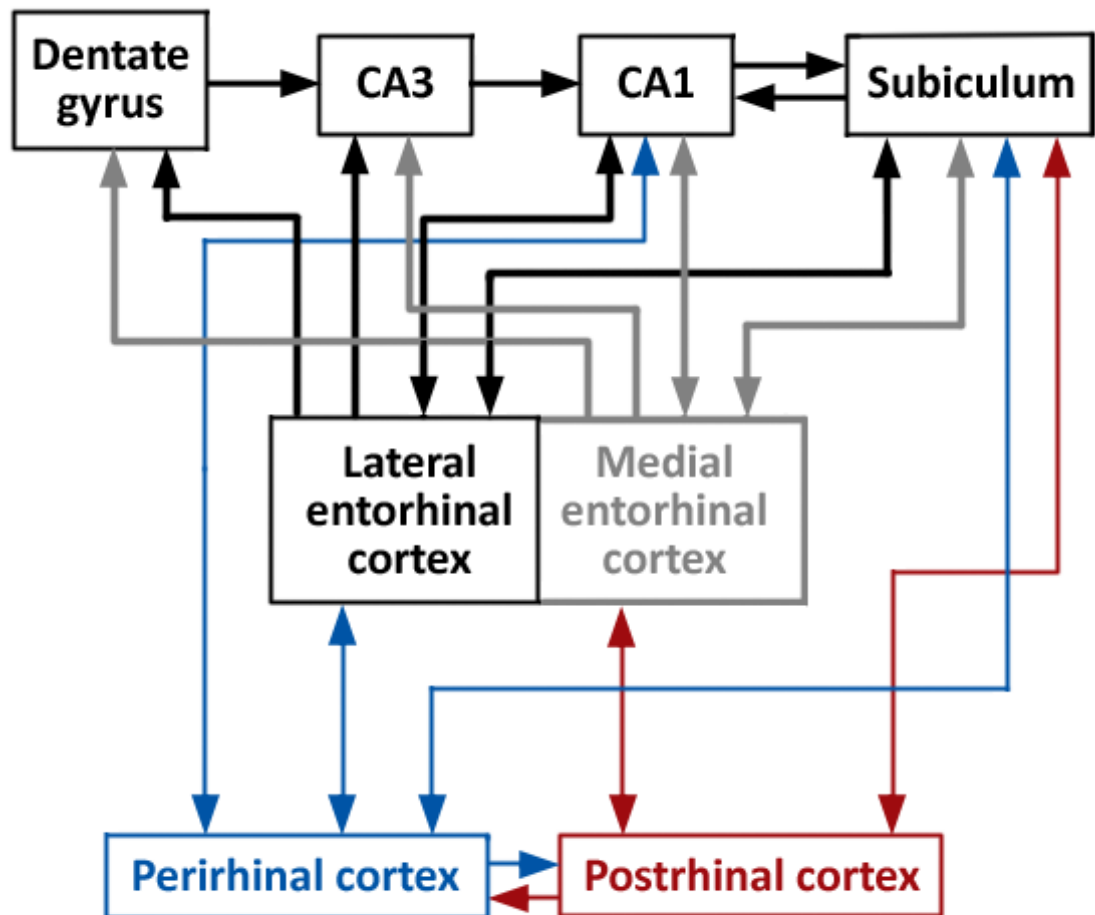
Figure 1.6: Schematic diagram showing the main projections within the hippocampal-parahippocampal circuit. Adapted from Witter et al. (2000) with supplemental information from Swanson & Cowan (1977) and Burwell (2000).

In addition to being connected to each other, these cortical areas also show large amounts of interconnectivity with the hippocampus. As described in section 1.2, the hippocampus forms reciprocal connections with the perirhinal (Swanson & Cowan, 1977; Deacon et al., 1983; Kosel et al., 1983; Van Groen & Wyss, 1990; McIntyre et al., 1996; Kloosterman et al., 2003b), entorhinal (Steward, 1976; Steward & Scoville, 1976; Swanson & Cowan, 1977; Swanson et al., 1981; Wyss, 1981; Tamamaki & Nojyo, 1993; 1995; Ohara et al., 2009) and the postrhinal cortices (Kloosterman et al., 2003b). Many of these projections have been shown to be capable of eliciting an electrophysiological response in their respective tissues. Electrical stimulation of the entorhinal (Naber et al., 1999) and postrhinal cortices (Naber et al., 2001b) results in the generation of fEPSPs in area CA1 and the

subiculum. Furthermore, the projection from the entorhinal cortex to area CA1 can sustain NMDA receptor-dependent LTP following 100 Hz HFS (Remondes & Schuman, 2003). Additionally, the return projection from the hippocampus to the entorhinal cortex can also carry electrophysiological information (Ivanco & Racine, 2000; Kloosterman et al., 2003a; 2004). Moreover, the CA1 to entorhinal (Craig & Commins, 2005) and subiculum to entorhinal projections (Craig & Commins, 2006) are both capable of sustaining short- and long-term changes in synaptic plasticity. Both projections can sustain LTP or LTD depending on previous electrophysiological activity as both show a frequency-dependent metaplasticity (Craig & Commins, 2007).

While the presence of projections from the perirhinal cortex to the hippocampus has proven to be controversial (Canning & Leung, 1997; Liu & Bilkey, 1998b; Canning & Leung, 1999; Witter et al., 1999; Canning et al., 2000), there is a large body of evidence to support the presence of direct perirhinal projections to the hippocampus. Stimulation of the perirhinal cortex can also result in the generation of fEPSPs in the CA1/subiculum (Naber et al., 1999). This monosynaptic projection from the perirhinal cortex to the area CA1 can sustain LTP (Liu & Bilkey, 1996b) and this LTP is NMDA receptor-depedent (Liu & Bilkey, 1996a). The indirect, polysynaptic projection from the perirhinal cortex to the dentate gyrus has also shown to be capable of sustaining LTP in the freely moving rat (Ivanco & Racine, 2000). In addition to this direct projection to the hippocampus, the perirhinal cortex can also exert an indirect electrophysiological influence in area CA1 through the entorhinal cortex (Naber et al., 1999); stimulation of the perirhinal cortex leads to an enhancement of entorhinal-induced fEPSPs in the area CA1 (Liu & Bilkey, 1996b). The monosynaptic return projection from the hippocampus to the perirhinal cortex is also capable of synaptic plasticity; both 300 Hz HFS in the freely moving rat (Ivanco & Racine, 2000) and 100 Hz theta-burst stimulation in the anaesthetised rat (Cousens & Otto, 1998) of area CA1 can induce LTP in the perirhinal cortex. However, Ivanco and Racine (2000) observed that synaptic changes in the perirhinal cortex following HFS of CA1 took several sessions to induce, indicating that this projection may be resistant to activity-dependent changes in synaptic plasticity but the findings of Cousens and Otto (1998) appear to contradict this view as the LTP

observed during their experiment was robust and easily induced with their stimulation protocol.

This extensive anatomical and physiological interconnectivity within the hippocampal-parahippocampal network has lead to postulation over its role in learning and memory (see section 1.4 for a review of behavioural functions in this network). The relationship between the perirhinal and entorhinal cortices within the hippocampal-parahippocampal network is not entirely clear; anatomical evidence would suggest that they act as relay areas between the hippocampus and the rest of the neocortex (Deacon et al., 1983; McIntyre et al., 1996; Burwell & Amaral, 1998a; 1998b; Bucci et al., 2000; Burwell, 2000) and the initial physiological findings would support this (Muir & Bilkey, 1998; Canning et al., 2000; Ivanco & Racine, 2000; Naber et al., 2001a; Kloosterman et al., 2003a; 2004) but electrophysiological recordings made in both the perirhinal and entorhinal cortices of sleeping and awake rats do not reveal a clear pattern of neuronal function expected of a relay system (Pelletier et al., 2004). More recent evidence has lead to the idea of the perirhinal and entorhinal cortices acting as a gating system to selectively transfer information from the neocortex to the hippocampus (Iijima et al., 1996; Koganezawa et al., 2008). Evidence for this comes from the $GABA_A$- and frequency-dependent communication observed in the entorhinal cortex to hippocampus projection; although activity was observed within the entorhinal cortex, not all of this activity was relayed to the hippocampus (Iijima et al., 1996).

Focussing on the characteristics of the electrophysiological projections within the hippocampal-parahippocampal network, we propose that there is a segregation of inputs which either have tendency towards LTP (electrophysiologically excitatory) or towards LTD (electrophysiologically inhibitory) from the hippocampus to the parahippocampal region which may underlie the roles of these neocortical areas in associative learning (see section 1.4). Note that "excitatory" and "inhibitory" in this context must not be confused with those terms as they are traditionally used in describing excitatory and inhibitory neurotransmission. Evidence for the perirhinal cortex as an associative area with capabilities for both electrophysiologically excitatory and inhibitory activity comes cortical areas adjacent to the perirhinal cortex tend to be a mix of both whereas

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those from cortical areas located further away from the perirhinal cortex are largely electrophysiologically excitatory in nature (Martina et al., 2001). Additionally, associative electrophysiologically excitatory activity from the perirhinal cortex is propagated to the entorhinal cortex following input from the amygdala (Kajiwara et al., 2003). Moreover, previous research from our lab has also shown differences in electrophysiologically excitatory activity between hippocampal inputs to the entorhinal cortex. Inputs from the distal CA1 and proximal subiculum to the lateral entorhinal cortex are largely electrophysiologically excitatory in nature whereas conversely the projection from the proximal CA1 to the medial entorhinal cortex is largely electrophysiologically inhibitory in nature (Craig & Commins, 2007). Furthermore, the distal CA1 and proximal subiculum project to the perirhinal cortex whereas the proximal CA1 and distal subiculum project to the postrhinal cortex (Kloosterman, 2003b). Moreover, the perirhinal cortex shows greatest interconnectivity with the lateral entorhinal cortex whereas the postrhinal cortex shows greatest interconnectivity with the medial entorhinal cortex (Burwell & Amaral, 1998a; Burwell, 2000). Furthermore, perirhinal and postrhinal cortices differ in their connections with other parts of the neocortex; the perirhinal is largely connected with areas associated with olfactory and auditory information whereas the postrhinal cortex is largely connected with areas associated with visual and visuospatial information (Furtak et al., 2007b). Taking the anatomical and physiological evidence into account, we posit that the perirhinal cortex forms part of an electrophysiologically excitatory circuit along with the lateral entorhinal cortex and the postrhinal cortex forms part of an electrophysiologically inhibitory circuit along with the lateral entorhinal cortex. We predict that the projections to the perirhinal cortex from the distal region of area CA1 and the proximal region of the subiculum will both be electrophysiologically excitatory in nature whereas the projection from the proximal region of area CA1 to the postrhinal cortex will be electrophysiologically inhibitory in nature (Figure 1.7). We propose that these circuits underlie the associative properties of the parahippocampal region.
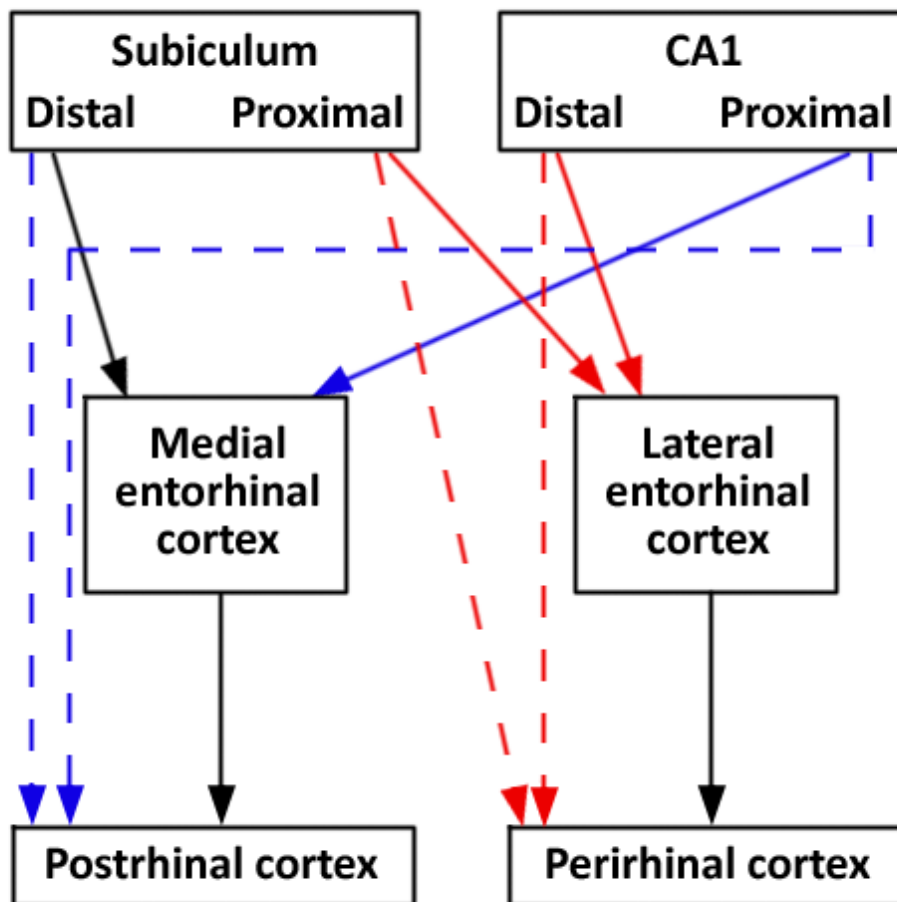
Figure 1.7: Proposed model of the segregation of electrophysiologically excitatory and inhibitory circuits in the hippocampal-parahippocampal network. Red arrows represent electrophysiologically excitatory projections and blue arrows represent electrophysiologically inhibitory projections. Dashed arrows of either colour represent the predicted electrophysiologically excitatory or inhibitory nature of a given projection. Black arrows are anatomically defined projections that have no electrophysiologically excitatory or inhibitory properties (in the case of the distal subiculum to medial entorhinal cortex projection; Craig, 2006) or have not yet been examined in detail. Based on the findings in Craig & Commins, 2007.

1.4 Function:

1.4.1 Functions of the perirhinal cortex:

The main behavioural roles identified for the perirhinal cortex are recognition memory (Mumby & Pinel, 1994; Liu & Bilkey, 2001; Mumby et al., 2002b; Winters & Bussey, 2005a; Hannesson et al., 2005; Mumby et al., 2007; Albasser et al., 2009), visual novelty detection (Zhu & Brown, 1995; Zhu et al., 1995b; 1997; Bogacz et al., 2001; VanElzakker et al., 2008) and temporal order memory (Hannesson et al., 2004; Barker et al., 2007). Although it is not required for learning spatial memory-dependent tasks like the Morris water maze (MWM; Machin et al., 2002; Burwell et al., 2004b; Moses et al., 2005) or the radial arm maze (Vann et al., 2000b; Machin et al., 2002; Moran et al., 2003), it has been implicated in other forms of spatial memory. For example, the perirhinal cortex has been implicated in object-in-place associative learning (Bussey et al., 2000; 2001; Barker & Warburton, 2008; 2009), spatial reference memory (Wiig & Bilkey, 1994; Abe et al., 2009) and long-term spatial memory (Glenn et al., 2003). The remainder of this section will review the evidence for the perirhinal cortex's role in object recognition memory and spatial memory. We will also evaluate the interactions between the perirhinal cortex and the hippocampus in these processes and we will review the molecular correlates that may implicate synaptic plasticity in the formation of these types of memory.

1.4.2 Object recognition memory:

Object recognition memory is an animal's capability to remember whether they have encountered an object before. The standard behavioural test for this form of memory is the object recognition task. In this task, animals are exposed to a set of objects in the training phase and in the testing phase, one of the objects from the training phase is replaced with a novel object. Animals should preferentially explore this novel object over the other familiar objects (Ennaceur & Delacour, 1988). Ennaceur and Delacour (1988) propose that the strength of this task is that it reflects spontaneous behaviour in the animal, therefore it is a "pure" memory test

without the need for any rule learning and as such, it is easily comparable across species. The validity of the task as a test of memory comes from initial studies which showed that nootropic drugs enhanced performance in the task (Ennaceur et al., 1989) and antagonism of cholinergic neurotransmission could cause a time-dependent impairment in object discrimination (Ennaceur & Meliani, 1992a). Unexpectedly, object recognition memory seemed to be spared following lesions of the fornix (Ennaceur & Aggleton, 1994). However, lesions of the perirhinal cortex resulted in impairments of the object recognition task (Ennaceur et al., 1996; Ennaceur & Aggleton, 1997). Recently, immediate early gene imaging studies have shown that the perirhinal cortex is activated following object recognition tasks (Wan et al., 1999; Aggleton & Brown, 2005; Warburton et al., 2005) and following the presentation of novel visual stimuli (Zhu et al., 1995b; 1996; 1997).

Molecular, pharmacological and physiological evidence have further strengthened the case for the importance of the perirhinal cortex in object recognition memory. Additionally, the case for synaptic plasticity being the physiological process underlying memory is also supported by these studies. Benzodiazepine-induced impairments in object recognition memory have been described, indicating that GABAergic neurotransmission is required for this form of learning (Longone et al., 1996; Bertaina-Anglade et al., 2006). In rats, administration of the benzodiazempine lorazepam impairs recognition memory and when applied to perirhinal slices, it also disrupts both LTP and LTD (Wan et al., 2004). Antagonism of L-type voltage-dependent Ca^{2+} channels (Seoane et al., 2009) and of CREB signalling (Warburton et al., 2005) have also been shown to impair perirhinal LTD and object recognition memory. Taken together, the evidence would suggest that long-term synaptic plasticity is involved in recognition memory formation.

There is much evidence to support this claim as glutamatergic signalling, crucial for synaptic plasticity (see section 1.3), is also required for object recognition memory (de Lima et al., 2005; Winters & Bussey, 2005b; Barker et al., 2006; Nilsson et al., 2007; Barker & Warburton, 2008). In rats, the perirhinal administration of AP-5 (Winters & Bussey, 2005b; Barker et al., 2006) or systemic administration of MK-801 (de Lima et al., 2005; Nilsson et al., 2007) impairs object recognition tasks performance by disrupting glutamatergic signalling via the NMDA receptor. Similar

pharmacological challenges of the NMDA receptor also disrupt perirhinal LTP and LTD (Ziakopoulos et al., 1999; Cho et al., 2000b; Cho & Bashir, 2002; Karasawa et al., 2008). The AMPA receptor has also been implicated in both object recognition memory (Winters & Bussey, 2005b) as well as baseline neuronal transmission (Park et al., 2006) and synaptic plasticity (Griffiths et al., 2008) in the perirhinal cortex. A role for the kainate receptor in object recognition memory (Barker et al., 2006) and perirhinal synaptic plasticity (Park et al., 2006) has also been proposed. Functional dissociations have already been shown between NMDA and kainate receptors; kainate receptors mediate recognition memory at short but not long delays whereas NMDA receptors mediate recognition memory at long but not short delays (Barker et al., 2006). As yet, there have been no studies investigating the role of perirhinal mGlu receptors in recognition memory but, bearing in mind their contribution to synaptic plasticity in the perirhinal cortex (McCaffery et al., 1999; Cho et al., 2000b; Cho & Bashir, 2002), we predict that interactions between perirhinal NMDA and mGlu receptors may underlie object recognition memory.

Simultaneous blockade of both NR2A and NR2B NMDA receptor subtypes in the perirhinal cortex lead to impairments an object recognition task (Barker et al., 2006). As the NR2A subunit is associated with LTP and the NR2B subunit is associated with LTD (Massey et al., 2004; Toyoda et al., 2005; Bartlett et al., 2007; Yashiro & Philpot, 2008), this implies that both perirhinal LTP- and LTD-like processes are required for the formation of object recognition memories. Furthermore, transgenic overexpression of the NR2B subunit in hippocampal and cortical neurons enhances performance in an object recognition task yet does not promote LTD in the area CA1 (Wang et al., 2009) which is a conflicting finding compared to the majority of research in this area. There are a large number of molecular mechanisms involved in object recognition memory that are correlated with LTD including L-type voltage-dependent Ca^{2+} channels (Seoane et al., 2009), CREB (Warburton et al., 2005), muscarinic cholinergic receptors (Warburton et al., 2003; Massey et al., 2008), GABA receptors (Wan et al., 2004) mGlu receptors (Barker et al., 2006; Moult et al., 2006), NMDA receptors (Cho et al., 2000b; Cho et al., 2002; Roberts et al., 2009; Winters et al., 2010), kainate receptors (Barker et al., 2006; Park et al., 2006; Holman et al., 2007) and AMPA receptor internalisation

(Griffiths et al., 2008). Therefore, although both LTP and LTD might be involved in object recognition memory, it would appear that an LTD-like process is the principle physiological mechanism underlying recognition memory.

1.4.3 The role of the perirhinal cortex in spatial memory:

Initial research into the roles of the hippocampus and the perirhinal cortex and their respective contributions to spatial memory portrayed a simple dissociation between the two areas; animals with perirhinal lesions are impaired on object recognition but not spatial memory tasks (Aggleton et al., 1997; Ennaceur & Aggleton, 1997; Glenn & Mumby, 1998; Bussey et al., 1999; Machin et al., 2002) and animals with hippocampal lesions are impaired on spatial but not object recognition memory tasks (Ennaceur et al., 1997; Mumby et al., 2002a). Double dissociation studies confirmed these findings showing that animals with hippocampal lesions showed impairments in recognition memory but not in spatial memory and those with perirhinal lesions showed impairments in spatial memory but not in recognition memory (Ennaceur et al., 1996; Bussey et al., 2000; Winters et al., 2004). This dissociation between hippocampal and perirhinal function was conserved across rat strains (Futter et al., 2006) and species (Pihlajamäki et al., 2004; Buckley, 2005; Köhler et al., 2005). Futhermore, immediate early gene imaging supports these lesion studies whereby c-Fos levels are increased in the hippocampus but not the perirhinal cortex during spatial learning tasks and they are increased in the perirhinal cortex but not the hippocampus during recognition tasks (Aggleton & Brown, 2005).

Yet, there are aspects of spatial memory that seem to require the perirhinal cortex (Aggleton & Brown, 2005) and situations involving spatial novelty may require dual activation of the hippocampus and the perirhinal cortex (VanElzakker et al., 2008). However, it has been shown that novel spatial locations for familiar objects (Jenkins et al., 2004) and temporal changes in spatial order (Amin et al., 2006) resulted in activation of the hippocampus and not the perirhinal cortex. This apparent confusion about perirhinal function may be explained by anatomical classifications; that different research groups use one of the two alternative

definitions of the perirhinal cortex (one that includes the postrhinal cortex as being part of the perirhinal cortex and the other that classifies the perirhinal and postrhinal cortices as separate regions, see section 1.2). Therefore, the variance in the literature may be due to reporting deficits associated with assigning postrhinal functions to the perirhinal cortex.

The majority of perirhinal lesion studies that spare the postrhinal cortex do not show deficits in spatial memory (Ennaceur et al., 1996; Ennaceur & Aggleton, 1997; Glenn & Mumby, 1998; Liu & Bilkey, 1998a; Machin et al., 2002; Moran & Dalrymple-Alford, 2003; Moses et al., 2005; Futter et al., 2006) but one study of perirhinal-only lesions has demonstrated deficits in object-place memory but not allocentric spatial memory (Bussey et al., 2001). Perirhinal lesion studies that do report spatial memory deficits also show some damage to the postrhinal cortex (Mumby & Glenn, 2000; Glenn et al., 2003) or also involve entorhinal cortex lesions (Nagahara et al., 1995). One combined perirhinal and postrhinal lesion study has reported impairments in spatial memory (Liu & Bilkey, 2001) but a number of other combined perirhinal and postrhinal lesion studies showed no deficits in spatial memory (Aggleton et al., 1997; Bussey et al., 1999; 2000; Winters et al., 2004). Functional differences between the perirhinal and postrhinal cortices have also been demonstrated; there is differential activity between the postrhinal and perirhinal cortices following spatial memory tasks (Vann et al., 2000a). Lesions of the postrhinal cortex do not result in substantial changes in place cell firing in the area CA1 (Nerad et al., 2009) whereas lesions of the perirhinal cortex result do cause disruption of CA1 place cell function (Muir & Bilkey, 2001). However, in this latter study there appeared to be some damage to the postrhinal cortex which prevents its role in spatial processing from being ruled out and as both of these investigations in parahippocampal effects on place cell function utilised different lesioning techniques which may have resulted in differential secondary effects in the hippocampus (Glenn et al., 2005). In spite of some contradictions, these studies indicate that the postrhinal cortex may form part of a separate functional circuit in the hippocampal-parahippocampal network compared to the perirhinal cortex (Aggleton et al., 2000; Furtak et al., 2007b; and see section 1.3.3 for physiological justification of this proposal). These perirhinal and postrhinal functional circuits

may govern different aspects of associative memory and as perirhinal cortex lesions more often than not spare spatial memory function, the postrhinal cortex may play a larger role in spatial memory. However, there does not appear to be a complete dissociation between the two cortical areas across all aspects of spatial memory.

The divergence in the literature as regards hippocampal and perirhinal contributions to spatial memory may also be due to the types of test utilised (Mumby & Glenn, 2000). Allocentric spatial memory tests like the MWM may exploit a completely different set of neuronal processes compared to tests of spatial memory that do not rely on navigation (Aggleton et al., 2000). Both the perirhinal and postrhinal cortex contribute to contextual associations for long-term memory (Burwell et al., 2004a) but this contextual association does not appear to be necessary for all forms of spatial learning; lesions of the hippocampus and the parahippocampal region suggest allocentric spatial memory can occur without the need for parahippocampal-dependent contextual associative learning (Burwell et al., 2004b) and rats with lesions of the perirhinal cortex are unimpaired in a delayed-matching-to-place task (Glenn & Mumby, 1998). Therefore, in studying the role of the perirhinal cortex in spatial memory, tasks that focus on the contextual properties and not on allocentric properties of spatial memory have been developed.

There are two main spatial variations of the object recognition task. In the object displacement task, animals are again exposed to a set of objects in the training phase but in the testing phase, instead of a novel object being introduced, one of the familiar objects is moved to a novel location where there was no object previously and animals should explore this moved object preferentially over the other objects (Ennaceur & Meliani, 1992b). Training regimes that can impair allocentric spatial memory can also impair performance in the object displacement task (Commins et al., 2003) and manipulation of distal and proximal cues can also result in object displacement task deficits (Craig et al., 2005). These findings suggest that the hippocampus may have a role in this type of spatial learning. This form of learning appears to require expression of neurotrophins (Calamandrei et al., 2002; Niewiadomska et al., 2006) and exercise-induced enhancement of task performance has been associated with increases in hippocampal and perirhinal

BDNF (Griffin et al., 2009). Futhermore, successful performance of the task is dependent on glutamatergic signalling through NMDA (Roullet et al., 1996; Usiello et al., 1998; Larkin et al., 2008) and AMPA receptors (Roullet et al., 2001). A dopaminergic component has also been identified (Roullet et al., 1996; Mele et al., 2004) and interactions between these glutamate and dopamine systems in the nucleus accumbens have been implicated in the consolidation of this task (Ferretti et al., 2005).

In the object-in-place task, two familiar objects switch their positions in the testing phase (although, unlike the object displacement, they are still occupying positions where an object had been previously located in the training phase) and animals should explore these moved objects preferentially over the other objects (Bussey et al., 2000). Although no lesion studies have determined the roles of the hippocampus and perirhinal cortex in the object displacement task, perirhinal lesions lead to impairments on a radial arm maze variation of the object-in-place task (Bussey et al., 2001). Additionally, perirhinal NMDA receptors have been demonstrated to underlie short-term object-in-place memory (Barker & Warburton, 2008). However, these findings do not rule out hippocampal contributions to the object-in-place task. Lesions of the hippocampus cause impairments in a similarly designed task in the monkey (Gaffan & Harrison, 1989) and also in the rat (Bussey et al., 2000; 2001). Hippocampal lesions in the rat have also shown that the hippocampus does not contribute to object recognition nor object context memories but only to the spatial location where an object has been previously encountered (Piterkin et al., 2008).

These findings suggest that, although the perirhinal cortex and hippocampus are normally associated with object recognition memory and spatial memory respectively, the contributions of the hippocampus and parahippocampal region to spatial memory are not clearly defined. Further analysis of the hippocampal-parahippocampal network is required in order to fully determine the roles of these various cortical and subcortical areas in the various forms of spatial memory needed to solve these different tasks.

1.5 Objectives of this thesis:

The main objective of this thesis is to characterise the electrophysiological, molecular and pharmacological properties this projection from the area CA1 to the perirhinal cortex projection in the anaesthetised rat. As discussed above, this projection from the area CA1 of the hippocampus to the perirhinal cortex (Swanson & Cowan, 1977; Deacon et al., 1983; Van Groen & Wyss, 1990) can undergo changes in synaptic plasticity following 100 Hz theta-burst stimulation (Cousens & Otto, 1998). We will firstly examine this projection's ability to sustain short-term synaptic plasticity (in the form of PPF and PTP) and long-term plasticity (in the form of LTP) following 250 Hz HFS. We will also analyse tissue samples from the area CA1 and perirhinal cortex to determine whether BDNF and ERK play a role in synaptic plasticity in this projection. Furthermore, we will utilise drugs that target the NMDA and AMPA/kainate glutamate receptors to determine what form of LTP is being induced in the CA1 to perirhinal cortex projection.

To ascertain whether the CA1 to perirhinal cortex projection forms part of an electrophysiologically excitatory circuit within the hippocampal-parahippocampal network (see section 1.3.3), we will examine long-term synaptic plasticity utilising a range of stimulation frequencies. By using both low (1 Hz, 5 Hz and 10 Hz) and high (50 Hz and 100 Hz) stimulation frequencies, we hope to plot a BCM curve and determine the value for θ_m (the frequency at which the transition from LTD to LTP occurs). We hypothesise that the CA1 to perirhinal cortex projection will show similar frequency-dependent changes in synaptic plasticity as has been demonstrated in the distal CA1 to lateral entorhinal cortex projection, i.e. LFS should induce LTD and HFS should induce LTP (Craig & Commins, 2007).

The final objective of this thesis is to determine the functional roles of the hippocampus and perirhinal cortex in the behaving animal. In particular their roles in recognition memory (using an object recognition task) and spatial memory (using an object displacement task) will be analysed. To complement our electrophysiological experiments, we will perform concurrent molecular and pharmacological analyses of these behavioural tasks. Molecular analysis will involve measuring BDNF levels in the hippocampus and perirhinal cortex where we expect

to see upregulation of BDNF in the hippocampus following spatial learning and upregulation in the perirhinal cortex following recognition learning. Pharmacological blockade of NMDA and AMPA/kainate glutamate receptors is expected to impair learning and we expect to find impairments in memory that correlate with any deficits we observe in synaptic plasticity following identical pharmacological intervention.

Chapter 2

Electrophysiological characterisation of the CA1 to perirhinal cortex projection: Depth profile, paired-pulse facilitation and long-term potentiation.

Abstract:

In order for consolidation of memories in the neocortex to occur, the hippocampus must be able to communicate with the neocortex. A number of projections have been identified but not all have been electrophysiologically characterised. The projection originating in area CA1 of the hippocampus and terminating in the perirhinal cortex has been shown to undergo changes in synaptic strength following stimulation of the area CA1. A stimulating electrode was inserted into the area CA1 and a recording electrode was inserted into the perirhinal cortex of urethane-anaesthetised Wistar rats (n = 10). Baseline recordings were made for 10 min by stimulating area CA1 (0.05 Hz stimulation protocol). High-frequency stimulation (HFS; 250 Hz) was performed and post-HFS fEPSP recordings were made for 1 hr (0.05 Hz, as above). In the baseline condition, PPF was observed at shorter intervals but not at longer intervals. 250 Hz HFS induced long-term potentiation (LTP) which lasted 1 hr. Post-HFS PPF recordings showed no significant changes compared to baseline PPF. We also further characterised the electrophysiological properties on the CA1 to perirhinal cortex projection by compiling depth profiles for the stimulating and recording electrodes. These findings confirm those from a previous study in that the CA1 to perirhinal cortex projection can sustain short- and long-term changes in synaptic strength. In addition, the PPF data suggests that the LTP observed is largely postsynaptic in nature. This supports current theories of memory consolidation as it allows for the hippocampus to communicate directly with the perirhinal cortex.

2.1 Introduction:

The hippocampus is a subcortical area of the brain that plays a crucial role in the formation of memories (Squire, 1992). The hippocampal formation can be divided into a number of subregions, namely the dentate gyrus (DG), CA1, CA3 and the subiculum. Area CA1 is the major output structure of the hippocampus and it projects to the subiculum and the entorhinal, perirhinal and postrhinal cortices (Swanson & Cowan, 1977; Witter et al., 1989; Van Groen & Wyss, 1990; Tamamaki & Nojyo, 1995; Naber at al., 2001a). The projections from area CA1 to the subiculum (Taube, 1993; Stewart, 1997; Commins et al., 1998), the entorhinal cortex (Craig & Commins, 2005; 2006) and the perirhinal cortex (Cousens & Otto, 1998) have been shown to be capable of changes in synaptic plasticity. The area CA1 receives reciprocal projections from the entorhinal (Naber et al., 2001a) and perirhinal cortices (Kosel et al., 1983). In addition, these reciprocal projections from the entorhinal cortex (Remondes & Schuman, 2003) and perirhinal cortex (Naber et al., 1999) have also shown to be capable of sustaining long-term potentiation (LTP).

Electrophysiologically active projections in the brain can sustain short- and long-term changes in synaptic strength. Short-term plasticity has been described in forms of paired-pulse facilitation (PPF) and post-tetanic potentiation (PTP). PPF is a presynaptic event whereby the first stimulus from the pair of stimuli primes the presynaptic neuron for firing (Foster & McNaughton, 1991), thus leading to facilitation of the second stimulus via a Ca^{2+}-dependent mechanism (Zucker, 1974; Thomson et al., 1993). Increased concentrations of Ca^{2+} in the presynaptic nerve terminal following the first stimulus results in greater amounts of Ca^{2+} present in the nerve terminal following the second stimulus (Katz & Miledi, 1970; Thomson, 2000). This, in turn, may lead to an increase in the amount of neurotransmitter in each quantum being released into the synapse (Foster & McNaughton, 1991) and thus a facilitation effect is seen following the second stimulus once it occurs within a number of milliseconds; the exact time

depending on the particular synapse being stimulated (Mallart & Martin, 1967; Thomson, 1997).

PTP, on the other hand, is a form of short-term plasticity lasting in the range of seconds to minutes (Liley, 1956; Hubbard, 1963). The enhancement of synaptic strength is believe to be as a result of mobilisation of reserve neurotransmitter vesicles following high-frequency stimulation in a Ca^{2+}-dependent manner (Landau, 1969; Stanley, 1997; Kasai & Takahashi, 1999). Unlike PPF, PTP requires a strong input in the form of high-frequency or tetanic stimulation in order to be evoked.

LTP is a form of long-term synaptic plasticity where high-frequency stimulation results in a long-lasting increase in synaptic strength (Bliss & Lømo, 1973). It is currently the accepted model for the physiological basis of memory as it is a Hebbian form of learning; repeated activation of a neuron by another leads to a strengthening of the connection between the two neurons (Hebb, 1949). This relatively simple hypothesis has been refined in the last 60 years to the synaptic plasticity and memory (SPM) theory that identifies activity-dependent synaptic plasticity as the as the process by which memories are encoded (Martin et al., 2000). LTP is usually observed over the course of hours but has been reported to last up to a year in one animal (Abraham, 2003). The mechanisms underlying LTP are different to those found in short-term plasticity (McNaughton, 1982) and involve a combination of pre- and postsynaptic components (Schultz et al., 1994; Isaac et al., 1995; Blundon & Zakharenko, 2008).

The perirhinal cortex provides excitatory inputs to the area CA1 (Naber et al., 1999). It can also connect with the hippocampus via the entorhinal cortex owing to the presence of reciprocal connections between the perirhinal and entorhinal cortices (Burwell and Amaral, 1998a; 1998b; Burwell, 2001), the entorhinal cortex is in turn connected to the hippocampus directly (Deacon et al., 1983; Kloosterman et al., 1999). A direct connection from area CA1 to the perirhinal cortex has been described in tracing studies (Swanson & Cowan, 1977; Deacon et al., 1983; Van Groen & Wyss, 1990; Naber et al., 1997) but there has been little research into the electrophysiological properties of this projection. To date, a single study (Cousens &

Otto, 1998) has identified the CA1 to perirhinal cortex projection as a monosynaptic projection and that stimulation of the area CA1 following a theta-burst stimulation (TBS; 100 Hz HFS delivered in bursts) protocol has been shown to induce LTP which can in turn be reversed using theta-pulse stimulation (TPS; 5 Hz) protocol.

While Cousens and Otto (1998) demonstrated LTP in the CA1 to perirhinal cortex projection, the aim of this experiment is to expand on this work and further characterise the electrophysiological properties of the CA1 to perirhinal cortex projection and attempt to investigate the abilities of the projection to sustain both short- and long-term synaptic plasticity. We will provide a depth profile of the CA1 to perirhinal cortex projection, we will characterise PPF over a range of interpulse intervals (IPIs) and we will describe PTP following high-frequency stimulation to examine short-term plasticity. We will also study LTP using a 250 Hz HFS protocol in order to explore long-term plasticity in this projection. We hypothesise that the CA1 to perirhinal projection can sustain PPF at lower IPIs and that the projection can sustain both PTP and LTP following HFS.

2.2 Methods:

2.2.1 Surgery:

Adult male Wistar rats (n=10; approximately 3 months old; weight: 300-400 g; Biomedical Unit; University College Dublin) were anaesthetised using urethane (ethyl carbamate; 1.5 mg/kg; i.p.; Sigma) and mounted on a stereotaxic frame. An incision was made to expose the skull and burr holes were made to allow the electrodes to be inserted into the correct coordinates (Paxinos & Watson, 2005).

A stainless bipolar stimulating electrode (50 µm diameter), insulated apart from the tip, was aimed at area CA1. Stainless wire recording electrodes (50 µm diameter) were aimed at the perirhinal cortex. Coordinates for both electrodes were based on the work of Cousens and Otto (1998); the stimulating electrode (CA1) was inserted 6.3 mm posterior to Bregma, 5.5 mm lateral to the midline and lowered to a depth of 2.2 mm measured from the surface of the brain. The recording electrode (perirhinal cortex) was inserted 5.2 mm posterior to Bregma, 4.3 mm lateral to the midline and lowered to a depth of 5.0 mm measured from the surface of the brain. The recording electrode was inserted at an angle of 17° to increase the chances of hitting the correct population of cells that are part of the monosynaptic CA1 to perirhinal projection described by Cousens and Otto (1998).

2.2.2 Stimulation and data acquisition:

Signals were filtered between 1 Hz and 1 kHz and then amplified (DAM-50 differential amplifier, World Precision Instruments, Hertfordshire, UK). Recordings were digitised using a PC running Spike2 (version 5.02, CED, Cambridge, UK) connected to a CED-1401 interface (CED, Cambridge, UK). In analysing the field excitatory postsynaptic potentials (fEPSPs), the slope of the response was measured (Gooney & Lynch, 2001). The slope

was calculated from the middle two-thirds of the downward-deflecting component of the response.

Depth profiles were recorded before data acquisition. In order to record a depth profile for the stimulating electrode, the recording electrode was slowly lowered into the perirhinal cortex and allowed to settle for 10 min (as detailed in section 2.1 above). The stimulating electrode was then lowered in 100 μm steps into the area CA1. At each step, two 1 mA stimuli were delivered with a 20 s interpulse interval (IPI). The second stimulus in each case was recorded, the first acting as a test only. This procedure was repeated until the maximal fEPSP was recorded. Similarly, a depth profile for the recording electrode was recorded by firstly lowering the stimulating electrode into the area CA1 and allowing it to settle for 10 min (as detailed in section 2.1 above). The recording electrode was then lowered into the perirhinal cortex in the same manner as above.

Baseline paired-pulse facilitation (PPF) effects were measured after the electrodes were allowed to settle for 10 min. Six different IPIs were used: 20 ms, 40 ms, 60 ms, 120 ms, 240 ms and 480 ms. Six pairs of stimuli were delivered at each IPI (i.e. six pairs at 20 ms followed by six pairs at 40 ms and so on) with a 30 s interval between each pair of stimuli. The PPF value was calculated by taking the value of six slopes values from the first stimuli out of the pairs (fEPSP1) for a given IPI and then normalising the average of six slope values from the second stimuli from the pairs (fEPSP2) with the resulting value expressed as a percentage (Commins et al., 1998).

After recording baseline responses to single stimuli (0.05 Hz) over 10 min, LTP was induced using a high-frequency stimulation (HFS) protocol. The HFS protocol consisted of three trains of stimuli (each lasting 200 ms) at 250 Hz with an inter-train interval of 30 s (Gooney et al., 2002). Following HFS, recordings were made for an hour at 0.05 Hz. Following this hour of recording, post-HFS PPF recordings were made using the same protocol as the pre-HFS PPF recordings.

2.2.3 Statistical analysis:

A series of dependent t-tests and repeated measures analyses of variance (ANOVAs) with the appropriate *post-hoc* test (Tukey at the 5 % level of significance) were used. A star-rated system was used where appropriate (*$*p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

2.2.4 Histology:

After each experiment, all rats were sacrificed by decapitation and their brains were immediately removed and stored in 4 % formaldehyde (Sigma) in 0.1 M sodium phosphate buffer. The brains were then sectioned into 50 μm coronal slices using a vibrating microtome (VibroSlice, Campden Instruments Ltd., UK). The slices were mounted on glass slides and stained with cresyl violet (Sigma) before being examined under a light microscope to confirm the final positions of the electrodes. Composite drawings summarising all electrode placements were produced using Paxinos and Watson (2005).

2.2.5 Ethical considerations:

Laboratory procedures for the maintenance and experimentation of animals conformed to the Department of Health (Ireland) guidelines and the European directive 86/609/EC. Every effort was made to minimise the suffering and the number of animals used in this study.

2.3 Results:

2.3.1 Basic properties of fEPSP responses in the CA1 to perirhinal cortex projection:

An initial characterisation of the evoked response obtained in the perirhinal cortex following a single pulse stimulation of CA1 delivered at 0.05 Hz over 10 min (n=10) revealed a mean amplitude of -6.484 mV (±0.197), a mean latency of peak amplitude of 7.456 ms (±0.259) and a mean slope of the downward deflection of -1.306 mV/s (±0.049). Although we found a significant increase in both fEPSP peak amplitude and slope during potentiation and facilitation (see below) we decided to limit our analyses to the slope measures (rather than the amplitude) consistent with the work of Cousens and Otto (1998) and Gooney and Lynch (2001).

2.3.2 Histology and depth profile:

In order to confirm that the electrodes were lowered into the correct locations in the rat brain, slices were examined to determine the location of the electrode tracts. The tracts for the stimulating electrode were found to terminate mainly in the pyramidal layer of area CA1 (Figure 2.1(a)) and those for the recording electrode were found to terminate in the perirhinal cortex, mainly in the deep layers thereof (Figure 2.1(b)).

To further characterise the electrophysiological properties of the CA1 to perirhinal cortex projection, depth profiles were performed for both the stimulating and recording electrodes. The stimulating electrode was lowered slowly and gradually towards area CA1 while the recording electrode remained in place in the perirhinal cortex (Figure 2.2 (a); left panel). No response was elicited from single pulse stimuli at the surface or 1.0 mm below surface (in the visual cortex). At 1.5 mm below the surface, a small downward deflection was observed as the stimulating electrode reached the deep cerebral white matter (-2.786 mV) and the size of the downward deflection increased at 2.0 mm (in the pyramidal layer of CA1; -4.542 mV) before finally

maximising at 2.2 mm in the septal region of area CA1 (-5.987 mV; Figure 2.2 (a); right panel).

