The perirhinal cortex (PRC), comprised of Brodmann areas 35 and 36, is a rostrocaudally-oriented strip of cortex in the medial temporal lobe, whose lesion produces memory impairments. Previous in vivo recording studies have revealed that a reduction in the responsiveness of perirhinal neurons to familiar stimuli underlies object recognition memory; whereas the opposite behavior, an increase in responsiveness, is required for associative memory formation between two paired stimuli. Both phenomena rely on long-term plasticity in the PRC. However, it is currently unclear how, as a result of experience, the same network could support these two seemingly opposite forms of long-term plasticity. PRC receives topographically organized projections from many high-order neocortical areas, mostly from the adjacent temporal neocortex; and it possesses an intrinsic network that distributes these neocortical inputs throughout its rostrocaudal axis. Previous studies suggest that neocortical inputs strongly recruit perirhinal inhibitory interneurons located at the same transverse level. In contrast, distant neocortical inputs only lead to excitation because longitudinal perirhinal connections engage few inhibitory interneurons. Given these facts, it is possible that the PRC acts as a Hebbian network to associate coincident but spatially distributed inputs. Consequently, the main objective of this thesis is to shed light on the mechanisms allowing neocortical inputs to undergo long-term depression (LTD) or potentiation (LTP), depending on to what extent they
recruit the intrinsic perirhinal connections. Thus, using electrophysiological and imaging techniques in the whole guinea pig brain \textit{in vitro}, I show that theta-frequency stimulation (TFS) at a single neocortical site leads to an input-specific group I mGluR-dependent LTD at all perirhinal levels, whereas paired TFS of two distant neocortical sites recruits the intrinsic circuit of the PRC and results in a NMDAR-dependent LTP to the paired inputs. Consistent with these results, utilizing anterograde tracing in rats and electron microscopic observations, this thesis shows that there are more inhibitory synapses formed by direct neocortical inputs to PRC and short-range perirhinal connections compared to long-range neocortical and perirhinal axons coursing in the PRC. Together, these findings suggest a mechanism whereby PRC associates temporally relevant but spatially distributed neocortical inputs.
Acknowledgements and Dedication

First, I express deep gratitude to my PhD mentor Denis Paré for his patient and diligent mentorship as well as friendship. The insights and knowledge I gained from him will always guide me through my career. I would also like to thank the members of my thesis committee: Ian Creese, Bart Krekelberg, György Buzsáki and Tibor Koós for their time, comments and guidance. I am indebted to many friends in the Paré lab for their help and camaraderie. I would like to thank Yoland Smith for his mentorship on electron microscopy and Jean-François Paré for teaching me this beautiful technique. Finally, I would especially like to thank my parents Mehmet and Aynur Ünal and my sister Yağmur for their endless support; and I dedicate this thesis to them.
Preface

The studies presented in Chapters III and IV are the result of a collaboration between myself and John Apergis-Schoute. This work is to be published in Cerebral Cortex (Unal et al., 2012). Chapter V describes work that is currently under preparation for publication.
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<tr>
<td>ABC</td>
<td>Avidin-biotin peroxidase complex</td>
</tr>
<tr>
<td>AIDA</td>
<td>(RS)-1-Aminoindan-1,5-dicarboxylic acid</td>
</tr>
<tr>
<td>AMPAR</td>
<td>(\alpha)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>AP</td>
<td>Anteroposterior axis</td>
</tr>
<tr>
<td>AP5</td>
<td>((2R))-amino-5-phosphonovaleric acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CA1</td>
<td><em>Cornu Ammonis</em> region 1</td>
</tr>
<tr>
<td>CA3</td>
<td><em>Cornu Ammonis</em> region 3</td>
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<tr>
<td>cAMP</td>
<td>3'-5'-cyclic adenosine monophosphate</td>
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<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>DV</td>
<td>Dorsoventral axis</td>
</tr>
<tr>
<td>EC</td>
<td>Entorhinal cortex</td>
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<tr>
<td>EPSP</td>
<td>Excitatory postsynaptic potential</td>
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<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>I.P.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol (1,4,5)-trisphosphate</td>
</tr>
<tr>
<td>IPSC</td>
<td>Inhibitory postsynaptic current</td>
</tr>
<tr>
<td>IPSP</td>
<td>Inhibitory postsynaptic potential</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>mGluR</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>ML</td>
<td>Mediolateral axis</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>MTL</td>
<td>Medial temporal lobe</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
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<td>NMDAR</td>
<td>(N)-methyl-D-aspartate receptor</td>
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<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<tr>
<td>PHAL</td>
<td><em>Phaseolus vulgaris</em> leucoagglutinin</td>
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<tr>
<td>PRC</td>
<td>Perirhinal cortex</td>
</tr>
<tr>
<td>SWS</td>
<td>Slow-wave sleep</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TFS</td>
<td>Theta-frequency stimulation</td>
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<tr>
<td>VDCC</td>
<td>Voltage-dependent calcium channel</td>
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CHAPTER I