The depth profile for the recording electrode was similarly detailed. With the stimulating electrode in place in area CA1, the recording electrode was lowered into the perirhinal cortex at an angle of 17° (Figure 2.2 (b); left panel). An initial small downward deflection was observed just below the surface and at 1.0 mm, 2.0 mm and 3.0 mm below the surface as the electrode was lowered through the visual cortex (-4.903 mV), deep cerebral white matter (-4.139 mV) and area CA2 of the hippocampus (-4.164 mV) respectively. At 4.0 mm the downward deflection decreased in size as the electrode reaches the border between the deep cerebral white matter and the ventral region of the secondary auditory cortex (-2.720 mV). The fEPSP was at its lowest amplitude at 4.6 mm deep in the ventral temporal association cortex (-2.138 mV) and maximising at 5.0 mm in the deep layers of the perirhinal cortex (-6.062 mV; Figure 2.2 (b); right panel).
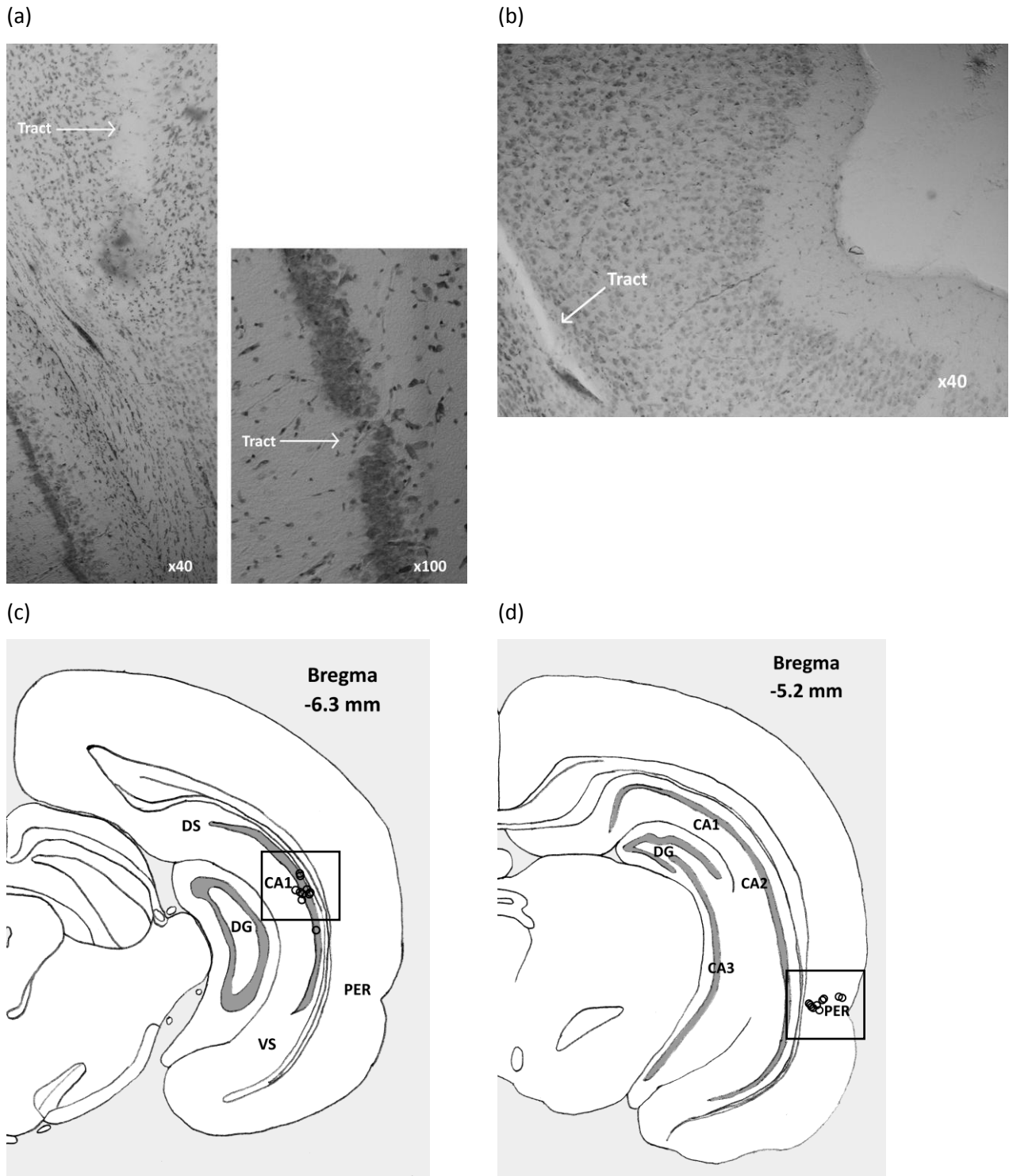
Figure 2.1: Representative coronal sections stained with cresyl violet showing electrode tracts of stimulating sites in (a) CA1 and (b) the perirhinal cortex. Drawings of approximate final positions of all electrode sites in (c) CA1 and (d) the perirhinal cortex (n=10).

(a)
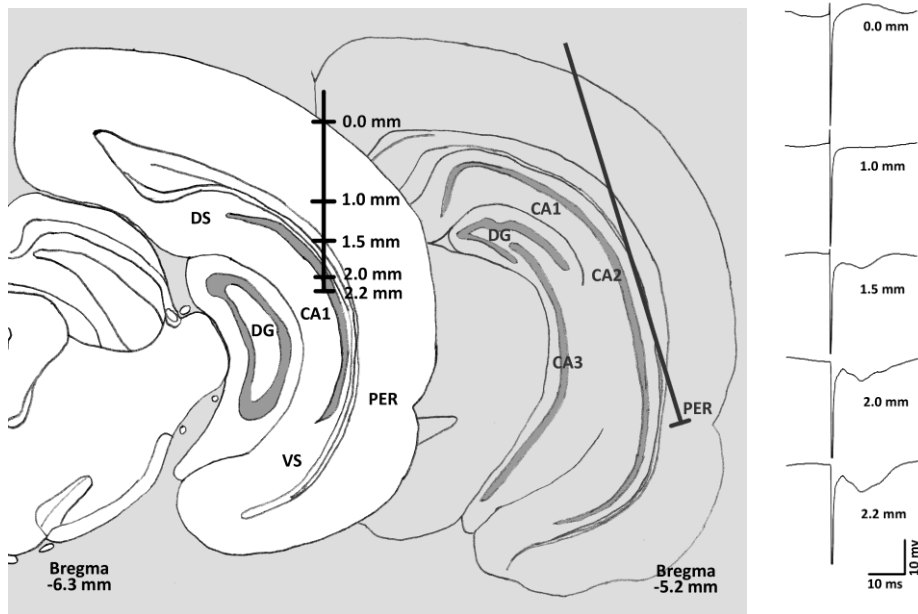
(b)
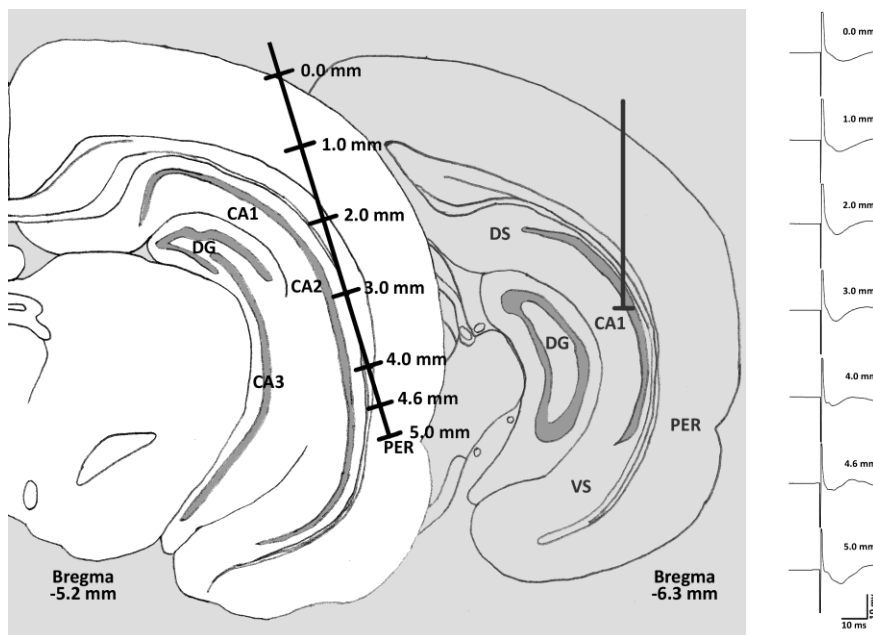
Figure 2.2: Depth profiles for the stimulating electrode and the recording electrode. (a) The recording electrode was lowered directly into the perirhinal cortex and the stimulating electrode was lowered incrementally into the area CA1, recording at each step. (b) Similarly the stimulating electrode was lowered directly into the area CA1 and the recording electrode was lowered incrementally into the perirhinal cortex. Sample fEPSP traces are given for the respective electrode depths. Drawings adapted from Paxinos and Watson (2005).

2.3.3 Baseline paired-pulse facilitation:

Having described the basic characteristics of the CA1 to perirhinal cortex projection, we then examined short-term plasticity in the projection. After allowing both electrodes to settle into their respective places and prior to 250 Hz HFS, PPF recordings were made at six different interpulse intervals (IPIs; 20 ms, 40 ms, 60 ms, 120 ms, 240 ms and 480 ms) and averaged over six trials (Figure 2.3 (a)). The values for PPF were determined by taking the average of the six slope values for Pulse 1 (the first fEPSP recording in each pair of stimuli for each IPI) and normalising the average of the six slope values for Pulse 2 (the second fEPSP in each pair of stimuli for each IPI). A 2 x 6 repeated measures ANOVA confirmed that there was a significant effect for interval (F = 14.819; df = 5, 45; p < 0.001) and a significant effect for facilitation (Pulse 1 vs. Pulse 2; F = 21.521; df = 1, 45; p < 0.001). There was also a significant interaction effect found for interval and facilitation (F = 14.819; df = 5, 45; p < 0.001).

Following a number of dependent t-tests, significant facilitation was found at the 20 ms (t = -3.748; df = 9; p < 0.01), 40 ms (t = -5.047; df = 9; p < 0.001), 60 ms (t = -7.084; df = 9; p < 0.001), 120 ms (t = -2.687; df = 9; p < 0.05) and 480 ms intervals (t = 2.799; df = 9; p < 0.05) but not at the 240 ms interval (t = -2.687; df = 9; p > 0.05; Figure 2.3 (a)). Facilitation peaked at the 40 ms and 60 ms intervals and tapered out at longer intervals with no facilitation seen at the 240 ms interval and a significant depression observed at the 480 ms interval.

In Chapters 4 and 5, we found discrepancies in the PPF data at some of the intervals (in particular the 20 ms, 240 ms and 480 ms intervals). To determine whether the observed PPF at the 20 ms and 240 ms intervals and paired-pulse depression at the 480 ms interval were real, we pooled the baseline data from Chapters 2, 3 (recorded for but not shown in Chapter 3) and Chapter 4 and ran a 2 x 6 repeated measures ANOVA (as described in the first paragraph of this section). There were significant effects found for interval (F = 43.983; df = 5, 225; p < 0.001) and for facilitation (Pulse 1 vs. Pulse 2; F = 88.352; df = 1, 45; p < 0.001). There was also a significant interaction

effect found for interval and facilitation (F = 43.994; df = 5, 225; p < 0.001). Dependent t-tests revealed significant facilitation at the 20 ms (t = -6.957; df = 45; p < 0.001), 40 ms (t = -11.611; df = 45; p < 0.001), 60 ms (t = -11.347; df = 45; p < 0.001), 120 ms (t = -6.267; df = 45; p < 0.001) and 240 ms intervals (t = -2.404; df = 45; p < 0.05). Significant depression was found at the 480 ms interval (t = 2.644; df = 45; p < 0.05). Facilitation peaked at the 40 ms interval and tapered out at longer intervals with a significant depression observed at the 480 ms interval.
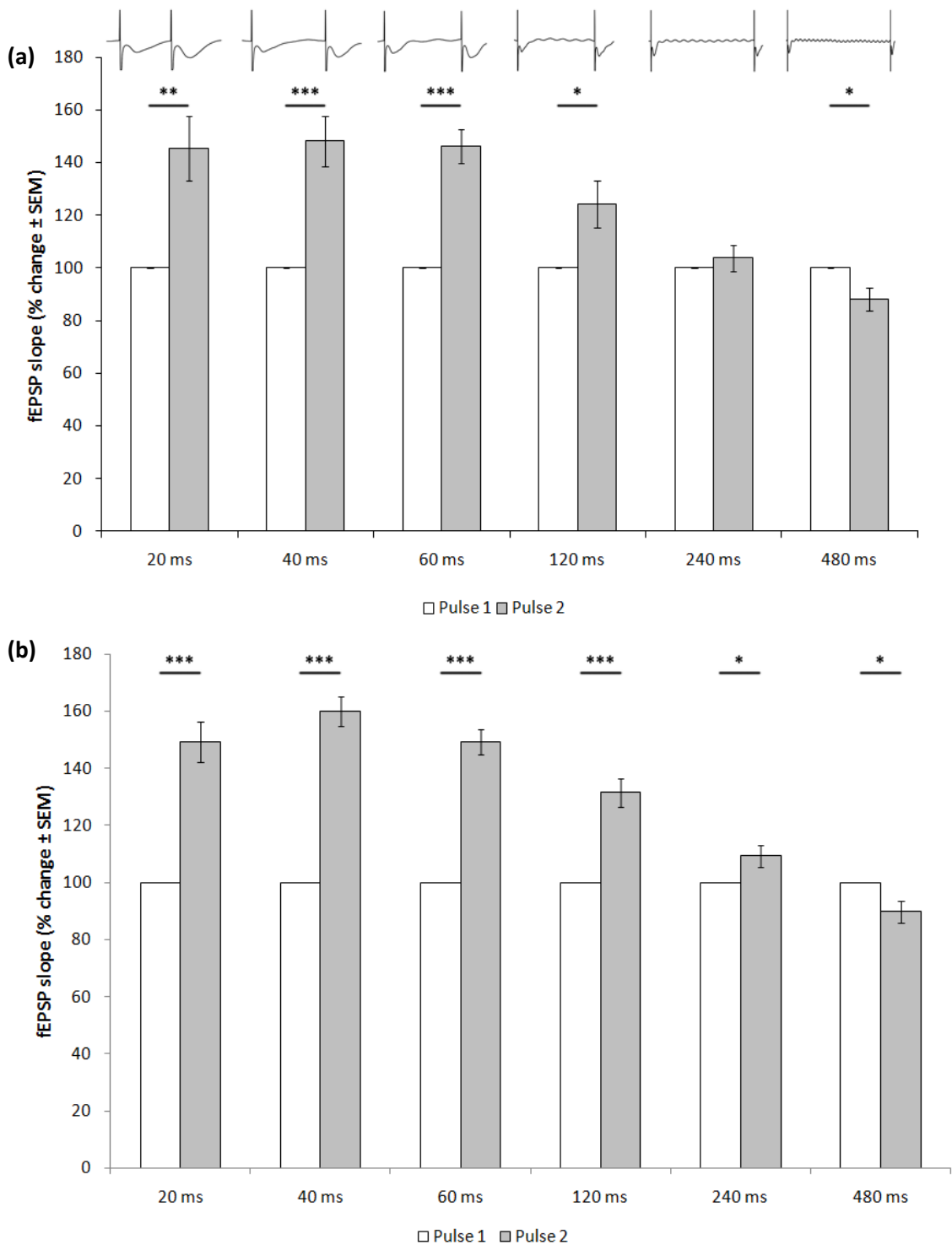
Figure 2.3: (a) Paired-pulse facilitation (PPF) and depression (PPD) at six interpulse intervals in the baseline condition (n = 10). PPF peaked at the 40 ms and 60 ms intervals and tapered off at longer intervals. Inset are representative fEPSP traces from each interval. (b) Pooled baseline PPF data (n = 46) which confirm that PPF peaks at the 40 ms interval and tapers off at longer intervals with PPD observed at 480 ms. *p < 0.05; **p < 0.01;***p < 0.001.

2.3.4 Effect of 250 Hz high-frequency stimulation on fEPSP strength:

Following baseline PPF recordings, we wanted to investigate the long-term plastic properties of the CA1 to perirhinal cortex projection. We initially recorded fEPSPs using a 0.05 Hz stimulation protocol (-10-0 min) before using a 250 Hz high-frequency stimulation protocol on area CA1 and then recording again at 0.05 Hz for 60 min (Figure 2.4). A repeated measures ANOVA with Bonferroni correction was used to analyse fEPSP slopes at four different times (-10-0 min, 0-10 min, 20-30 min and 50-60min). There was a significant effect for time (F = 108.034; *df* = 3, 27; *p* < 0.001) and *post-hoc* analysis revealed that, compared to baseline levels, fEPSP slopes were significantly higher in the 0-10 min (*p* < 0.001), 20-30 min (*p* < 0.001) and 50-60 min intervals (*p* < 0.001). In addition, it was found that the fEPSP slopes in the 0-10 min interval were significantly higher than in the 20-30 min (*p* < 0.01) and 50-60 min intervals (*p* < 0.001), indicating that PTP had been induced. Finally, as mean fEPSP slopes in the 0-10 min (135.914 % ±2.570) and 20-30 min (122.943 % ±1.037) were both above 120 %, they were classified as exhibiting LTP (see Chapter 1). The mean fEPSP slope value in the 50-60 min interval (113.478 % ±0.989) was lower than 120 % but remained significantly higher than baseline levels (see above).
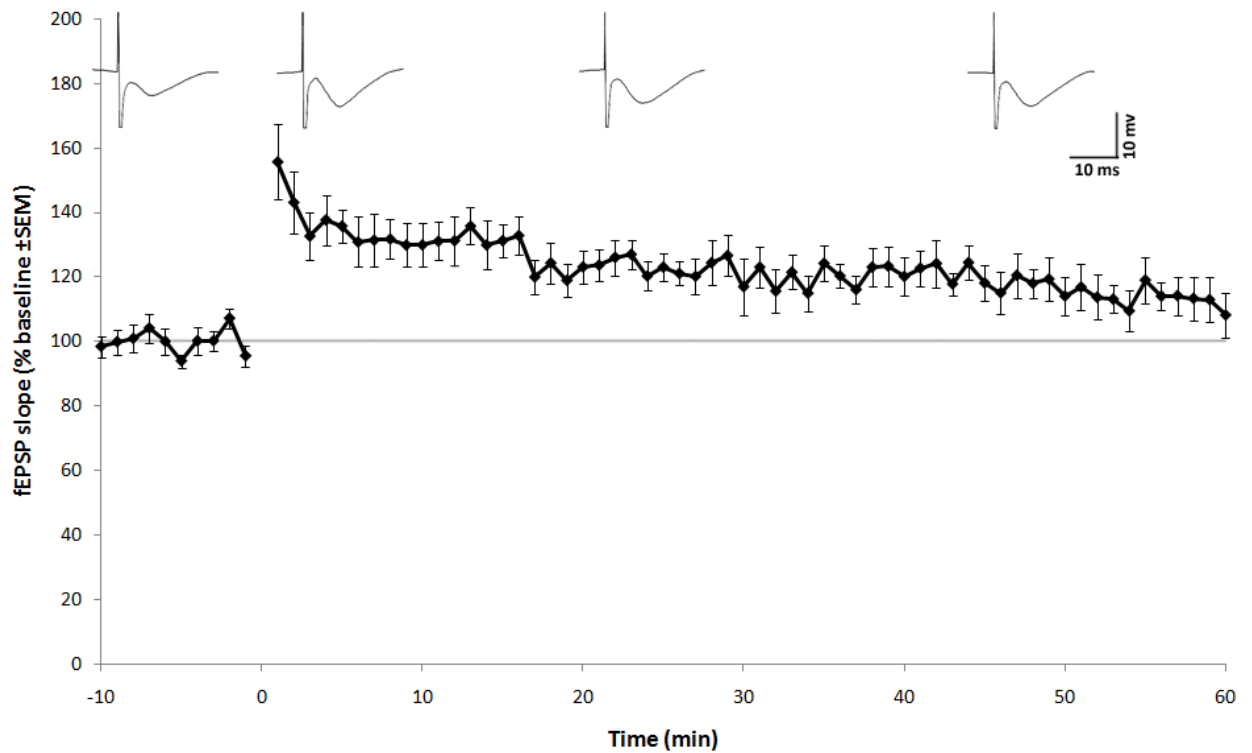
Figure 2.4: Induction of long-term potentiation (LTP) following 250 Hz high-frequency stimulation (HFS) in the CA1 to perirhinal cortex projection (n = 10). LTP lasted for at least 1 h, fEPSPs remaining significantly higher than baseline at all times. Each point represents an average of three fEPSP slopes recorded over 1 min and data is expressed as a percentage of baseline fEPSP slopes where baseline is approximately 100 %. Inset are representative fEPSP traces showing typical fEPSPs at baseline, 0-10 min, 20-30 min and 50-60 min.

2.3.5 Effect of 250 Hz HFS on paired-pulse facilitation:

One hour after 250 Hz HFS, PPF recordings were again made at the same six different IPIs (20 ms, 40 ms, 60 ms, 120 ms, 240 ms and 480 ms) and analysed in the same way as detailed in Section 2.3.2. As with the pre-HFS PPF recordings, a 2 x 6 repeated measures ANOVA confirmed that there was a significant effect for interval (F = 11.060; df = 5, 45; $p < 0.001$) and a significant effect for facilitation (Pulse 1 vs. Pulse 2; F = 18.766; df = 5, 45; $p < 0.01$). There was also a significant interaction effect found for interval and facilitation (F = 11.060; df = 5, 45; $p < 0.001$).

Following dependent t-tests, significant facilitation was again found at the 20 ms (t = -2.726; df = 9; p < 0.01), 40 ms (t = -10.711; df = 9; p < 0.001), 60 ms (t = -8.338; df = 9; p < 0.001), 120 ms (t = -3.218; df = 9; p < 0.05) and 480 ms intervals (t = 2.889; df = 9; p < 0.05) but not at the 240 ms interval (t = -0.731; df = 9; p > 0.05; Figure 2.5). Facilitation again peaked at the 40 ms and 60 ms intervals and tapered out at longer intervals with no facilitation seen at the 240 ms interval and again a significant depression observed at the 480 ms interval.

In order to compare the amounts of facilitation observed in the baseline condition (Pre-HFS) to those observed in the post-HFS condition (Figure 2.5), the average normalised slopes of all the recordings for Pulse 2 from each ISI in the pre-HFS group were compared to the average normalised slopes for Pulse 2 from the post-HFS group. A 2 x 6 repeated measures ANOVA revealed that there was a significant effect for interval (F = 17.528; df = 5, 45; p < 0.001) but there was no significant effect for time (pre-HFS vs. post-HFS; F = 1.107; df = 1, 45; p > 0.05) and no interaction effect between interval and time (F = 0.364; df = 5, 45; p > 0.05).
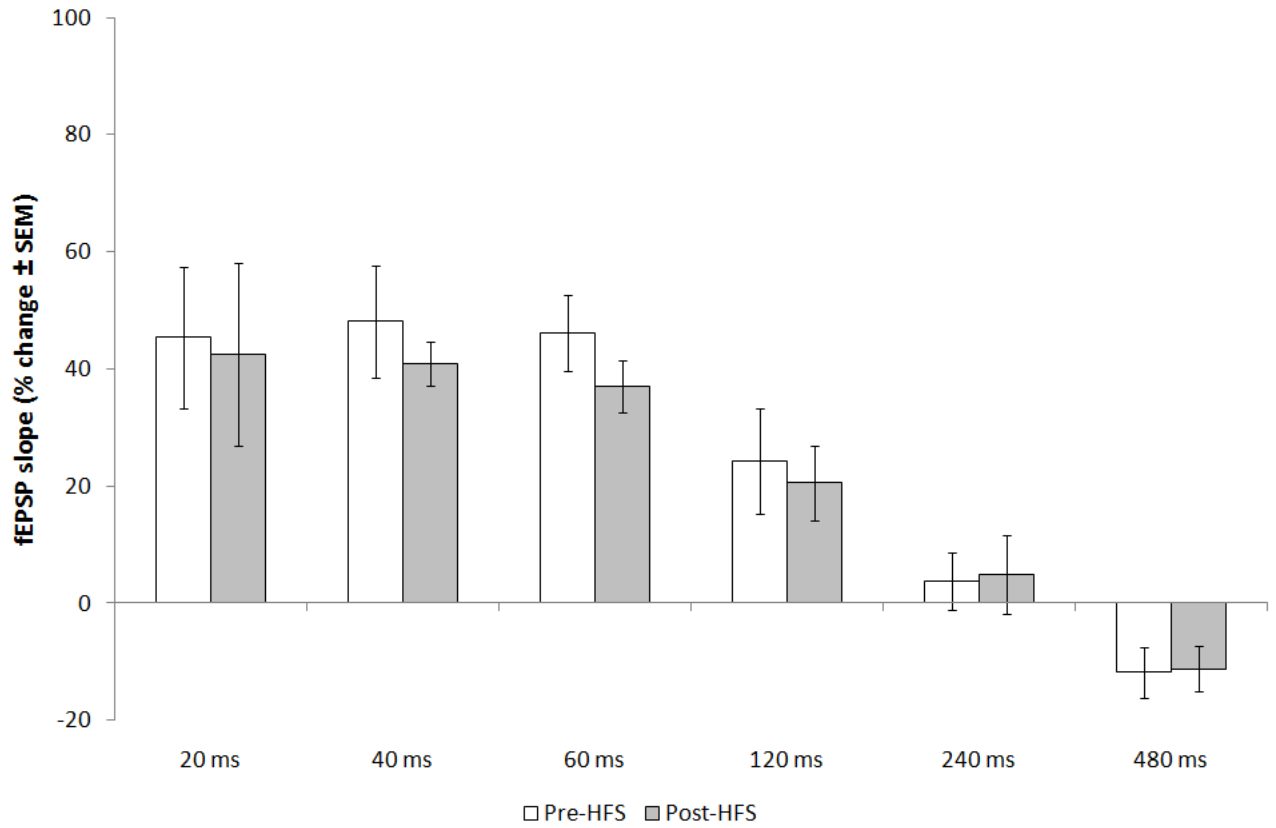
Figure 2.5: The effect of 250 Hz high frequency stimulation (HFS) on paired-pulse facilitation (PPF) at six interpulse intervals (IPIs; n = 10). PPF peaked at the 40 ms and 60 ms intervals and tapered off at longer intervals in both the pre-HFS and post-HFS conditions. There were no significant differences in facilitation between the pre- and post-HFS conditions.

2.4 Discussion:

The evidence from the experiment detailed above indicates that the CA1 to perirhinal cortex projection can sustain both short- and long-term changes in synaptic plasticity. This confirms the findings of Cousens and Otto (1998) where they found that this projection could sustain LTP following a TBS protocol. The ability of this projection to undergo plastic changes following electrophysiological activity suggests that the projection can transmit information from the area CA1 to the perirhinal cortex, providing another connection in the network between the hippocampal formation and the parahippocampal region (Witter et al., 2000a; 2000b).

In viewing this projection in the context of the hippocampal-parahippcampal network (Witter et al., 2000a; 2000b), it is important to determine to what extent the entorhinal cortex plays in modulating the flow of information from the hippocampus to the perirhinal cortex. The entorhinal cortex has traditionally been seen as being an intermediate in all hippocampal to perirhinal communication (Witter et al., 2000a) and the direct connection between the perirhinal cortex and the hippocampus is often left out of discussions on the hippocampal-parahippocampal network. However, the CA1 to perirhinal cortex projection has been shown to be a monosynaptic projection (Cousens & Otto, 1998) which takes the entorhinal cortex out of the equation. In addition, the mean latency of peak amplitude of fEPSPs in the CA1 to lateral entorhinal cortex projection is 11.45 ms (±0.61) and in the CA1 to medial entorhinal cortex projection is 16.82 ms (±1.38; Craig & Commins, 2005) which are both larger latencies compared to the 7.456 ms (±0.259) we have found in the CA1 to perirhinal cortex projection. This suggests that the responses observed here are the product of stimulating the area CA1 and it communicating with the perirhinal cortex directly rather than through the entorhinal cortex.

The two forms of short-term synaptic plasticity investigated in this study were PPF, a fast, Ca^{2+}-dependent form of presynaptic plasticity (Mallart & Martin, 1967; Zucker, 1974; Thomson, 1997) and PTP, a slower, Ca^{2+}-dependent form of presynaptic

plasticity (Liley, 1956; Landau, 1969; Stanley, 1997; Kasai & Takahashi, 1999). In this projection, PPF was observed at a number of IPIs with significant amounts of facilitation observed in the 20 ms, 40 ms, 60 ms and 120 ms intervals and no facilitation seen at longer intervals. PPF was strongest in the 20 ms, 40 ms and 60 ms intervals and the facilitation effect at 120 ms, although significant, was lower than the three shorter intervals. This peaking of facilitation with shorter intervals and tapering off with longer intervals is similar to the patterns of PPF seen in other projections. The CA1 to subiculum projection shows PPF at a range of IPIs from 10 ms to 500 ms with facilitation peaking in the 50 ms interval (Commins et al., 1998). In addition, the CA1 to entorhinal cortex projection shows PPF at IPIs ranging from 20 ms to 240 ms with facilitation peaking in the 60 ms interval (Craig & Commins, 2005).

A second form of short-term plasticity was detected following 250 Hz HFS; PTP was observed in the initial 10 min recording post-HFS. PTP is due to increased levels of neurotransmitter being made available presynaptically following sustained trains of tetani (Hubbard, 1963). fEPSP slope averages at this time were significantly higher than both baseline (-10-0 min) and later time points (20-30 min; 50-60 min) with PTP fEPSP slope averages being approximately 41 % higher than baseline levels. This agrees in part with the findings of Cousens and Otto (1998) who found PTP lasting 2 min in 25 % of their animals following TBS. The more common PTP in our study may be due to the higher frequency of stimulation used. However, 250 Hz HFS failed to induce PTP in the CA1 to entorhinal cortex projection (Craig & Commins, 2005; 2006). In addition, neither the CA1 to lateral entorhinal cortex nor the CA1 to medial entorhinal cortex exhibited PTP following 50 Hz or 100 Hz HFS (Craig & Commins, 2007). However, the extent to which pre- and postsynaptic processes play a role in plasticity in the CA1 to entorhinal cortex projection compared to the CA1 to perirhinal cortex projection may vary (see below).

Long-term synaptic plasticity in the form of LTP was observed following a 250 Hz HFS protocol with LTP lasting for up to 1 hr post-HFS. Overall, fEPSP slope averages remained significantly higher than baseline fEPSP slope averages for the entire

recording period (1 hr). After the initial period of PTP, fEPSP slope averages decreased compared to PTP but remaining significantly higher than baseline levels, fEPSP slope averages remaining approximately 22 % higher than baseline levels. Comparing these findings to those of Cousens and Otto (1998), levels of potentiation observed in our study following 250 Hz HFS were lower than those elicited by Cousens and Otto's use of TBS (22 % in our study compared to approximately 86 % in Cousens & Otto, 1998). As Cousens and Otto used TBS because of its similarity to theta rhythms observed in the behaving animal, it may be a more appropriate form of stimulation for the projection and be able to induce higher levels of LTP compared to 250 Hz HFS. Future studies should investigate the effects of different stimulation frequencies on LTP strength.

Finally, in order to determine the locus of change for LTP, we investigated the effects of 250 Hz HFS on short-term plasticity by comparing pre-HFS PPF recordings to post-HFS recordings. Changes in PPF strength following HFS are understood to reflect a presynaptic component to LTP (Schultz et al., 1994; Commins et al., 1998). Possible presynaptic modifications following HFS include changes in the size of quanta being released (Foster & McNaughton, 1991). Therefore, no change in PPF strength following HFS suggests that there is no significant or at least a minimal presynaptic component to the LTP we have observed. In this study, there were no significant differences in the amounts of facilitation observed in post-HFS PPF recordings compared to pre-HFS PPF recordings. This indicates that the LTP observed in the CA1 to perirhinal cortex is mainly postsynaptic in nature. This, along with the absence of PTP, is another difference between the CA1 to perirhinal cortex and CA1 to entorhinal cortex projections as post-HFS differences in PPF are seen in the latter case (Craig & Commins, 2005). This indicates that presynaptic processes play a larger role in LTP in the CA1 to entorhinal cortex projection and may explain why PTP is not observed in that projection but is in the CA1 to perirhinal cortex projection.

The theory that the hippocampus is an association area that outputs information to the neocortex for long-term storage (McClelland et al., 1995;

McClelland, 1998) requires hippocampal-cortical projections to be able to sustain changes in synaptic strength (Rolls, 1996; McClelland & Goddard, 1996). Therefore, the ability of the CA1 to perirhinal cortex projection to sustain short- and long-term synaptic plasticity supports the current theories of memory consolidation as it allows for transfer of information from the hippocampus to the parahippocampal region (Hasselmo & McClelland, 1999). It has been hypothesised that synaptic plasticity is the process by which initial encoding, storage and consolidation but not retrieval of memories occurs (Martin et al., 2000) and for that reason the ability of the CA1 to perirhinal projection to exhibit LTP may mean it is involved in consolidation of memories that are dependent on the perirhinal cortex, i.e. recognition memory (Liu & Bilkey, 2001; Winters & Bussey, 2005a; 2005b) and time-dependent aspects of spatial memory (Nagahara et al., 1995; Liu & Bilkey, 1998a; Ramos, 2002; Ramos & Vaquero, 2005).

In summary, in this study we have confirmed the findings of Cousens & Otto (1998) and demonstrated that the CA1 to perirhinal cortex can sustain both short-and long-term synaptic plasticity. This indicates that the area CA1 may signal directly to the perirhinal cortex during memory consolidation. In the following chapter, we investigate the molecular underpinnings of these changes in synaptic strength in order to further characterise the CA1 to perirhinal cortex projection.

Chapter 3

Electrophysiological characterisation of the CA1 to perirhinal cortex projection: Roles for brain-derived neurotrophic factor and extracellular signal-regulated kinase.

Abstract:

The CA1 to perirhinal cortex projection has been shown to sustain short- and long-term changes in synaptic strength. In other pathways, changes in synaptic strength have been shown to rely on intracellular signalling. Two molecules, namely brain-derived neurotrophic factor (BDNF) and extracellular signal-regulated kinase (ERK), have been both implicated in modulating changes in synaptic strength and they are both involved in learning and memory. In this study, we have attempted to characterise the molecular properties underpinning long-term potentiation (LTP) in the CA1 to perirhinal cortex projection by measuring BDNF and ERK levels. As in the previous experiment, LTP lasting 1 hr was induced by stimulating area CA1 and recording in the perirhinal cortex using a 250 Hz high-frequency stimulation (HFS) protocol. Tissue samples of area CA1 and the perirhinal cortex were then taken from both the stimulated and unstimulated (control) hemispheres of the brain. In each hemisphere, BDNF levels were assayed using an ELISA and ERK levels were assayed using western blot analysis. Area CA1 had significantly higher levels of BDNF compared to the perirhinal cortex ($p < 0.001$) but there were no significant differences observed when comparing stimulated to control hemispheres. There were no significant differences found for ERK and the effect sizes for stimulation were small. These findings do not rule out BDNF and ERK as modulators of synaptic strength in this pathway but as no significant findings were reported, their roles remain unidentified.

3.1 Introduction:

As discussed the preceding chapter, in order for the hippocampus to act as an association area for transferring information to the neocortex for long-term storage (McClelland et al., 1995; McClelland, 1998), any projections from the hippocampus to the neocortex must be capable of sustaining changes in synaptic strength (Rolls, 1996; McClelland & Goddard, 1996; Hasselmo & McClelland, 1999). We (Chapter 2) and others (Cousens & Otto, 1998) have demonstrated that the CA1 to perirhinal cortex projection can sustain both short- (paired-pulse facilitation, PPF, and post-tetanic potentiation, PTP) and long-term changes in synaptic plasticity (long-term potentiation, LTP). LTP is a long-lasting increase in synaptic strength (Bliss & Lømo, 1973; Abraham, 2003) following activity-dependent modifications of the presynaptic (Schultz et al., 1994) and postsynaptic nerve terminals (Isaac et al., 1995; Blundon & Zakharenko, 2008). One area of intense investigation over the last number of years is an attempt to correlate molecular changes that occur with LTP to changes in behaviour, especially learning. To date there has been limited research in examining the molecular changes that occur with LTP in the CA1 to perirhinal cortex projection. This chapter examines two specific molecules, brain-derived neurotrophic factor (BDNF) and extracellular signal-regulated kinase (ERK). Both have been strongly implicated in plasticity and memory processes.

Neurotrophins are a family of proteins that include BDNF, nerve growth factor (NGF), neurotrophin-3 (NT3) and neurotrophin-4 (NT4). BDNF (Barde et al., 1982) is a protein that is necessary for neuronal growth in the developing brain (see Klein, 1994 for review) and also plays a role in synaptic plasticity (Korte et al., 1995; Kang and Schuman, 1995a; 1995b; Bramham and Messaoudi, 2005; Lynch et al., 2007). BDNF signals via its specific receptor, the tropomyosin receptor kinase B (TrkB; Squinto et al., 1991, Klein et al., 1991; Bramham et al., 2008), or the low affinity neurotrophin receptor, p75 (Rodriguez-Tébar et al., 1990). Learning (Falkenberg et al., 1992b; Kesslak

et al., 1998; 2003; Mizuno et al., 2000; Gooney et al., 2002), exercise (Neeper et al., 1995; 1996; Widenfalk et al., 1999) and high-frequency stimulation of the brain (Patterson et al., 1992; Castrén et al., 1993; Dragunow et al., 1993; Gooney & Lynch, 2001) all give rise to increases in BDNF expression. For example, stimulation protocols that induce late phase LTP are known to correlate with sustained BDNF release, indeed, late phase LTP is impaired following disruption of BDNF-TrK interactions (Aicardi et al., 2004; Santi et al., 2006). In addition, BDNF knockout mice show significantly weaker LTP in area CA1 of the hippocampus compared to wild types (Korte et al., 1995). However, deficits in LTP in BDNF knockout mice can be reversed by the application of exogenous recombinant BDNF (Korte et al., 1996b; Patterson et al., 1996). Inhibition of Trk receptors prevents the induction of LTP in the dentate gyrus (Maguire et al., 1999). Blockade of the TrkB receptor in the hippocampus can also impair the induction of LTP following theta-burst stimulation (TBS) and post-tetanic blockade of the TrkB receptor disrupts LTP maintenance (Kang et al., 1997).

The mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway is an intracellular signalling cascade also implicated in the formation of memories (Bozon et al., 2003; Maher et al., 2006; Giovannini, 2006); learning (Crow et al., 1998; Atkins et al., 1998; Selcher et al., 1999; Walz et al., 1999) and high-frequency stimulation of the brain (Maguire et al., 1999; Gooney & Lynch, 2001) are associated with increases in total ERK or phospho-ERK (pERK). BDNF and ERK are interrelated, for example ERK can be activated (pERK) by BDNF-activated tyrosine kinases (Marsh et al., 1993; Hetman et al., 1999; McCarthy & Feinstein, 1999), pERK can subsequently activate cAMP response element-binding protein (CREB; Curtis & Finkbeiner, 1999; Davis et al., 2000) and CREB in turn can modulate transcription of other proteins, including BDNF (Finkbeiner et al., 1997; Tao et al., 1998; Shieh et al., 1998; Finkbeiner, 2000b). Long-term spatial memory is associated with increases in pERK levels in the CA1/CA2 field of dorsal hippocampus (Blum et al., 1999). Blum and colleagues (1999) also showed that inhibition of the MAPK cascade lead to an impairment of long-term spatial memory. Phosphorylation of ERK in the dentate gyrus

and entorhinal cortex is associated with consolidation of recognition memory whereas reconsolidation of recognition memory is associated with phosphorylation of ERK in area CA1 and the entorhinal cortex (Kelly et al., 2003). In addition to the MAPK/ERK cascade's role in learning and memory, it has also been found to be necessary for LTP induction (Liu et al., 1999; Wu et al., 1999). It has been suggested that LTP might be mediated by ERK signalling following TrkB activation by BDNF; inhibition of ERK by UO126 blocked BDNF-induced changes in glutamate release in the dentate gyrus (Gooney & Lynch, 2001). More specifically, the induction of hippocampal LTP has been shown to induce phosphorylation of ERK (Welsby et al., 2008). Furthermore, paradoxical sleep-induced deficits in LTP in dorsal CA1 are associated with concurrent deficits in pERK levels (Ravassard et al., 2009). Therefore it is likely that phosphorylation of ERK plays a role in LTP induction.

As suggested above, there has been limited research looking at the molecular correlates of LTP in the CA1 to perirhinal cortex projection and as such we have decided to examine both BDNF and ERK in this pathway. The aim of this experiment is to determine whether there are increases in BDNF, ERK and pERK in the CA1 to perirhinal cortex projection following 250 Hz HFS. We will induce LTP with 250 Hz HFS and then compare the unstimulated (control) and stimulated (250 Hz) hemispheres of the animals' brains by measuring levels of BDNF with an enzyme-linked immunosorbant assay (ELISA) and levels of ERK-2 and pERK by western blot analysis in the area CA1 and perirhinal cortex. We hypothesise that there will be higher levels of BDNF and pERK in the stimulated hemisphere compared to the unstimulated hemisphere of each animal.

3.2 Methods:

3.2.1 Surgery:

Adult male Wistar rats (n=6; approximately 3 months old; weight: 300-400 g; Biomedical Unit; University College Dublin) were anaesthetised using urethane (ethyl carbamate; 1.5 mg/kg; i.p.; Sigma) and mounted on a stereotaxic frame. A stimulating electrode was inserted into the area CA1 and a recording electrode was inserted into the perirhinal cortex as detailed in Chapter 2.

3.2.2 Stimulation and data acquisition:

Baseline fEPSP recordings, 250 Hz HFS and post-HFS fEPSP recordings were performed as detailed in Chapter 2.

3.2.3 Brain-derived neurotrophic factor enzyme-linked immunosorbant assay (ELISA):

After each experiment, all animals were sacrificed by decapitation and their brains were immediately removed. The brains were then dissected on ice. An area of approximately 3 mm^3 was removed from both area CA1 and perirhinal cortex that contained the final resting place of both the stimulating and recording electrode respectively. Equivalent sized areas in the unstimulated hemisphere were also removed for subsequent analysis. These samples were frozen and stored in Krebs-CaCl$_2$/dimethyl sulfoxide (DMSO) (Krebs solution was made using pre-prepared formula; all from Sigma, Ireland). All samples were later washed in Krebs-CaCl$_2$ and homogenised in 400 µl Krebs-CaCl$_2$. 10 µl aliquots of each sample were analysed using a Bradford protein assay (Bradford, 1976) in order to determine total protein concentration. Utilising these results, the sample solutions were normalised to be equal in concentration (1069.2 µg/ml for hippocampal samples and 135.6 µg/ml for

perirhinal cortex samples) and an enzyme-linked immunosorbent assay (ELISA) for BDNF was performed using a BDNF Emax$^©$ ImmunoAssay System kit (Promega Corporation, U.S.A.), following the manufacturer's protocol.

3.2.4 Western blotting:

Using the previously normalised tissue samples from the area CA1 and perirhinal cortex, 10 µl of each sample was added to 10 µl of Laemmli sample buffer (BioRad, U.S.A.) and boiled for 2 min. 10 µl of each sample solution was then loaded onto 10 % sodium dodecyl sulfate (SDS) polyacrylamide gels. A molecular weight marker was loaded onto the end lanes in each gel (Kaleidoscope Prestained Standards, BioRad, U.S.A.) in order to confirm the size of the target proteins. Proteins were separated by application of 55 mA constant current (for a double rig of two 10 % SDS gels) for 45 min. The proteins were transferred onto nitrocellulose membranes by application of a 350 mA constant current for 1 hr.

The nitrocellulose membranes were blocked overnight at 4° C with a solution of 6 % non-fat milk (Marvel, Cadbury, U.K.) in phospho-buffered saline-Tween (PBS-T; 0.005 % Tween-20, BioRad, U.S.A.). Following 4 x 15 min washes in PBS-T, the nitrocellulose membranes were incubated for 2 hr at room temperature with the respective primary antibody: mouse anti-ERK-2 IgG (Santa Cruz Biotechnology, U.S.A.; 1:1000 dilution in 2 % non-fat milk in PBS-T); mouse anti-pERK IgG (Santa Cruz Biotechnology, U.S.A.; 1:2000 dilution in 2 % non-fat milk in PBS-T); and mouse anti-β-actin (Santa Cruz Biotechnology, U.S.A.; 1:1000 dilution in 2 % non-fat milk in PBS-T). Following incubation with the primary antibody, the nitrocellulose membranes were given another 4 x 15 min washes in PBS-T. The nitrocellulose membranes were then incubated for 1 hr at room temperature with the secondary antibody, rabbit anti-mouse IgG-peroxidase conjugate (Sigma; 1:1000 dilution in 2 % non-fat milk in PBS-T for ERK-2 and β-actin; 1:6000 dilution in 2 % non-fat milk in PBS-T for pERK), for 1 hr at room temperature and again given 4 x 15 min washes in PBS-T. The proteins on the

nitrocellulose membranes were visualised with a chemiluminescent solution (ProteoQwest™, Sigma) and exposed to CL-Xposure X-ray film (Pierce) for 1 min for ERK and 10 min for pERK and β-actin. The exposed film was developed and fixed by hand before being scanned for analysis. The protein bands were quantitated by densitometric analysis using ImageJ software (NIH, U.S.A.). Nitrocellulose membranes were stripped with ReBlot Plus Strong stripping solution (Chemicon International, U.S.A.) before probing for the next protein.

3.2.5 Statistical analysis:

A series of dependent t-tests and repeated measures analyses of variance (ANOVAs) with the appropriate *post-hoc* test (Tukey at the 5 % level of significance) were used. A star-rated system was used where appropriate (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). In addition, effect sizes were calculated using Cohen's *d*.

3.2.6 Ethical considerations:

Laboratory procedures for the maintenance and experimentation of animals conformed to the Department of Health (Ireland) guidelines and the European directive 86/609/EC. Every effort was made to minimise the suffering and the number of animals used in this study.

3.3 Results:

3.3.1 Basic properties of fEPSP responses in the CA1 to perirhinal cortex projection:

An initial characterisation of the evoked response obtained in the perirhinal cortex following a single pulse stimulation of CA1 delivered at 0.05 Hz over 10 min (n=6) revealed a mean amplitude of -8.212 mV (±0.048), a mean latency of peak amplitude of 7.340 ms (±0.041) and a mean slope of the downward deflection of -1.795 mV/s (±0.019). Although we found a significant increase in both fEPSP peak amplitude and slope during potentiation we decided to limit our analyses to the slope measures consistent with the work of Cousens and Otto (1998) and the previous chapter.

3.3.2 Effect of 250 Hz high-frequency stimulation on fEPSP strength:

As in Chapter 2, we initially recorded fEPSPs using a 0.05 Hz stimulation protocol (-10-0 min) before using a 250 Hz high-frequency stimulation protocol on area CA1 and then recording again at 0.05 Hz for 60 min (Figure 3.1). A repeated measures ANOVA with Bonferroni correction was used to analyse fEPSP slopes at four different times (-10-0 min, 0-10 min, 20-30 min and 50-60min; Figure 3.1). There was a significant effect for time (F = 103.113; df = 3, 27; p < 0.001) with LTP lasting 1 hr post-HFS being observed. *Post-hoc* analysis revealed that, compared to baseline levels, fEPSP slopes were significantly higher in the 0-10 min (176.582 % ±5.871; p < 0.001), 20-30 min (139.278 % ±1.722; p < 0.001) and 50-60 min intervals (121.598 % ±0.830; p < 0.001). Furthermore, fEPSP slopes in the 0-10 min interval were significantly higher than in the 20-30 min (p < 0.001) and 50-60 min intervals (p < 0.001), indicating that PTP had occurred.
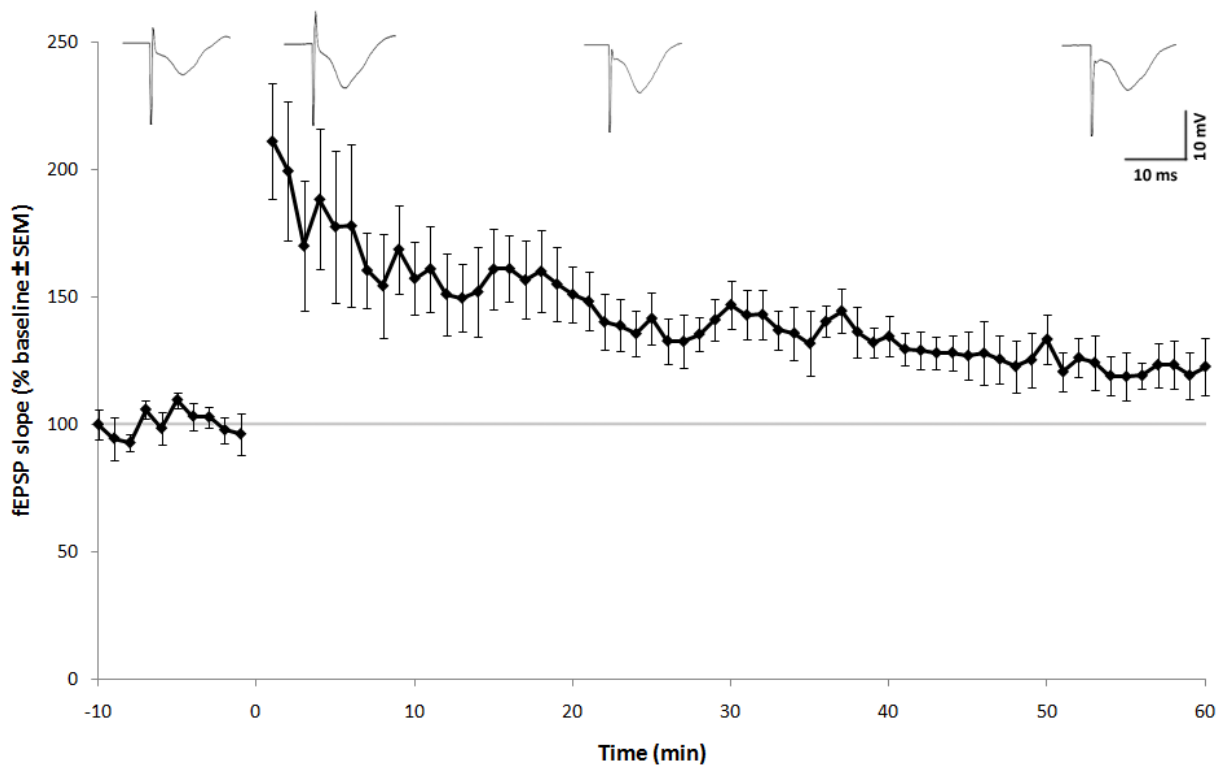
Figure 3.1: Induction of long-term potentiation (LTP) following 250 Hz high-frequency stimulation (HFS) in the CA1 to perirhinal cortex projection (n = 6). LTP lasted for at least 1 h, fEPSPs remaining significantly higher than baseline at all times. Each point represents an average of three fEPSP slopes recorded over 1 min and data is expressed as a percentage of baseline fEPSP slopes where baseline is approximately 100 %. Inset are representative fEPSP traces showing typical fEPSPs at baseline, 0-10 min, 20-30 min and 50-60 min.

3.3.3 Effect of 250 Hz HFS on brain-derived neurotrophic factor:

Firstly, we compared total BDNF levels in the area CA1 to those in the perirhinal cortex by using a 2 x 2 ANOVA with area analysed on two levels (CA1 and perirhinal cortex) and stimulation analysed on two levels (stimulated and unstimulated hemispheres). There was a significant effect for area (F = 1360; df = 1, 10; p < 0.001); area CA1 had significantly higher levels of BDNF compared to the perirhinal cortex (Figure 3.2). However, there was no significant effect for stimulation (F = 2.428; df = 1, 10; p > 0.05) and no interaction effect between the factors (F = 3.156; df = 1, 10; p > 0.05).

Although there was no significant effect for stimulation, we determined the effect size for stimulation in the area CA1 and the perirhinal cortex using Cohen's d

value (Cohen, 1992; Thalheimer & Cook, 2002). In CA1, the stimulated hemisphere contained 370.05 pg/mg$^{\text{total protein}}$ BDNF (±18.69) compared to 315.66 pg/mg$^{\text{total protein}}$ BDNF (±15.02) in the unstimulated hemisphere and there was a medium effect size for stimulation (d = 0.642). In the perirhinal cortex, the stimulated hemisphere contained 99.42 pg/mg$^{\text{total protein}}$ BDNF (±6.30) compared to 102.98 pg/mg$^{\text{total protein}}$ BDNF (±1.86) in the unstimulated hemisphere and a small effect size was observed (d = -0.153).
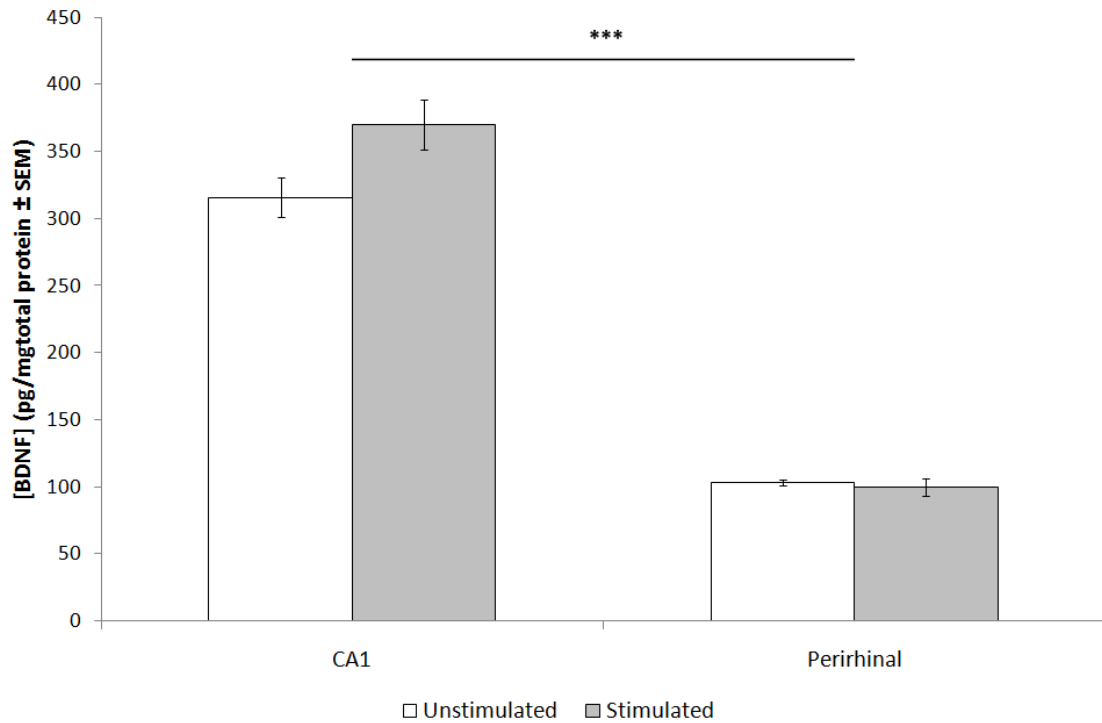
Figure 3.2: Comparison of brain-derived neurotrophic factor (BDNF) levels across the stimulated (250 Hz HFS) and unstimulated (control) hemispheres (n = 6) in area CA1 and the perirhinal cortex. There is significantly more BDNF in area CA1 compared to the perirhinal cortex. ***$p < 0.001$.

3.3.4 Effect of 250 Hz HFS on extracellular signal-regulated kinase:

Following western blot analysis, we compared ERK-2 and pERK levels in the area CA1 and perirhinal cortex (Figure 3.3). Using β-actin as a control measure, we first determined whether there were any changes in β-actin levels following 250 Hz HFS by

comparing stimulated and unstimulated hemispheres using dependent t-tests. There were no significant differences found for β-actin in either the area CA1 (t = 1.083; df = 5; p > 0.05) or in the perirhinal cortex (t = 0.720; df = 5; p > 0.05). As there were no differences in β-actin levels, ERK-2 and pERK levels were then analysed in two ways: firstly they were compared separately as standardised against β-actin levels (i.e. ERK-2/β-actin and pERK/β-actin) and secondly they were compared as a ratio of each other (i.e. pERK/ERK-2). In all cases, arbitrary units are used and paired t-tests were used to compare results from the stimulated hemisphere to the unstimulated hemisphere and effect sizes were calculated using Cohen's d value.

In the area CA1 (Figure 3.3 (a)), there were no significant differences found between the stimulated and unstimulated hemispheres for ERK-2/β-actin (t = -1.179; df = 5; p > 0.05) or for pERK/β-actin (t = -1.569; df = 5; p > 0.05). Small effect sizes were found for both ERK-2/β-actin (d = -0.365) and pERK/β-actin (d = -0.394). In addition, there was no significant difference found for pERK/ERK-2 between the two hemispheres (t = -0.309; df = 5; p > 0.05). Similarly, there were no significant differences found in the perirhinal cortex (Figure 3.3(b)) between the two hemispheres for ERK-2/β-actin (t = -0.047; df = 5; p > 0.05), pERK/β-actin (t = -0.800; df = 5; p > 0.05) or for pERK/ERK-2 (t = 1.037; df = 5; p > 0.05). Finally there were small effect sizes found for ERK-2/β-actin (d = -0.014) and pERK/β-actin (d = 0.269).

Figure 3.3 (overleaf): Comparison of extracellular signal-regulated kinase (ERK) levels across the stimulated (250 Hz HFS) and unstimulated (control) hemispheres (n = 6) in (a) area CA1 and (b) the perirhinal cortex. There were no significant differences found between stimulated and unstimulated hemispheres in ERK/β-actin, pERK/β-actin or ERK/pERK.

(a)
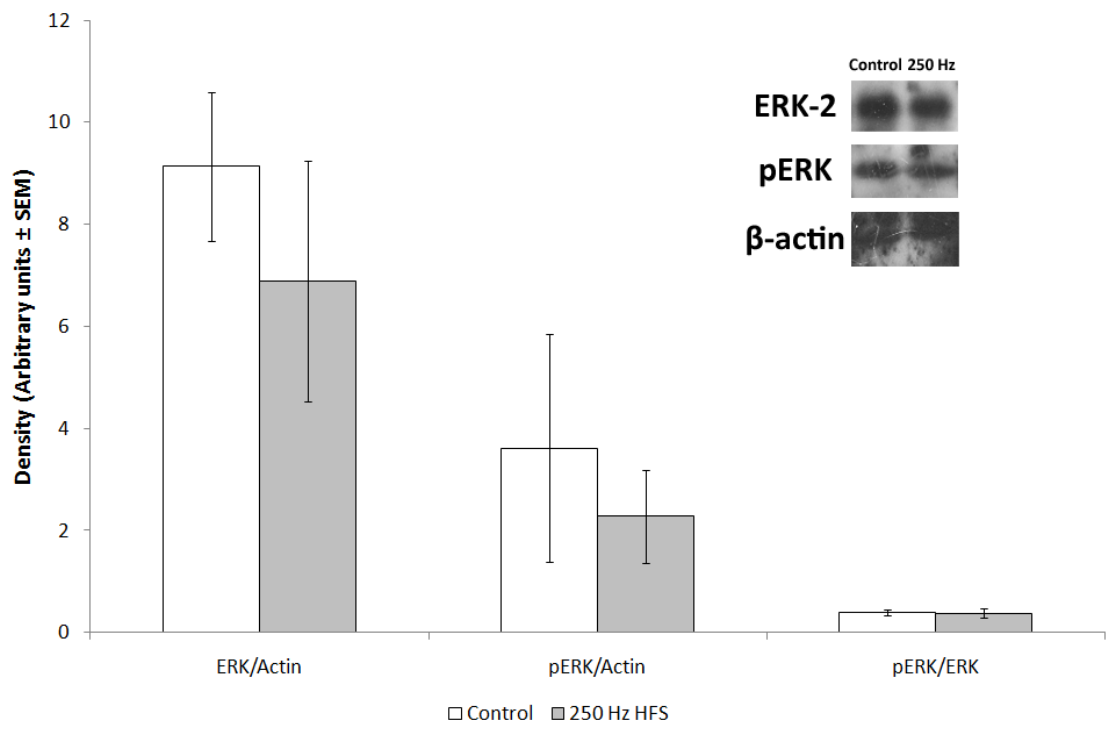
(b)
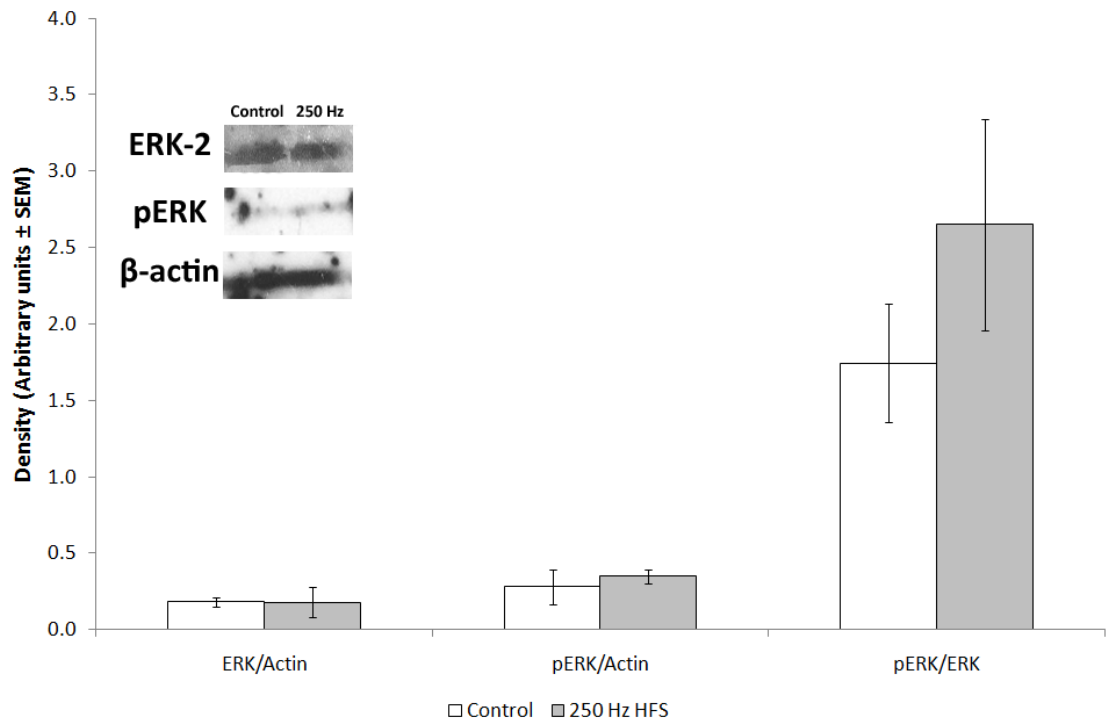
3.4 Discussion:

This experiment confirms the findings of our previous chapter and those of Cousens and Otto (1998) where in showing that the CA1 to perirhinal cortex projection can sustain long-term synaptic plasticity in the forms of PTP and LTP respectively. However, we failed to demonstrate whether BDNF or ERK play a significant role underlying these synaptic changes.

After inducing LTP (Figure 3.1), we analysed tissue samples from the area CA1 (site of the stimulating electrode) and the perirhinal cortex (site of the recording electrode). After BDNF analysis via ELISA, the levels of BDNF observed in our experiment in the area CA1 and perirhinal cortex were in accordance for the levels observed in other studies (Eagleson et al., 2001; Greisen et al., 2005; Xapelli et al, 2008; Kealy & Commins, 2009). We hypothesised that there would be an increase in BDNF activation following 250 Hz HFS in the area CA1 as previous research has shown that increases in BDNF levels are associated with stimulation of area CA1 (Patterson et al., 1992; Kealy & Commins, 2009) and the dentate gyrus (Castrén et al., 1993; Dragunow et al., 1993; Gooney & Lynch, 2001). Despite these previous findings, we did not find a significant difference in BDNF levels between stimulated and unstimulated hemispheres in this present study. The stimulated hemisphere did have with a medium to large effect size, indicating that significance might be observed with a larger group of subjects. Another possibility is that 250 Hz HFS caused a concurrent increase in BDNF levels in both hemispheres (Bramham et al., 1996), obscuring the effect of HFS on BDNF levels. In addition, hippocampal neurons have been shown to recycle BDNF rather than synthesise new BDNF following LTP (Santi et al., 2006) which may mean that levels of BDNF are not changing but perhaps the efficacy of BDNF signalling through the TrkB receptor is being altered in some other way or that another signalling molecule is responsible for the synaptic plasticity underlying LTP in this projection.

250 Hz HFS has been shown previously to increase levels of activated ERK in the dentate gyrus (Maguire et al., 1999; Gooney & Lynch, 2001). We therefore

hypothesised that stimulation of area CA1 utilising the same 250 Hz HFS protocol would cause a significant increase in ERK levels. However, analysis of ERK-2 and pERK levels in the area CA1 suggests that 250 Hz HFS has no significant effect on MAPK signalling in this experiment. The small effect size for these changes suggest that there is no strong effect of stimulation on ERK levels, indicating that even with a larger group the effect would be negligible. These results suggest that either ERK activation does not occur to a great extent in CA1 following stimulation or that the effects of stimulation are not present 1 hr post-HFS. Maguire and colleagues (1999) and Gooney and Lynch (2001) both report increased levels of pERK 40 min post-HFS, indicating that ERK activation is still observable at that time. It is possible that levels of activated ERK have returned to baseline levels after 1 hr. However, a form of pERK-independent LTP has been described; 250 Hz HFS in the perforant path in mice can induce LTP but without any observed increases in ERK phosphorylation (Steward et al., 2007).

Activation of certain areas of the brain has been shown to induce molecular changes in their efferent targets. Pharmacological activation of the entorhinal cortex can induce changes in hippocampal BDNF (Lindefors et al., 1992; Falkenberg et al., 1993) and trkB levels (Falkenberg et al., 1992a). Electrophysiological stimulation of the entorhinal cortex can also increase levels of hippocampal BDNF (Springer et al., 1994). Similarly, stimulation of the hippocampus can induce changes in entorhinal BDNF levels (Hernández et al., 2008). As such, we wished to investigate whether 250 Hz HFS in area CA1 could induce changes in BDNF levels in the perirhinal cortex. Following 250 Hz HFS in area CA1, there was no significant difference between the stimulated and unstimulated hemispheres in the perirhinal cortex and as there was only a small effect size for stimulation, it is likely that either the 250 Hz HFS in the area CA1 has no great effect on perirhinal BDNF levels or there is a time-dependent effect on BDNF. It has been demonstrated in perirhinal cortex slices that BDNF secretion occurs over the course of 5-12 min (Aicardi et al., 2004). As we recorded for 1 hr before harvesting tissue samples, this gives ample time for BDNF levels to return to baseline levels.

Finally, in order to determine whether intracellular signalling in the perirhinal cortex was affected by 250 Hz HFS in the area CA1, we analysed ERK-2 and pERK levels in the perirhinal cortex. Again, there were no significant differences observed between the two hemispheres in either protein and the effect sizes were small. Taken together with the ERK data for the area CA1, it is unlikely that changes in ERK levels play a big role in LTP in the CA1 to perirhinal cortex projection. However, it is impossible at this stage to rule out other factors such as enhancement of downstream signalling by other methods whereby the same amount of pERK has a greater effect. Equally, it may be that 1 hr is too late after 250 Hz HFS to observe any significant changes in MAPK signalling.

In summary, in this study we have confirmed both our findings in chapter 2 and those of Cousens & Otto (1998) in demonstrating that the CA1 to perirhinal cortex can sustain both short-and long-term synaptic plasticity. We have also shown that there are no significant changes in BDNF, ERK-2 or pERK levels following 250 Hz HFS but that the lack of observed changes does not rule out their involvement in changes in synaptic plasticity in the CA1 to perirhinal cortex projection. In the following chapter, we further investigate the electrophysiological and molecular underpinnings of these changes in synaptic strength by using an array of different stimulation frequencies instead of a 250 Hz HFS protocol.

Chapter 4

Electrophysiological characterisation of the CA1 to perirhinal cortex projection: Frequency-dependent changes in synaptic plasticity and brain-derived neurotrophic factor.

Abstract:

The CA1 to perirhinal cortex projection can sustain long-term potentiation (LTP) following 250 Hz high-frequency stimulation (HFS) and this LTP is associated with increases in brain-derived neurotrophic factor (BDNF) levels. The CA1 to entorhinal cortex projections can sustain both LTP following HFS and long-term depression (LTD) following low-frequency stimulation (LFS) and this study aims to determine whether the CA1 to perirhinal projection shows a similar frequency-dependent pattern of synaptic plasticity. Five groups of Wistar rats (n = 6 in each) were anaesthetised with urethane and paired-pulse faciliatation (PPF) over six interpulse intervals (IPIs). Baseline recordings at 0.05 Hz were then made over 10 min by stimulating the area CA1 and recording in the perirhinal cortex. The five groups each underwent one of five different stimulation protocols (1 Hz, 5 Hz, 10 Hz, 50 Hz or 100 Hz) and post-stimulation recordings were again made at 0.05 Hz for 1 hr. PPF recordings were again made after this recording period over the same six IPIs. The animals were sacrificed and tissue samples were taken from the area CA1 and perirhinal cortex from the unstimulated and stimulated hemispheres of each brain. These tissue samples were analysed by ELISA for BDNF. No LTD was observed in any of the stimulation frequency groups but LTP was observed following 50 Hz and 100 Hz HFS. Based on pre- and post-stimulation PPF recordings, there was no difference in the locus of change in synaptic strength for any stimulation frequency group. Finally, increases in BDNF are positively correlated with stimulation frequency in the area CA1 but the same pattern was not observed in the perirhinal cortex. These findings suggest that the CA1 to perirhinal cortex projection is largely electrophysiologically excitatory in nature and that the relationship between LTP/LTD and BDNF levels may involve changes in downstream BDNF signalling.

4.1 Introduction:

In the previous two chapters, we have described short- and long-term synaptic plasticity in the CA1 to perirhinal cortex projection utilising only a single stimulation protocol (250 Hz high-frequency stimulation, HFS; Gooney & Lynch, 2001). We have determined that long-term potentiation (LTP) can be induced in this projection by stimulating the area CA1 using a 250 Hz HFS protocol. We now wish to investigate whether different stimulation frequencies have an effect on synaptic plasticity in this pathway and whether the CA1 to perirhinal cortex projection is capable of sustaining long-term depression (LTD) and thus conform to the Bienenstock-Cooper-Munro (BCM) model of biphasic synaptic modification (Bienenstock et al., 1982; see Chapter 1).

LTP is just one aspect of long-lasting changes in synaptic plasticity representing an increase in synaptic strength and a converse process has been described in the literature. LTD is a long-lasting reduction in synaptic strength usually induced following low-frequency stimulation (LFS; Bramham & Srebro, 1987). The existence of both LTP and LTD is expected as the ability of a synapse to be modulated both positively and negatively by different frequencies supports the case for changes in synaptic strength being the physiological basis for learning and memory (Bear et al., 1987; Thiels et al., 1996). Indeed, the molecular correlates of both LTP and LTD are found to be important for memory formation. AMPA glutamate receptor trafficking has been found to underpin both types of synaptic plasticity, learning and memory formation (Griffiths et al., 2008; Kessels & Malinow, 2009).

In the area CA1, a number of studies have shown that LTD can be induced by LFS (Thiels et al., 1994; Doyère et al., 1996; Citri et al., 2009; Hosseinmardi et al., 2009). NMDA-dependent homosynaptic LTD in area CA1 can be induced with LFS (1-3 Hz) of the Schaffer collateral (Dudek & Bear, 1992). Dudek and Bear (1993) later showed that this synaptic plasticity was bidirectional, LTP and LTD could be induced in the same synapses following a series of HFS and LFS protocols and these findings were later reproduced *in vivo* (Heynen et al., 1996). The CA1 to entorhinal cortex projection has

been shown to sustain LTP following 50 Hz, 100 Hz and 250 Hz HFS protocols (Craig &
Commins, 2005; 2007) and LTD following 1 Hz, 5 Hz and 10 Hz LFS protocols (Craig &
Commins, 2007), indicating that hippocampal output can be modified in an activity
dependent manner.

In the perirhinal cortex, activity-dependent LTD has been described following 1
Hz LFS that is reliant on metabotropic glutamate (mGlu) receptors (Cho et al., 2000b;
Cho et al., 2002). Kainate glutamate receptor-dependent LTD has also been identified
in the perirhinal cortex and it seems to be induced via a different level of activity
compared to AMPA-dependent LTD (Park et al., 2006). A role for perirhinal LTD in
object recognition memory has been suggested as antagonism of L-type voltage-
dependent calcium channels (VLDCCs) blocks object recognition memory and prevents
the induction of LTD but not LTP (Seoane et al., 2009). Furthermore, viral blockade of
mGlu and AMPA receptor interaction block perirhinal LTD *in vitro* and recognition
memory *in vivo* (Griffiths et al., 2008). Strengthening the case for LTD having a
functional role in the behaving animal, recent research has shown that perirhinal LTD is
experience-dependent. Jo and colleagues (2006) found that in the developing
perirhinal cortex of the rat that LTD switched from being dependent on mGlu receptors
to being dependent on muscarinic acetylcholine (mACh) receptors and that this switch
depended on visual experience; dark-reared rats did not show this switch in LTD form.
In perirhinal cortex slices prepared from rats who had been exposed to visual stimuli in
an object learning task, it has been shown that multiple exposures to these visual
stimuli prevents the subsequent induction of LTD but not LTP (Massey et al., 2008).
Massey and colleagues (2008) also showed that this experience-dependent impairment
of LTD was dependent on mACh receptors. These experiments suggest that LTD and
LTP may play differential roles in recognition memory, with an LTD-like mechanism
being suggested as the process underlying object recognition memory (Warburton et
al., 2003; Barker et al., 2006).

There is increasing molecular evidence to suggest that LTP and LTD are different
extremes of the same process (Bienenstock et al., 1982; Bear, 2003; Yu et al., 2008).

There are a number of molecular processes found to be important in LTP that are also implicated in LTD including Ca^{2+} (Mulkey & Malenka, 1992; Cummings et al., 1996), protein phosphatases (Mulkey et al., 1993; 1994; Dickonson et al., 2009), BDNF (Aicardi et al., 2004), extracellular signal-regulated kinase (ERK; Norman et al., 2000; Thiels et al., 2002; Gallagher et al., 2004), NMDA glutamate receptors (Dudek & Bear, 1992; Kirkwood et al., 1993; Peng et al., 2009) and AMPA glutamate receptors (Lüscher et al., 1999; Wang & Linden, 2000). The most studied model of the LTP/LTD dichotomy is the cycling of AMPA glutamate receptors to and from the postsynaptic membrane. The insertion of AMPA receptors into the postsynaptic membrane has been implicated in the induction of LTP (Shi et al., 1999; 2001; Barry & Ziff, 2002) and conversely, the endocytosis of AMPA receptors has been suggested to be the mechanism underlying LTD (Lüscher et al., 1999; Wang & Linden, 2000; Holman et al., 2007). This AMPA receptor endocytosis has been shown to be Ca^{2+}-dependent (Beattie et al., 2000), mGlu receptor-dependent (Snyder et al., 2001; Xiao et al., 2001), protein kinase C-dependent (Czarnecki et al., 2007) and Arc-dependent (Bramham et al., 2010).

The presence of BDNF can alter the effect observed at different stimulation frequencies, suggesting that neurotrophins may play a role in modulating metaplasticity (prior synaptic activity resulting in changes in the potential for synaptic plasticity; Abraham & Bear, 1996). It has been suggested that BDNF may shift the synaptic modulation threshold, preventing the induction of LTD *in vivo* (Jiang et al., 2003). For example, at lower frequencies where LTP is normally not observed, LTP can be induced in the presence of BDNF (Figurov et al., 1996; Huber et al., 1998). Huber and colleagues (1998) also demonstrated that at frequencies that would normally induce strong LTD; there is an attenuation of depression in the presence of BDNF. Other studies have shown that application of BDNF in visual cortex slices can completely block LTD (Akaneya et al., 1996; Kumura et al., 2000) and that this attenuation of LTD by BDNF is phospholipase C-dependent (Ikegaya et al., 2002). Furthermore, in the hippocampus there is an activity-dependent effect for BDNF expression. LFS shows little or no changes in BDNF levels whereas the use of HFS

results in significant increases in BDNF levels (Springer et al., 1994; Hartmann et al., 2001; Balkowiec & Katz, 2002; Gärtner & Staiger, 2002). In the perirhinal cortex, levels of HFS sufficient to induce LTP lead to significant increases in BDNF levels lasting 5-12 min and conversely levels of LFS sufficient to induce LTD lead to a significant but transient decrease in BDNF levels (Aicardi et al., 2004). LTP Similarly, surface expression of the TrkB receptor also has also been shown to be activity-dependent with LFS resulting in no increase in TrkB surface expression but there is a significant increase in TrkB surface expression following HFS (Du et al., 2000).

In this study, we will examine the plastic properties of the CA1 to perirhinal cortex projection by using five different stimulation frequency protocols (1 Hz, 5 Hz, 10 Hz, 50 Hz and 100 Hz). We will investigate the effect of each stimulation frequency on synaptic strength; we expect HFS protocols to induce LTP and LFS protocols to induce LTD. Therefore we expect to determine the BCM threshold for this projection, i.e. the frequency at which LTD becomes LTP. In addition, we will determine whether these changes in stimulation frequencies affect short-term plasticity in this projection by analysing paired-pulse facilitation (PPF). Furthermore, we will determine whether the locus of change in synaptic strength is pre- or postsynaptic by comparing PPF levels before and after stimulation. Finally, we will analyse brain-derived neurotrophic factor (BDNF) levels in the area CA1 and perirhinal cortex using ELISA. We hypothesise that following a HFS protocol, there will be significantly higher levels of BDNF in the stimulated hemisphere compared to the unstimulated hemisphere and that following a LFS protocol, there will be significantly lower levels of BDNF in the stimulated hemisphere compared to the unstimulated hemisphere. We hope to show that the electrophysiological BCM threshold can be replicated in BDNF levels.

4.2 Methods:

4.2.1 Surgery:

Adult male Wistar rats (n = 30; approximately 3 months old; weight: 300-400 g; Biomedical Unit; University College Dublin) were anaesthetised using urethane (ethyl carbamate; 1.5 mg/kg; i.p.; Sigma) and mounted on a stereotaxic frame. A stimulating electrode was inserted into the area CA1 and a recording electrode was inserted into the perirhinal cortex as detailed in Chapter 2.

4.2.2 Stimulation and data acquisition:

Pre-HFS PPF recordings, baseline fEPSP recordings, post-stimulation fEPSP recordings and post-stimulation PPF recordings were performed as detailed in Chapter 2. After recording baseline responses to single stimuli (0.05 Hz) over 10 min, rats were treated with a particular stimulation protocol depending on the group. Five groups (n = 6 in each) were respectively treated using 1 Hz, 5 Hz, 10 Hz, 50 Hz and 100 Hz stimulation protocols. Each stimulation protocol had a constant number of stimuli (900 pulses) and only varied in the frequency by which the pulses were delivered. Post-stimulation recordings were again made at 0.05 Hz over 60 min as in previous chapters. Immediately after the post-stimulation PPF recordings, each animal was sacrificed and tissue samples were taken from the area CA1 and perirhinal cortex as described in Chapter 3.

4.2.3 Brain-derived neurotrophic factor enzyme-linked immunosorbant assay (ELISA):

Analysis of BDNF in the area CA1 and perirhinal cortex was performed as described in Chapter 3 via an enzyme-linked immunosorbent assay (ELISA) for BDNF using a BDNF Emax$^{©}$ ImmunoAssay System kit (Promega Corporation).

4.2.4 Statistical analysis:

A series of dependent t-tests and repeated measures analyses of variance (ANOVAs) with the appropriate *post-hoc* test (Tukey at the 5 % level of significance) were used. A star-rated system was used where appropriate (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

4.2.5 Ethical considerations:

Laboratory procedures for the maintenance and experimentation of animals conformed to the Department of Health (Ireland) guidelines and the European directive 86/609/EC. Every effort was made to minimise the suffering and the number of animals used in this study.

4.3 Results:

4.3.1 Basic properties of fEPSP responses in the CA1 to perirhinal cortex projection:

An initial characterisation of the baseline evoked response obtained in the perirhinal cortex following a single pulse stimulation of CA1 delivered at 0.05 Hz over 10 min (n = 30) was performed in each group. In the 1 Hz group there was a mean amplitude of -8.214 mV (±0.063), a mean latency of peak amplitude of 6.777 ms (±0.029) and a mean slope of the downward deflection of -2.204 mV/s (±0.036). In the 5 Hz group there was a mean amplitude of -6.213 mV (±0.062), a mean latency of peak amplitude of 6.132 ms (±0.030) and a mean slope of the downward deflection of -1.382 mV/s (±0.020). In the 10 Hz group there was a mean amplitude of -3.869 mV (±0.079), a mean latency of peak amplitude of 5.929 ms (±0.051) and a mean slope of the downward deflection of -1.177 mV/s (±0.028). In the 50 Hz group there was a mean amplitude of -8.046 mV (±0.075), a mean latency of peak amplitude of 5.937 ms (±0.029) and a mean slope of the downward deflection of -1.820 mV/s (±0.050). Finally, in the 100 Hz group there was a mean amplitude of -4.857 mV (±0.087), a mean latency of peak amplitude of 5.469 ms (±0.032) and a mean slope of the downward deflection of -1.455 mV/s (±0.026).

4.3.2 Effect of different stimulation frequencies on synaptic plasticity:

The main aim of this experiment was to determine what effect various stimulation frequencies have on synaptic plasticity in the CA1 to perirhinal cortex projection. In all groups, we initially recorded fEPSPs using a 0.05 Hz stimulation protocol (-10-0 min) before using one of five different stimulation protocols (1 Hz, 5 Hz, 10 Hz, 50 Hz and 100 Hz) on area CA1 and then recording again at 0.05 Hz for 60 min (Figure 4.1). To examine the effects of the different stimulation frequencies on LTP, a 5 x 4 mixed factorial ANOVA was used with time analysed on 4 levels (−10–0 min, 0–10 min, 20–30

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min and 50–60 min) as a within-groups measure. Stimulation frequency was analysed on 5 levels (1 Hz, 5 Hz, 10 Hz, 50 Hz and 100 Hz) as a between-groups measure. Overall, there was a significant effect of time (F = 126.536; df = 3, 135; $p < 0.001$). Stimulation frequency was also shown to have a significant effect (F = 5689.724; df = 4, 45; $p < 0.001$). In addition, an interaction effect between stimulation frequency and time was shown (F = 13.076; df = 12, 135; $p < 0.001$).

Differences between the groups were determined using Tukey *post-hoc* analysis (Figure 4.1). Across the full length of the experiment, the 1 Hz group showed significantly lower levels of potentiation compared to 5 Hz, 50 Hz and 100 Hz groups ($p < 0.001$ in each case) but not the 10 Hz group ($p > 0.05$). The 5 Hz group showed significantly more potentiation compared to the 1 Hz ($p < 0.001$) and 10 Hz groups ($p < 0.05$) and significantly less potentiation compared to the 50 Hz and 100 Hz groups ($p < 0.001$ in both cases). The 10 Hz group showed significantly less potentiation compared to the 5 Hz ($p < 0.05$), 50 Hz ($p < 0.001$) and 100 Hz groups ($p < 0.001$) and, as described above, there was no significant difference observed between the 1 Hz and 10 Hz groups ($p > 0.05$). The 50 Hz group showed significantly more potentiation compared to the 1 Hz, 5 Hz and 10 Hz groups ($p < 0.001$ in each case) but there was no significant difference between the 50 Hz and 100 Hz groups ($p > 0.05$). Finally, the 100 Hz group showed significantly more potentiation compared to the 1 Hz, 5 Hz and 100 Hz groups ($p < 0.001$ in each case).
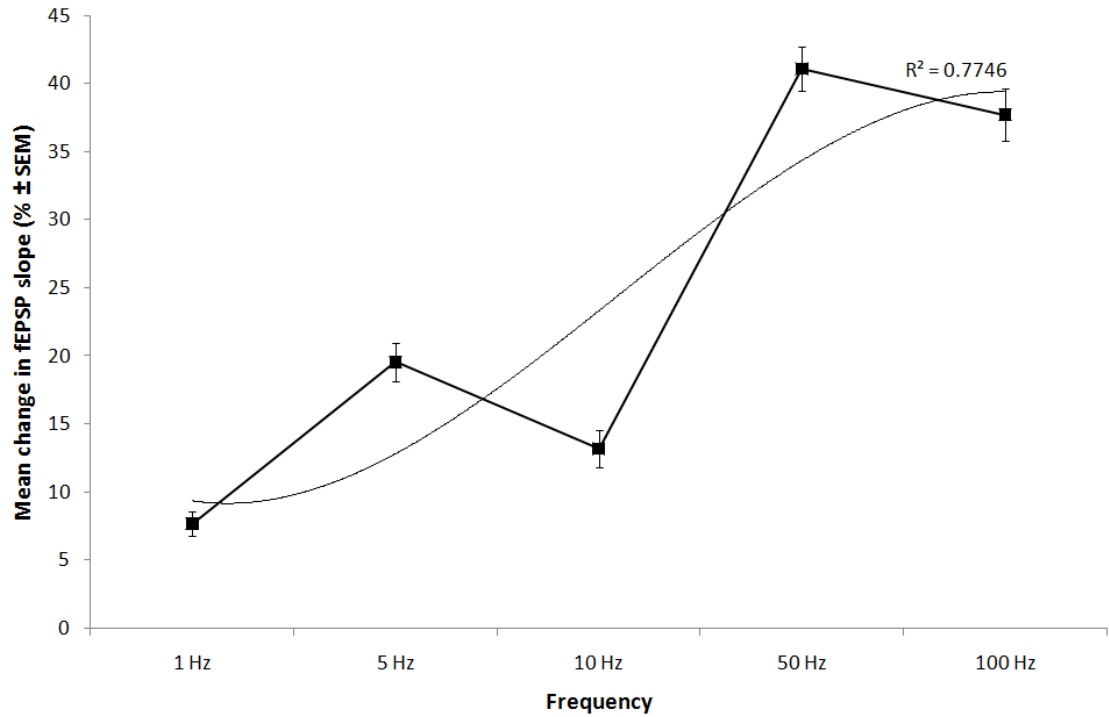
Figure 4.1: Comparison of potentiation levels for the five stimulation frequencies averaged over 1 hr.