Introduction
1.1 The medial temporal lobe and memory

Scientific investigation of memory dates back to Wilhelm Wundt, one of the founders of modern psychology. Although Wundt mostly avoided use of the term memory, he designed the first controlled study to understand how humans store short-term semantic information: he asked his subjects to recall a string of letters immediately after they were briefly exposed. He found that this type of memory was limited to approximately six letters, and did not change with practice (Wundt, 1912).

Neural correlates of what Wundt was exploring remained unknown for years, until the medial temporal lobe (MTL) started to be the focus of attention in memory research with the seminal article published by Scoville and Milner (1957). This article explained the case of Henry Gustav Molaison (known as HM) along with other temporal lobectomy patients. HM had severe epilepsy localized to MTL in both hemispheres. When his neurosurgeon Scoville removed most of the MTL of HM in both hemispheres, it became apparent that this part of the brain was required for long-term declarative memories. After the surgery, HM suffered from severe anterograde amnesia and he was unable to learn new semantic information. In other words, HM could not acquire long-term declarative memories, although he had intact working memory and procedural memory. Removal of both MTLs resulted in an inability to form new memories of facts and events as well as recollecting declarative memories that had been acquired shortly before the lobectomy. On the other hand, HM, along with other lobectomy patients, could easily remember semantic information that had been learned long time before the lobectomy (Milner et al., 1968). This indicated that the MTL converts short-term declarative memories to long-term, sometimes permanent memories.
The main components of the MTL include the hippocampus and rhinal cortices. Rhinal cortices refer to the entorhinal, perirhinal, and postrhinal (known as parahippocampal in primates) cortices. The perirhinal cortex (PRC), consisting of five-layered Brodmann area 35 and six-layered area 36, is the first stop of processed sensory information propagating from the secondary, or associative, cortices to the hippocampus. The PRC receives highly processed sensory information from these associative cortices and projects to the entorhinal cortex (EC), the main source of inputs to the hippocampus (Fig. 1.1; Deacon et al., 1983; Room and Groenewegen, 1986). The PRC also represents the main return path for hippocampo-entorhinal efferents to the neocortex (Fig. 1.1). However, the PRC is not only a simple relay station. Much data indicates that the PRC is itself required for the acquisition of recognition and associative memory (Gaffan and Murray, 1992; Meunier et al., 1993, 1996; Zola-Morgan et al., 1989; Suzuki et al., 1993).

![Fig. 1.1 Location and connectivity of the rhinal cortices.](image)

(A) Cresyl violet-stained coronal section of guinea pig rhinal cortices showing relative position of the rhinal sulcus (rh) and perirhinal areas 35 and 36; as well as the adjacent neocortex (NC), entorhinal cortex (EC), dentate gyrus of hippocampus (DG), and the lateral ventricle (V). Cross indicates orientation (L, lateral; M, medial; D, dorsal; V, ventral). (B1) Stepwise progression of impulses through the rhinal cortices. Most principal cells of the temporal neocortex and perirhinal cortex project to the adjacent cortical field. (B2) Leap mode shows a minority of cells projecting beyond the adjacent cortical field.
1.2 *Network properties of the PRC*