Each stimulation frequency group was analysed separately in order to determine the effect of each stimulation frequency on synaptic plasticity. Repeated-measures ANOVAs were ran for each group with Bonferroni correction to examine the specific differences at four different times (−10–0 min, 0–10 min, 20–30 min and 50–60 min) within each group. There was a significant effect for time in the 1 Hz group (F = 5.922; df = 3, 27; p < 0.01; Figure 4.2). *Post-hoc* analysis showed that 1 Hz stimulation failed to induce post-tetanic potentiation (PTP) as there was no significant difference in fEPSP slope size between baseline (-10-0 min pre-HFS) and the 0-10 min interval (103.005 % ±1.606; p > 0.05). Similarly, there was no significant difference in fEPSP slopes observed between baseline levels and the 20-30 min interval (103.487 % ±2.622; p > 0.05). However, fEPSP slopes during the 50-60 min interval (113.17 % ±5.75) were significantly higher compared to baseline levels (p < 0.05).
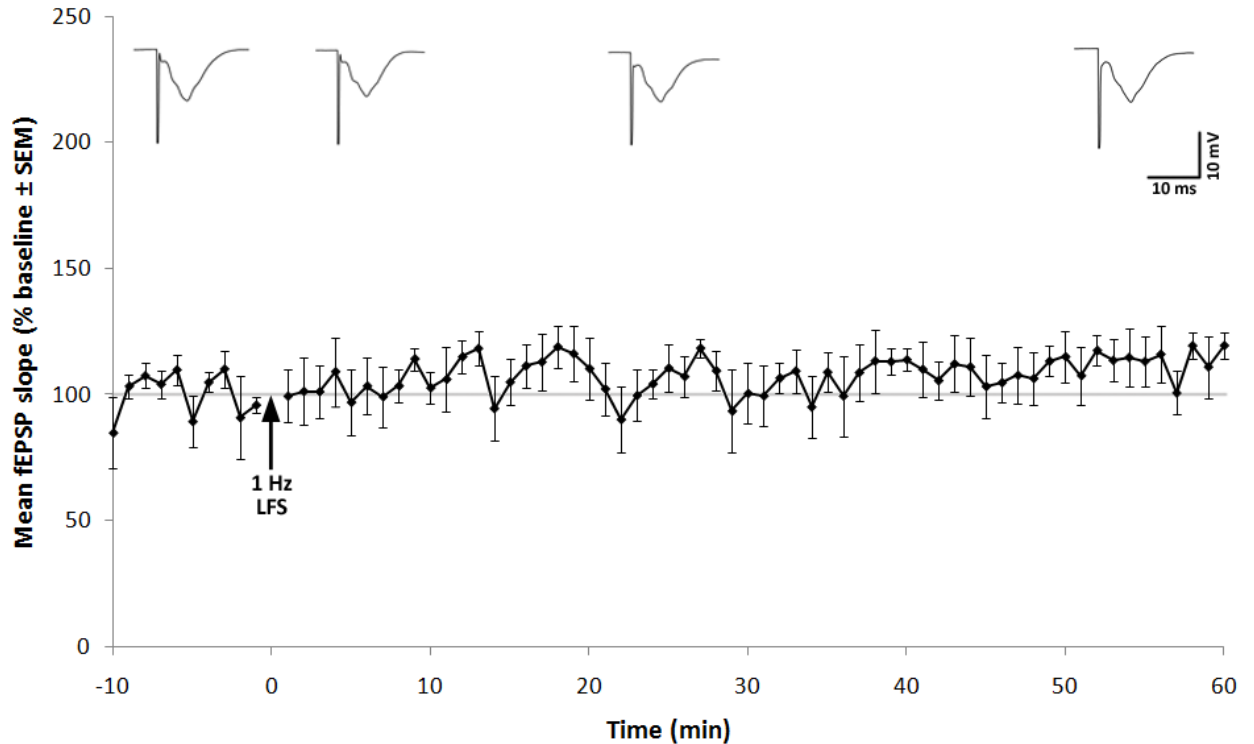
Figure 4.2: Plot of fEPSP slopes before and after a 1 Hz stimulation protocol in the CA1 to perirhinal cortex projection (n = 6). Each point represents an average of three fEPSP slopes recorded over 1 min and data is expressed as a percentage of baseline fEPSP slopes where baseline is approximately 100 %. Inset are representative fEPSP traces showing typical fEPSPs at baseline, 0-10 min, 20-30 min and 50-60 min.

In the 5 Hz group, there was a significant effect found for time (F = 90.090; df = 3, 27; p < 0.001; Figure 4.3). The 5 Hz stimulation protocol induced potentiation lasting approximately 25 min before dropping to below 120 % of baseline. The fEPSPs recorded in the 0-10 min interval (135.614 % ±1.237) were significantly higher than baseline levels (p < 0.001) and they were also significantly higher than fEPSP averages at the 20-30 min (119.173 % ±1.867; p < 0.001) and 50-60 min intervals (110.494 % ±1.750; p < 0.001). However, even though fEPSPs in the 0-10 min interval were significantly higher than those in the 20-30 min (p < 0.001) and the 50-60 min intervals (p < 0.001), this was not classified as PTP as the potentiation seen in the first 10 minutes remains at approximately the same level throughout that time, as opposed to PTP's characteristic large increase in fEPSP slope over the first 2-3 min post-HFS followed by a sharp decline in fEPSP slope size.
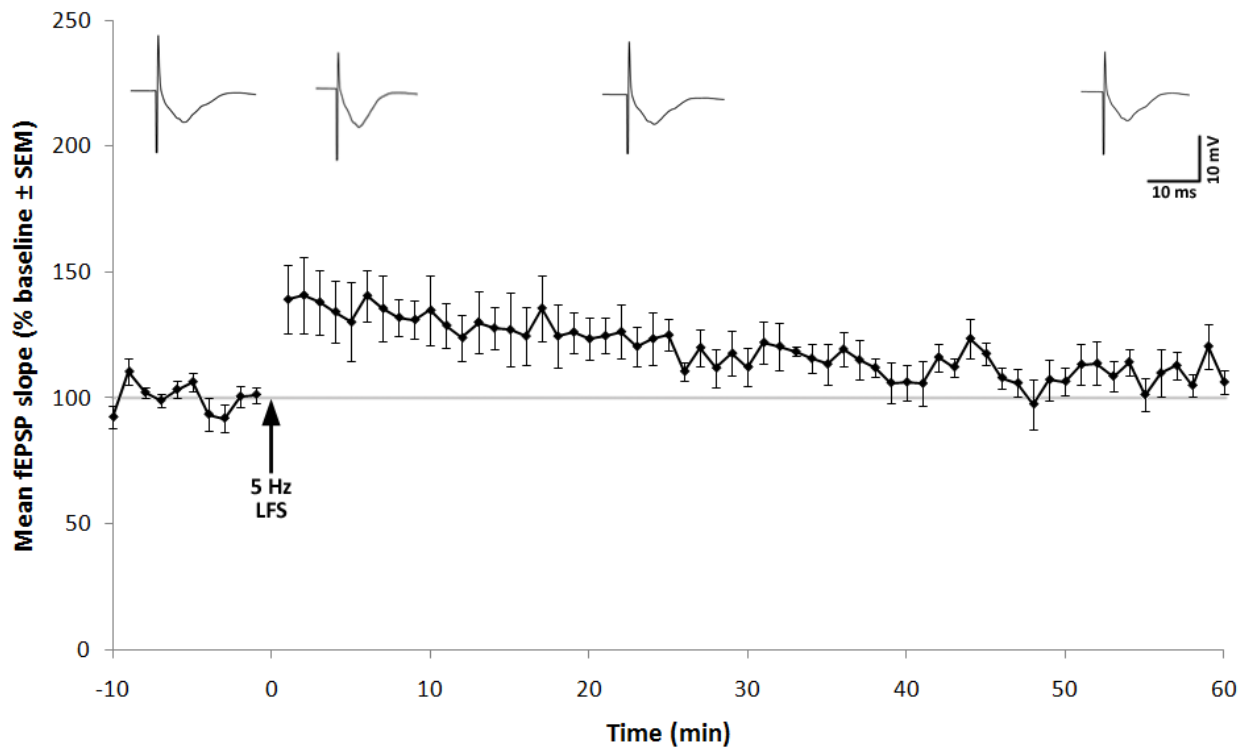
Figure 4.3: Plot of fEPSP slopes before and after a 5 Hz stimulation protocol in the CA1 to perirhinal cortex projection (n = 6). Each point represents an average of three fEPSP slopes recorded over 1 min and data is expressed as a percentage of baseline fEPSP slopes where baseline is approximately 100 %. Inset are representative fEPSP traces showing typical fEPSPs at baseline, 0-10 min, 20-30 min and 50-60 min.

A significant effect for time was also found in the 10 Hz group (F = 26.725; df = 3, 27; p < 0.001; Figure 4.4). There was a significant increase in fEPSP slope size compared to baseline levels at the 0-10 min (123.392 % ±2.261; p < 0.001) and 20-30 min intervals (115.477 % ±2.090; p < 0.05) but there was no significant difference between baseline fEPSP slope values and those in the 50-60 min interval (102.857 % ±1.008; p > 0.05). In addition, there was no significant difference between the 0-10 min interval and the 20-30 min (p > 0.05) which indicates that there was no PTP following 10 Hz stimulation.

Figure 4.4: Plot of fEPSP slopes before and after a 10 Hz stimulation protocol in the CA1 to perirhinal cortex projection (n = 6). Each point represents an average of three fEPSP slopes recorded over 1 min and data is expressed as a percentage of baseline fEPSP slopes where baseline is approximately 100 %. Inset are representative fEPSP traces showing typical fEPSPs at baseline, 0-10 min, 20-30 min and 50-60 min.

As with the three previous groups, there was a significant effect for time in the 50 Hz group (F = 40.712; df = 3, 27; p < 0.001; Figure 4.5). Unlike the previous groups, 50 Hz stimulation induced PTP and LTP. PTP was observed in the first 3 minutes of recording before fEPSP slopes decreased in size. However, fEPSP slopes in the 0-10 min interval (156.365 % ±4.549) remained significantly higher compared to baseline (p < 0.001). fEPSP slopes remained significantly higher compared to baseline levels in both the 20-30 min (138.416 % ±2.862; p < 0.001) and the 50-60 min intervals (130.550 % ±2.069; p < 0.001). Finally, the fEPSP slope values in the 0-10 min interval were significantly higher compared to the 50-60 min interval (p < 0.01) which indicates that PTP had been induced.
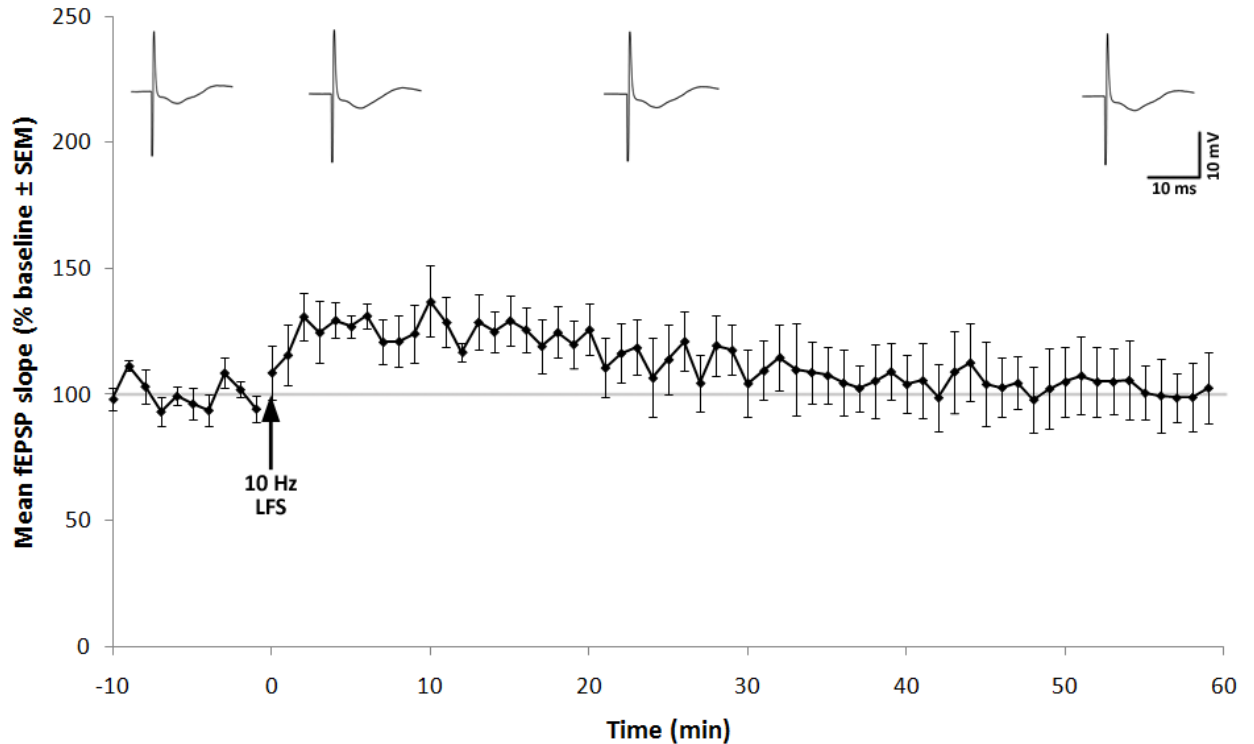
Figure 4.5: Plot of fEPSP slopes before and after a 50 Hz stimulation protocol in the CA1 to perirhinal cortex projection (n = 6). Each point represents an average of three fEPSP slopes recorded over 1 min and data is expressed as a percentage of baseline fEPSP slopes where baseline is approximately 100 %. Inset are representative fEPSP traces showing typical fEPSPs at baseline, 0-10 min, 20-30 min and 50-60 min.

Finally, there was also a significant effect for time in the 100 Hz group (F = 35.836; df = 3, 27; $p < 0.001$; Figure 4.6). Like the 50 Hz group, the 100 Hz group also exhibited PTP and LTP. Again PTP was observed in the first 3 minutes of recording before fEPSP slopes decreased in size. Overall, fEPSP slopes in the 0-10 min interval (156.742 % ±6.381) remained significantly potentiated compared to baseline levels ($p < 0.001$). fEPSP slopes also remained significantly higher compared to baseline levels in the 20-30 min (140.941 % ±3.271; $p < 0.001$) and the 50-60 min intervals (125.336 % ±2.895; $p < 0.001$). Finally, the fEPSP slope values in the 0-10 min interval were significantly higher compared to the 50-60 min interval ($p < 0.01$) indicating that PTP had been induced.
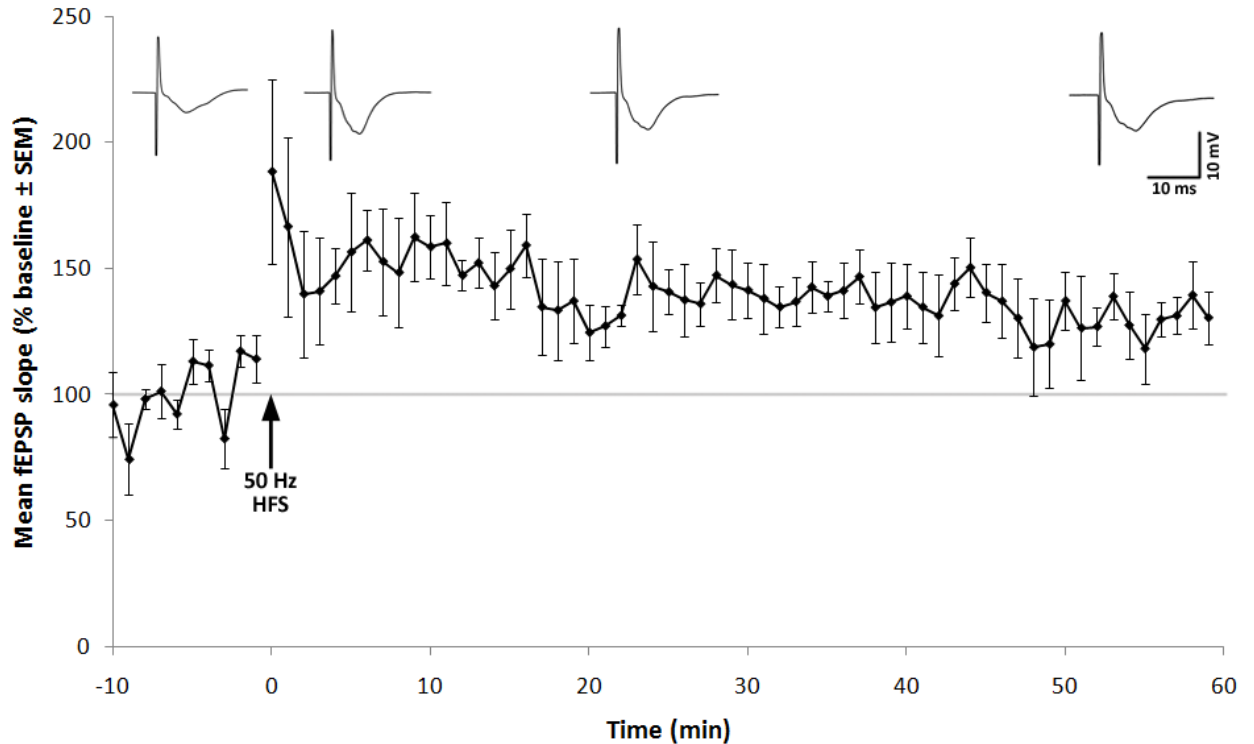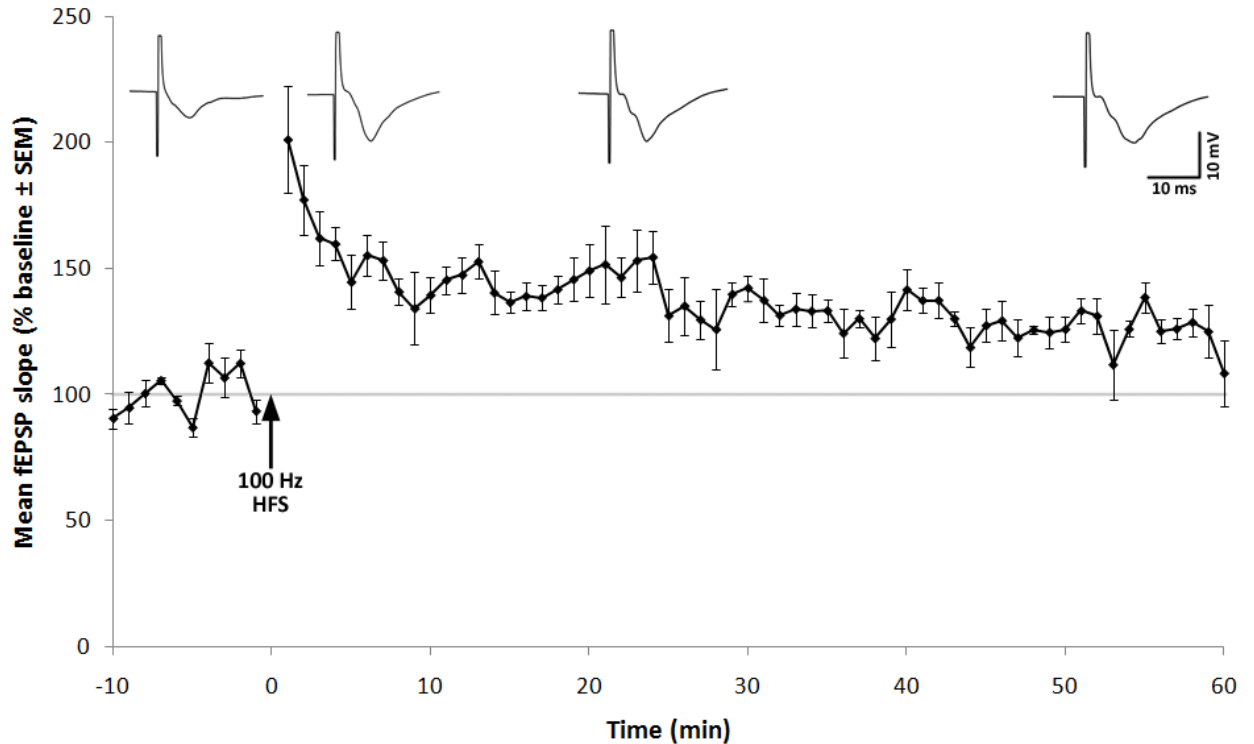
Figure 4.6: Plot of fEPSP slopes before and after a 100 Hz stimulation protocol in the CA1 to perirhinal cortex projection (n = 6). Each point represents an average of three fEPSP slopes recorded over 1 min and data is expressed as a percentage of baseline fEPSP slopes where baseline is approximately 100 %. Inset are representative fEPSP traces showing typical fEPSPs at baseline, 0-10 min, 20-30 min and 50-60 min.

We performed a more detailed analysis of the differences between each group at the four specified time points (−10–0 min, 0–10 min, 20–30 min and 50–60 min). We utilised a series of one-way ANOVAs with Tukey *post-hoc* analysis which revealed further differences between the groups. Baseline levels (10 to 1 min pre-HFS) showed no significant differences between the five different stimulation frequencies ($F < 0.001$; *df* = 4, 45; $p > 0.05$). However, there were significant differences observed between the five stimulating frequencies immediately after HFS (0 to 10 min post-HFS; $F = 36.953$; *df* = 4, 45; $p < 0.001$; Table 4.1). Tukey *post-hoc* analysis revealed that the 1 Hz group showed significantly lower levels of potentiation compared to all other groups ($p < 0.001$ for all groups apart from the 10 Hz group where $p < 0.01$). The 5 Hz group showed significantly higher levels of potentiation compared to the 1 Hz group ($p < 0.001$), significantly lower levels of potentiation compared to the 50 Hz and 100 Hz

groups ($p < 0.01$ in both cases) and there were no significant differences between the 5 Hz and 10 Hz groups ($p > 0.05$). In addition, the 10 Hz group showed significantly higher levels of potentiation compared to the 1 Hz group ($p < 0.01$) and significantly lower levels of potentiation compared to the 50 Hz and 100 Hz groups ($p < 0.001$ in both cases). The 50 Hz group showed significantly higher levels of potentiation compared to the 1 Hz ($p < 0.001$), 5 Hz ($p < 0.01$) and 10 Hz groups ($p < 0.001$) but there were no significant differences between the 50 Hz and 100 Hz groups ($p > 0.05$). Finally, the 100 Hz group also showed significantly higher levels of potentiation compared to the 1 Hz ($p < 0.001$), 5 Hz ($p < 0.01$) and 10 Hz groups ($p < 0.001$). Figure 4.7 summarises the findings that in the initial ten minute recording period that fEPSP slopes were significantly higher in the 50 Hz and 100 Hz groups compared to the other three groups. In addition, fEPSP slopes in the 5 Hz and 10 Hz groups were significantly higher than the 1 Hz group. These results show that low (but not the lowest) stimulation frequencies can induce PTP in the CA1 to perirhinal cortex pathway.

Table 4.1: Differences between each group during the initial 10 minute recording period following stimulation (1 to 10 min post-stimulation). **$p < 0.01$; ***$p < 0.001$.

	1 Hz	5 Hz	10 Hz	50 Hz	100 Hz
1 Hz	-	***	**	***	***
5 Hz		-	-	**	**
10 Hz			-	***	***
50 Hz				-	-
100 Hz					-
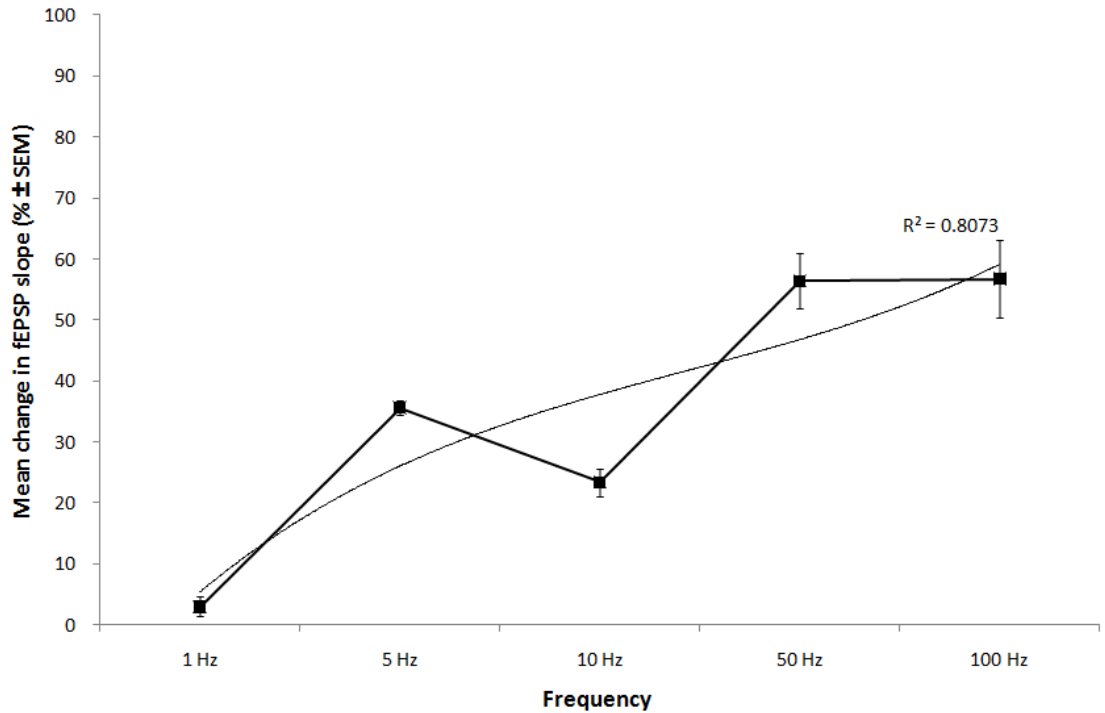
Figure 4.7: Comparison of potentiation levels for the five stimulation frequencies averaged over the initial 10 min recording period (1 to 10 min post-stimulation).

Looking at the middle time period after HFS (21 to 30 min post-HFS; Table 4.2) again showed a significant effect for stimulation frequency ($F = 37.558$; $df = 4, 45$; $p < 0.001$). Tukey *post-hoc* analysis showed that the 1 Hz group had significantly lower levels of potentiation compared to all other groups (5 Hz, $p < 0.001$; 10 Hz, $p < 0.05$; 50 Hz, $p < 0.001$; 100 Hz, $p < 0.001$). The 5 Hz group showed significantly higher levels of potentiation compared to the 1 Hz group ($p < 0.001$), significantly lower levels of potentiation compared to the 50 Hz and 100 Hz groups ($p < 0.001$ in both cases) and no significant differences were observed between the 5 Hz and 10 Hz group. The 10 Hz group also showed significantly higher levels of potentiation compared to the 1 Hz group ($p < 0.05$) and lower levels of potentiation compared to the 50 Hz and 100 Hz groups ($p < 0.001$ in both cases). The 50 Hz group showed significantly higher levels of potentiation for the 1 Hz, 5 Hz and 10 Hz groups ($p < 0.001$ in all cases) but there were again no differences between the 50 Hz and 100 Hz groups ($p > 0.05$). Lastly, the 100 Hz group showed significantly higher levels of potentiation for the 1 Hz, 5 Hz and 10 Hz

groups ($p < 0.001$ in all cases). Figure 4.8 summarises the findings that in the middle ten minute recording period that fEPSP slopes were significantly higher in the 50 Hz and 100 Hz groups compared to the other three groups, showing that higher stimulation frequencies are required to induce LTP in the CA1 to perirhinal cortex pathway.

Table 4.2: Differences between each group during the middle 10 minute recording period following stimulation (21 to 30 min post-stimulation). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

	1 Hz	5 Hz	10 Hz	50 Hz	100 Hz
1 Hz	-	***	*	***	***
5 Hz		-	-	***	***
10 Hz			-	***	***
50 Hz				-	-
100 Hz					-

Figure 4.8: Comparison of potentiation levels for the five stimulation frequencies averaged over the middle 10 min recording period (21 to 30 min post-stimulation).

A one-way ANOVA analysing the final ten minutes of the recording period (51 to 60 min post-HFS; Table 4.3) revealed further significant differences between the groups (F = 31.720; df = 4, 45; p < 0.001). At this stage, Tukey *post-hoc* analysis showed that the 1 Hz group had significantly higher levels of potentiation compared to the 10 Hz (p < 0.01) and significantly lower levels of potentiation compared to the 50 Hz and 100 Hz groups (p < 0.001 in both cases) and there were no significant differences between the 1 Hz and 5 Hz groups (p > 0.05). The 5 Hz group showed significantly lower levels of potentiation compared to the 50 Hz and 100 Hz groups (p < 0.001 in both cases) and no significant differences were found between the 5 Hz group and either the 1 Hz or 10 Hz groups (p > 0.05 in both cases). However, the 10 Hz group showed significantly lower levels of potentiation compared to the 1 Hz (p < 0.01), 50 Hz (p < 0.001) and 100 Hz groups (p < 0.001). The 50 Hz group showed significantly higher levels of potentiation compared to the 1 Hz, 5 Hz and 10 Hz groups (p < 0.001 in all cases) and there were no significant differences found between the 50 Hz and 100 Hz groups (p > 0.05). Finally, the 100 Hz group also showed significantly higher levels of potentiation compared to the 1 Hz, 5 Hz and 10 Hz groups (p < 0.001 in all cases). Figure 4.9 summarises the findings that in the final ten minutes of recording that fEPSP slopes were significantly higher in the 50 Hz and 100 Hz groups compared to the other three groups, showing that higher stimulation frequencies are required to induce LTP in the CA1 to perirhinal cortex pathway.

Table 4.3: Differences between each group during the final 10 minute recording period following stimulation (51 to 60 min post-stimulation). *p < 0.05; **p < 0.01; ***p < 0.001.

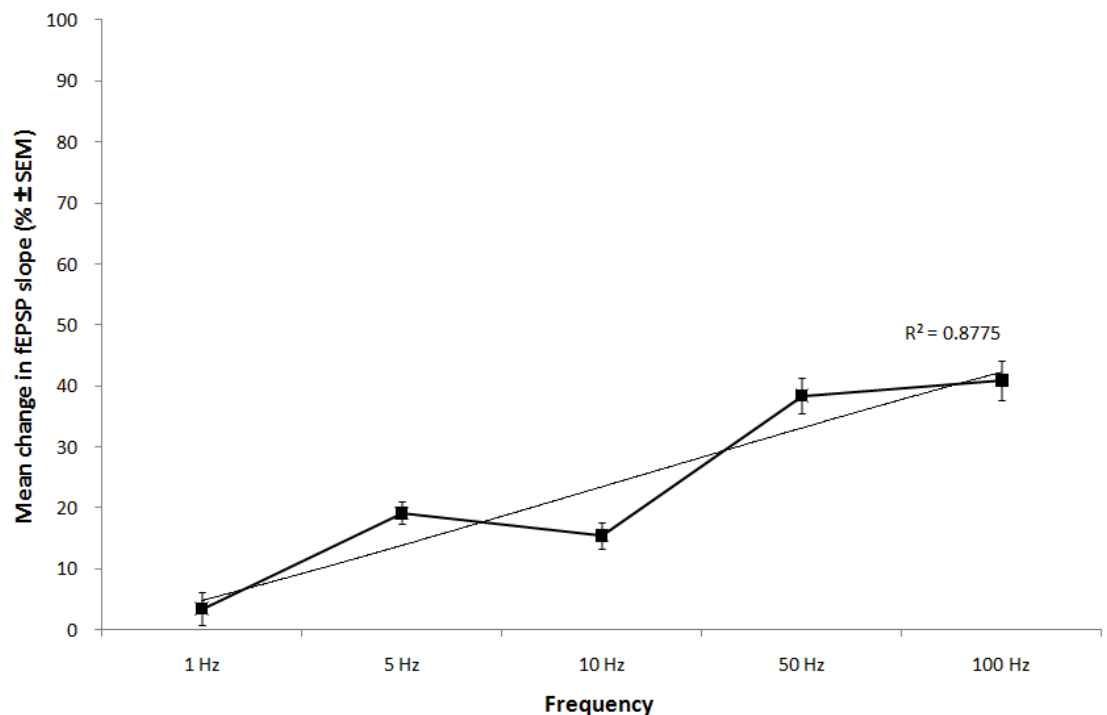|	1 Hz	5 Hz	10 Hz	50 Hz	100 Hz
1 Hz	-	-	**	***	***
5 Hz		-	-	***	***
10 Hz			-	***	***
50 Hz				-	-
100 Hz					-
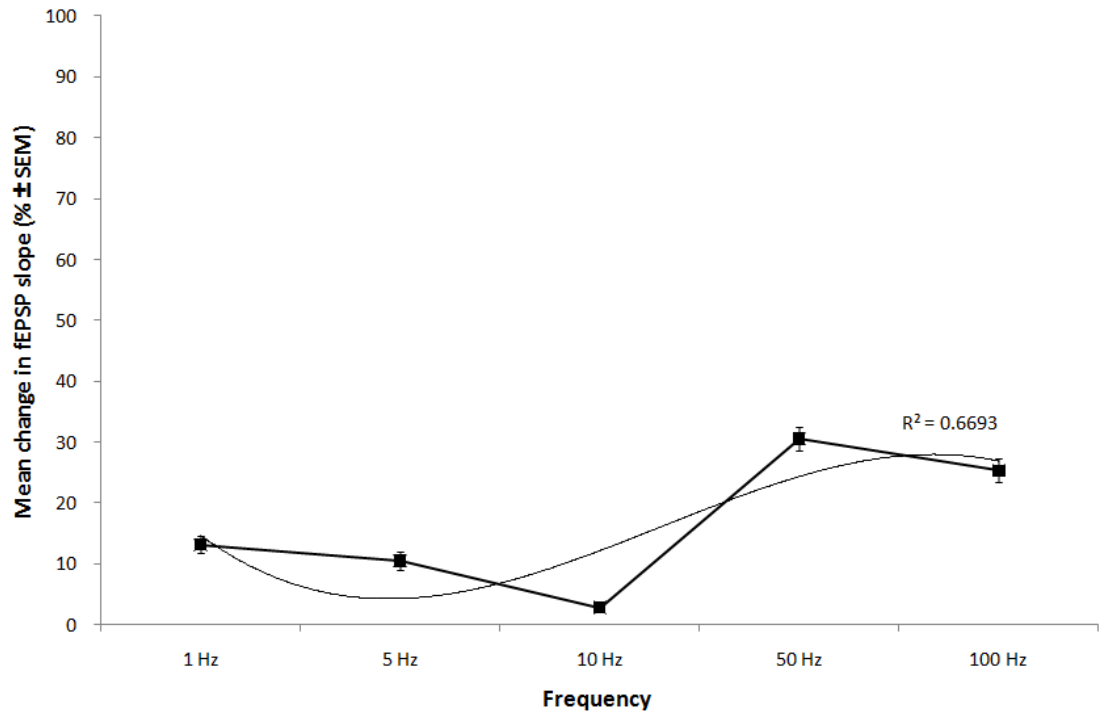
Figure 4.9: Comparison of potentiation levels for the five stimulation frequencies averaged over the final 10 min of recording (51 to 60 min post-stimulation).

4.3.3 Effect of different stimulation frequencies on pre- and post-stimulation paired-pulse facilitation:

PPF values from the baseline (pre-HFS) condition were compared to those recorded 1 hr post-HFS in order to determine whether any changes in synaptic strength could be localised to either the presynaptic or the postsynaptic neurons. Baseline versus post-HFS PPF values were analysed using a 2 x 6 x 5 mixed factorial ANOVA with time (baseline and post-HFS) and interpulse interval (IPI) (20 ms, 40 ms, 60 ms, 120 ms, 240 ms and 480 ms) as within-group measures and stimulation frequency (1 Hz, 5 Hz, 10 Hz, 50 Hz and 100 Hz) as a between-group measure (Figure 4.8). There was no significant effect found for time (F = 3.896; df = 1, 25; p > 0.05) or for stimulation frequency (F = 1.344; df = 4, 25; p > 0.05) but there was a significant effect found for IPI (F = 43.096; df = 5, 125; p < 0.001). Finally, no significant interaction effects were found between time and stimulation frequency (F = 0.416; df = 4, 25; p > 0.05), time and IPI (F = 2.217; df = 5, 125; p > 0.05) or for IPI and stimulation frequency (F = 1.549, df = 20, 125; p > 0.05).
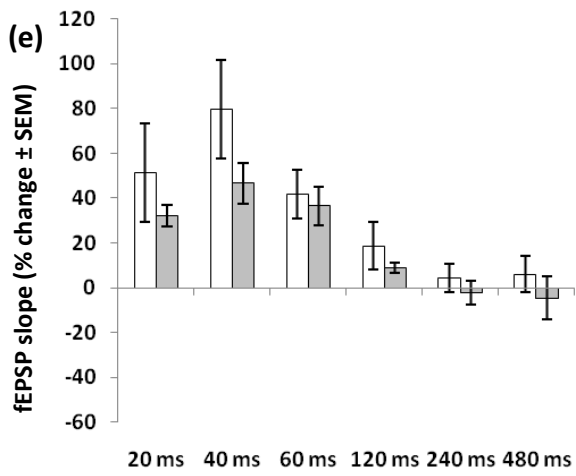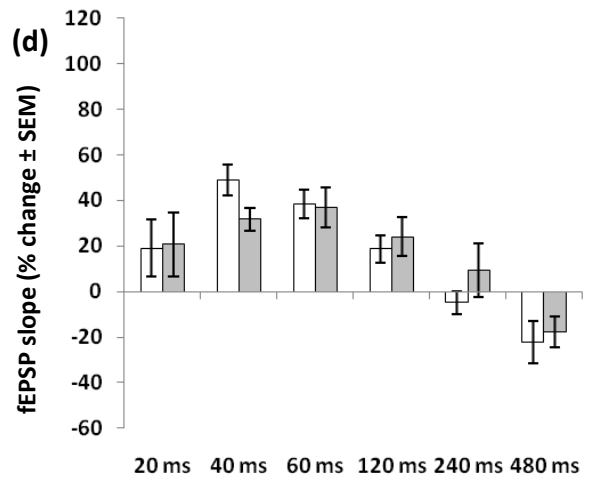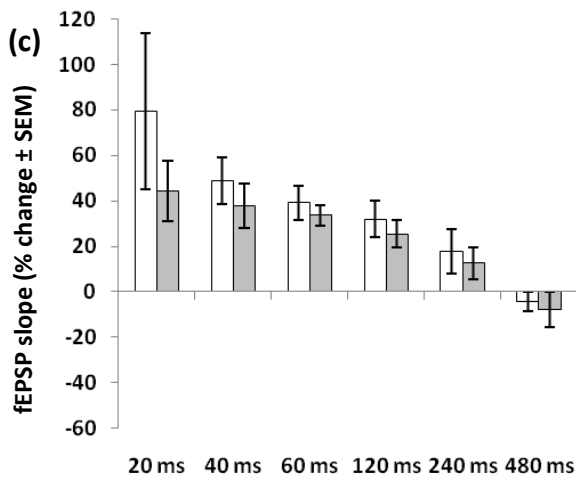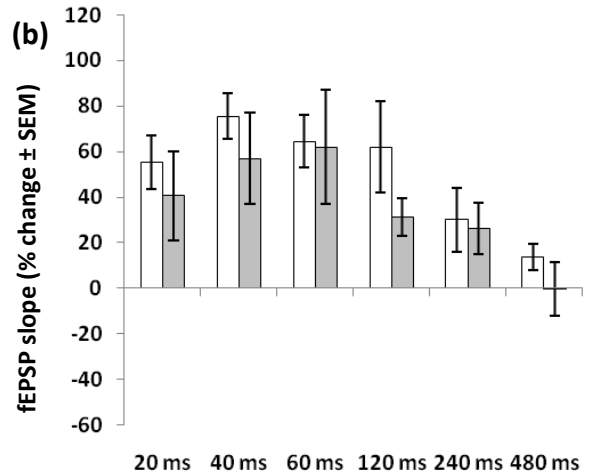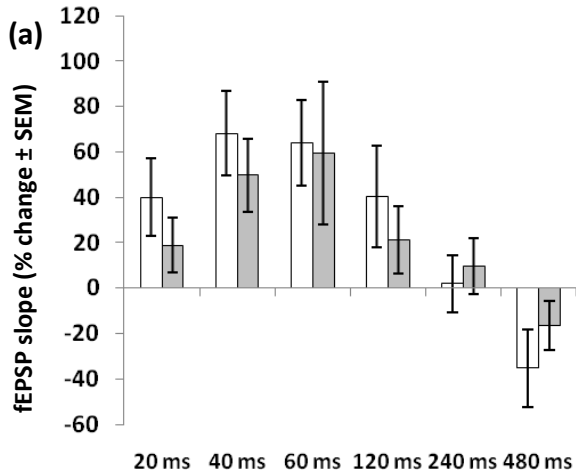
Figure 4.8: Comparison of pre-stimulation paired-pulse facilitation (PPF) values to post-stimulation PPF values in the CA1 to perirhinal cortex projection in the five different stimulation frequency groups: (a) 1 Hz; (b) 5 Hz; (c) 10 Hz; (d) 50 Hz; and (e) 100 Hz. There were no significant effects found for time in any group, indicating that the locus of change for synaptic plasticity was largely postsynaptic in nature. There were no significant effects for stimulation frequency, indicating that the locus of change for synaptic plasticity did not alter with different stimulation frequencies.

4.3.4 Effect of different stimulation frequencies on BDNF levels:

We first compared BDNF levels in the area CA1 of the hippocampus using a 2 × 5 mixed factorial ANOVA, with hemisphere (stimulated and unstimulated hemispheres) as the within-group measure and stimulation frequency (1 Hz, 5 Hz, 10 Hz, 50 Hz and 100 Hz) as the between-groups measure (Figure 4.9(a)). There was no significant effect for hemisphere (F = 3.534; df = 1, 25; p > 0.05). However, there was an overall effect for stimulation frequency (F = 5.319; df = 4, 25; p < 0.01) but there was no interaction effect found between hemisphere and stimulation frequency (F = 1.350; df = 4, 25; p > 0.05). Tukey *post-hoc* analysis showed that the 100 Hz group overall exhibited significantly lower levels of BDNF compared to the 5 Hz (p < 0.01), 10 Hz (p < 0.05) and 50 Hz groups (p < 0.05). There were no other significant effects found.

BDNF levels in the perirhinal cortex were again compared using a 2 x 5 mixed factorial ANOVA with hemisphere (stimulated and unstimulated hemispheres) as the within-group measure and stimulation frequency (1 Hz, 5 Hz, 10 Hz, 50 Hz and 100 Hz) as the between groups measure (Figure 4.9(b)). There was no significant effect found for hemisphere (F = 0.723; df = 1, 25; p > 0.05) but there was a significant effect found for stimulation frequency (F = 5.111; df = 4, 25; p < 0.01) and there was also a significant interaction effect found between hemisphere and stimulation frequency (F = 5.901; df = 4, 25; p < 0.01). Tukey *post-hoc* analysis revealed that there were significantly higher levels of BDNF in the 10 Hz group compared to the 1 Hz and 5 Hz groups (p < 0.01 in both cases). Dependent t-tests demonstrated that in the 1 Hz group, stimulation lead to a significant decrease in BDNF levels (t = 3.567; df = 5; p < 0.05). Additionally, stimulation lead to a significant increase in BDNF levels in the 10 Hz group (t = -10.563; df = 5; p < 0.001).

Finally, the percentage difference in BDNF levels between the stimulated and control hemispheres was calculated for each stimulation frequency in each brain area (Figure 4.10). There was a strong positive correlation between stimulation frequency

and BDNF levels in the area CA1 (R^2 = 0.903) but the correlation between stimulation frequency and BDNF levels in the perirhinal cortex was weaker (R^2 = 0.636).

Figure 4.9 (overleaf): Comparison of brain-derived neurotrophic factor (BDNF) levels across the stimulated and unstimulated (control) hemispheres for each stimulation frequency (1 Hz, 5 Hz, 10 Hz, 50 Hz and 100 Hz; n = 6 in each group) in the area CA1 and the perirhinal cortex. (a) In area CA1, there were no significant effects found for hemisphere but there were significantly lower levels of BDNF in the 100 Hz group overall compared to the 5 Hz, 10 Hz and 50 Hz groups. (b) In the perirhinal cortex, there were significant effects found for hemisphere; in the 1 Hz group there were significantly lower levels of BDNF in the stimulated hemisphere compared to the control and in the 10 Hz group there were significantly higher levels of BDNF in the stimulated hemisphere compared to the control. In addition, there were significantly higher levels of BDNF in the 10 Hz group overall compared to the 1 Hz and 5 Hz groups. *$p <$ 0.05; **$p <$ 0.01; ***$p <$ 0.001.

(a)
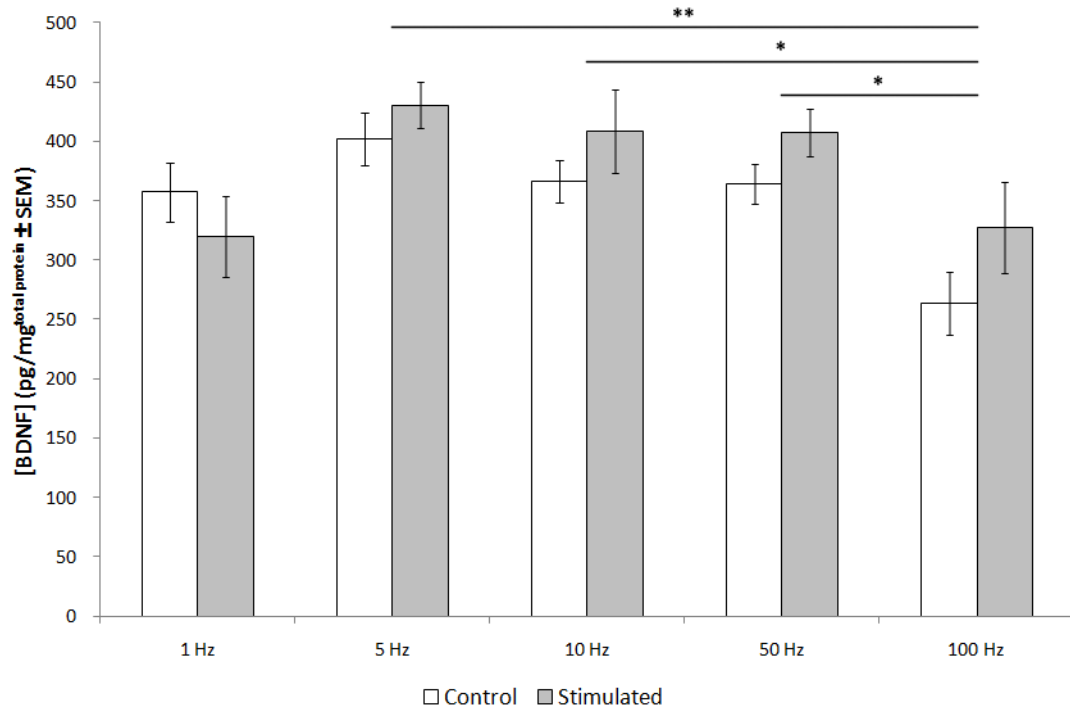
(b)
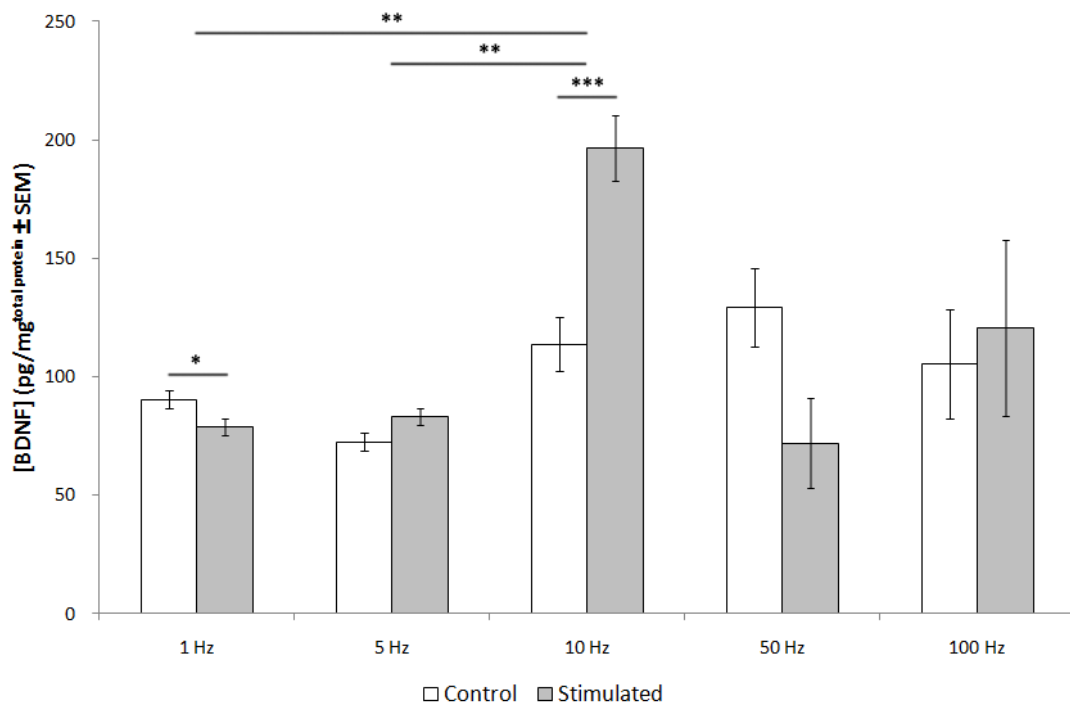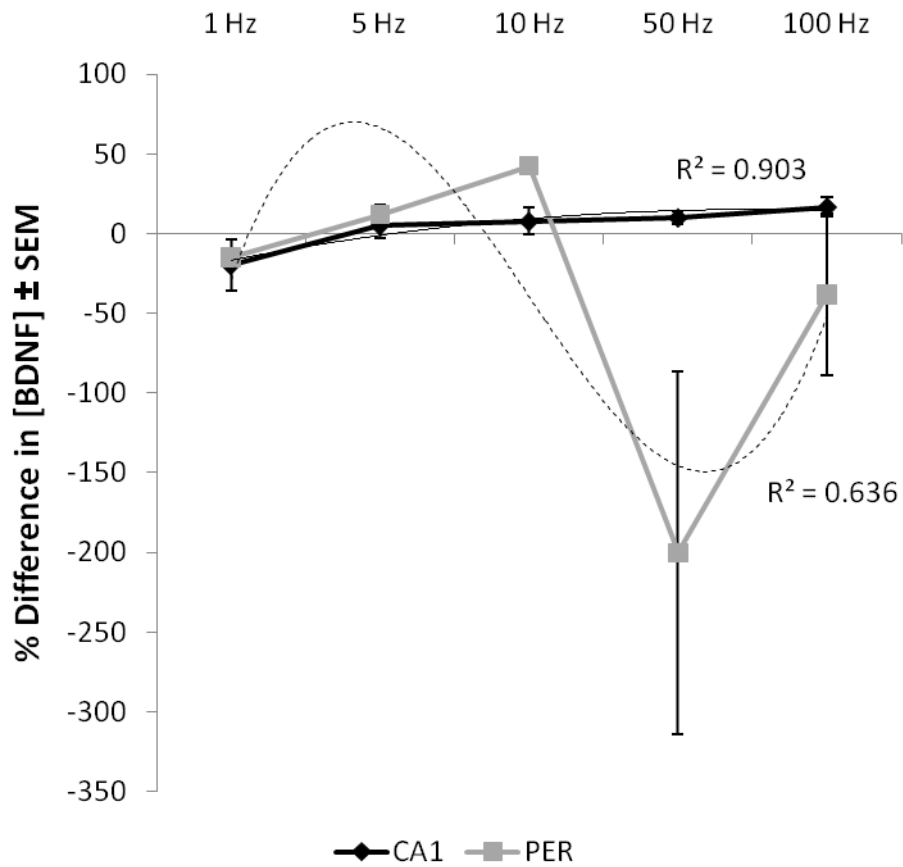
Figure 4.10: Percentage differences between stimulated and unstimulated hemispheres in CA1 and perirhinal brain-derived neurotrophic factor (BDNF) levels for each stimulation frequency group. There was a strong positive correlation between stimulation frequency and BDNF levels in the CA1 (solid line). There was a weaker positive correlation between stimulation frequency and BDNF levels in the perirhinal cortex (dashed line).

4.4 Discussion:

As shown in the previous two chapters, HFS of the area CA1 results in LTP being induced in the CA1 to perirhinal cortex pathway and here we have demonstrated that LTP can be induced at a number of different stimulation frequencies in an activity-dependent manner. Higher frequencies (50 Hz and 100 Hz) induced robust LTP of a similar level to LTP observed following 250 Hz HFS. Short-term plasticity in the form of PTP was also observed in these groups. LFS either failed to induce LTP (10 Hz) or low levels of potentiation were seen at the end of the 1 hr recording period (1 Hz and 5 Hz). In all three LFS groups, there was no PTP observed which indicates that PTP requires a rigorous stimulation protocol in order to be induced. We also demonstrated that because there was no change in post-stimulation PPF compared to baseline PPF in any stimulation group, therefore any changes observed in these groups were predominantly postsynaptic in nature and there was no differing pre- and postsynaptic effect for frequency.

The most striking finding of this study was the absence of LTD induction following LFS. This is unexpected as LTD has been demonstrated in other areas of the neocortex; in the entorhinal cortex, both LFS and pairing of presynaptic LFS and postsynaptic depolarisation can induce LTD (Deng & Lei, 2007) and in the perirhinal cortex there have been multiple reports of LTD being induced (Cho et al., 2000b; Cho et al., 2002). In addition, LFS of the area CA1 results in the induction of LTD in the CA1 to entorhinal cortex projection (Craig & Commins, 2007). Although LTD has not been described in the CA1 to perirhinal cortex projection, theta-pulse stimulation (TPS) has been shown to transiently depotentiate EPSPs that were previously exhibiting LTP (Cousens & Otto, 1998).

However, the induction of LTD has a proven track record of being difficult with other studies showing that it is not always possible to induce *in vivo*; LFS in area CA1 and the dentate gyrus failed to result in LTD in the adult rat (Errington et al., 1995) and LFS and two-pulse stimulation of the area CA1 fails to induce LTD in the CA1 to

subiculum projection (Anderson et al., 2000). The findings of Anderson and colleagues (2000) are pertinent to our findings presented here as they found that 1 Hz LFS lead to a late-developing potentiation similar to the potentiation seen in the final 10 min recording period in our 1 Hz group. In the perirhinal cortex, it has been shown that LTD is dependent on the size of intracellular Ca^{2+} increases (Cho et al., 2000a) which suggests that that a certain threshold of Ca^{2+} release must be reached in order for a given stimulation protocol to induce either LTD or LTP. In addition, Cho and colleagues (2000a) found that perirhinal LTD and LTP were induced via separate mechanisms and as such LTD and LTP in the perirhinal cortex may not represent two ends of the same activity-dependent process. In addition, perirhinal LTD can be blocked by previous exposure to learning (Massey et al., 2008) and is dependent on previous visual experience (Jo et al., 2006). These examples show how sensitive the BCM threshold is to shifting following experience and this sensitivity may explain why LFS in this projection failed to induce LTD. Changes in methodology might overcome this such as using two trains of LFS to induce LTD (one train to alter the metaplasticity of the projection, perhaps by reducing available BDNF and altering glutamate receptor distribution and another to induce LTD; Craig & Commins, 2007) or using younger animals (Errington et al., 1995).

Based on our findings, the lack of LTD observed at lower frequencies could be explained by the presence of BDNF. BDNF and TrkB expression has been to be regulated by AMPA receptor activation (Mackowiak et al., 2002) and BDNF has been shown to alter the threshold for synaptic plasticity (Ikegaya et al., 2002). The presence of BDNF alone is enough to induce potentiation (Kang & Schumann, 1995a; 1995b) and the application of BDNF *in vitro* can prevent the induction of LTD (Akaneya et al., 1996; Huber et al., 1998; Kinoshita et al., 1999). Furthermore, LTD induction has been associated with a concurrent reduction of BDNF in the perirhinal cortex (Aicardi et al., 2004). Taken together, these studies suggest that BDNF signalling must drop below a certain level in order for LTD to be induced, either by reductions in BDNF levels or by attenuating BDNF signalling downstream (e.g. inactivation of TrkB or MAPK signalling).

In this study, levels of BDNF in the area CA1 of the LFS groups was not significantly lower than the HFS groups exhibiting LTP (the LFS groups had more BDNF overall compared to the 100 Hz group) but there was a strong correlation between stimulation frequency and the magnitude of the stimulation-induced changes in BDNF levels in the area CA1. There was a significant reduction in BDNF in the perirhinal cortex of the 1 Hz group but we postulate that this was not big enough to induce LTD. Interestingly, a large significant increase in BDNF levels in the perirhinal cortex following 10 Hz LFS was not accompanied by LTP. The possibility that LFS or HFS could be causing contralateral as well as ipsilateral changes in BDNF levels (Bramham et al., 1996) cannot be ruled out. By using sham-stimulated controls (inserting the electrodes into the area CA1 and perirhinal cortex but not stimulating with either LFS or HFS), it may be possible to show whether changes in BDNF levels are restricted to the side being stimulated or whether contralateral effects are also occurring which may be masking any increases or decreases in BDNF following stimulation in these experiments.

In the previous chapter, we demonstrated that LTP could be induced using a 250 Hz HFS protocol without a concurrent increase in BDNF. Yet, we show in the next chapter that 250 Hz HFS can induce LTP and concurrently increase BDNF levels (Kealy & Commins, 2009). To explain these findings, we posit that these changes in BDNF levels may underlie metaplasticity in this projection and that BDNF levels may not actually be directly predictive of LTP/LTD.

This BDNF hypothesis is supported by the finding in the visual cortex where BDNF-induced blockade of LTD is prevented by tyrosine kinase (including TrkB) antagonism (Kumura et al., 2000) and inhibition of TrkB signalling *in vitro* subsequently lead to the induction of LTD (Jiang et al., 2003) showing that regulation of LTD may occur downstream of BDNF itself. Endocannabinoid-induced LTD can be impaired with BDNF application and it was confirmed that LTD could be induced by blocking the TrkB receptor (Huang et al., 2008). In the developing visual cortex in the rat, blockade of the TrkB receptor prevents the induction of LTP but the strength and form of LTD in the same tissue were unaffected (Sermasi et al., 2000). This suggests that the BDNF and its

TrkB receptor are required for LTP and their absence allows for LTD induction to occur more readily.

Complementing these findings, deletion of the gene for the p75 neurotrophin receptor (the universal neurotrophin receptor; see Chapter 1) in mice results in an impairment of LTD in the area CA1 (Woo et al., 2005) suggesting that neurotrophins, although perhaps not BDNF but other members of the protein family which have been suggested to play different roles in synaptic plasticity (Castrén et al., 1993), are still involved in the induction of LTD through the p75 receptor. As blockade of tyrosine kinase receptors in general still allowed for LTD induction (Kumura et al., 2000), the case for p75 playing a greater role in LTD is made stronger. Future research in this projection could look at the roles played by BDNF and its receptors in modulating synaptic plasticity.

Lastly, it could be that this projection is electrophysiologically excitatory in nature and that LTD is naturally difficult to induce. Evidence for this comes from the projection's readiness to induce LTP (Chapters 2 and 3; Cousens & Otto, 1998) and the lack of LTD using LFS protocols shown previously to induce LTD. In the projections going from the hippocamus to the entorhinal cortex, the projections originating in the distal CA1 and proximal subiculum and terminating in the lateral entorhinal cortex show similar excitability whereas those originating in the proximal CA1 and terminating in the medial entorhinal cortex showed a greater tendency for electrophysiologically inhibitory activity (Craig & Commins, 2007). As the lateral entorhinal cortex shows strong interconnectivity with the perirhinal cortex and the medial entorhinal cortex receives input from the postrhinal cortex (Insausti et al., 1997; Burwell & Amaral, 1998a) and there seems to be a functional difference between the circuits connecting the hippocampus and the entorhinal cortex (Craig & Commins, 2007), we suggest that the CA1 to perirhinal cortex projection forms part of the CA1/subiculum to lateral entorhinal cortex circuit. Therefore a tendency towards potentiation over depression may be expected. We predict that the postrhinal cortex is most likely associated with

the hippocampus to medial entorhinal cortex circuit and we hypothesise projections from the hippocampus to the postrhinal cortex to exhibit depression more readily.

In summary, in this chapter we demonstrated that the CA1 to perirhinal cortex projection can sustain PTP and LTP following 50 Hz and 100 Hz HFS. In addition, we showed that LFS did not induce LTD but in fact caused a range of different types of potentiation depending on the stimulation frequency. We suggest that the failure to induce LTD may be due to the presence of BDNF and TrkB signalling, possibly mediated through glutamate receptors. In the following chapter, we investigate the role of glutamate receptors in the induction of LTP and their effect on BDNF levels.

Chapter 5

Electrophysiological characterisation of the CA1 to perirhinal cortex projection: Roles of the NMDA and AMPA glutamate receptors.

Abstract:

Glutamate signalling is known to play role in the induction and maintenance of long-term potentiation (LTP). The NMDA glutamate receptor underlies LTP in the Schaffer collateral neurons but in mossy fibre neurons LTP is dependent on the AMPA/kainate glutamate receptors. In this chapter, we examined the pharmacological properties underpinning the plasticity observed in CA1 to perirhinal cortex projection by challenging the NMDA and AMPA/kainate receptors and investigating their effects on synaptic strength and BDNF levels. A stimulating electrode was inserted into the area CA1 and a recording electrode was inserted into the perirhinal cortex of urethane-anaesthetised Wistar rats. Rats (n = 6 in each drug group) were administered with either saline (0.09 %), MK-801 (NMDA antagonist; 0.1 mg/kg) or CNQX (AMPA/kainate antagonist; 1.5 mg/kg). Baseline recordings were made for 10 min by stimulating area CA1 (0.05 Hz stimulation protocol). High-frequency stimulation (HFS; 250 Hz) was performed and post-HFS fEPSP recordings were made for 1 hr (0.05 Hz, as above). Baseline and post-HFS paired-pulse facilitation (PPF) recordings were performed across six different interpulse intervals. CA1 and perirhinal cortex tissue samples were taken from the stimulated and unstimulated hemispheres of each rat brain and analysed using a brain-derived neurotrophic factor (BDNF) ELISA. Results indicate that LTP was induced in the saline and MK-801 groups but not in the CNQX group; fEPSPs in the latter group rapidly returned to baseline levels following a short period of post-tetanic potentiation. Drug treatment and HFS had no effect on PPF levels. Drug treatment significantly reduced concentrations of both CA1 and perirhinal BDNF and prevented stimulation-induced increases in BDNF in CA1. This molecular and electrophysiological data suggests that LTP in the CA1–perirhinal cortex projection may require activation of postsynaptic AMPA/kainate receptors in order to sustain LTP.

5.1 Introduction:

In the first two chapters, we demonstrated that the CA1 to perirhinal cortex projection is capable of sustaining short- and long-term changes in synaptic plasticity which may be in accordance with the role of the hippocampus acting as an association area for transferring information to the neocortex for long-term storage (McClelland et al., 1995; Rolls, 1996; McClelland & Goddard, 1996; McClelland, 1998; Hasselmo & McClelland, 1999). We have failed to find a discernible role for brain-derived neurotrophic factor (BDNF) and extracellular signal-regulated kinase (ERK) but are unable to rule out their contributions to plasticity in this projection.

As discussed in previous chapters, long-term potentiation (LTP) is a long-lasting increase in synaptic strength (Bliss & Lømo, 1973; Abraham, 2003) resulting from activity-dependent modifications of the presynaptic (Schultz et al., 1994) and postsynaptic nerve terminals (Isaac et al., 1995; Blundon & Zakharenko, 2008). In addition to molecular changes associated with both LTP and learning (see Chapter 1), there is also a strong pharmacological link between the two processes in that both learning and LTP can be affected by augmentation of glutamate signalling (Collingridge et al., 1983; 1988a; Morris et al., 1986; Vickery et al., 1997; Day et al., 2003).

As regards the glutamatergic nature of LTP, two different mechanisms for the induction of LTP have been described in the literature (Nicoll & Malenka, 1995). The first was identified in Schaffer collateral neurons in the hippocampus where it was found that LTP is dependent on the N-methyl-D-aspartic acid glutamate receptor (NMDA receptor; Collingridge et al., 1983). The second mechanism for LTP was identified in hippocampal mossy fibre neurons, where LTP can occur in the absence of functional NMDA receptors (Harris & Cotman, 1986). This non-NMDA receptor-dependent form of LTP has since been found to be mediated by the kainate glutamate receptor (Castillo et al., 1997; Vignes & Collingridge, 1997; Bortolotto et al., 1999). Kainate-induced potentiation in mossy fibre neurons can be induced independently of

the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor (Vignes & Collingridge, 1997).

The interactions between glutamate signalling and BDNF during hippocampal LTP have been investigated in several different studies. For example, Dragunow and colleagues (1993) have demonstrated that LTP can be blocked in the dentate granule cells of the hippocampus using the use-dependent NMDA receptor blocker (+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]cyclo-hepten-5,10-imine-maleate (MK-801) and this blockade of LTP induction also prevents associated increases in BDNF expression. Although application of BDNF in the hippocampus has been shown to potentiate synaptic transmission via the TrkB receptor, this BDNF-induced potentiation appears to occur independently of NMDA receptor activation (Kang & Schuman, 1995a; 1995b). It has however also been demonstrated that BDNF enhances phosphorylation of the hippocampal NMDA subunits NR1 and NR2B (Lin et al., 1998; Suen et al., 1997) in the postsynaptic density (PSD) and that the extent of PSD NR2B phosphorylation is similar to that of post-LTP induction suggesting that BDNF may directly modulate LTP via the NMDA receptor. Indeed, there is also evidence of BDNF interaction with the AMPA receptor during synaptic transmission and plasticity. Wu et al. (2004), for example, have demonstrated that BDNF may also enhance phosphorylation of the AMPA receptor GluR1 via changes in the NMDA receptor function (Wu et al., 2004). While the AMPA-type glutamate receptor modulators (the ampakines) have been shown to both increase BDNF protein levels and attenuates the aged-related impairment of LTP observed in hippocampal slices of middle-aged animals (Rex et al., 2006). However, in a more recent study examining the effects of BDNF on synaptic transmission, Madara and Levine (2008) suggest that BDNF does not act on postsynaptic AMPA receptors but rather it simultaneously modulates both pre and postsynaptic NMDA receptors.

The current study aims to extend our previous findings by examining the pharmacological properties of the CA1 to perirhinal cortex projection using the use-dependent NMDA receptor blocker MK-801 and the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Honoré et al., 1988). These particular

drugs have been chosen because both have been shown previously to impair performances in memory tasks following intraperitoneal administration (de Lima et al., 2005; Bäckström & Hyytiä, 2006; Nilsson et al., 2007). Further, we will investigate whether these drug treatments have an effect on paired-pulse facilitation (PPF) in order to determine whether the potentiation observed in the CA1 to perirhinal cortex projection is pre- or postsynaptic in nature. Finally, given the strong role of neurotrophins in learning and memory, especially BDNF, drug-induced changes in plasticity will be correlated with changes in BDNF concentrations in the area CA1 and in the perirhinal cortex using an enzyme-linked immunosorbent assay (ELISA).

5.2 Methods:

5.2.1 Surgery:

Adult male Wistar rats (n = 18; approximately 3 months old; weight: 300-400 g; Biomedical Unit; University College Dublin) were anaesthetised using urethane (ethyl carbamate; 1.5 mg/kg; i.p.; Sigma) and mounted on a stereotaxic frame. A stimulating electrode was inserted into the area CA1 and a recording electrode was inserted into the perirhinal cortex as detailed in Chapter 2.

One hour pre-tetanus (Morgan & Teyler, 1999), animals in the control group (n = 6) were administered with physiological saline (0.1 ml/100 g body weight of 0.9 % NaCl; Sigma, Ireland) and animals in the two experimental groups were given a glutamate receptor antagonist. The first group (n = 6) was administered with MK-801 (0.1 mg/kg body weight; Sigma, Ireland) and the second group (n = 6) was administered with CNQX (1.5 mg/kg body weight; Sigma, Ireland). The doses used in both the MK-801- and CNQX-treated groups were based on experiments where i.p. injection at the same concentrations resulted in a significant change in behaviour (de Lima et al., 2005; Bäckström & Hyytiä, 2006; Nilsson et al., 2007). All drugs were administered via i.p. injection. All animals were given approximately 40 min for the drug to enter their system before running the baseline PPF protocol, which meant that high-frequency stimulation (HFS) could be performed approximately 1 hr after drug administration (based on Leung & Shen, 1999; Habib & Dringenberg, 2009). All drugs were made up fresh each week of the experiment and dose-sized aliquots were frozen for use throughout the week.

5.2.2 Stimulation and data acquisition:

Pre-HFS PPF recordings, baseline fEPSP recordings, 250 Hz HFS, post-HFS fEPSP recordings and post-HFS PPF recordings were performed as detailed in Chapter 2.

5.2.3 Brain-derived neurotrophic factor enzyme-linked immunosorbant assay (ELISA):

Analysis of BDNF in the area CA1 and perirhinal cortex was performed as described in Chapter 3 via an enzyme-linked immunosorbent assay (ELISA) for BDNF using a BDNF Emax$^{©}$ ImmunoAssay System kit (Promega Corporation, U.S.A.).

5.2.4 Statistical analysis:

A series of dependent t-tests and repeated measures analyses of variance (ANOVAs) with the appropriate *post-hoc* test (Tukey at the 5 % level of significance) were used. A star-rated system was used where appropriate (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

5.2.5 Ethical considerations:

Laboratory procedures for the maintenance and experimentation of animals conformed to the Department of Health (Ireland) guidelines and the European directive 86/609/EC. Every effort was made to minimise the suffering and the number of animals used in this study.

5.3 Results:

5.3.1 Basic properties of fEPSP responses in the CA1 to perirhinal cortex projection:

An initial characterisation of the evoked response obtained in the perirhinal cortex following a single pulse stimulation of CA1 delivered at 0.05 Hz (n = 10) revealed a mean amplitude of −6.78 mV (±0.74), mean slope of the downward deflection of −0.825 mV/s (±0.091) with a latency of the peak amplitude of 8.27 ms (±0.68).

In the classic NMDA receptor-dependent model of LTP, the AMPA receptor has been shown to be the main receptor active during baseline conditions (Collingridge, 1985; Davies & Collingridge, 1989). Therefore we initially conducted a small set of control experiments to examine the effect of AMPA/kainate receptor antagonism on baseline fEPSP responses in the CA1 to perirhinal cortex projection (n = 3). We recorded baseline fEPSPs for 10 min (100 % ±0.96) and then injected each rat with CNQX (dose: 1.5 mg/kg; concentration of solution: 1.5 mg/ml; actual volume of injection: 1 ml/kg). We continued to record fEPSPs for a further 3 hr. fEPSP slopes (averaged over 10 min) at 1, 2 and 3 hr post-injection were 97.30 % (±1.85), 96.14 % (±1.73) and 98.26 % (±1.83) respectively. A repeated measures ANOVA was conducted on these data demonstrating no significant change in baseline response over the recording period (F = 1.063; df = 3, 27; p > 0.05).

5.3.2 Effects of glutamate receptor blockade on LTP:

To examine the effects of glutamate blockade on LTP, a 3 × 4 mixed factorial ANOVA was used with time analysed on 4 levels (−10–0 min, 0–10 min, 20–30 min and 50–60 min) as a within-groups measure. Drug treatment was analysed on 3 levels (saline, MK-801 and CNQX) as a between-groups measure. Overall, there was a significant effect of time (F = 96.652; df = 3, 81; p < 0.001). Drug treatment was also shown to have a significant effect (F = 78.723; df = 2, 27; p < 0.001). In addition, an interaction effect

between drug treatment and time was shown (F = 13.581; df = 6, 81; p < 0.01). Differences between the groups were determined using Tukey *post-hoc* analysis. Across the full length of the experiment (Figure 5.1), saline-treated animals showed significantly higher levels of potentiation compared to CNQX-treated animals (p < 0.001). Similarly, MK-801-treated animals showed significantly more potentiation compared to CNQX-treated animals (p < 0.001). There was no overall significant difference between saline-treated and MK-801-treated animals (p > 0.05).

Repeated measure ANOVAs with Bonferroni correction were used to analyse LTP at the four different time intervals within each group. In the saline-treated group, there was a significant effect for time (F = 51.494; df = 3, 27; p < 0.001) with LTP lasting for at least 1 hr post-HFS. Compared to baseline levels, fEPSP slopes were significantly higher in the 0-10 min (145.444 % ± 4.121; p < 0.001), 20-30 min (133.093 % ±2.775; p < 0.001) and the 50-60 min intervals (136.983 % ±2.154; p < 0.001). PTP was observed in the first 2 min of recording post-HFS but overall there were no significant differences observed between the 0-10 min group and either the 20-30 min (p > 0.05) or the 50-60 min intervals (p > 0.05). Therefore, 250 Hz HFS in saline-treated animals can induce PTP and LTP lasting for at least 1 hr as normal.

In the MK-801-treated group, there was also a significant effect for time (F = 33.041; df = 3, 27; p < 0.001) with LTP lasting for at least 50 min post-HFS. Compared to baseline levels, fEPSP slopes were significantly higher in the 0-10 min (151.944 % ±3.636; p < 0.001) and the 20-30 min intervals (131.603 % ± 2.958; p < 0.001). However, there was no significant difference between baseline levels and fEPSP slope values in the 50-60 min interval (113.232 % ±3.147; p > 0.05). Furthermore, fEPSP slopes in the 0-10 min interval were significantly higher than the 20-30 min (p < 0.05) and 50-60 min intervals (p < 0.001) which indicates that PTP was induced (PTP was observed only in the first 3 min of recording post-HFS). Finally, fEPSP slopes in the 20-30 min interval were significantly higher than in the 50-60 min interval (p < 0.05) which suggests that fEPSP slopes were beginning to decrease in size as time increased. In

short, 250 Hz HFS in MK-801-treated animals could induce PTP and LTP but this LTP may not be as robust as in control animals.

In the CNQX-treated group, there was again a significant effect found for time (F = 57.911; df = 3, 27; $p < 0.001$) but with potentiation being much shorter in duration than described in the other two groups. fEPSP slopes in the 0-10 min group (122.335 % ±3.082) were significantly higher compared to baseline levels ($p < 0.001$) but by the 20-30 min interval (104.283 % ±1.158) there were no significant differences compared to baseline ($p > 0.05$). However, in the 50-60 min interval the fEPSP slopes (91.654 % ± 1.473) were significantly lower than baseline levels ($p < 0.001$). In addition, fEPSP slopes in the 0-10 min interval were significantly higher than both the 20-30 min ($p < 0.001$) and 50-60 min intervals ($p < 0.001$). Finally, fEPSP slopes in the 20-30 min interval were significantly higher compared to the 50-60 min interval ($p < 0.001$). Taken together, 250 Hz HFS in CNQX-treated animals could induce PTP but no long-lasting increases in synaptic strength were observed.

Using one-way ANOVAs to look in more detail at the effects of drug treatment at different times during recording showed some significant differences (Figure 5.1). Baseline levels (10 to 1 min pre-HFS) showed no significant differences ($F < 0.001$; df = 2, 27; $p > 0.05$) but there were significant differences observed between drug treatments during post-tetanic potentiation (PTP; 0 to 10 min post-HFS; F = 12.970; df = 2, 27; $p < 0.001$). Tukey *post-hoc* analysis revealed that CNQX-treated animals showed significantly lower levels of PTP compared to saline-treated animals ($p < 0.01$) and MK-801-treated animals ($p < 0.001$). There were no significant differences between saline- and MK-801-treated animals ($p > 0.05$).

Looking at middle-LTP (21 to 30 min post-HFS) showed a significant effect for drug treatment (F = 49.979; df = 2, 27; $p < 0.001$) and Tukey *post-hoc* analysis revealed that CNQX-treated animals exhibited significantly lower levels of potentiation compared to saline-treated animals ($p < 0.001$) and MK-801-treated animals ($p < 0.001$). Late-LTP (51 to 60 min post-HFS) showed further significant differences between the groups (F = 92.254; df = 2, 27; $p < 0.001$). At this stage, Tukey *post-hoc*

analysis again showed that CNQX-treated animals exhibited significantly lower levels of potentiation compared to saline-treated animals ($p < 0.001$) and MK-801-treated animals ($p < 0.001$). In addition, MK-801-treated animals also showed significantly lower levels of potentiation compared to saline-treated animals ($p < 0.001$).
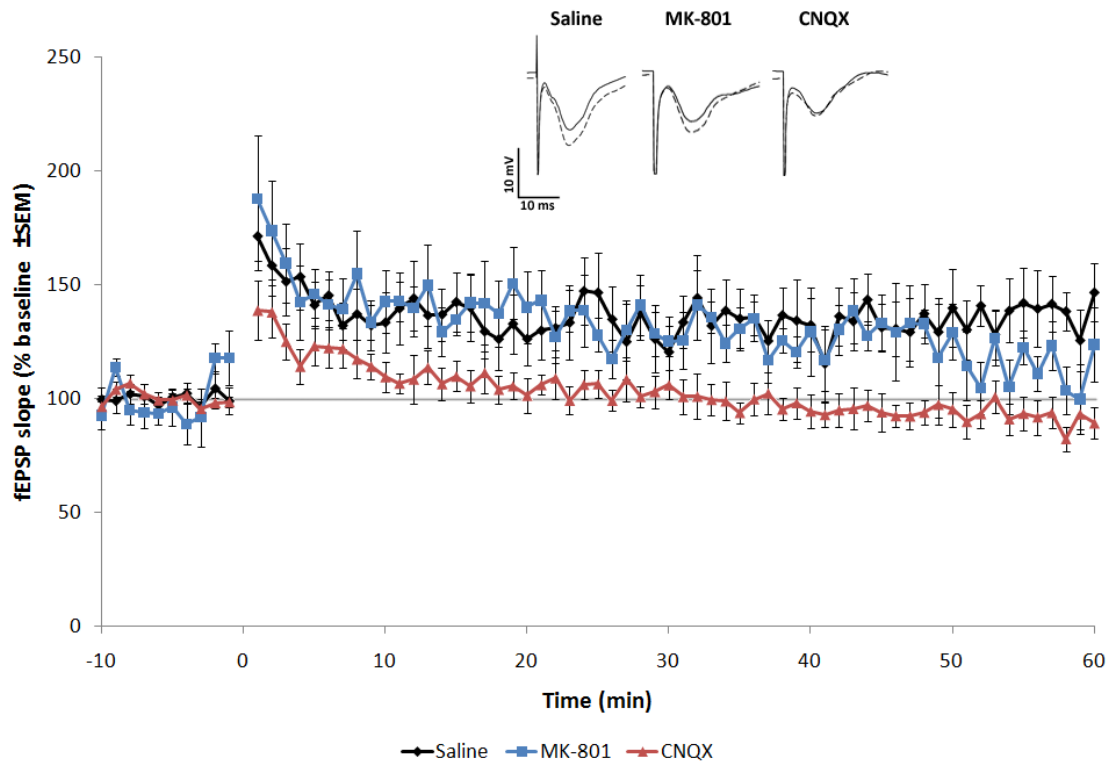
Figure 5.1: Effect of glutamate receptor antagonism on long-term potentiation (LTP) induced by 250 Hz high-frequency stimulation (HFS). Saline-treated animals (n = 6, black diamonds) showed LTP lasting for at least 1 hr. MK-801-treated animals (n = 6, blue squares) showed LTP lasting approximately 50 min before beginning to deteriorate. CNQX-treated animals (n = 6, red triangles) showed LTP lasting only approximately 25 min before dropping below baseline levels. Each point represents an average of three fEPSP slopes recorded over 1 min and data is expressed as a percentage of baseline fEPSP slopes where baseline is approximately 100 %. Inset are representative fEPSP traces from the three drug groups showing baseline fEPSPs (−10 min; solid line) and post-HFS fEPSPs (+30 min; dashed line).

5.3.3 Effects of glutamate receptor blockade on baseline and post-HFS PPF:

Prior to 250 Hz HFS, PPF recordings were made at six different interpulse intervals (IPI; 20 ms, 40 ms, 60 ms, 120 ms, 240 ms and 480 ms; a within-groups measure) in all three

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drug groups (saline, MK-801 and CNQX; a between groups measure). A $3 \times 6 \times 2$ mixed factorial ANOVA confirmed significant effects for interval ($F = 25.961$; $df = 5, 75$; $p < 0.001$) and for facilitation (pulse1 vs pulse2; $F = 60.019$; $df = 1, 15$; $p < 0.001$) but there were no significant effects found for drug treatment ($F = 0.143$; $df = 2, 15$; $p > 0.05$). There were no interaction effects for interval and drug treatment ($F = 0.809$; $df = 10, 75$; $p > 0.05$) and facilitation and drug treatment ($F = 0.143$; $df = 2, 15$; $p > 0.05$) but there was an interaction effect for interval and facilitation ($F = 25.961$; $df = 5, 75$; $p < 0.001$). The results showed that significant facilitation occurs at shorter intervals, peaking with a 40 ms interval in saline- and MK-801-treated animals and with a 60 ms interval in CNQX-treated animals (Figure 5.2(a)). Following dependent t-tests, significant facilitation was found for 40 ms, 60 ms and 120 ms IPIs in all drug treatment groups ($p < 0.05$), for 20 ms IPI for the saline- and CNQX-treated groups ($p < 0.05$) and for 240 ms IPI for CNQX-treated animals alone ($p < 0.05$) PPF tapered out at longer intervals; there was no significant facilitation seen in the second fEPSP response to the second stimulus compared to the first for the 240 ms (in saline- and MK-801-treated animals) and 480 ms intervals (all drug treatment groups; Figure 5.2(a)).

We then compared the percentage facilitation obtained in each treated group and at each interval 60 min post-HFS to the baseline facilitation levels (Figure 5.2(b)). A $2 \times 3 \times 6$ mixed factorial ANOVA was employed, again with IPI as a within-groups measure and drug treatment as a between-groups measure and also with time (baseline and post-HFS) as a within-groups measure. No overall effects were found for drug treatment ($F = 1.608$; $df = 1, 15$; $p > 0.05$) but significant effects were found for time ($F = 7.002$; $df = 1, 15$; $p < 0.05$) and interval ($F = 28.474$; $df = 5, 75$; $p < 0.001$). However, no interaction effects were found for time and drug treatment ($F = 2.523$; $df = 2, 15$; $p > 0.05$), for interval and group ($F = 28.474$; $df = 10; 75$; $p > 0.05$) or for time and interval ($F = 1.586$; $df = 5, 75$; $p > 0.05$). Dependent t-tests revealed that there were no significant changes observed in the saline- and CNQX-treated groups and in the MK-801-treated group there was only a significant attenuation in facilitation seen following HFS at the 20 ms interval (Figure 5.2(b)).

(a)
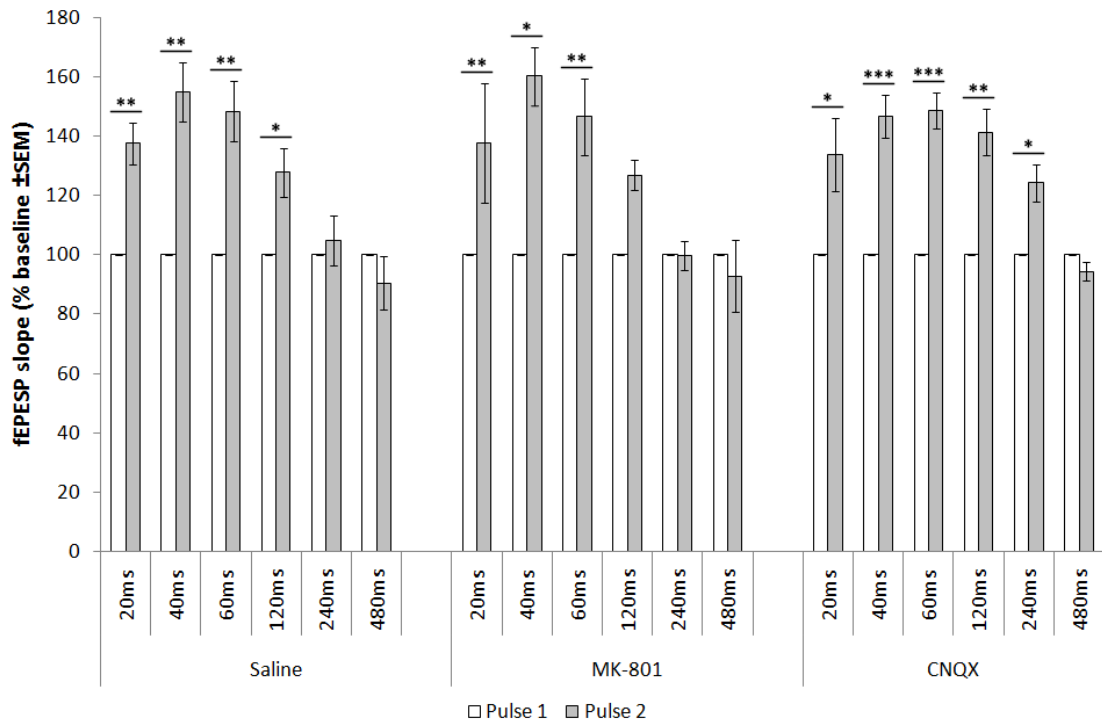
(b)
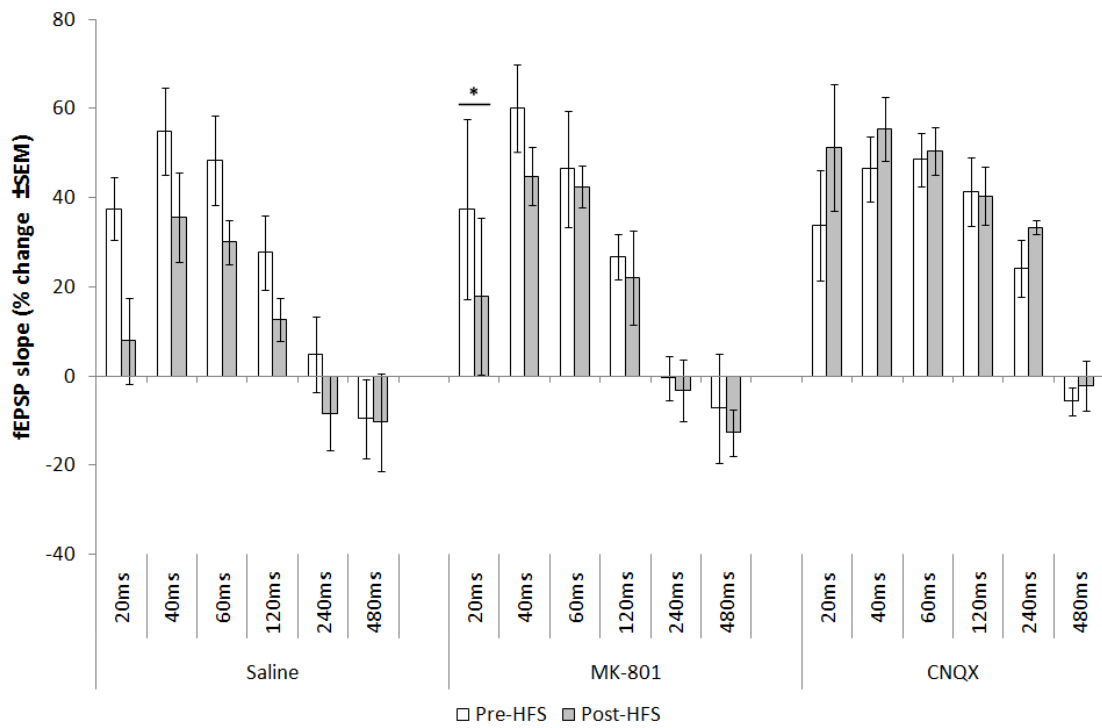
Figure 5.2 (previous page): (a) The effect of paired-pulse facilitation (PPF) at six interpulse intervals (IPIs) in the baseline condition. PPF peaked at 40 ms in the saline- and MK-801-treated groups and at 60 ms in the CNQX-treated group. PPF tapered off at longer intervals. (b) Comparison between baseline and post-HFS levels of facilitation at the six IPIs. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

5.3.4 Effects of glutamate receptor blockade on BDNF levels:

We first compared BDNF levels in the area CA1 of the hippocampus using a 2 × 3 mixed factorial ANOVA, with hemisphere (stimulated and unstimulated hemispheres) as the within-group measure and drug treatment (saline, MK-801 and CNQX) as the between-groups measure (Figure 5.3(a)). There was an overall effect for hemisphere with the area CA1 of the stimulated hemisphere showing significantly higher levels of BDNF compared to the area CA1 of the unstimulated hemisphere ($F = 12.787$; $df = 1, 15$; $p < 0.01$). We also found an overall effect for drug treatment ($F = 66.884$; $df = 2, 15$; $p < 0.001$). *Post-hoc* analysis (Tukey test) showed that the MK-801- and CNQX-treated groups showed significantly lower levels of BDNF in the area CA1 compared to the saline-treated group ($p < 0.001$). There was also an interaction effect between drug treatment and hemisphere in the area CA1 ($F = 3.564$; $df = 2, 15$; $p = 0.05$). Further dependent t-tests demonstrated that in the saline-treated group there was a significant increase in BDNF levels in the stimulated side of the area CA1 compared to the unstimulated side ($t = -4.708$; $df = 5$; $p < 0.01$). No other differences were noted.