The PRC is a rostrocaudally-elongated strip of cortex located in the fundus (area 35) and lateral bank (area 36) of the rhinal sulcus (Fig. 1.1A). PRC forms one of the densest connections between the neocortex and hippocampus. Highly processed sensory information from the neocortical association cortices is relayed to PRC, which transmits this information to the hippocampus mostly via the lateral entorhinal cortex. Hippocampal and entorhinal projections arrive at the neocortex via PRC as well (Fig. 1.1B; Witter et al., 1986). Most of these projections follow a stepwise progression, forming synapses only with adjacent cortical areas (Fig. 1.1B1). However, a minority of cells projects beyond the adjacent cortical field (Fig. 1.1B2). Anatomical data indicate that the PRC forms dense reciprocal connections with many neocortical areas. Most of these inputs arise from the temporal neocortex bordering the PRC laterally (Fig. 1.2). These connections are organized topographically: anterior neocortical areas mostly contact rostral perirhinal cells and caudal neocortical areas project to more caudal parts of the PRC (Fig. 1.2; Deacon et al., 1983; Room and Groenewegen, 1986; Suzuki and Amaral, 1994; Saleem and Tanaka, 1996; Burwell and Amaral, 1998a). The most posterior region of the PRC is now identified as the postrhinal cortex (Burwell et al., 1995; Burwell, 2001). This region receives inputs mostly from caudal parts of the adjacent temporal neocortex and projects to the medial entorhinal cortex (Burwell, 2001; Furtak et al., 2007; Agster and Burwell, 2009; Biella et al., 2010).

Based on the connections summarized above, it has long been assumed that the PRC faithfully transmits signals between the neocortex and hippocampus in both directions. In fact, the two-stage model of episodic memory is based on this assumption.
According to this model, in the wake state, information of neocortical origin is rapidly stored in the hippocampus via changes in the efficacy of synapses between CA3 pyramidal neurons. During slow-wave sleep (SWS), the synchronized discharges of CA3 neurons in relation to sharp waves would “replay” representations stored in CA3, activate the rhinal cortices, which would in turn activate neocortical neurons representing the events of interest (Buzsáki, 1989). As a result, “replay” of waking activities during SWS would lead to long-term synaptic changes in the associative cortical networks that store episodic memories (Buzsáki, 1989; Pennartz et al., 2002).

Surprisingly, information transfer from the neocortex to the EC through PRC occurs with a low probability (see de Curtis and Paré, 2004). Both in vitro (de Curtis et al., 1999; Biella et al., 2001, 2002, 2003; Martina et al., 2001) and in vivo (Pelletier et al., 2004, 2005) studies have revealed that impulse transmission via the PRC is subjected to potent local inhibition observed as large, biphasic IPSPs (Biella et al., 2001; Martina et al., 2001). Part of this inhibition has been shown to arise from perirhinal GABAergic interneurons activated by neocortical inputs in a feedforward manner (Pinto et al., 2006) as well as long-range neocortical and perirhinal GABAergic neurons that project to principal perirhinal and entorhinal cells, respectively (Pinto et al., 2006; Apergis et al., 2007). In addition to the topography in its connections with the neocortex, the PRC possesses dense intrinsic connections in its rostrocaudal axis (Witter et al., 1986). Unlike neocortical inputs, the long-range intrinsic (longitudinal) connections of the PRC appear to recruit very few inhibitory interneurons, since neocortical stimulation evokes pure excitatory responses at rostrocaudally distant perirhinal sites (Fig. 1.2).
Fig. 1.2 Organization of neocortical inputs and intrinsic perirhinal connections, as inferred from previous electrophysiological and tracing studies. (A) Neocortical projections to the PRC are dominated by feedforward inhibition. In addition to glutamatergic projection cells ending on perirhinal interneurons, there are GABAergic neocortical cells projecting to principal perirhinal neurons. (B) Long-range intrinsic perirhinal connections originate from projection cells that contact few interneurons.

1.3 Relation between perirhinal activity-dependent plasticity in vitro and unit activity in vivo

Lesion studies show that the PRC is required for simple forms of recognition and associative memory. Recognition memory refers to the ability to remember a previously encountered stimulus, whereas associative memory refers to the formation of links between stimuli that are paired in time and space. Lesions of the PRC cause more severe recognition memory deficits than the impairment caused by hippocampal lesions (Gaffan and Murray, 1992; Meunier et al., 1993, 1996; Zola-Morgan et al., 1989; Suzuki et al., 1993; Aggleton et al., 1986; Murray and Mishkin, 1986; Leonard et al., 1995). Lesion
studies also showed that the PRC is required for forming and maintaining associative memories between stimuli of the same or different sensory modalities (Murray et al., 1993; Higuchi and Miyashita, 1996; Buckley and Gaffan, 1998; Parker and Gaffan, 1998; Goulet and Murray, 2001). Clearly, local plasticity in PRC can support various forms of memory, independent of the hippocampus.