We compared the effects of drug treatment within each hemisphere using a one-way ANOVA. Within the area CA1 of the unstimulated hemisphere there was a significant effect found for drug treatment ($F = 87.178$; $df = 2, 15$; $p < 0.001$). Tukey *post-hoc* tests revealed that BDNF levels were significantly higher in the saline-treated group compared to the MK-801- and CNQX-treated groups ($p < 0.001$ for both) and that BDNF levels in the CNQX-treated group were significantly higher than those in the MK-801-treated group ($p < 0.05$). Similarly there was an effect for drug treatment in the area CA1 of the stimulated hemisphere ($F = 30.680$; $df = 2, 15$; $p < 0.001$). Again, Tukey *post-hoc* tests showed that there were significantly higher levels of BDNF in the

saline-treated group compared to the MK-801- and CNQX-treated groups ($p < 0.001$ for both) but no significant differences between MK-801- and CNQX-treated groups ($p > 0.05$).

We next compared BDNF levels in the perirhinal cortex, again using a 2×3 mixed factorial ANOVA with hemisphere (stimulated and unstimulated hemispheres) as the within-group measure and drug treatment (saline, MK-801 and CNQX) as the between-groups measure (Figure 5.3(b)). There was an overall effect for hemisphere with the perirhinal cortex of the stimulated hemisphere showing significantly higher levels of BDNF compared to the perirhinal cortex of the unstimulated ($F = 10.952$; $df = 1, 15$; $p < 0.01$).

Again, an overall effect for drug treatment on BDNF levels was observed ($F = 23.083$; $df = 2, 15$; $p < 0.001$). *Post-hoc* analysis (Tukey test) showed that compared to the saline-treated group, the MK-801- and CNQX-treated groups again showed significantly lower levels of perirhinal BDNF ($p < 0.001$ and $p < 0.01$ respectively). In addition, overall BDNF levels in the MK-801-treated group were significantly lower than the CNQX-treated group ($p < 0.05$). There was an interaction effect between drug treatment and HFS in the perirhinal cortex ($F = 5.036$; $df = 2, 15$; $p < 0.05$). Dependent t-tests demonstrated that in the MK-801 group there were significantly higher levels in the stimulated hemisphere compared to the unstimulated hemisphere ($t = -2.762$; $df = 5$; $p < 0.05$). A similar pattern was observed in the CNQX-treated group ($t = -3.480$; $df = 5$; $p < 0.05$).

We again compared the effects of drug treatment within each hemisphere using a one-way ANOVA. Within the perirhinal cortex of the unstimulated hemisphere there was a significant effect found for drug treatment ($F = 30.522$; $df = 2, 15$; $p < 0.001$). Tukey *post-hoc* analysis revealed that BDNF levels were significantly higher in the saline-treated group compared to the MK-801- and CNQX-treated groups ($p < 0.001$ for both) but no significant differences were found between the MK-801- and the CNQX-treated groups ($p < 0.05$). Similarly there was an effect for drug treatment in the perirhinal cortex of the stimulated hemisphere ($F = 12.055$; $df = 2, 15$; $p < 0.01$).

Tukey *post hoc* analysis showed that there were significantly higher levels of BDNF in the stimulated side of the perirhinal cortex in the saline-treated group compared to the MK-801-treated group ($p < 0.01$) but there were no significant differences between saline- and CNQX-treated groups ($p > 0.05$). However, there were significantly higher levels of perirhinal BDNF in the stimulated hemisphere in the CNQX-treated group compared to the MK-801-treated group ($p < 0.05$).

Figure 5.3 (overleaf): Effect of glutamate receptor antagonism and 250 Hz high-frequency stimulation (HFS) on BDNF levels in (a) the area CA1 and (b) the perirhinal cortex. Saline-treated animals showed a significant increase in BDNF levels in the area CA1 following 250 Hz HFS. No significant increases in BDNF in the area CA1 were found in either drug treated group. In the perirhinal cortex there were significant increases in BDNF levels in both the MK-801- and CNQX-treated groups but not in the saline-treated group following HFS. There was no significant difference between perirhinal BDNF levels in the stimulated hemisphere in the CNQX group compared to the saline group. *$p < 0.05$; **$p < 0.01$.
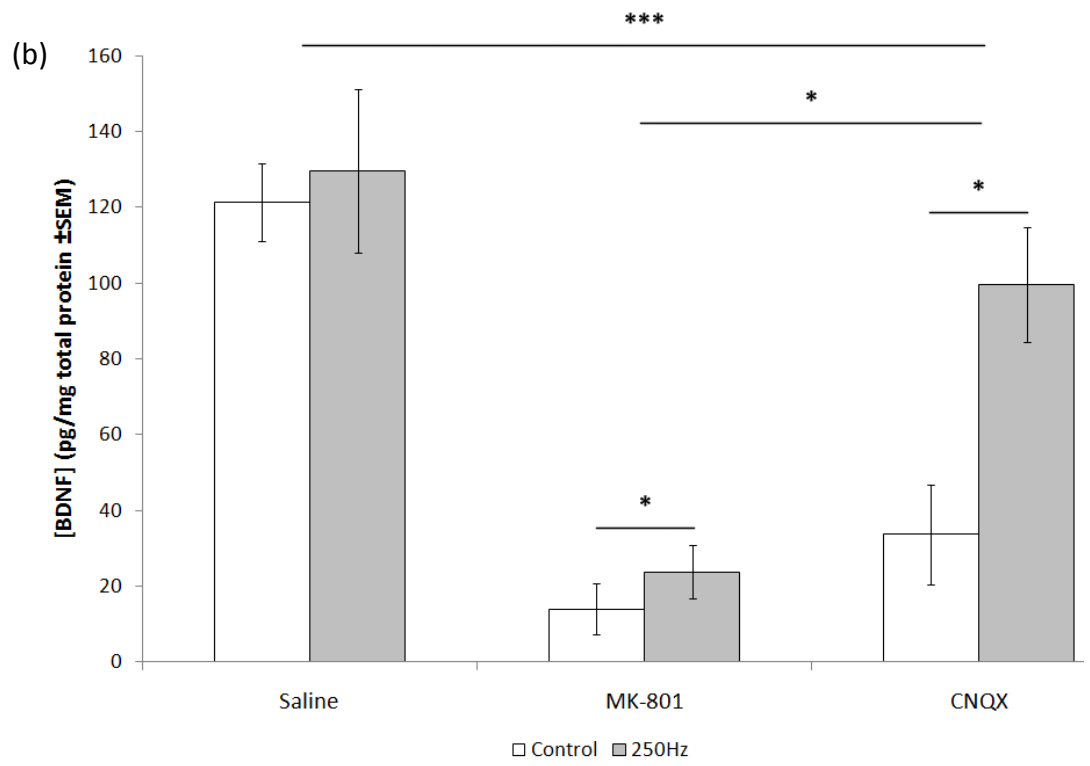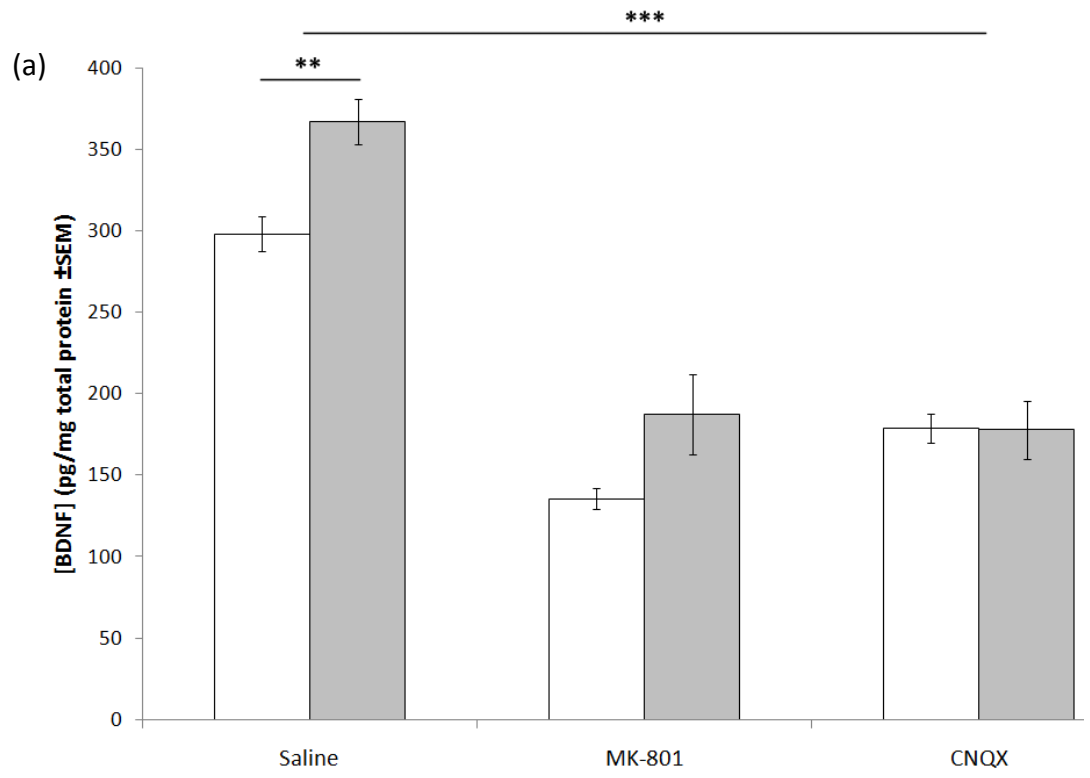
5.4 Discussion:

Here we again demonstrate that the monosynaptic projection from the area CA1 to the perirhinal cortex is capable of sustaining LTP following 250 Hz HFS (Chapters 2 and 3; Cousens & Otto, 1998). As area CA1 is one of the primary output structures of the hippocampus (Van Groen & Wyss, 1990; Amaral et al., 1991; Naber et al., 2001a; Amaral & Lavenex, 2007), demonstrating plasticity (short-term and long-term) here may provide a possible route and mechanism for information to be consolidated to long-term memory and determining the roles of the glutamate receptors that underlie this plasticity may reveal the way in which this consolidation can occur.

Glutamate receptors of various kinds have been shown to have a central role in LTP (Collingridge et al., 1983; Castillo et al., 1997) and in memory formation (Morris et al., 1986). In the current studies, we demonstrate that the potentiation induced in this pathway can be blocked by CNQX and not MK-801, perhaps suggesting that the potentiation observed in this pathway is AMPA/kainate receptor-mediated rather than NMDA receptor-mediated. Although it should be noted that we observed a lowering of potentiation in the MK-801-treated group at 50 min post-HFS, indicating that there may also be some NMDA receptor influence at a longer time period. This finding that NMDA and AMPA/kainate receptors may perform different roles controlling plasticity in this projection demonstrates the complexity of the hippocampal circuit in terms of the types of potentiation observed in the various pathways and may suggest different functions for the different pathways. For example, NMDA receptor-mediated pathways include the Schaffer collateral (Collingridge et al., 1983; 1988a), the perforant pathway (Wu et al., 2001) and the CA1 to subiculum pathway (Wozny et al., 2008) while kainate receptors are known to mediate potentiation in the mossy fibre pathway (Bortolotto et al., 1999). Bearing in mind that CNQX is an antagonist of both AMPA and kainate receptors (Honoré et al., 1988), we are as yet unable to say whether the CA1 to perirhinal cortex pathway should be classed with the mossy fibre pathway as a kainate

receptor-mediated pathway (Bortolotto et al., 1999) or as an AMPA receptor-mediated pathway.

In addition to the type of LTP that is induced, the locus of change following potentiation has been much debated. One method in determining the locus of change following potentiation is by comparing levels of PPF post-HFS to baseline levels of facilitation (McNaughton, 1982; Larkman et al., 1992; Schulz et al., 1994; Commins et al., 1998). PPF is considered to be mainly presynaptic in nature; therefore if with potentiation there is a change in the facilitation strength, then it is possible that this potentiation contains at least some component of a presynaptic mechanism (McNaughton, 1982; Larkman et al., 1992; Schulz et al., 1994). Here we again report no significant change in facilitation 1 hr post-HFS in the saline group (see Chapter 2), suggesting that the potentiation observed in this pathway may be largely due to postsynaptic rather than presynaptic factors (Commins et al., 1998).

We initially observed that baseline PPF remained similar irrespective of drug treatment, suggesting that this short-term facilitation effect is not mediated by glutamate receptors. This would be expected as PPF is known to result from residual calcium in the presynaptic terminal following rapid activation of ion-gated Ca^{2+} channels (Wu & Saggau, 1994). Furthermore, at 60 min post-HFS we also found no significant change in facilitation in any of the drug treated groups, again suggesting that the potentiation observed is mainly postsynaptic in nature (Commins et al., 1998). Bearing this in mind, we did observe an attenuation of the PTP response (0–10 min post-HFS, Figure 5.1) in the CNQX-treated group. PTP is also known to be a presynaptic event where the strength of the fEPSP is increased due to raised levels of neurotransmitter being released (Hubbard, 1963). This attenuation of PTP in the CNQX group suggests a small AMPA-dependent presynaptic component to the potentiation observed in the CA1 to the perirhinal cortex pathway.

Molecularly, saline-treated animals showed LTP and increased BDNF levels in area CA1 in the stimulated hemisphere. This was not seen in our previous study characterising the molecular nature of CA1 to perirhinal cortex LTP (Chapter 3) but the

large effect size observed in our previous work conforms with the findings of this study. Although some authors demonstrate that unilateral LTP can trigger BDNF, trkC and nerve growth factor increases bilaterally in the hippocampal region (Bramham et al., 1996), we cannot rule out the possibility of an increase in BDNF in the unstimulated hemisphere in our experiment as well, we can only suggest for now that the BDNF expression was enhanced in the stimulated hemisphere compared to the unstimulated side. Conversely, i.p. injection of either MK-801 or CNQX reduced BDNF concentrations in both the area CA1 and the perirhinal cortex generally. Stimulation of the hippocampal formation has been shown in many other studies to lead to an increase in BDNF levels (Castrén et al., 1993; Gooney & Lynch, 2001). It has also been shown that BDNF (Korte et al., 1995; 1996; Patterson et al., 1996; Gooney & Lynch, 2001) and activation of the TrkB receptor (Kang et al., 1997; Minichiello et al., 2002) play substantial roles in the induction of LTP. As BDNF production was inhibited equally in both of our drugs groups, this suggests that glutamatergic signalling is required for BDNF expression (Dragunow et al., 1993; Wetmore et al., 1994; Falkenberg et al., 1996, Kesslak et al., 2003). Our findings compare favourably with those of Dragunow et al. (1993) who found that not only did MK-801 block the induction of LTP in the perforant path but that it also lowered the expression of BDNF. In addition, we found that not only did these glutamate receptor antagonists reduce global levels of BDNF but that they also blocked stimulation-induced increases in BDNF concentrations in the area CA1. This mirrors the attenuation of learning-induced increases in BDNF expression following administration of MK-801 in rats (Kesslak et al., 2003). This again points to a possible necessity for glutamatergic signalling in BDNF expression.

In the perirhinal cortex, where evoked responses occurred, BDNF synthesis was also generally inhibited in both the MK-801- and CNQX-treated groups. Interestingly, in the saline-treated group there were no significant differences observed between the stimulated and unstimulated hemispheres. This may be explained by the work by Aicardi et al. (2004) where increases in the secretion of perirhinal BDNF levels following TBS in slices have been described. In their study, the induction of LTP leads to an

increase in BDNF secretion in the 5–12 min following TBS (Aicardi et al., 2004); any increases in BDNF in the perirhinal cortex of the saline group may have been secreted by the time we took our tissue samples for analysis (i.e. over an hour post-HFS).

Nevertheless both MK-801- and CNQX-treated groups showed significantly higher levels of BDNF in the stimulated hemisphere compared to the unstimulated hemisphere. Of particular interest are the perirhinal BDNF levels in the stimulated hemisphere of the CNQX-treated group where they increased to a level not significantly different to those of the saline controls (i.e. reversing the drug-induced decreases in BDNF levels). This differs from Aicardi et al.'s experiment (2004) detailed above where, converse to the situation in the saline group, increased levels of BDNF were detected over an hour post-HFS in our study (Figures 5.3(a) and (b)) compared to an increase lasting only 5–12 min in Aicardi et al.'s study. These differences might be explained by the fact that Aicardi et al. (2004) measured secreted BDNF from slices while we analysed homogenised tissue samples and as such may have detected unsecreted BDNF.

Overall, these findings suggest that the NMDA receptor and the AMPA/kainate receptors not only seem to have different functions in terms of electrophysiology in this pathway but that these differences extend to the expression of BDNF in these two brain areas. As some studies have shown behavioural differences between NMDA receptor blockade and AMPA/kainate receptor antagonism (de Lima et al., 2005; Winters & Bussey, 2005b; Nilsson et al., 2007), the electrophysiological and molecular differences between the drug groups are expected. Furthermore, our results demonstrating an AMPA-mediated role in plasticity and BDNF changes perhaps should not be too surprising given that activation particularly of the non-NMDA receptors (AMPA & Kainate) result in an increased expression of neurotrophic factors, including BDNF (Zafra et al., 1990, 1992), while reciprocally BDNF via the TrkB receptor can increase the expression levels of AMPA receptors (Narisawa-Saito et al., 2002) which can enhance glutamatergic transmission in hippocampal cells (Lessmann et al., 1994; Levine et al., 1998). It is also known that the kinetics of AMPA-mediated BDNF

activation are consistent with the known properties of AMPA receptors (Arai et al., 1996, 2002; Lockhart et al., 2007). In addition, sub-chronic administration of CX691 (an AMPA modulator) has been shown to elevate BDNF mRNA expression in both the whole and CA1 sub-region of the hippocampus (Woolley et al., 2009), while administration of another AMPA modulator (S18986) has been shown to enhance AMPA-mediated BDNF mRNA and protein expression in rat primary cortical neuronal cultures (Lockhart et al., 2007). These results along with ours suggest a close coupling of the AMPA receptor, BDNF and plasticity in the hippocampal–perirhinal projection.

In conclusion, this study suggests that the projection from area CA1 to the perirhinal cortex can undergo changes in synaptic strength and the induction of LTP in this projection is reliant on AMPA/kainate receptors. Moreover, from our PPF data, the changes in plasticity appear to be mainly postsynaptic in nature. Finally, BDNF does appear to play a role in these changes in plasticity.

Chapter 6

The effect of NMDA and AMPA glutamate receptor antagonism on spatial and recognition memory: A behavioural and molecular analysis.

Abstract:

The hippocampus has been shown to be necessary for tests of spatial memory and the perirhinal cortex has been shown to be necessary for tests of recognition memory. Glutamatergic signalling has been implicated in both forms of memory and the synaptic plasticity that underlies these learning processes is believed to rely on neurotrophins. In Experiment 1, we compared hippocampal and perirhinal brain-derived neurotrophic factor (BDNF) levels in three different tasks: an object recognition task (a test of recognition memory); an object displacement task (a test of spatial memory); and a control task with no changes in the retention trial. There were significantly higher levels of hippocampal BDNF in the object displacement task compared to the other two tasks. This perhaps reflects the role of the hippocampus in spatial memory. In Experiment 2, we compared performance in an object recognition task following treatment with saline, MK-801 (an NMDA receptor blocker) or CNQX (an AMPA/kainate receptor antagonist) and analysed hippocampal and perirhinal BDNF levels in all groups. The CNQX-treated group was impaired in the object recognition task, indicating that AMPA/kainate receptors are required for recognition memory. There were no significant differences in BDNF levels between the groups. In Experiment 3, we compared performance in an object displacement task following treatment with saline, MK-801 or CNQX and again analysed hippocampal and perirhinal BDNF levels in all groups. Both MK-801- and CNQX-treated rats were impaired in the object displacement task, indicating that both NMDA and AMPA/kainate receptors are required for spatial memory. CNQX-treatment caused a significant increase in hippocampal BDNF compared to the other two groups, suggesting that disrupted BDNF signalling may underlie the deficit in spatial memory caused by AMPA/kainate receptor antagonism. These experiments demonstrate the importance of glutamatergic signalling in these tasks, suggesting that changes in synaptic plasticity are required for learning and memory.

6.1 Introduction:

In the previous chapters, we have demonstrated that the CA1 to perirhinal cortex projection is capable of sustaining short- and long-term changes in synaptic plasticity in an activity-dependent manner. We have also showed that long-term potentiation (LTP) in this projection seems to be AMPA glutamate receptor-dependent and that brain-derived neurotrophic factor (BDNF) may play a role in modulating synaptic plasticity in this projection. In this chapter, we attempt to connect our electrophysiological and molecular findings to behaviour in the rat by investigating the role of NMDA and AMPA glutamate receptors in spatial and recognition memory tasks.

The roles of the hippocampus and the perirhinal cortex in the behaving animal have largely been determined; the perirhinal cortex has been shown to be necessary for object recognition memory (Mumby & Pinel, 1994; Liu & Bilkey, 2001; Mumby et al., 2002b; Winters & Bussey, 2005a; Hannesson et al., 2005; Mumby et al., 2007; Albasser et al., 2009) whereas the hippocampus is required in tasks involving spatial memory (O'Keefe & Nadel, 1978; Handelmann & Olton, 1981; Morris et al., 1982; Sutherland et al., 1983; Talpos et al., 2008). Recordings made in the unanaesthetised rat show that neurons in the perirhinal cortex underwent changes in activity during a visual recognition task with familiar stimuli eliciting a smaller response than novel stimuli (Zhu et al., 1995a) and similarly recordings in the hippocampal neurons show increased activity activated during spatial tasks (O'Keefe, 2007). Rats with perirhinal lesions have no difficulties with object discrimination but they do have difficulty in recognising objects to which they have been previously exposed to (Davies et al., 2007).

In support of a functional difference between these two areas of the brain, animals with perirhinal lesions are impaired on object recognition but not on spatial memory tasks (Aggleton et al., 1997; Ennaceur & Aggleton, 1997; Glenn & Mumby, 1998; Bussey et al., 1999; Machin et al., 2002) and animals with hippocampal lesions are impaired on spatial but not object recognition memory tasks (Ennaceur et al., 1997). Double dissociation studies confirmed the findings of these disparate studies with fornix lesions sparing recognition memory but not spatial memory and

perirhinal lesions showing no spatial impairments but recognition memory was impaired (Ennaceur et al., 1996; Bussey et al., 2000). Further, a combined perirhinal and postrhinal lesion led to both short- and long-term deficits in recognition but not spatial memory whereas hippocampal lesions led to deficits in spatial memory alone (Winters et al., 2004) and the dissociation of perirhinal cortex and spatial memory appears to be conserved across different strains of rat (Futter et al., 2006). However, a more complex relationship between the hippocampus and perirhinal cortex has been suggested (Mumby & Glenn, 2000; Brown & Aggleton, 2001) with both structures being implicated in place-object learning (Bussey et al., 2000; 2001) and spatial reference memory (Abe et al., 2009). In addition, a time-dependent role for the perirhinal cortex in spatial memory has been proposed (Glenn et al., 2003).

Glenn and colleagues (2005) in a more recent study have shown that different types of lesioning in the perirhinal cortex induce differential expression of the transcription factor Fos; excitotoxic and electrolytic lesions lead to increases in c-Fos expression in the neocortex whereas excitotoxic and aspiration lesions result in increases in c-Fos expression in the dentate gyrus. These alterations in the areas activated following different types of lesions may account for the confusion in the lesion study literature surrounding the function of the perirhinal cortex. Similarly, lesions of the fornix result in decreased expression of c-Fos in the dentate gyrus, CA1, CA3, subiculum, postrhinal cortex and multiple other subcortical and cortical fields (although not in the perirhinal cortex; Vann et al., 2000b). A unilateral lesion of the hippocampus leads to decreased expression of c-Fos and zif268 across a number of different subcortical and cortical areas involved in solving a spatial memory task, including the perirhinal cortex (Jenkins et al., 2006). As such, the effects of lesion studies cannot be relied on as being totally restricted to the area of brain removed and may affect other downstream structures and networks. The difficulties observed in lesion studies have led to a focus on immediate early gene imaging studies in discerning the roles of the hippocampus and perirhinal cortex in learning and memory (Aggleton & Brown, 2005). Many studies have reported differential expression of c-Fos in the hippocampus and perirhinal cortex; increased expression of c-Fos in the perirhinal cortex being associated with recognition memory (Zhu et al., 1995b; 1996; Wan et al., 1999) and in the hippocampus being

associated with spatial memory (Vann et al., 2000a; Jenkins et al., 2004; Amin et al., 2006).

Another transcription factor known to be involved in learning and memory is cAMP responsive element-binding protein (CREB). In rats, hippocampal CREB has been shown to be necessary for spatial learning (Guzowski & McGaugh, 1997; Pittenger et al., 2002) and phosphorylation of CREB is associated with acquiring a spatial novelty task (Vianna et al., 2000). In addition, perirhinal CREB is needed for recognition memory in the rat (Warburton et al., 2005). Furthermore, both c-Fos (Nakagawara et al., 1994; Gaiddon et al., 1996; Nonomura et al., 1996) and CREB (Finkbeiner et al., 1997) are regulated by BDNF, which itself has also been implicated in both spatial and recognition memory. Both upregulation of BDNF in the hippocampus (Falkenberg et al., 1992; Kesslak et al., 1998; Mizuno et al., 2000; Schaaf et al., 2001) and of its receptor TrkB (Gómez-Pinilla et al., 2001) have been associated with spatial learning. Reduced BDNF availability is associated with deficits in spatial learning; BDNF knock-out mice have impaired spatial memory (Linnarsson et al., 1997) and rats treated with an anti-BDNF antibody were impaired compared to control rats (Mu et al., 1999). Moreover, application of BDNF following ischemia reverses the ischemia-induced deficits in spatial memory (Kiprianova et al., 1999; Almli et al., 2000). There is little research as to whether BDNF levels in the perirhinal cortex have a bearing on recognition memory but recently BDNF mRNA has been shown to be upregulated during the learning and reconsolidation of an object recognition task (Romero-Granados et al., 2009). There has been evidence to suggest that expression of BDNF in the area CA1 is required for consolidation of object recognition tasks (Rossato et al., 2007; Furini et al., 2009) and the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) transduction pathway (associated with BDNF signalling; Marsh et al., 1993; Hetman et al., 1999; McCarthy & Feinstein, 1999) is needed in the hippocampus but not the perirhinal cortex for consolidation and reconsolidation of object recognition memories (Kelly et al., 2003). Taken together, these findings suggest that the relationship between hippocampal and perirhinal function is more complex than originally supposed.

Pharmacological evaluation of hippocampal and perirhinal function has also proven useful. Treatment of the perirhinal cortex with the benzodiazepine lorazepam leads not only to impairments in recognition memory but also negatively affects perirhinal synaptic plasticity (Wan et al., 2004) and blockade of glutamate receptors has led to impairments in a variety of memory tasks. For example, the NMDA receptor is implicated in both spatial memory (Morris et al., 1986; Larkin et al., 2008) and object recognition memory (Barker & Warburton, 2008). Perirhinal administration of AP-5 (Winters & Bussey, 2005b; Barker et al., 2006) or systemic administration of MK-801 (de Lima et al., 2005; Nilsson et al., 2007) either pre- or post-training, for example, has been shown to impair rats' performance in object recognition tasks. There is also evidence for the involvement of the AMPA receptor in object recognition memory, perirhinal administration of 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline (CNQX) to rats either pre- or post-training results in an impairment in performance in an object recognition task (Winters & Bussey, 2005b). In addition, it seems that NMDA and kainate receptors may also have different functional roles in object recognition memory; with kainate receptors mediating object recognition memory at a short but not a long delay and NMDA receptors mediating such memory at a long but not a short delay (Barker et al., 2006). Barker and Warburton (2008) have also demonstrated that perirhinal NMDA receptors are required for acquisition of short-term object-in-place associative memories. Systemic administration of either MK-801 (Dragunow et al., 1993; Kealy & Commins, 2009) or CNQX (Kealy & Commins, 2009) has also been associated with deficits in synaptic plasticity and reductions in BDNF levels in the hippocampus and the perirhinal cortex, perhaps indicating that the synaptic processes underlying learning and memory are NMDA and AMPA/kainate receptor-dependent and that BDNF may play a role in their functioning.

In this chapter, we will investigate the roles of the hippocampus and the perirhinal cortex in a test of spatial memory (object displacement task) and recognition memory (object recognition task) by analysing levels of BDNF protein in each area after testing (Experiment 1). We expect to find BDNF expression to be upregulated in the hippocampus following the object displacement task (due to task's reliance on spatial memory) and upregulated in the perirhinal cortex

following the object recognition task (due to the task's reliance on recognition memory). We shall then determine the effects of NMDA and AMPA/kainate glutamate receptor blockade on performance in the object displacement task (Experiment 2) and the object recognition task (Experiment 3). In both Experiments 2 and 3, we will also analyse BDNF protein levels in order to see if glutamate signalling regulates BDNF expression in the hippocampus and perirhinal cortex and we also expect any impairment in recognition or displacement memory following drug treatment to be associated with a concurrent decrease in BDNF expression in the hippocampus and the perirhinal cortex.

6.2 Methods:

6.2.1 Experiment 1 – Comparisons in behaviour and brain-derived neurotrophic factor levels between object recognition and object displacement memory tasks:

Adult male Wistar rats (n = 18; approximately 3 months old; weight: 300-400 g; Biomedical Unit; University College Dublin) were assigned to three different experimental groups (n = 6 in each): no change control, Object Recognition and Object Displacement groups. All animals were trained on Day 1 and tested on Day 2 in the same circular arena (Ø 135 cm) with the arena and objects cleaned between each trial and each animal. All animals were given an initial trial prior to training which consisted of 1 min exploring the arena with no objects present. Training consisted of allowing each rat to explore a circular arena containing 4 distinct objects (with 2 distal cues located outside the arena) across 4 x 2 min trials with a 1 min interval between each trial (Figure 1(a)). Testing on Day 2 depended on which experimental group the animals belonged to. Animals in the control group were given a single 2 min exploratory trial in the same arena with no change in the configuration of the objects (i.e. identical to the training condition; Figure 1(a)). Animals in the Object Recognition group were given a single 2 min exploratory trial in the same arena but with one of the objects from Day 1 replaced with a novel object (Figure 1(b)). Finally, animals in the Object Displacement group were given a single 2 min exploratory trial in the same arena with one object moved to a new position within the arena compared to Day 1 (Figure 1(c)). All movements were recorded by using EthoVision (Noldus, Wageningen, Netherlands). Exploratory behaviour in each animal was also recorded by counting the number of nose contacts on each object and the length of time spent in nose contact with each object (measured by a stopwatch). Animals were sacrificed immediately after the experiment and the hippocampus and perirhinal cortex were dissected out of each hemisphere and frozen in Krebs-Ca^{2+}/dimethyl sulfoxide for molecular analysis later (see section 6.2.4).

6.2.2 Experiment 2 – Effect of glutamate receptor blockade on performance in the object recognition task:

Adult male Wistar rats (n = 24; approximately 3 months old; weight: 300-400 g; Biomedical Unit; University College Dublin) were assigned to three different experimental groups (n = 8 in each): saline control (0.1 ml/100 g body weight of 0.9 % NaCl; Sigma, Ireland), MK-801-treated (0.1 mg/kg body weight; Sigma, Ireland) and CNQX-treated groups (1.5 mg/kg body weight; Sigma, Ireland). All animals were trained on Day 1 and tested on Day 2 in the same circular arena (Ø 135 cm) with the arena and objects cleaned between each trial and each animal. Drugs were administered in all groups 20 min before training on Day 1. All animals were given a single trial prior to training which consisted of 1 min exploring the arena with no objects present. Training consisted of allowing each rat to explore a circular arena containing 4 distinct objects (with 2 cues outside the arena) across 4 x 2 min trials with a 1 min interval between each trial (Figure 1(a)). Testing on Day 2 consisted of animals being given a single 2 min exploratory trial in the same arena but with one of the objects from Day 1 replaced with a novel object (Figure 1(b)). All movements were recorded by using EthoVision (Noldus, Wageningen, Netherlands). Exploratory behaviour in each animal was also recorded by counting the number of nose contacts on each object and the length of time spent in nose contact with each object (measured by a stopwatch). Animals were sacrificed immediately after the experiment and the hippocampus and perirhinal cortex were dissected out of each hemisphere and frozen in Krebs-Ca^{2+}/dimethyl sulfoxide for molecular analysis later (see section 6.2.4).

6.2.3 Experiment 3 – Effect of glutamate receptor blockade on performance in the object displacement task:

Adult male Wistar rats (n = 20; approximately 3 months old; weight: 300-400 g; Biomedical Unit; University College Dublin) were assigned to three different experimental groups: saline control (n = 7; 0.1 ml/100 g body weight of 0.9 % NaCl; Sigma, Ireland), MK-801-treated (n = 6, originally n = 7 but the data from one

animal was dropped from the study because it was discovered to have severe hydrocephalus; 0.1 mg/kg body weight; Sigma, Ireland) and CNQX-treated groups (n = 7; 1.5 mg/kg body weight; Sigma, Ireland). All animals were trained on Day 1 and tested on Day 2 in the same circular arena (Ø 135 cm) with the arena and objects cleaned between each trial and each animal. Drugs were administered in all groups 20 min before training on Day 1. All animals were given a single trial prior to training which consisted of 1 min exploring the arena with no objects present. Training consisted of allowing each rat to explore a circular arena containing 4 distinct objects (with 2 cues outside the arena) across 4 x 2 min trials with a 1 min interval between each trial (Figure 1(a)). Testing on Day 2 consisted of animals being given a single 2 min exploratory trial in the same arena with one object moved to a new position within the arena compared to Day 1 (Figure 1(c)). Again, all movements were recorded by using EthoVision (Noldus, Wageningen, Netherlands). Exploratory behaviour in each animal was also recorded by counting the number of nose contacts on each object and the length of time spent in nose contact with each object (measured by a stopwatch). Animals were sacrificed immediately after the experiment and the hippocampus and perirhinal cortex were dissected out of each hemisphere and frozen in Krebs-Ca^{2+}/dimethyl sulfoxide for molecular analysis later (see section 6.2.4).

(a)
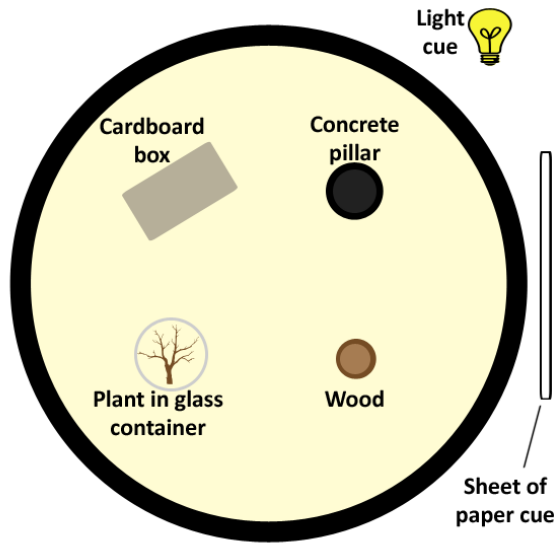
(b)
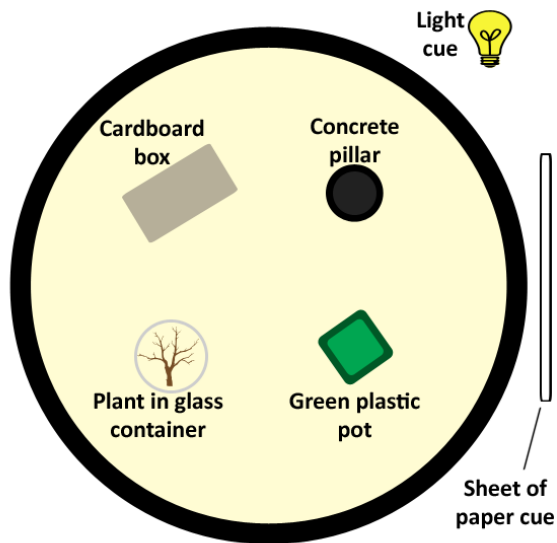
(c)
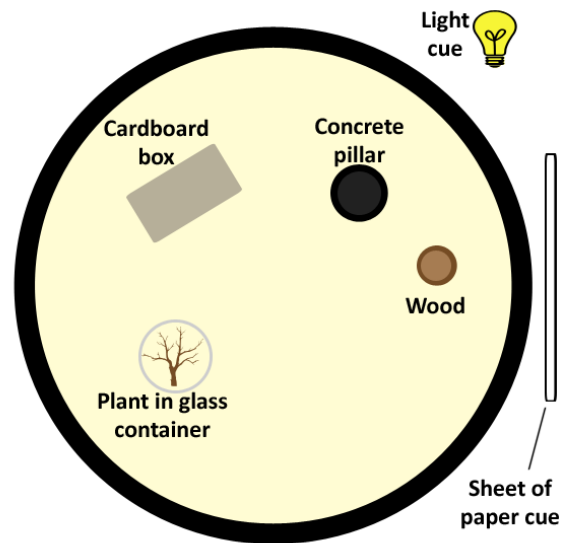
Figure 6.1: Diagrams depicting arena configurations for the three different experimental conditions. (a) Animals in all groups across all three experiments were trained on Day 1 with the four objects (cardboard box, concrete pillar, plant and wood) and two distal cues (light and sheet of paper) in the illustrated positions (left). Photo of training configuration given on right. Animals in the no change control group in Experiment 1 were tested on Day 2 in the same arena configuration. (b) Animals from the Object Recognition group in Experiment 1 and all three drug-treated groups in Experiment 2 were tested on Day 2 in this arena configuration. The wood from Day 1 was replaced with a green plastic pot. (c) Animals from the Object Displacement group in Experiment 1 and all three drug-treated groups in Experiment 3 were tested on Day 2 in this arena configuration. The wood from Day 1 was moved to the position illustrated here.

6.2.4 Brain-derived neurotrophic factor enzyme-linked immunosorbant assay (ELISA):

Analysis of BDNF in the hippocampus and perirhinal cortex was performed as described in Chapter 3 via an enzyme-linked immunosorbent assay (ELISA) for BDNF using a BDNF Emax$^{©}$ ImmunoAssay System kit (Promega Corporation, U.S.A.).

6.2.5 Statistical analysis:

A series of dependent t-tests and repeated measures analyses of variance (ANOVAs) with the appropriate *post-hoc* test (Tukey at the 5 % level of significance) were used. A star-rated system was used where appropriate (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

6.2.6 Ethical considerations:

Laboratory procedures for the maintenance and experimentation of animals conformed to the Department of Health (Ireland) guidelines and the European directive 86/609/EC. Every effort was made to minimise the suffering and the number of animals used in this study.

6.3 Results:

6.3.1 Experiment 1 – Comparisons in behaviour and brain-derived neurotrophic factor levels between object recognition and object displacement memory tasks:

To determine the effect of training on the average time spent exploring the objects, a repeated measures ANOVA was used (Figure 6.2). The training data from the three groups were pooled together and training was analysed on 4 levels (Trial 1, Trial 2, Trial 3 and Trial 4) as a within-group. Overall, there was a significant effect for training (F = 4.716; df = 3, 51; p < 0.01) indicating that animals spent less time exploring the objects in the later trials compared to the early trials.
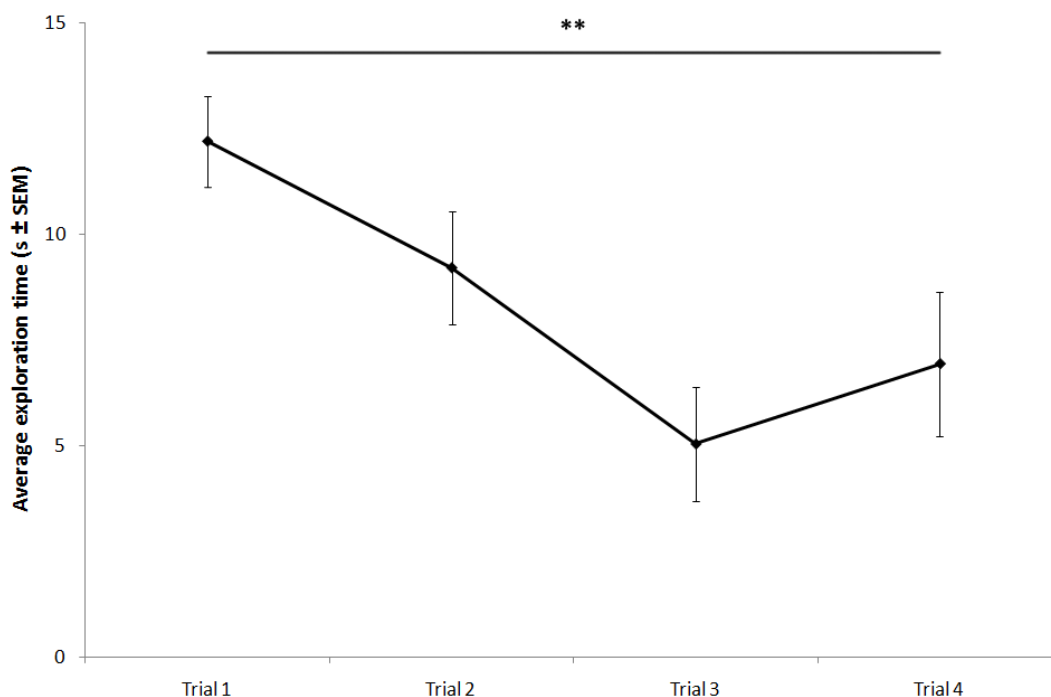
Figure 6.2: The effect of training on average exploration time on all objects. There was a significant overall effect for training across the four trials indicating that animals were spending less time exploring all objects in trial 4 compared to trial 1. **p < 0.01.

Examining retention in more detail, overall exploration (across all objects) was compared between trial 4 and the retention trial in each group. A 2 x 3 mixed factorial ANOVA was used with trial analysed on 2 levels (trial 4 and retention) as a within-group measure and type of retention test was analysed on 3 levels (no

change, recognition and displacement) as a between-groups measure. There was no significant effect found for trial (F = 2.286; df = 1, 15; p > 0.05) and no significant effect was found for the type of retention (F = 0.840; df = 2, 15; p > 0.05). There was also no interaction effect found between trial and type of retention (F = 0.603; df = 2, 15; p > 0.05).

In the control group (Figure 6.3), the exploration time for each object on trial 4 of training was compared to the exploration time in the retention trial using a 2 x 4 repeated measures ANOVA. Trial was analysed on 2 levels (trial 4 and retention) as a within-groups measure and objects were analysed on 4 levels (cardboard box, concrete pillar, plant and wood) as a within-groups measure. There was no significant effect for trial (F = 0.595; df = 1, 15; p > 0.05), no significant effect for object (F = 1.784; df = 3, 15; p > 0.05) and there was no significant interaction effect observed (F = 6.007; df = 3, 15; p > 0.05).
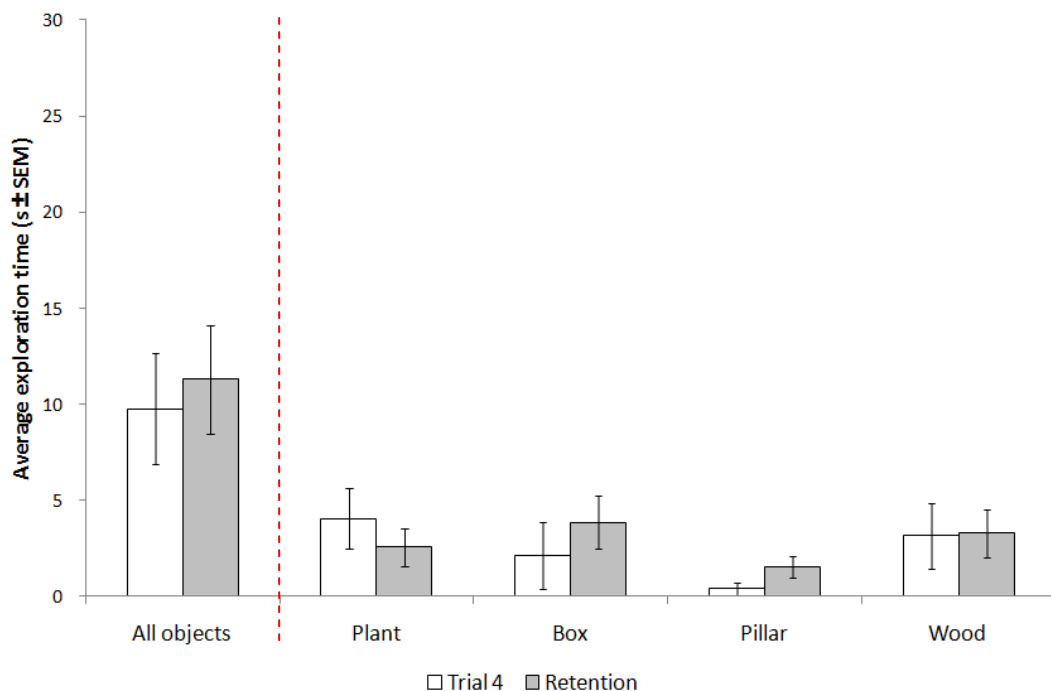
Figure 6.3: Average exploration of objects between trial 4 of training and the retention trial in the control group (no change between trial 4 and the retention trial). There was no significant increase in exploration for all objects between trial 4 and the retention trial (left panel). No significant differences in exploration were observed for any of the four objects when analysed separately (right panel).

In the Object Recognition group, the exploration time for each object on trial 4 of training was compared to the exploration time in the retention trial using a 2 x 4 repeated measures ANOVA (Figure 6.4). Trial was analysed on 2 levels (trial 4 and retention) as a within-groups measure and objects were analysed on 4 levels (cardboard box, concrete pillar, plant and wood/pot) as a within-groups measure. There was no significant effect for trial (F = 3.006; df = 1, 15; p > 0.05) but there was a significant effect for object (F = 19.491; df = 3, 15; p < 0.001). However, there was no significant interaction effect observed (F = 3.081; df = 3, 15; p > 0.05). Bonferroni analysis revealed that animals spent significantly more time exploring the wood/pot on both trials compared to the cardboard box (p < 0.05), concrete pillar (p < 0.01) and the plant (p < 0.05).
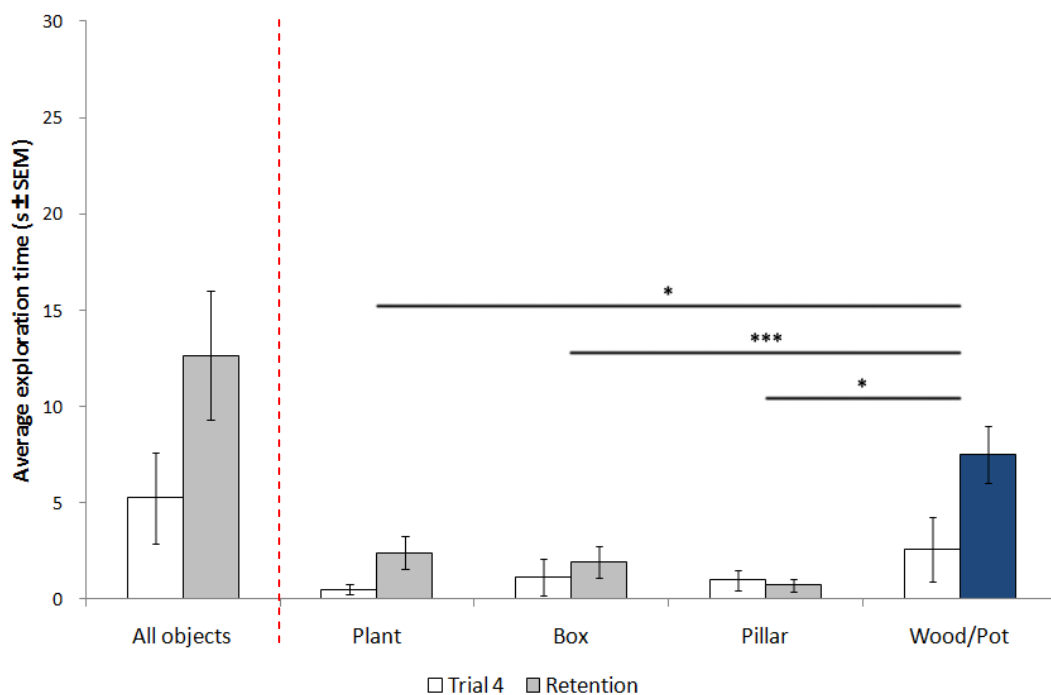
Figure 6.4: Average exploration of objects between trial 4 of training and the retention trial in the Object Recognition group (wood changed to plastic pot in retention trial). There was no significant increase in exploration for all objects between trial 4 and the retention trial (left panel). Animals explored the wood/pot significantly more than the other objects across both trials (right panel). However, there were no significant differences in the animals' exploration of the wood and pot between trial 4 and the retention trial. *p <0.05; ***p < 0.001.

In the Object Displacement group, the exploration time for each object on trial 4 of training was compared to the exploration time in the retention trial using a 2 x 4 repeated measures ANOVA (Figure 6.5). Trial was analysed on 2 levels (trial 4 and retention) as a within-groups measure and objects were analysed on 4 levels (cardboard box, concrete pillar, plant and wood) as a within-groups measure. There was no significant effect for trial ($F = 0.137$; $df = 1, 15$; $p > 0.05$) and no significant effect for object ($F = 2.115$; $df = 3, 15$; $p > 0.05$). Additionally, there was also no significant interaction effect observed ($F = 0.236$; $df = 3, 15$; $p > 0.05$).
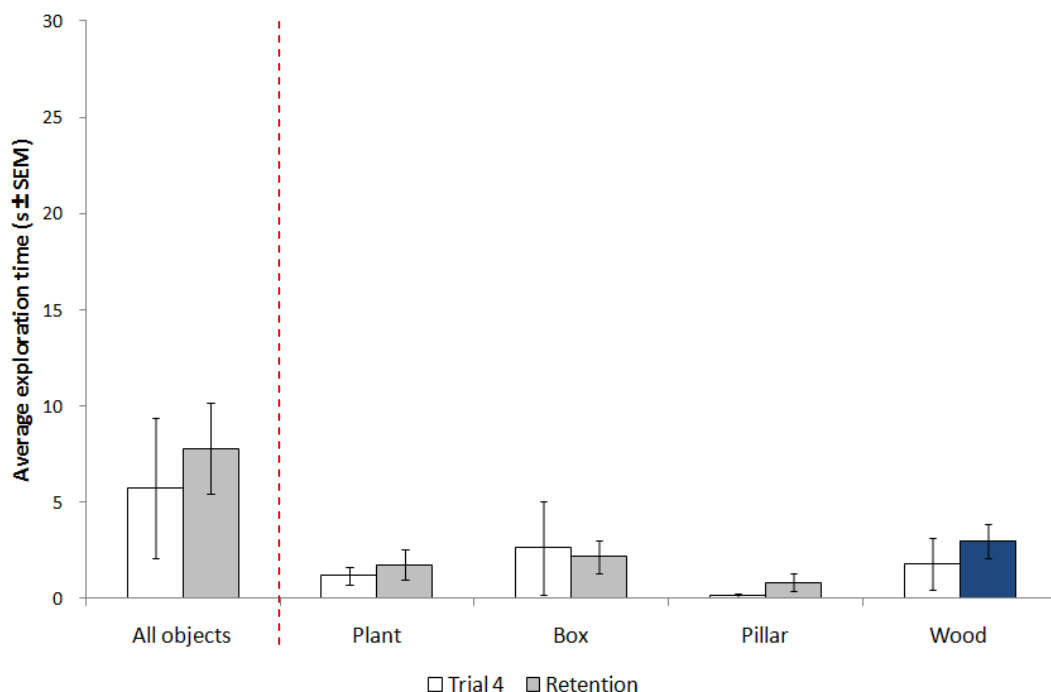
Figure 6.5: Average exploration of objects between trial 4 of training and the retention trial in the Object Displacement group (wood moved to a novel position in retention trial). There was no significant increase in exploration for all objects between trial 4 and the retention trial (left panel). No significant differences in exploration were observed for any of the four objects when analysed separately (right panel).

Levels of hippocampal and perirhinal BDNF were compared between the three groups using a 2 x 3 mixed factorial ANOVA. Area was analysed on 2 levels (hippocampus and perirhinal cortex) as a within-groups measure and type of retention test was analysed on 3 levels (no change, recognition and displacement) as a between-groups measure (Figure 6.6). There was a significant effect observed for area with the hippocampus exhibiting significantly higher levels of BDNF compared to the perirhinal cortex ($F = 86.083$; $df = 1, 15$; $p < 0.001$) and there was a

significant effect found for group (F = 5.152; df = 2, 15; $p < 0.01$) but there was no interaction effect observed (F = 2.512; df = 2, 15; $p > 0.05$). Tukey *post-hoc* analysis revealed that overall the recognition group had significantly lower levels of BDNF compared to the displacement group ($p < 0.05$). Comparisons between retention groups were made within each brain area using one-way ANOVAs with Tukey *post-hoc* analysis. There was a significant effect for retention type in the hippocampus (F = 15.036; df = 2, 17; $p < 0.001$) with the displacement group exhibiting significantly higher levels of BDNF compared to both the control and recognition groups ($p < 0.05$ in both cases). In the perirhinal cortex however, there were no significant differences found between the three retention groups (F = 1.040; df = 2, 17; $p > 0.05$).
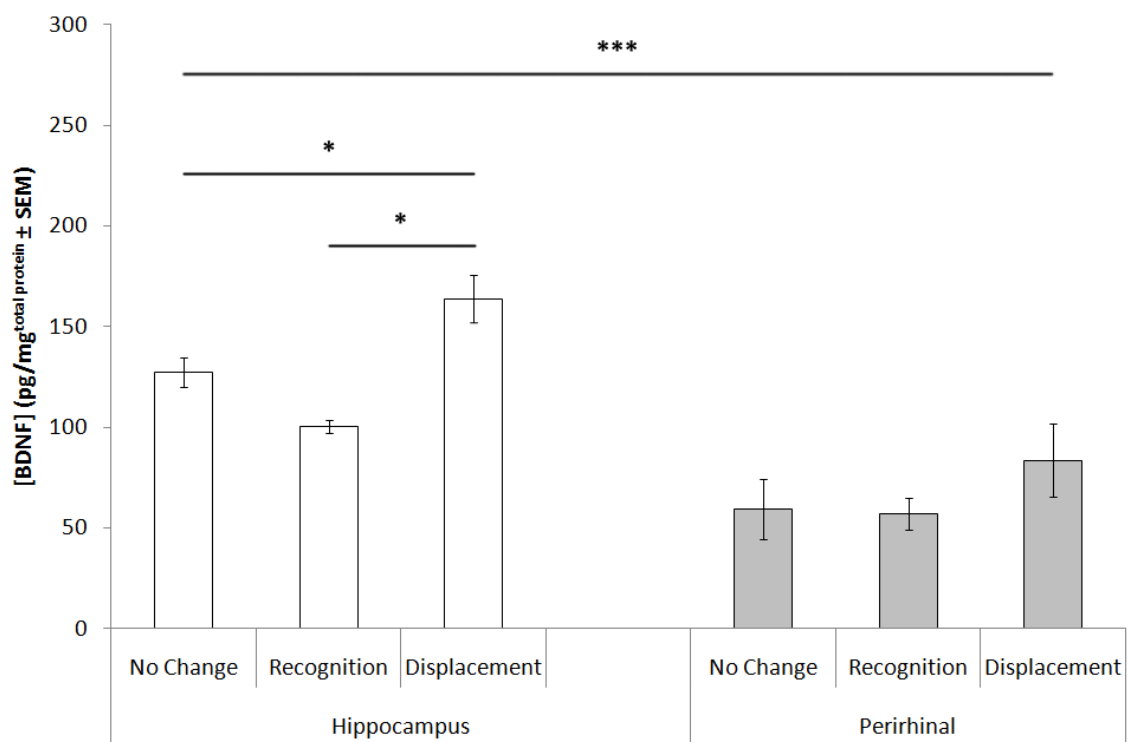
Figure 6.6: Average brain-derived neurotrophic factor (BDNF) levels for each retention group in the hippocampus and the perirhinal cortex. There were significantly higher levels of BDNF overall in the hippocampus compared to the perirhinal cortex and within the hippocampus there were significantly higher levels of BDNF in the displacement group compared to the no change control and recognition groups. There were no significant differences within the perirhinal cortex. *$p < 0.05$; ***$p < 0.001$.

6.3.2 Experiment 2 – Effect of glutamate receptor blockade on performance in the object recognition task:

To determine the effect of training on the average time spent exploring the objects, a 3 x 4 mixed factorial ANOVA was used (Figure 6.7). Training was analysed on 5 levels (Trial 1, Trial 2, Trial 3, and Trial 4) as a within-group measure and drug treatment was analysed on 3 levels (saline, MK-801 and CNQX) as a between-groups measure. Overall, there was a significant effect for training ($F = 9.841$; $df = 3$, 63; $p < 0.001$) but no significant effect was found for drug treatment ($F = 0.861$; $df = 2$, 21; $p > 0.05$) indicating that drug treatment did not significantly affect exploration and there was no interaction effect observed between training and drug treatment ($F = 0.547$; $df = 6$, 63; $p > 0.05$).
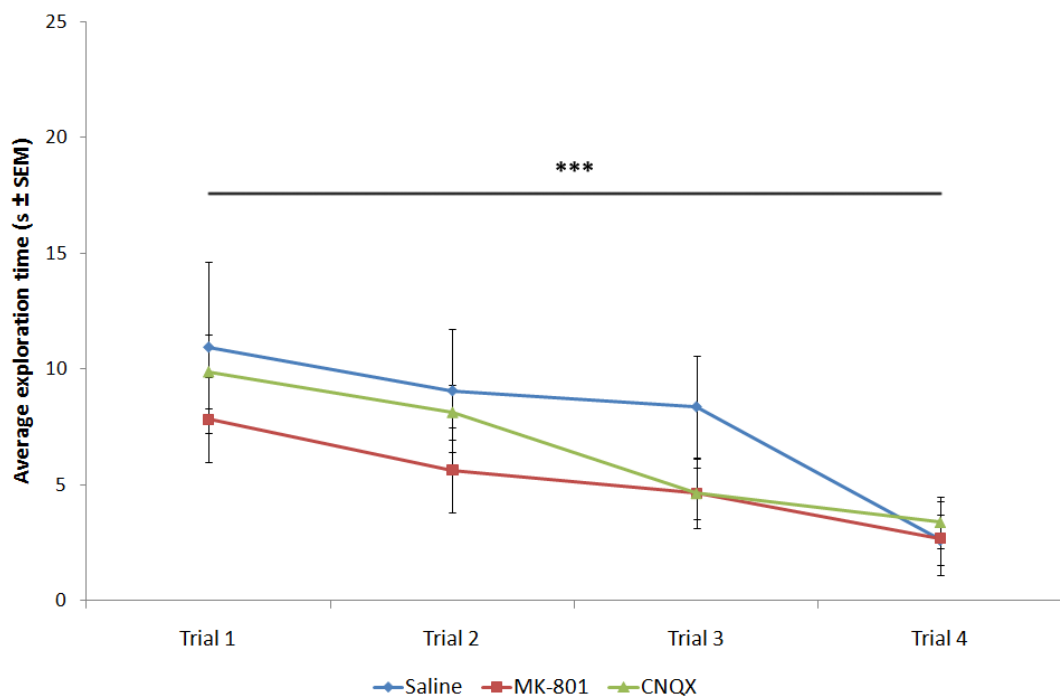
Figure 6.7: The effect of training on average exploration time on all objects. There was a significant overall effect for training across the four trials indicating that animals were spending less time exploring all objects in trial 4 compared to trial 1. There were no significant differences observed between the drug groups. ***$p < 0.001$.

Looking at each drug treatment group separately, the effects for training were determined using a repeated measures ANOVA with Bonferroni correction. Training analysed on 4 levels as a within-groups measure (Trial 1, Trial 2, Trial 3 and Trial 4). In the saline group, there was a significant effect found for training ($F =$

3.113; *df* = 3, 21; *p* < 0.05). In the MK-801 group, there was also a significant effect found for training (*F* = 3.847; *df* = 3, 21; *p* < 0.05). Finally, there was a significant effect found for training in the CNQX group (*F* = 4.642; *df* = 3, 21; *p* < 0.05).

Examining retention in more detail, overall exploration (across all objects) was compared between trial 4 and the retention trial in each group. A 2 x 3 mixed factorial ANOVA was used with trial analysed on 2 levels (trial 4 and retention) as a within-group measure and drug treatment was analysed on 3 levels (saline, MK-801 and CNQX) as a between-groups measure. There was a significant effect found for trial (*F* = 71.842; *df* = 1, 21; *p* < 0.001) but there was no significant effect found for drug treatment (*F* = 2.064; *df* = 2, 21; *p* > 0.05). However, there was a significant interaction effect found between trial and drug treatment (*F* = 5.188; *df* = 2, 21; *p* < 0.05). Dependent t-tests were ran on the three drug groups comparing overall object exploration in trial 4 of training to overall object exploration in the retention trial. There were significantly higher levels of overall object exploration found in the retention trial compared to trial 4 of training in all three drug treatment groups: saline (*t* = -5.194; *df* = 7; *p* < 0.001; Figure 6.8), MK-801 (*t* = -7.283; *df* = 7; *p* < 0.001; Figure 6.9) and CNQX (*t* = -2.433; *df* = 7; *p* < 0.05; Figure 6.10). This indicates a renewed interest in exploration for all objects during the retention trial.

In the three drug treatment groups, the effects of NMDA or AMPA/kainate blockade on object recognition memory were analysed. The average exploration time for the target object in the retention trial (green plastic pot) was compared to the average exploration time for the piece of wood in trial 4 of training using dependent t-tests. A significant effect was found for trial (*F* = 25.163; *df* = 1, 21; *p* < 0.001) but no significant effect was found for drug treatment (*F* = 0.895; *df* = 2, 21; *p* > 0.05) and no significant interaction effect was found between trial and drug treatment (*F* = 2.385; *df* = 2, 21; *p* > 0.05). Dependent t-tests were ran on the three drug groups comparing target object exploration in trial 4 of training (piece of wood) to target object exploration in the retention trial (green plastic pot). There were significantly higher levels of target object exploration found in the retention trial compared to trial 4 of training in all the saline- (*t* = -2.732; *df* = 7; *p* < 0.05; Figure 6.8) and MK-801-treated groups (*t* = -4.390; *df* = 7; *p* < 0.01; Figure 6.9) but not in the CNQX-treated group (*t* = -1.570; *df* = 7; *p* > 0.05; Figure 6.10). As overall

object exploration was significantly increased in all three groups in the retention trial the lack of increased exploration of the target object in the CNQX group indicates that CNQX-treatment blocked object recognition memory but both saline- and MK-801-treatment spared object recognition memory.
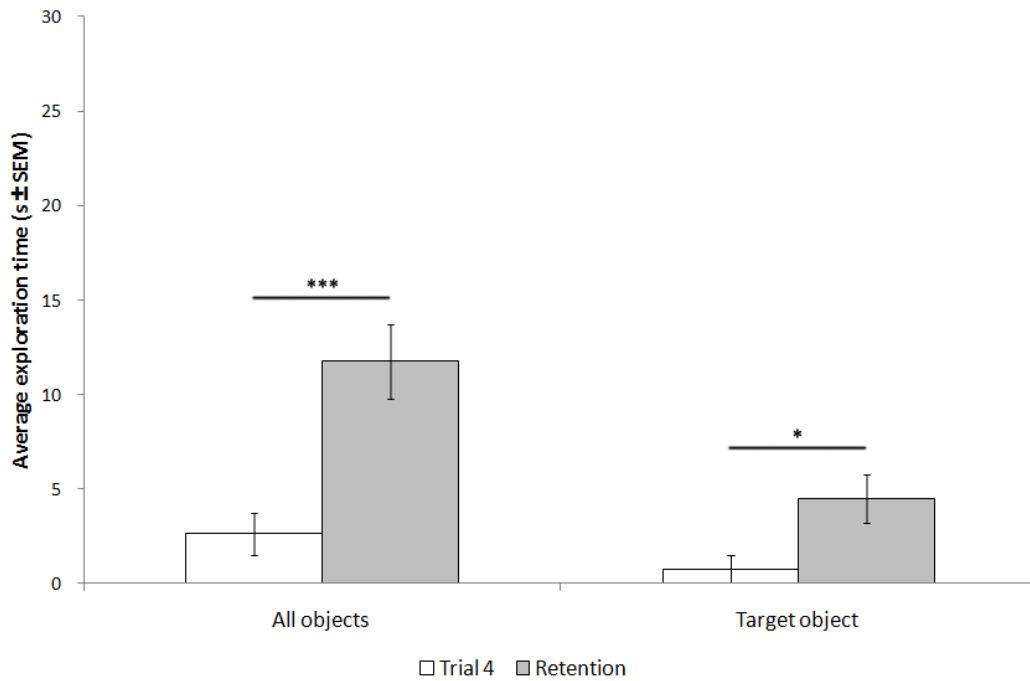
Figure 6.8: Average exploration of objects between trial 4 of training and the retention trial in the saline-treated group in the object recognition task (wood changed to plastic pot in retention trial). There was a significant increase in exploration for all objects in the retention trial compared to trial 4. There was also increased exploration of the target object in the retention trial compared to trial 4. *p <0.05; ***p < 0.001.
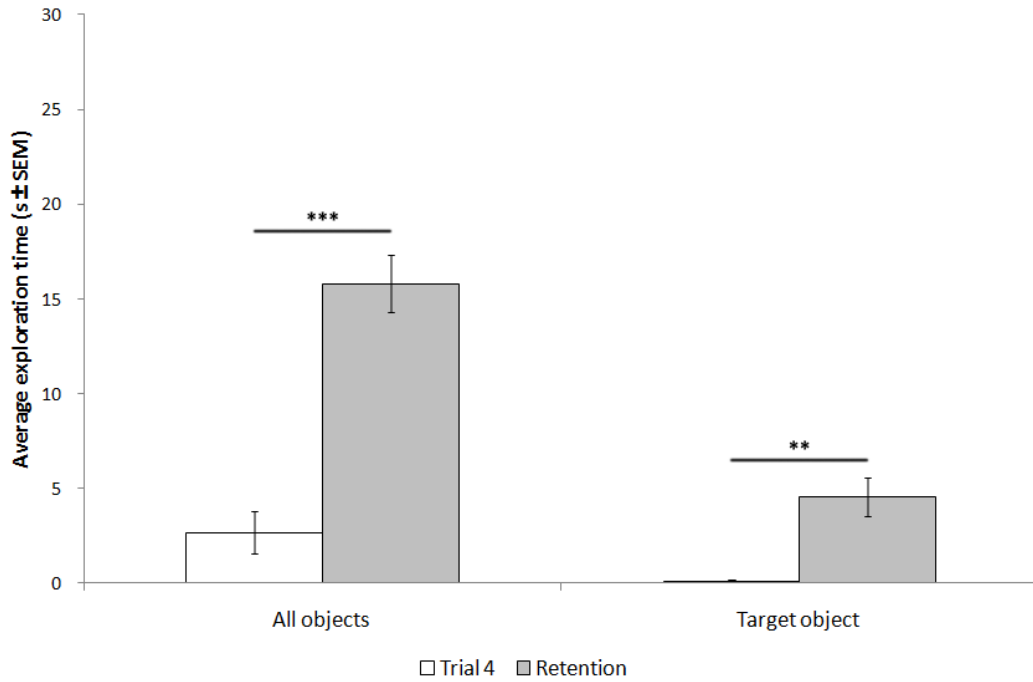
Figure 6.9: Average exploration of objects between trial 4 of training and the retention trial in the MK-801-treated group in the object recognition task (wood changed to plastic pot in retention trial). There was a significant increase in exploration for all objects in the retention trial compared to trial 4. There was also increased exploration of the target object in the retention trial compared to trial 4. **$p <0.01$; ***$p < 0.001$.
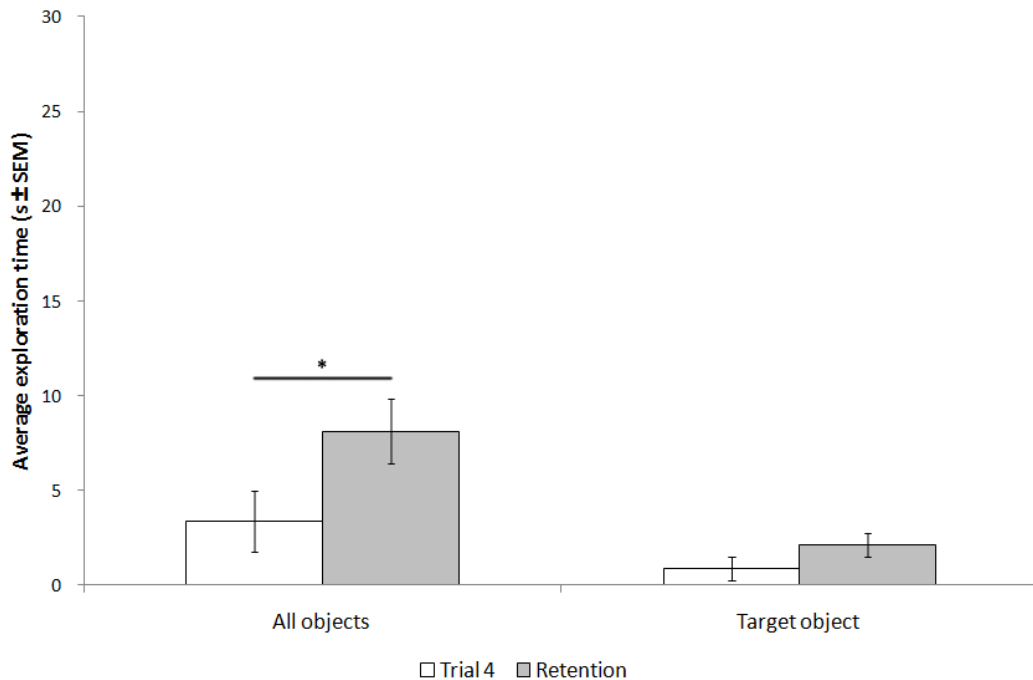
Figure 6.10: Average exploration of objects between trial 4 of training and the retention trial in the CNQX-treated group in the object recognition task (wood changed to plastic pot in retention trial). There was a significant increase in exploration for all objects in the retention trial compared to trial 4. However, there were no changes in exploration of the target object in the retention trial compared to trial 4. *$p <0.05$.

Levels of hippocampal and perirhinal BDNF were compared between the three groups using a 2 x 3 mixed factorial ANOVA. Area was analysed on 2 levels (hippocampus and perirhinal cortex) as a within-groups measure and drug treatment was analysed on 3 levels (saline, MK-801 and CNQX) as a between-groups measure (Figure 6.11). There was a significant effect observed for area with the hippocampus exhibiting significantly higher levels of BDNF compared to the perirhinal cortex (F = 151.654; df = 1, 21; p < 0.001) but there was no significant effect found for drug treatment (F = 1.906; df = 2, 21; p > 0.05) and no interaction effect was observed between area and drug treatment (F = 1.471; df = 2, 21; p > 0.05).
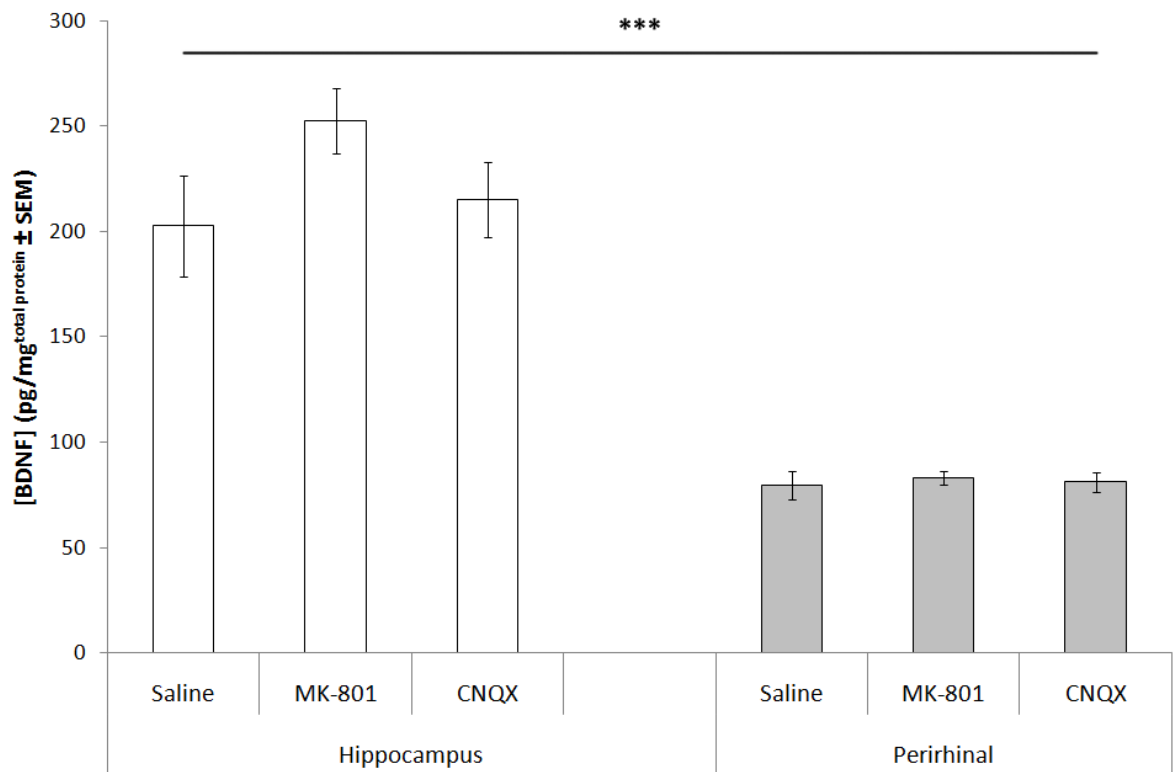
Figure 6.11: Average brain-derived neurotrophic factor (BDNF) levels for each drug treatment group in the hippocampus and the perirhinal cortex following an object recognition task. There were significantly higher levels of BDNF overall in the hippocampus compared to the perirhinal cortex but no significant differences were found between the drug treatment groups in either the hippocampus or the perirhinal cortex. ***p < 0.001.

6.3.3 Experiment 3 – Effect of glutamate receptor blockade on performance in the object displacement task:

To determine the effect of training on the average time spent exploring the objects, a 3 x 4 mixed factorial ANOVA was used (Figure 6.12). Training was analysed on 5 levels (Trial 1, Trial 2, Trial 3 and Trial 4) as a within-group measure and drug treatment was analysed on 3 levels (saline, MK-801 and CNQX) as a between-groups measure. Overall, there was a significant effect for training ($F = 5.746$; $df = 3$, 51; $p < 0.01$) but no significant effect was found for drug treatment ($F = 2.035$; $df = 1$, 17; $p > 0.05$) indicating that drug treatment did not significantly affect exploration. However, there was an interaction effect observed between training and drug treatment ($F = 2.976$; $df = 6$, 51; $p < 0.05$).

Figure 6.12: The effect of training on average exploration time on all objects. There was a significant overall effect for training across the four trials indicating that animals were spending less time exploring all objects in trial 4 compared to trial 1. There were no significant differences observed between the drug groups. ***$p < 0.001$.

Looking at each drug treatment group separately, the effects for training were determined using a repeated measures ANOVA with Bonferroni correction. Training analysed on 4 levels as a within-groups measure (Trial 1, Trial 2, Trial 3 and

Trial 4). In the saline group, there was a significant effect found for training ($F = 5.367$; $df = 3, 18$; $p < 0.01$) with a significant difference observed between trial 4 and trials 2 and 3 ($p < 0.05$ in both cases). In the MK-801 group, there was no significant effect found for training ($F = 1.286$; $df = 3, 15$; $p > 0.05$). Finally, there was a significant effect found for training in the CNQX group ($F = 4.746$; $df = 3, 18$; $p < 0.05$).

Examining retention in more detail, overall exploration (across all objects) was compared between trial 4 and the retention trial in each group (Figure 6.13). A 2 x 3 mixed factorial ANOVA was used with trial analysed on 2 levels (trial 4 and retention) as a within-group measure and drug treatment was analysed on 3 levels (saline, MK-801 and CNQX) as a between-groups measure. There was a significant effect found for trial ($F = 32.237$; $df = 1, 17$; $p < 0.001$) and a significant effect found for drug treatment ($F = 5.875$; $df = 2, 17$; $p < 0.05$). In addition, there was also a significant interaction effect found between trial and drug treatment ($F = 3.613$; $df = 2, 17$; $p < 0.05$). Tukey *post-hoc* analysis showed that there was a significant difference in overall object exploration between the MK-801- and CNQX-treated groups ($p < 0.05$). Next, dependent t-tests were ran on the three drug groups comparing overall object exploration in trial 4 of training to overall object exploration in the retention trial. There were significantly higher levels of overall object exploration found in the retention trial compared to trial 4 of training in both the saline- ($t = -3.238$; $df = 6$; $p < 0.05$; Figure 6.13) and MK-801-treated groups ($t = -4.092$; $df = 5$; $p < 0.01$; Figure 6.14) but not the CNQX-treated group ($t = -1.945$; $df = 6$; $p > 0.05$; Figure 6.15). This indicates a renewed in object exploration during the retention trial in the saline- and MK-801-treated groups but not in the CNQX-treated group.

In the three drug treatment groups, the effects of NMDA or AMPA/kainate blockade on object displacement memory were analysed. The average exploration time for the target object in the retention trial (moved piece of wood) was compared to the average exploration time for the piece of wood in trial 4 of training using dependent t-tests (Figure 6.14). No significant effect was found for trial ($F = 1.472$; $df = 1, 17$; $p > 0.05$) but there was a significant effect was found for drug treatment ($F = 4.218$; $df = 2, 17$; $p < 0.05$). However, there was no significant

interaction effect found between trial and drug treatment ($F = 2.428$; $df = 2, 17$; $p > 0.05$). Tukey *post-hoc* analysis revealed that there were significantly lower levels of target object exploration in the CNQX-treated group compared to the MK-801-treated group ($p < 0.05$). Next, dependent t-tests were ran on the three drug groups comparing target object exploration in trial 4 of training (piece of wood) to target object exploration in the retention trial (moved piece of wood). There was a significantly higher level of target object exploration found in the retention trial compared to trial 4 of training in the saline-treated group ($t = -2.922$; $df = 6$; $p < 0.05$; Figure 6.13) but not in either the MK-801- ($t = -0.549$; $df = 5$; $p > 0.05$; Figure 6.14) or the CNQX-treated groups ($t = -2.222$; $df = 6$; $p > 0.05$; Figure 6.15). As overall object exploration was significantly increased in the saline- and MK-801-treated groups in the retention trial (Figure 6.13) the lack of increased exploration of the target object in the MK-801 group indicates that MK-801-treatment blocked object displacement memory but saline-treatment spared object recognition memory. In the case of the CNQX-treated group, there was no significant increase in overall exploration or in target object exploration which suggests that perhaps CNQX-treatment affected the animals' acquisition of the task on a more fundamental level than the MK-801 group.
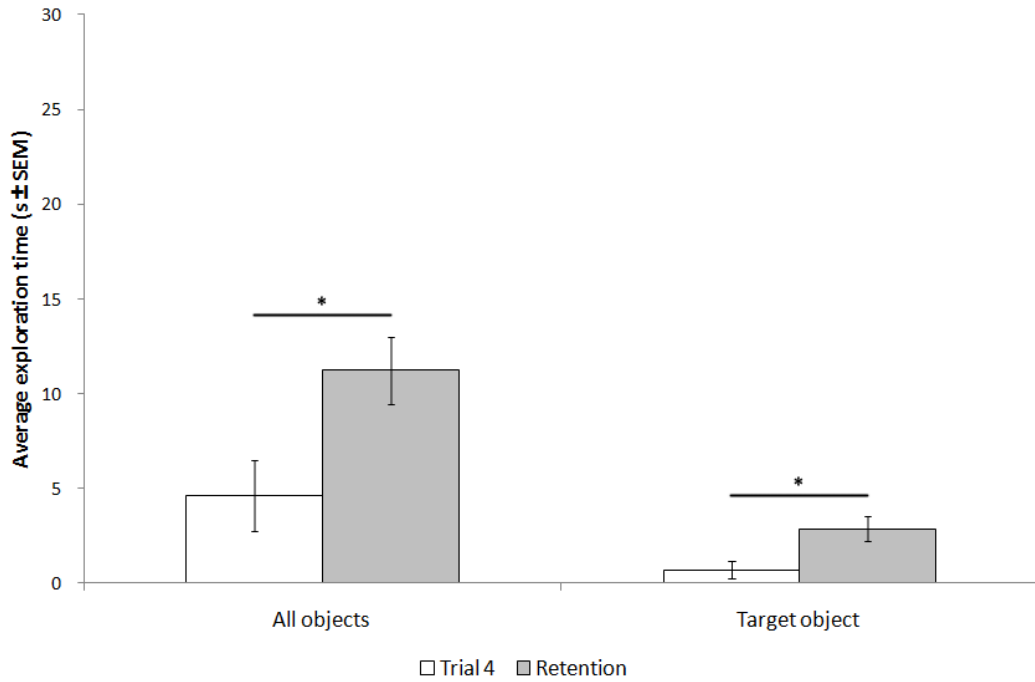
Figure 6.13: Average exploration of objects between trial 4 of training and the retention trial in the saline-treated group in the object displacement task (wood moved to a novel position in retention trial). There was a significant increase in exploration for all objects in the retention trial compared to trial 4. There was also increased exploration of the target object in the retention trial compared to trial 4. *p <0.05.
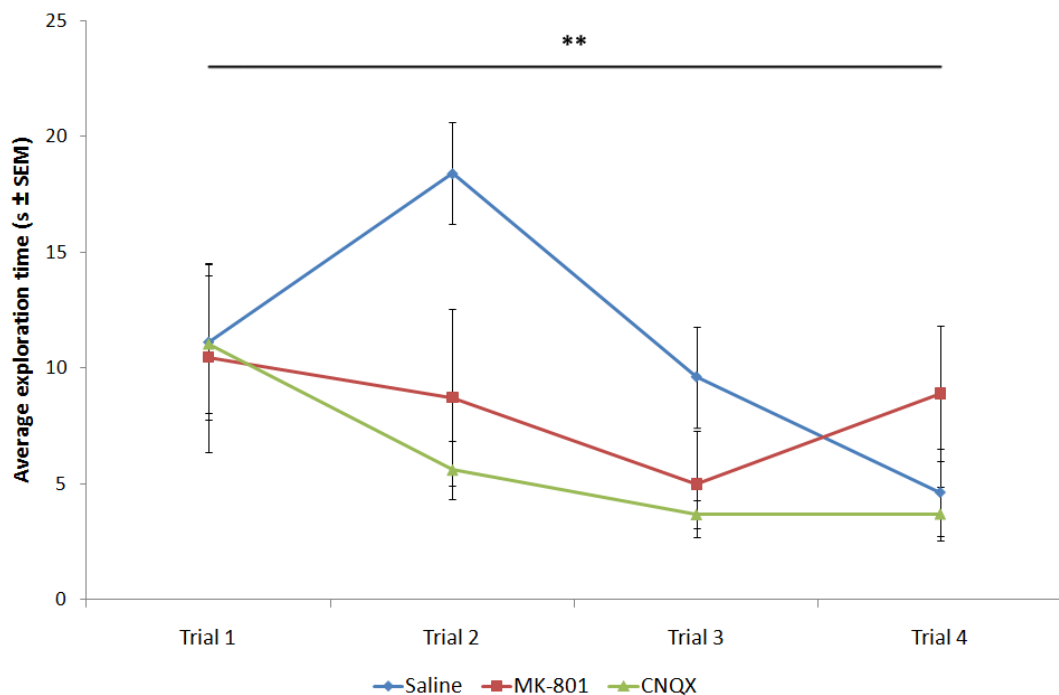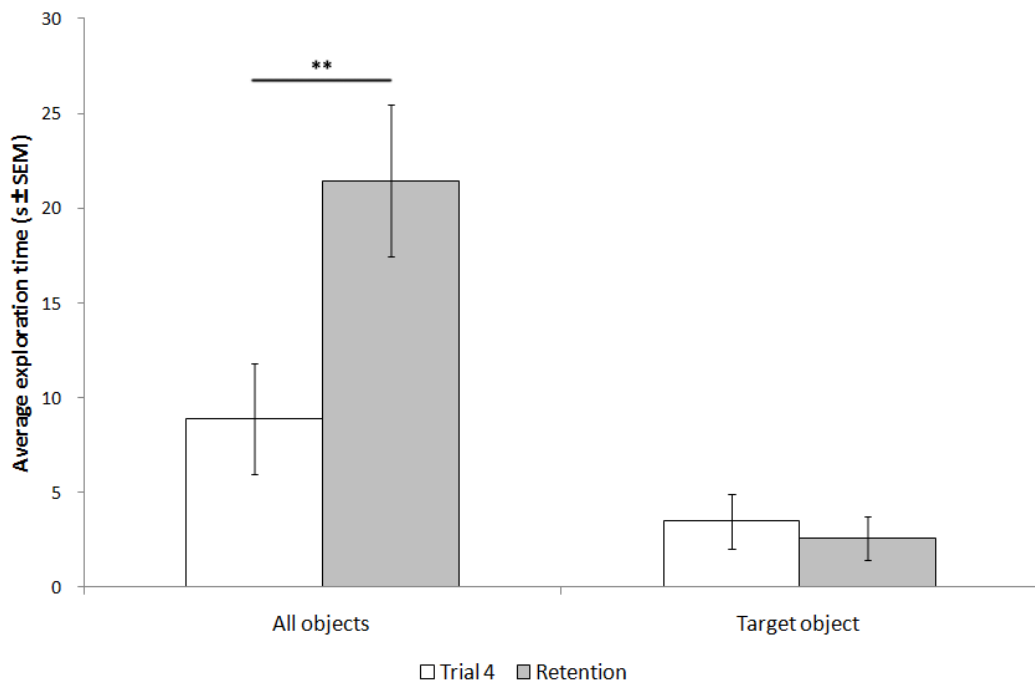

Figure 6.14: Average exploration of objects between trial 4 of training and the retention trial in the MK-801-treated group in the object displacement task (wood moved to a novel position in retention trial). There was a significant increase in exploration for all objects in the retention trial compared to trial 4. However, there were no changes in exploration of the target object in the retention trial compared to trial 4. **p <0.01.
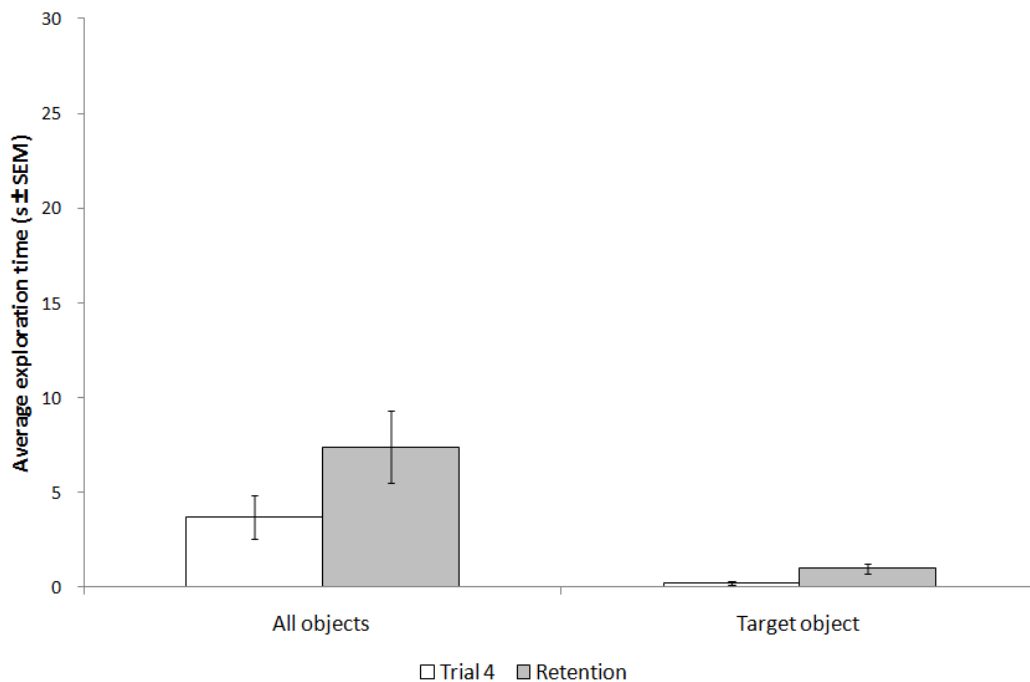
Figure 6.15: Average exploration of objects between trial 4 of training and the retention trial in the CNQX-treated group in the object displacement task (wood moved to a novel position in retention trial). There were no changes in exploration for all objects in the retention trial compared to trial 4 and there were no changes in exploration of the target object in the retention trial compared to trial 4.