The PRC can undergo activity-dependent LTD or LTP depending on the stimulation parameters and membrane potential of perirhinal cells. High-frequency stimulation (100 Hz) leads to an NMDAR-dependent potentiation of evoked responses in perirhinal slices kept in vitro (Bilkey, 1996; Ziakopoulos et al., 1999), whereas low-frequency stimulation (1 Hz) produces either a Ca$^{2+}$-dependent LTD or LTP of perirhinal neurons depending on the holding potential of the perirhinal cells. LTD is observed at -70mV and LTP is induced if the cells are kept at -10mV (Cho et al., 2001).

Long-term plasticity in the PRC can also be observed in vivo. Single-unit recording studies performed in both rats and monkeys show that about a quarter of perirhinal cells display reduced firing to previously presented visual stimuli in object-recognition tasks (Brown et al., 1987; Fahy et al., 1993; Miller et al., 1993; Rolls et al., 1993; Sobotka and Ringo, 1993; Waburton et al., 2003; Eichenbaum et al., 1996). This stimulus-specific decrement in activity is observed within 90 ms of the stimulus presentation. In addition, this decrease in firing develops with a single exposure and persists for more than 24 hours. Furthermore, this stimulus-specific reduction in perirhinal activity is reflected by decreased levels of perirhinal cells expressing c-Fos, an immediate early gene widely used to assess neuronal activity (Waburton et al., 2003).
It was suggested that there is a direct link between perirhinal LTD induced \textit{in vitro} and familiarity-induced suppression observed \textit{in vivo}. For instance, muscarinic antagonists, which block LTD induction in the PRC \textit{in vitro}, also reduce the familiarity-induced suppression of perirhinal firing and impair recognition memory (Waburton et al., 2003). Therefore perirhinal LTD is thought to underlie recognition memory.

On the other hand, the opposite behavior, namely an increase in firing, has been observed in other \textit{in vivo} single-unit studies utilizing associative memory tasks (Messinger et al., 2001; Naya et al., 2003a). About one-third of perirhinal neurons display increased firing in monkeys trained to form associations between two arbitrary visual stimuli during associative memory tasks. This behavior is known as pair-coding.

1.4 \textit{Hypothesis}

Returning to the network properties of the PRC, the unique intrinsic connections of the PRC might be the source of the apparent contradiction between LTD and LTP observed in object-recognition and associative memory tasks, respectively. The above considerations have important implications for the associative functions of the PRC. First, activation of a restricted area in the adjacent temporal neocortex recruits local interneurons, which might be the source of decreased responsiveness to familiar stimuli. Second, distant neocortical inputs travel through the rostrocaudal axis of the PRC and converge on different subsets of neurons. Importantly, since rostrocaudal perirhinal connections do not engage inhibitory interneurons, concurrent activation of two or more neocortical sites can shift the balance of perirhinal neurons from inhibition to excitation, as in pair-coding. In other words, the extent to which the stimulation parameters recruit
the intrinsic perirhinal connections might determine the polarity of the long-term plasticity in the PRC.

Thus, we hypothesize that this unique anatomical organization allows the PRC to associate coincident but spatially distributed neocortical inputs. The present thesis will test this hypothesis by focusing on (aim 1) the neocortical input parameters that differentially recruit the intrinsic perirhinal connections and induce long-term plasticity, (aim 2) the induction and expression mechanisms of the neocortically-evoked perirhinal LTD and LTP, and (aim 3) the proportion of inhibitory synapses formed by direct neocortical inputs vs. long-range perirhinal and neocortical axons coursing in the PRC as observed at the electron microscopic level.

1.5 Specific aims

Aim 1: to assess whether neocortical input patterns that differentially recruit intrinsic longitudinal perirhinal connections lead to contrasting forms of synaptic plasticity.

Rationale. It is known that neocortical inputs to PRC can undergo long-term depression (LTD) or potentiation (LTP) depending on the membrane potential of the postsynaptic perirhinal neurons. However, a more realistic method to induce LTD and LTP would be to alter the spatiotemporal profile of neocortical activity.

Hypothesis. The polarity of neocortically-induced plasticity (depression vs. potentiation) in the PRC depends on the spatiotemporal distribution of neocortical inputs. Specifically, LTD or LTP will develop depending on whether neocortical inputs recruit the intrinsic longitudinal connections of the perirhinal network.
Methods. In the whole guinea pig brain kept in vitro, theta-frequency stimulation (TFS) was utilized to induce activity-dependent long-term plasticity. Field potential recordings and optical imaging of voltage-dependent signals were used to assess LTD and LTP. Results. It was found that TFS at a single neocortical site leads to LTD at all perirhinal recording sites; whereas simultaneous TFS at two distant neocortical sites results in LTP at all rostrocaudal levels of the PRC. Both the LTD and LTP were specific to the TFS induction sites.

Aim 2: to determine the induction and expression mechanisms of the activity-dependent LTD and LTP induced by single-site vs. paired-site TFS, respectively.

Rationale. To shed light on the induction and expression mechanisms of the neocortically-induced LTD and LTP, pharmacological manipulations are combined with field potential recordings and optical imaging.

Hypothesis. The LTD induced by TFS at a single neocortical site depends on the activation of group I metabotropic glutamate receptors (mGluRs). On the other hand, the LTP induced by concurrent TFS of two rostrocaudally distant neocortical sites depends on the recruitment of the intrinsic perirhinal connections and is mediated by NMDAR activation. Therefore, the LTP should be abolished when longitudinal perirhinal connections are inactivated.

Methods. Lidocaine microinjections were made into the PRC or neocortex to block longitudinal propagation of inputs in the PRC and the polarity of long-term plasticity was assessed after TFS as in aim 1. In another set of experiments, we perfused the brain with the general NMDAR antagonist AP5 and/or the group I mGluR antagonist AIDA
Results. Experiments with inactivation of the intrinsic perirhinal connections indicated that propagation of inputs through the rostrocaudal axis of PRC is required for LTP. The LTP is NMDAR-dependent, whereas the LTD requires group I mGluR activation.

Aim 3: to compare the proportion of inhibitory synapses formed by neocortical axons vs. intrinsic perirhinal axons with perirhinal neurons.

Rationale. The general hypothesis tested in this thesis is based on the assumption that long-range axons in the PRC contact much fewer local-circuit cells compared to neocortical inputs contacting perirhinal cells in transverse register. However, this notion is based on electrophysiological studies; there is no direct anatomical evidence that this is the case. Therefore in this aim, this assumption was tested directly using anatomical methods.

Hypothesis. We hypothesized that direct neocortical inputs and short-range perirhinal connections recruit significantly more inhibition in the PRC than the long-range axons of perirhinal and neocortical neurons coursing in the rostrocaudal axis of the PRC (refer to the introduction of Chapter V for a detailed description of the various ultrastructural correlates of this inhibition).

Methods. This idea was tested by combining anterograde tracing with immunocytochemistry at the light and electron microscopic levels. We compared the proportion of symmetric and asymmetric synapses formed by neocortical vs. intrinsic perirhinal axons.
Results. Long-range perirhinal axons predominantly form asymmetric synapses almost exclusively with the dendritic spines of principal perirhinal neurons. In contrast, a higher proportion of excitatory terminals contributed by short-range neocortical and perirhinal axons form synapses with the dendrites of local-circuit neurons. In addition, short-range neocortical and perirhinal axons form a substantially higher proportion of symmetric synapses with the dendrites of perirhinal neurons.
CHAPTER II

General Methods
2.1 Whole brain in vitro experiments

Experiments were conducted on Hartley guinea pigs (200-250 g, 3-5 weeks old; Charles River, Wilmington, MA) in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee of Rutgers University. All animals were kept in a 12 hour light/dark cycle and had ad libitum access to food and water. It should be noted that the cytoarchitectural organization of the whole parahippocampal region in guinea pig is similar to that of other mammals (Reep, 1984; Uva et al., 2004).

This chapter explains general methods used in most experiments. More detail on methods and techniques specific to certain experiments are described in data chapters.

2.1.1 Whole brain preparation

Prior to the experiments, two cold (15°C) extracellular solutions were prepared: a perfusate and a superfusate. Their composition was identical except for the addition of the volume expander Dextran 70 (3%) to the perfusate. Both solutions contained (in mM) 126 NaCl; 3 KCl; 26 NaHCO3; 1.2 KH2PO4; 1.3 MgSO4; 2.4 CaCl2, 10 glucose, and 5 HEPES. The solutions were saturated with an oxygen-based gas mixture (95% O2, 5% CO2).