Levels of hippocampal and perirhinal BDNF were compared between the three groups using a 2 x 3 mixed factorial ANOVA. Area was analysed on 2 levels (hippocampus and perirhinal cortex) as a within-groups measure and drug treatment was analysed on 3 levels (saline, MK-801 and CNQX) as a between-groups measure (Figure 6.16). There was a significant effect observed for area with the hippocampus exhibiting significantly higher levels of BDNF compared to the perirhinal cortex ($F = 86.785$; $df = 1, 17$; $p < 0.001$) and there was also a significant effect found for drug treatment ($F = 5.673$; $df = 2, 17$; $p < 0.05$). Additionally, there was an interaction effect observed between area and drug treatment ($F = 7.372$; $df = 2, 17$; $p < 0.01$). Tukey *post-hoc* analysis showed that there were significantly higher levels of BDNF overall in the CNQX-treated group compared to the saline-treated group ($p < 0.05$). Comparisons between drug treatment groups were made within each brain area using one-way ANOVAs with Tukey *post-hoc* analysis. There was a significant effect for drug treatment in the hippocampus ($F = 9.894$; $df = 2, 19$;

$p < 0.001$) with the CNQX-treated group exhibiting significantly higher levels of BDNF compared to the saline-treated group ($p < 0.001$). In the perirhinal cortex however, there were no significant differences found between the three drug treatment groups ($F = 1.464$; $df = 2, 19$; $p > 0.05$).
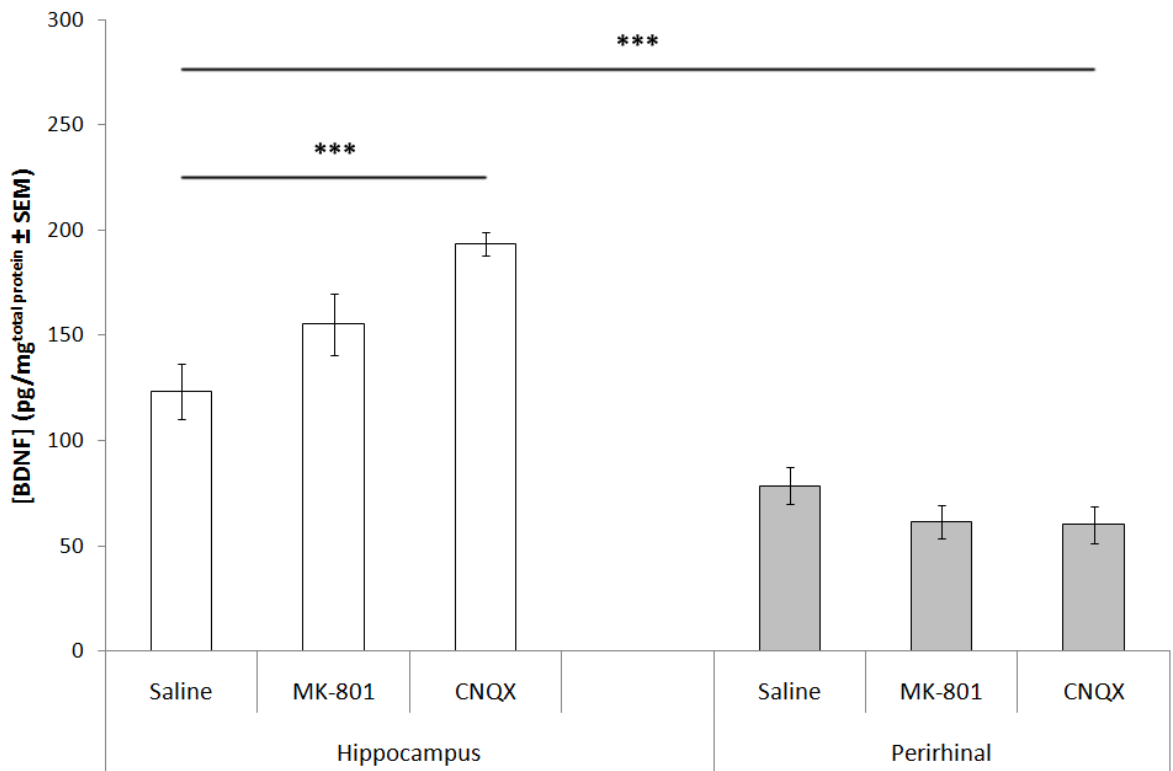
Figure 6.16: Average brain-derived neurotrophic factor (BDNF) levels for each drug treatment group in the hippocampus and the perirhinal cortex following an object displacement task. There were significantly higher levels of BDNF overall in the hippocampus compared to the perirhinal cortex and within the hippocampus there were significantly higher levels of BDNF in the CNQX-treated group compared to the saline-treated group. There were no significant differences found between the drug treatment groups in the perirhinal cortex. ***$p < 0.001$.

6.4 Discussion:

In this chapter, we have shown that the systemic administration of CNQX, an AMPA/kainate glutamate receptor antagonist, leads to impairments in both an object recognition task and an object displacement task when given before training. On the other hand, pre-training systemic administration of the use-dependent NMDA receptor channel blocker MK-801 impairs performance on the object displacement task but not the object recognition task. The observed impairment of object recognition memory by CNQX-treatment was expected as it has been shown that perirhinal administration of CNQX impaired performance in an object recognition task (Winters & Bussey, 2005b) and other studies have directly implicated the kainate receptor in object recognition memory (Barker et al., 2006). That the NMDA and AMPA/kainate receptors were involved in object displacement memory was also expected as several studies have shown the importance of both receptors in many different tests of spatial memory; for example, in the Morris water maze there has been evidence that the NMDA receptor (Morris et al., 1986; Davis et al., 1992; Watanabe et al., 1992; Liang et al., 1994; Shi et al., 2006; Ferretti et al., 2007) and the AMPA/kainate receptor are involved in learning this task (Liang et al., 1994; Filliat et al., 1998; Ferretti et al., 2007). More specifically, research focussing on spatial memory for objects as opposed to navigation has shown that the NMDA (Usiello et al., 1998; Roullet et al., 2001; Ferretti et al., 2005; Barker & Warburton, 2008; Larkin et al., 2008) and AMPA receptors (Roullet et al., 2001; Ferretti et al., 2005) are also involved in object displacement memory. However, our findings point to perhaps a more crucial role for the AMPA/kainate receptor in object displacement memory. As there was no increase in overall exploration in the retention trial for the CNQX-treated group, this suggests that these animals may not have perceived that there was a novel spatial configuration of the objects possibly due to CNQX-treatment disrupting acquisition or consolidation of the objects' spatial arrangements in Day 1 (Roullet et al., 2001).

The findings regarding the role of the NMDA receptor in object recognition memory presented here conflict with previous research. Although repeated administration of MK-801 in mice has been shown to impair object displacement

but not object recognition memory (Mandillo et al., 2003), the majority of the literature has shown that disruption of NMDA receptor function impairs performance on object recognition tasks (Winters & Bussey; 2005b; Barker et al., 2006). While many studies utilised the NMDA receptor antagonist AP-5, some researchers have also shown that the use-dependent NMDA receptor blocker MK-801 can impair object recognition memory. Nilsson and colleagues (2007) also found that systemic administration of MK-801 at the same dose that we used (0.1 mg/kg body weight) in mice resulted in no impairments on a retention test 1.5 hrs post-training. It was only with higher doses (0.2 mg/kg body weight) that impairments were observed. The work of de Lima and colleagues (2005) found that systemic administration of MK-801 (0.01 mg/kg body weight and 0.1 mg/kg body weight) in Wistar rats pre-training resulted in impairments both 1.5 and 24 hrs post-training. Both these studies used a simpler object configuration than that utilised in the current study; training with two objects and replacing one with a novel object for the retention trial compared to the configuration used here where training was with four objects and with one being replaced with a novel object for the retention trial. This means that our object recognition task should have been more difficult due to the increased number of items and therefore any memory impairments due to drug-treatment should have been more evident.

Our findings suggest that the NMDA and the AMPA/kainate receptors may play different functional roles in object recognition and object displacement memory. We suggest that although NMDA receptors have been implicated in object recognition memory (Winters & Bussey, 2005b; Barker et al., 2006; Barker & Warburton, 2008), activity-dependent blockade of NMDA receptors is not always enough to block acquisition of the object recognition task whereas AMPA receptor antagonism alone is enough to block acquisition. This fits with our electrophysiological data where long-term potentiation (LTP) could be induced in the CA1 to perirhinal cortex of MK-801-treated animals but the induction of LTP in the same projection in CNQX-treated animals was blocked (Chapter 5; Kealy & Commins, 2009). However, it has been proposed that object recognition memory depends on long-term depression (LTD) in the perirhinal cortex (Warburton et al., 2003) and if so, the idea of potentiation as reported in the CA1 to perirhinal cortex

may be counterintuitive in terms of being a physiological mechanism for recognition memory. Yet, later work by Barker and colleagues (2006) points to a dual LTP/LTD process underlying recognition memory due to the need for blockade of both NR2A and NR2B subunit-containing NMDA receptors to impair object recognition memory 24 hrs after training. As MK-801 is not selective for either subunit (Murray et al., 2000), it would be expected that it should cause an impairment in object recognition memory by inhibiting both types of NMDA receptor but MK-801 has been shown to be unpredictable in behavioural tests due to dose-dependent fluctuations in behavioural responses; lower doses lead to increased activity on a number of different lever press tasks compared to controls whereas higher doses result in decreased activity compared to controls on the same tasks (Gilmour et al., 2009). Gilmour and colleagues (2009) speculate that MK-801 may have altered selectivity for NR2A and NR2B subunits depending on dose which might explain the differences observed between our current study and those that previously report a deficit following MK-801 treatment (de Lima et al., 2005; Nilsson et al., 2007).

Leading on from the pharmacology of object recognition and object displacement memory, the role of BDNF in these tasks is less clear. In Experiment 1, we found that in the hippocampus there are significantly higher levels of BDNF following the object displacement task, which is what is expected due to the central role of the hippocampus in spatial memory (O'Keefe & Nadel, 1978; Talpos et al., 2008). This fits with imaging studies in the rat where immediate early gene expression is increased in the hippocampus following a spatial memory task (Vann et al., 2000a; Jenkins et al., 2004; Amin et al., 2006). However, we did not find the expected increases in BDNF levels in the perirhinal cortex following the object recognition task as predicted by immediate early gene expression studies that indicate that there is increased activation of BDNF-related molecules such as c-Fos (Nakagawara et al., 1994; Gaiddon et al., 1996; Nonomura et al., 1996) in the perirhinal cortex following an object recognition task (Zhu et al., 1995b; 1996; Wan et al., 1999). In Experiments 2 and 3, we did not find the expected decreases in BDNF following MK-801- and CNQX-treatment in either the hippocampus or the perirhinal cortex. Previously we have shown that treatment with both drugs at the

same dose leads to significant decreases in BDNF levels (Chapter 5; Kealy & Commins, 2009) and MK-801 has been shown to result in decreased BDNF levels in the hippocampus (Dragunow et al., 1993). However, these reported decreases were found after analysing tissue samples taken after electrophysiological experiments whereas we had to wait 24 hrs post-drug treatment to analyse samples, at which time levels of BDNF may have returned to normal levels (Aicardi et al., 2004). This may explain why we did not observe any increases in perirhinal BDNF following the recognition task in Experiment 1 and why we did observe any decreases in BDNF levels following drug-treatment in Experiments 2 and 3.

In conclusion, we have demonstrated that glutamate receptors play a part in both object recognition and object displacement memory. We have shown that the NMDA receptor is necessary in performing the object displacement task and that AMPA/kainate receptors are necessary for both object recognition and object displacement memory. Therefore we propose that these different types of glutamate receptors perform different roles in these forms of learning and memory. Furthermore, we have shown that BDNF is upregulated in the hippocampus following learning an object displacement task which suggests that synaptic plasticity in the hippocampus may underpin learning in this spatial memory task.

Chapter 7

Hippocampal output to neocortex: General discussion.

7.1 Summary of the findings of this thesis:

In this thesis, our main objectives were to characterise the projection originating in area CA1 of the hippocampus and terminating in the perirhinal cortex in the anaesthetised rat and to characterise the roles of the hippocampus and perirhinal cortex in the behaving animal. We examined the electrophysiological properties of the CA1 to perirhinal cortex projection using a range of high-frequency stimulation (HFS) and low-frequency stimulation (LFS) protocols as well as using short-term electrophysiological measures such as paired-pulse facilitation (PPF; Chapters 2 and 4). We also examined the roles of brain-derived neurotrophic factor (BDNF) and extracellular signal-regulated kinase (ERK) using enzyme-linked immunosorbant assays (ELISAs) and western blotting respectively (Chapter 3). We then investigated the effects of N-methyl-D-aspartic acid (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate glutamate receptor blockade on synaptic plasticity and BDNF levels in this projection (Chapter 5). In Chapter 6, we examined the behavioural roles of the hippocampus and perirhinal cortex in an object recognition task (a test of recognition memory) and an object displacement task (a test of spatial memory). We again analysed levels of hippocampal and perirhinal BDNF and challenged these behaviours pharmacologically using the same drugs used in our electrophysiological experiments.

It has previously been shown that the CA1 to perirhinal cortex monosynaptic projection was capable of sustaining changes in synaptic plasticity (Cousens & Otto, 1998). We have confirmed and expanded on these findings in demonstrating that this projection is capable of short-term and long-term changes in activity-dependent synaptic plasticity. We have demonstrated that this projection can sustain short-term synaptic plasticity in the forms of PPF and post-tetanic potentiation (PTP). PPF peaked consistently at the 40 ms interpulse interval, confirming that this projection is monosynaptic in nature. PTP was found to be activity-dependent and only HFS resulted in PTP induction. Activity-dependent long-term synaptic plasticity was also observed in this projection; HFS but not LFS of the area CA1 induced long-term potentiation (LTP) in the perirhinal cortex lasting at

least 1 hr. However, LFS of the CA1 to perirhinal cortex projection failed to induce long-term depression (LTD).

Furthermore, we have shown that glutamatergic neurotransmission is required for synaptic plasticity to occur in this pathway and it is also required in the behaving animal in the performance of hippocampal- and perirhinal-dependent memory tasks. Antagonism of AMPA/kainate glutamate receptor by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) blocked the induction of LTP following HFS but blockade of the NMDA receptor by (+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]cyclo-hepten-5,10-imine-maleate (MK-801) had no effect on LTP induction. Similarly, AMPA/kainate receptor antagonism but not NMDA receptor blockade impaired performance in an object recognition task (a test of perirhinal-dependent recognition memory). However, we demonstrated that both the AMPA/kainate and NMDA receptors were required for successful performance in an object displacement task (a test of hippocampal-dependent spatial memory).

Lastly, both changes in synaptic plasticity in the CA1 to perirhinal cortex projection and hippocampal-dependent learning and memory may be somewhat reliant on neurotrophins. HFS of the area CA1 could induce increases in BDNF, a molecule implicated in synaptic plasticity (Korte et al., 1995; Kang and Schuman, 1995a; 1995b; Bramham and Messaoudi, 2005; Lynch et al., 2007) and learning and memory (Falkenberg et al., 1992; Linnarsson et al., 1997; Kesslak et al., 1998; Mu et al., 1999; Mizuno et al., 2000; Schaaf et al., 2001; Rossato et al., 2007; Furini et al., 2009; Romero-Granados et al., 2009). However, this stimulation-induced increase in BDNF levels was not consistent across all electrophysiological experiments; HFS did not reliably increase BDNF levels in the area CA1. LFS of the area CA1 could induce increases in perirhinal BDNF levels but not at all of the LFS protocols tested. We also found that increases in hippocampal BDNF were associated with learning in the object displacement task and that AMPA/kainate-induced deficits in the object displacement task were associated with significantly higher levels of hippocampal BDNF compared to control animals. These findings suggest that the hippocampus is involved in object displacement memory and that the BDNF's role in this form of learning may be dependent on AMPA/kainate glutamatergic signalling.

Overall, these findings point towards an electrophysiologically excitatory role for the CA1 to perirhinal cortex projection in the hippocampal-parahippocampal network (see section 7.2). In addition, as glutamatergic signalling is required for both changes in synaptic plasticity and learning and memory, these data provide further evidence to support the idea that synaptic plasticity underlies memory processes (see section 7.3; Martin et al., 2000).

7.2 Significance of findings:

Our electrophysiological findings support the theory that the role of the hippocampus in learning and memory is to output associative information to the neocortex for long-term storage (McClelland et al., 1995; McClelland, 1998). In order for this theory to be true, it is crucial that projections from the hippocampus to the neocortex must be able to sustain changes in synaptic strength in activity-dependent manner (Rolls, 1996; McClelland & Goddard, 1996) as synaptic plasticity is believed to be required for the encoding, storage and consolidation of memories (Martin et al., 2000). Our findings that the CA1 to perirhinal cortex projection can sustain both short- and long-term changes in activity-dependent synaptic plasticity indicate that this projection may therefore play a role in the storage or consolidation of perirhinal-dependent memories (Hasselmo & McClelland, 1999).

These findings fit into our proposed model of electrophysiologically excitatory and inhibitory projections within the hippocampal-parahippocampal network (see Chapter 1; Figure 7.1). Based on anatomy (Swanson & Cowan, 1977; Wyss, 1981; Kosel et al., 1982; Deacon et al., 1983; Kosel et al., 1983; McIntyre et al., 1996; Naber et al., 1997; Burwell & Amaral, 1998a; Naber et al., 1999; 2000; 2001a; 2001b; Kloosterman et al., 2003b) and physiology (Canning et al., 2000; Ivanco & Racine, 2000; de Curtis & Biella, 2002; Garden et al., 2002; Kloosterman et al., 2003a; 2004; Craig & Commins, 2005; 2006; 2007; Kealy & Commins, 2009), the hippocampal-parahippocampal network has been proposed as a relay of parallel pathways between the hippocampus and the constituents of the parahippocampal region that may play a role in learning and memory (Witter et al., 2000a; 2000b; Witter, 2002). Our lab have shown previously that the projections originating in the distal CA1 and proximal subiculum which terminating in the lateral entorhinal cortex show a greater tendency to sustain electrophysiologically excitatory synaptic plasticity whereas those originating in the proximal CA1 and terminating in the medial entorhinal cortex show a greater tendency to sustain electrophysiologically inhibitory synaptic plasticity (Craig & Commins, 2007). Looking at perirhinal-entorhinal interconnectivity; the lateral entorhinal cortex has multiple reciprocal projections with the perirhinal cortex whereas the medial entorhinal cortex is

connected reciprocally with the postrhinal cortex (Insausti et al., 1997; Burwell & Amaral, 1998a). Taking into account these anatomical findings, there seems to be differences also in electrophysiological activity between two main circuits connecting in the hippocampal-parahippocampal network, with the circuit centred around the lateral entorhinal cortex (which includes the perirhinal cortex) being electrophysiologically excitatory in nature and the circuit centred around the medial entorhinal cortex (including the postrhinal cortex) being electrophysiologically inhibitory in nature. From this model, we predicted that the CA1 to perirhinal cortex projection should be electrophysiologically excitatory in nature as it forms part of the CA1/subiculum to lateral entorhinal cortex circuit (Figure 7.2). Based on our investigation into frequency-dependent changes in synaptic plasticity in the CA1 to perirhinal cortex projection (Chapter 4), our findings fit with the proposed model (Figure 7.2). HFS induced LTP as predicted and LFS did not induce LTD. We did observe low levels of potentiation (rather than depression) following 1 Hz and 5 Hz LFS protocols which we would not expect if this was an electrophysiologically inhibitory pathway.
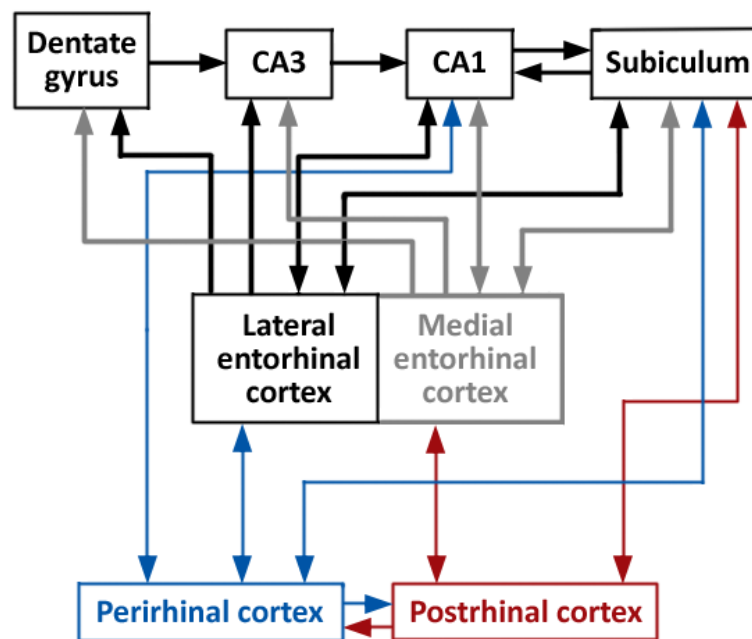
Figure 7.1: Schematic diagram showing the main projections within the hippocampal-parahippocampal circuit. Adapted from Witter et al. (2000) with supplemental information from Swanson & Cowan (1977) and Burwell (2000).
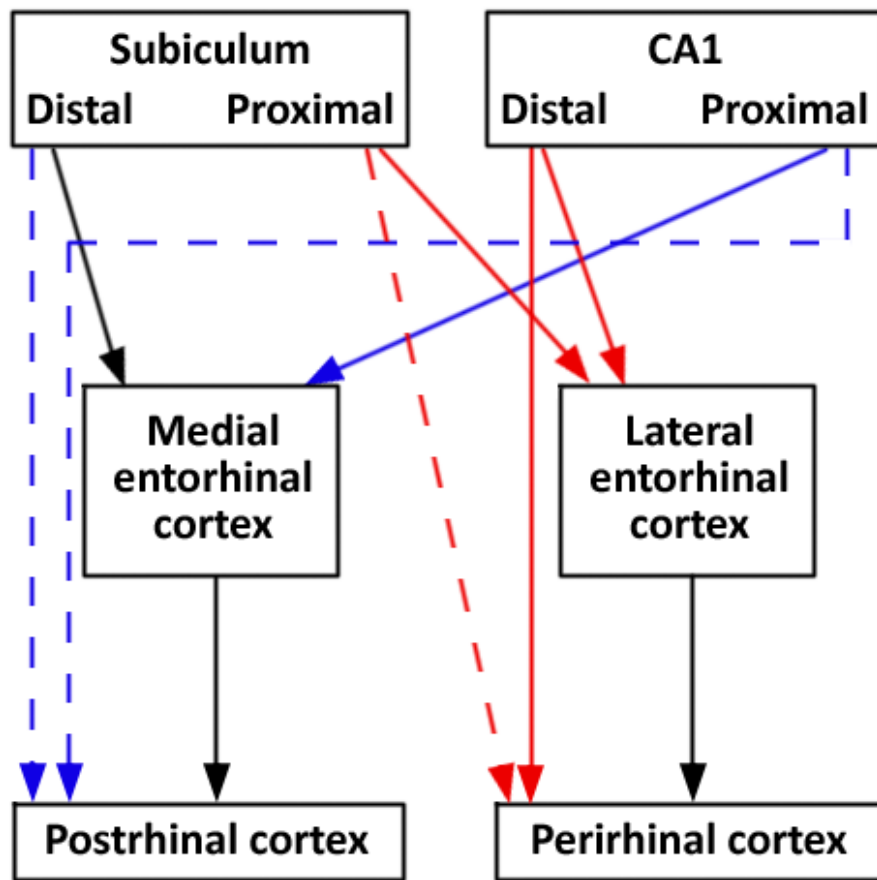
Figure 7.2: Proposed model of the segregation of electrophysiologically excitatory and inhibitory circuits in the hippocampal-parahippocampal network. Red arrows represent electrophysiologically excitatory projections and blue arrows represent electrophysiologically inhibitory projections. Dashed arrows of either colour represent the predicted electrophysiologically excitatory or inhibitory nature of a given projection. Black arrows are anatomically defined projections that have no electrophysiologically excitatory or inhibitory properties (in the case of the distal subiculum to medial entorhinal cortex projection; Craig, 2006) or have not yet been examined in detail. Based on the findings in Chapter 4 and Craig & Commins, 2007.

From this and previous work (Burwell, 2000; Witter et al., 2000; Craig & Commins, 2007), clear predictions can be made in terms of the remaining projections and future work should focus on determining the electrophysiologically excitatory and inhibitory capabilities of the other projections in the hippocampal-parahippocampal network. We suggest, for example, that the projections from the proximal subiculum and lateral entorhinal to the perirhinal cortex will both be electrophysiologically excitatory in nature. We also predict that the projections

from the proximal CA1, distal subiculum and medial entorhinal cortex will be electrophysiologically inhibitory in nature. Further electrophysiological examination of the distal CA1 to the perirhinal cortex (the focus of this thesis) may reveal that LTD can be induced following changes in metaplasticity as metaplastic effects have been described in other projections within the hippocampal-parahippocampal network (Craig & Commins, 2007).

If a clear distinction between hippocampal output projections were found, what would be the implications of this? Firstly, it may represent a means for separate forms of sensory information to be processed differentially. For example, this would allow visuospatial information from the postrhinal cortex (Burwell & Amaral, 1998b; Burwell & Hafeman, 2003; Furtak et al., 2007b) to be processed while keeping olfactory information from the perirhinal cortex (Herzog & Otto, 1998; Canning et al., 2000; Biella et al., 2003; Santiago et al., 2004) in a separate circuit, perhaps allowing the hippocampus to associate the disparate sensory information if necessary. Secondly, these separate electrophysiologically excitatory and inhibitory circuits may be involved in regulating neuronal activity. Evidence for this comes from epilepsy research, where abnormal perirhinal functioning has been identified as a possible mechanism underlying epileptogenesis (Ferland et al., 1998; Schwabe et al., 2000). Failure of electrophysiologically inhibitory interactions between the perirhinal, postrhinal and entorhinal cortices and the hippocampus leads to increased electrophysiological excitability that may drive epileptogenesis (de Curtis & Paré, 2004). Input from olfactory areas into the perirhinal cortex (Kemppainen et al., 2002; Majak & Pitkänen, 2003) has been shown to increase electrophysiologically excitatory activity within the hippocampal-parahippocampal region (Kelly & McIntyre, 1996). There have been analogous findings in epilepsy studies in humans whereby epilepsy patients exhibit lower tissue volumes in the parahippocampal region compared to controls (Bernasconi et al., 2003a; 2003b).

There could be an important role for these electrophysiologically excitatory and inhibitory projections from the hippocampus in epileptogenesis. Uncontrolled electrophysiologically excitatory activity may play a role medial temporal lobe epilepsy and as such, further electrophysiological examination of the interconnectivity within the hippocampal-parahippocampal network would be a

vital step in understanding how epileptiform activity is propagated through the medial temporal lobe. Disconnection studies between the cortices of the parahippocampal region in the rat may provide a way to control kindling activity and prevent seizures from spreading to other neocortical areas. Equally, pharmacological dampening of glutamatergic signalling may prevent surplus electrophysiologically excitatory activity from manifesting itself as epileptiform activity. As AMPA receptor antagonism successfully impaired LTP in the CA1 to perirhinal projection and kainate receptors have been implicated in perirhinal functioning (Barker et al., 2006), focussed research on this system may yield beneficial results for epilepsy treatments.

Evidence for the functional roles of the hippocampus and the perirhinal cortex in the behaving animal come from our results in the three behavioural experiments described in Chapter 6. In Experiment 1, we demonstrated that hippocampal BDNF was upregulated following performance of an object displacement task when compared to animals who performed an object recognition task and animals who performed a control task with no changes. This supports findings in the literature that suggest that the hippocampus and not the perirhinal cortex plays a greater role in spatial memory (Ennaceur et al., 1996; 1997; Bussey et al., 2000; Winters et al., 2004). However, we did not observe any changes in perirhinal BDNF levels following an object recognition task, object displacement task or a control task; an increase in perirhinal BDNF following the object recognition task was expected due to its role in recognition memory (Aggleton et al., 1997; Ennaceur & Aggleton, 1997; Glenn & Mumby, 1998; Bussey et al., 1999; Machin et al., 2002; Winters et al., 2004). Other molecules associated with cellular activity have been shown to be increased following learning in both spatial and recognition memory tasks, for example there is increased c-Fos expression in the hippocampus associated with spatial memory (Vann et al., 2000a; Jenkins et al., 2004; Amin et al., 2006) and there is also increased c-Fos expression in the perirhinal cortex associated with recognition memory (Zhu et al., 1995b; 1996; Wan et al., 1999).

However, increased expression of transcription factors in the perirhinal cortex is not predictive of changes in BDNF expression. BDNF may not be as

fundamental in perirhinal functioning as it is in hippocampal functioning. This idea is supported by the higher overall levels of BDNF relative to total protein found in the hippocampus compared to the perirhinal cortex (Chapters 3, 4, 5 and 6) and the entorhinal cortex (Harvey et al., 2008) perhaps suggesting there is more BDNF in the hippocampus compared to the neocortex because BDNF is required for more aspects of hippocampal rather than neocortical functioning. There is another possible explanation for the apparent lack of changes observed in perirhinal BDNF levels: electrophysiological stimulation and learning-related input may be modulating BDNF function in ways that do not change the total levels of BDNF. BDNF already present in stores in the perirhinal cortex may be secreted in an activity-dependent manner which would increase the amount of available BDNF for signalling (Aicardi et al., 2004) and this secreted BDNF may be later endocytosed and recycled for later use (Santo et al., 2006). Neither of these processes would result in changes in total BDNF levels in the tissue.

As the primary candidate for a physiological mechanism for learning and memory is synaptic plasticity (Martin et al., 2000), we examined the effects of glutamatergic blockade on both synaptic plasticity in the CA1 to perirhinal cortex projection (Chapter 5) and hippocampal- and perirhinal-dependent learning and memory (Chapter 6). Glutamatergic signalling has been implicated in modulating synaptic plasticity in many ways; NMDA receptors have been shown to be involved in both LTP (Collingridge et al., 1983; 1988a; Dragunow et al., 1993; Bashir et al., 1994; Wu et al., 2001; Wozny et al., 2008) and LTD (Aroniadou & Teyler, 1991; Xiao et al., 1995; Bartlett et al., 2007; Yashiro & Philpot, 2008) and similarly AMPA and kainate receptors have also roles to play in LTP (Harris & Cotman, 1986; Castillo et al., 1997; Vignes & Collingridge, 1997; Bortolotto et al., 1999; Yu et al., 2008) and LTD (Xiao et al., 1995; Beattie et al., 2000; Holman et al. 2007; Yu et al., 2008). These receptors may also underpin learning and memory; both NMDA (Morris et al., 1986; Liang et al., 1994 Barker et al., 2006; Ferretti et al., 2007; Barker & Warburton, 2008) and AMPA/kainate receptor antagonism (Liang et al., 1994; Filliat et al., 1998; Barker et al., 2006; Ferretti et al., 2007; Kessels & Malinow, 2009) can impair performance in learning and memory tasks.

The findings in this thesis support the case for the role of synaptic plasticity in learning and memory. We demonstrated that AMPA/kainate glutamate receptor antagonism prevented the induction of LTP whereas use-dependent blockade of the NMDA glutamate receptor had no effect on LTP induction in the CA1 to perirhinal cortex projection (Chapter 5; Kealy & Commins, 2009). Neither CNQX (AMPA/kainate antagonist) nor MK-801 (NMDA blocker) had any effect on short-term plasticity. However, there is always the caveat in this form of electrophysiological research that HFS is not a naturally occurring physiological event and HFS-induced LTP may not be the same as synaptic plasticity induced by other methods such as other electrophysiological stimulation protocols or indeed learning (Shors & Matzel, 1997; Hölscher, 1999) or that certain forms of LTP may not underlie all types of memory formation (Hölscher, 1997). Yet, there is a wealth of evidence and theory that supports the role of synaptic plasticity, if not specifically HFS-induced LTP, in learning and memory (Morris & Frey, 1997; Martin et al., 2000; Morris et al., 2003).

Some of our behavioural findings were in parallel with the electrophysiological data (Chapter 6); in Experiment 2, for example, we demonstrated that the performance of rats treated with CNQX was impaired in the object recognition task compared to the MK-801-treated group and saline-treated controls. However, in Experiment 3 we demonstrated that both CNQX- and MK-801-treated animals were impaired in the object displacement task. These findings suggest that both NMDA and AMPA/kainate receptors are required for the object displacement task whereas only AMPA/kainate receptors are needed for the object recognition task and for the induction of LTP in the CA1 to perirhinal cortex projection. This requirement for glutamatergic signalling in both synaptic plasticity and learning fits with recent experiments in the perirhinal cortex which identify changes in synaptic plasticity, particularly AMPA/kainate-dependent plasticity, as being necessary for recognition memory (Barker et al., 2006) and for spatial memory (in an object-in-place task; Barker & Warburton, 2008).

Based on both our electrophysiological and behavioural studies, we also suggest that there is an interaction between neurotrophic and glutamatergic signalling that underlies both synaptic plasticity and learning and memory.

However, this interaction appears to be quite complex. Early work into the role of BDNF in synaptic plasticity found that BDNF could enhance glutamatergic neurotransmission (Lessman et al., 1994; Kang & Schuman, 1995a; 1995b), LTP induction is associated with increases in BDNF levels (Castrén et al., 1993; Bramham et al., 1996; Hartmann et al., 2001) and BDNF could promote the release of glutamate in a Ca^{2+}-dependent manner (Matsumoto et al., 2001; Numakawa et al., 2001). There is also evidence that blockade of glutamatergic signalling can attenuate BDNF levels (Dragunow et al., 1993; Wetmore et al., 1994) whereas enhancement of glutamatergic signalling can cause an increase in BDNF expression (Zafra et al., 1990; Mackowiak et al., 2002). A simple explanation for these findings is that glutamatergic signalling and BDNF form a positive feedback system. However, the results from both our electrophysiological and behavioural studies do not fit with this explanation.

As described above, AMPA/kainate glutamate receptor antagonism prevented the induction of LTP whereas use-dependent blockade of the NMDA glutamate receptor had no effect on LTP induction in this projection (Chapter 5; Kealy & Commins, 2009). Yet, both AMPA/kainate and NMDA glutamate receptor blockade caused significant decreases in overall levels of BDNF in the area CA1 and the perirhinal cortex. As both groups exhibited attenuated levels of BDNF yet only the CNQX-treated group showed a deficit in synaptic plasticity, the effects of BDNF in synaptic plasticity cannot be explained alone by the level of BDNF present in the tissue. This is supported by the finding in the CNQX-treated group that 250 Hz HFS causes CA1 BDNF levels to return to the same level as the saline-treated control group. A similar observation was made in the behavioural study where CNQX-treatment caused a significant increase in hippocampal BDNF compared to saline-treated controls in the object displacement task. No deficits were observed in BDNF levels the following behavioural tasks but as tissue samples were taken 24 hrs after drug administration, reductions in BDNF at the time of training cannot be ruled out.

Future work in elucidating the roles of the hippocampus and perirhinal cortex in these forms in memory should focus on time-dependent effects on molecular activity within these structures; for example, a more systematic analysis of BDNF should be conducted whereby BDNF levels from tissue samples taken

immediately post-training are compared to BDNF levels from tissue samples taken immediately after the retention trial. A similar experiment from our lab examining neurotrophin expression during activity in the Morris water maze has shown training- and retention-induced changes in BDNF levels in the hippocampus and entorhinal cortex (Harvey et al., 2008). Such an experiment may reveal that BDNF is downregulated immediately after drug administration, at which point the glutamatergic blockade may cause changes in downstream signalling affecting retention. Subsequent increases in BDNF levels may not reverse the process because encoding or consolidation of that particular experience for the animal has been disrupted. However, this will only determine one aspect of glutamatergic blockade on learning and memory, parallel investigations examining expression of downstream molecules in BDNF signalling, such as ERK, and markers of neuronal activity, such as c-Fos, should also be performed. Additionally, direct application of blockers for BDNF signalling such as TrkB-IgG (Vaynman et al., 2004; Griesbach et al., 2009) or tyrosine kinase inhibitors (Inagaki et al., 2008) to the hippocampus and the perirhinal cortex could also determine the role of BDNF in learning and memory.

Taken together, these results indicate that the interaction between BDNF and glutamatergic signalling might involve regulation of BDNF levels, hence the abnormal levels observed in both the electrophysiological experiment and in the CNQX-treated group in Experiment 3 of the behavioural study. Regulation of BDNF by glutamate receptors is not a new concept; glutamate-dependent BDNF secretion has long been established (Canossa et al., 2001) and the attenuation of BDNF levels following MK-801- and CNQX-treatment in the electrophysiology study (Chapter 5) may explained by this. However, the increases in BDNF levels observed 24 hrs following CNQX administration in Experiment 3 of the behavioural study (Chapter 6) do not fit with these previous findings. Recent research has shown that the regulation of glutamatergic signalling through the AMPA receptor and the expression of BDNF are dissociable (Lauterborn et al., 2008) and this dissociation may explain our seemingly aberrant findings. As most studies into the interactions between glutamatergic signalling and neurotrophin expression are conducted over

a range of hours and not days, there needs to be long-term studies investigating the long-lasting effects of drug administration on neurotrophin levels.

Another conclusion that can be taken from our findings is that glutamate receptors may modulate BDNF signalling at a level below the regulation of the BDNF protein itself, for example glutamate may modulate the TrkB or p75 receptors (Huang & Reichardt, 2003) or act at some level further downstream such as ERK. However, we did not find any significant changes in ERK signalling in Chapter 3 following 250 Hz HFS which suggests that ERK may not play a large role in this projection. This is surprising considering the close relationship between BDNF and ERK signalling in LTP but it could be that BDNF signalling in this pathway is dependent on the phosphoinositide 3-kinase or phospholipase C secondary messenger cascades (Bramham & Messaoudi, 2005). As regards glutamatergic modulation of neurotrophin receptors; TrkB receptor activation has been shown to regulate the expression of NMDA receptor subunits (Margotti et al., 2002) and controls the expression of LTP in developing visual cortex (Sermasi et al., 2000). However, tetanic stimulation (as used throughout this thesis) has been shown to induce LTP independently of the TrkB receptor in adult hippocampal slices (Kang et al., 1997). As such, further research might be better to focus on neurotrophins other than BDNF as LTP in this projection might be induced via a different Trk receptor or perhaps through activation of p75 by pro-BDNF (which was not measured with the BDNF ELISA used here).

7.3 Concluding remarks:

The experiments outlined in this thesis have shown that the projection from the area CA1 to the perirhinal cortex is capable of both short- and long-term activity-dependent changes in synaptic plasticity. We also demonstrated that LTP in this projection is AMPA receptor-dependent and that BDNF signalling does not play a clear role in the synaptic plasticity observed here. Furthermore, we have shown that this projection is electrophysiologically excitatory in nature. Finally, we have established that the hippocampus and not the perirhinal cortex is activated following a spatial task, that AMPA receptors are required for recognition memory and that both NMDA and AMPA receptors are required for spatial memory. We post that the CA1 to perirhinal cortex may play a role in consolidating information into long-term memory, most likely during learning that involves determining the familiarity of stimuli.

Chapter 8

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