Our approach to the preparation of the whole guinea pig brain kept in vitro is based on initial descriptions (de Curtis et al., 1991; Muhlethaler et al., 1993). Animals were first deeply anesthetized with pentobarbital (60 mg/kg, i.p.), ketamine (80 mg/kg, i.p.) and xylazine (12 mg/kg, i.p.) and then perfused transcardially with the cold, oxygenated perfusate. During the perfusion, a craniotomy was performed on the dorsal
surface of the brain and the \textit{dura mater} was cut on the midline. Then, the perfusion was stopped, and the cranial nerves and carotid arteries were cut. The brain was then quickly transferred to the recording chamber filled with the superfusate. The brain was positioned with its ventral surface up and a cannula (GELoader, Eppendorf, Hauppauge, NY) was inserted into the basilar artery, which continuously delivered the perfusate to the brain (see Fig. 2.1). The rate of this perfusion was kept constant at 7 ml/min by a peristaltic pump. At this point, any leaking arteries were tied.

Once the leaking arteries were tied, the temperature of the recording chamber was increased to 30$^\circ$C at a rate of 0.4$^\circ$C per minute. A thermostat connected to a heater plate below the recording chamber controlled the temperature of the perfusate and superfusate.

\textbf{Fig. 2.1} Whole brain \textit{in vitro} preparation. A guinea pig brain is positioned with its ventral surface up in the recording chamber. The chamber is filled with the superfusate and a cannula is inserted to the basilar artery to continuously deliver the perfusate.
2.1.2  *Electrophysiological recordings*

Stimulating and recording electrodes were positioned under visual control while the temperature of the brain was gradually increased. For electrical stimulation, pairs of stimulating electrodes (tip separation: 200 µm) or concentric stimulating electrodes (in microinjection experiments) were inserted in the temporal neocortex. To record field potentials and unit activity, tungsten recording microelectrodes (5MΩ) or concentric electrodes (in microinjection experiments) were positioned at three different rostrocaudal levels of area 36 of the PRC (aligned with the stimulating electrodes) at a depth of 300-400 µm, corresponding to layer III (Fig. 2.2A2, empty circles). This depth was chosen because earlier field potential and current-source density analyses have revealed that neocortical stimuli evoke maximal field potential responses in layer III (Biella et al., 2002). Note that in microinjection experiments, the same concentric electrodes were used for both stimulating and recording. In all cases, the signals picked up by the recording electrodes were observed on an oscilloscope, digitized at 20 kHZ, and stored on a hard-disk for off-line analysis with Igor (IGOR, WaveMetrics, Lake Oswego, OR). To facilitate histological reconstruction of electrode tracks, at the end of the experiment, small electrolytic lesions (0.5 mA for 5 sec) were made to mark recording sites of interest.
2.1.3 Optical recordings

Optical recordings of the fluorescent signal generated by neurons stained with a voltage-sensitive dye were performed according to the technique described previously (Tominaga et al., 2000). When the temperature of the brain reached 23°C, the voltage-sensitive dye di-2-ANEPEQ (Invitrogen, Eugene, OR; 1.5 µg dissolved in 100 ml of perfusate) was applied to the brain via arterial perfusion for 10 minutes. Then, the dye-containing solution was filtered and applied again for another 10 minutes. This dye is rapidly taken up by neurons and changes its fluorescence intensity with changes in membrane voltage of neurons. This dye application allowed recording of large optical signals for at least 2 hours. The optical signal was observed through an epifluorescence microscope (Leica Micorsystems Inc., Bannaockburn, IL) positioned above the perirhinal
region, and collected with a CCD camera (MiCAM02, SciMedia, Costa Mesa, CA) at a rate of 200 Hz. Optical data is expressed as delta F/F.

2.1.4 Recording and stimulating procedures

We attempted to induce activity-dependent plasticity only once in each brain. In the control phase, 10 electrical stimuli (300 µsec, 0.5 mA; 0.1 Hz) were applied at each neocortical or perirhinal (in some microinjection experiments) site and evoked responses were averaged separately for each stimulation site. Then, in the induction phase, theta-frequency stimulation (TFS) was applied by giving thirty 1-sec trains of stimuli (8 Hz), each followed by a 0.5 sec stimulus-free period. Five and thirty minutes after the TFS, single shocks were applied at each stimulation site as in the control phase. In microinjection experiments, the responses were also checked one hour post-induction, when the effects of lidocaine had dissipated. When two neocortical sites were stimulated with the TFS pattern, the stimuli were either applied simultaneously or, as a control, with a delay of 62.5 ms (half a theta-cycle). In other control experiments, we reduced the stimulation intensity at the paired sites by decreasing shock durations from 300 to 100 µsec during induction.

2.1.5 Pharmacology

For pharmacological experiments, the NMDAR antagonist AP5 ((2R)-amino-5-phosphonovaleric acid; 100 µM), the group I mGluR antagonist AIDA ((RS)-1-Aminoindan-1,5-dicarboxylic acid; 100 µM), or their mixture were applied in a separate perfusate. After measuring the baseline activity in the control phase, the perfusate was
replaced with the drug-containing perfusate. TFS was applied ten minutes after changing to the drug-containing perfusate, which was replaced with the regular perfusate immediately after the induction. Five and thirty minutes after the TFS, 10 single shocks were applied at each stimulation site, as usual.

In control experiments, which were designed to determine whether sole application of AP5 or AIDA alters baseline responses, no TFS was applied while drugs were perfused for 10 minutes. Neocortically-evoked perirhinal responses were recorded both during and 5 minutes after perfusion.

2.1.6 Microinjections of lidocaine or vehicle

Microinjections of the Na\(^+\) channel blocker lidocaine or saline (vehicle) were done by using two Hamilton microsyringes (1 µl microsyringe, Hamilton Company, Reno, NV) and a microinjection pump (0.1 µl/min). Lidocaine or vehicle were infused at mid-rostrocaudal levels of either the PRC or adjacent neocortex by two microsyringes initially placed at a depth to reach the external capsule. In the case of perirhinal microinjections, one microsyringe was aimed at area 35, while the other was placed at area 36 to cover the whole perirhinal cortex.

2.1.7 Data analysis

All field potentials and optical responses presented in this study are averages of 10 stimuli applied at 0.1 Hz. The amplitude of field potential signals were measured and reported as the percent change produced by the theta-frequency stimulation or lidocaine microinjection. In optical recordings, square regions of interest (17x17 pixels) centered
on each recording electrode were identified, and the fluorescence signal in all the pixels was averaged using BrainVision Analyzer (Brain Products, Munich, Germany). The data are expressed as means ± standard errors. Statistical analyses consisted of t-tests and repeated measures ANOVAs followed by Bonferroni-corrected t-tests. For all statistical tests, the level of significance was defined as p < 0.05.

2.1.8 Histological verification of electrode positions

At the end of the experiments, brains were removed from the incubation chamber and fixed by immersion in a 2% paraformaldehyde and 1% glutaraldehyde solution for at least one week. The brains were then washed a few times in phosphate buffer saline (PBS, 0.1 M, pH 7.4), and 100 µm slices were cut with a vibrating microtome. The sections were washed five times (5 min each) in PBS (0.1 M, pH 7.4), mounted on gelatin-coated slides, air dried, counterstained with cresyl violet for electrode placement verification, dehydrated in a graded series of alcohol, and coverslipped.

2.2 Tract-tracing experiments

Experiments were conducted on adult male Sprague-Dawley rats (300-350 g; Charles River, Wilmington, MA) in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with the approval of the Institutional Animal Care and Use Committee of Emory University. All animals were kept in a 12 hour light/dark cycle with ad libitum access to food and water.
2.2.1 **Tract-tracing with PHAL**

Animals were anesthetized with isoflurane and placed in a stereotaxic apparatus. The anterograde tracer *Phaseolus vulgaris* leucoagglutinin (PHAL, 2.5% in 0.02M PBS, pH 8.0; Vector Laboratories, Burlingame, CA) was injected iontophoretically (5 µA, 7 sec ON/OFF for 15 min) through a glass pipette (tip diameter: 35-40 µm).

The injections were aimed at four sites (coordinates relative to bregma): a rostral level of PRC (AP, -3.3; ML, 6.3; DV, -4.6); a caudal level of PRC (AP, -5.2; ML, 7.1; DV, -4.1); a rostral level of the adjacent temporal neocortex (AP, -3.3; ML, 6.3; DV, -3.2); and finally a caudal level of the adjacent temporal neocortex (AP, -5.2; ML, 7.1; DV, -3.1). These coordinates were determined using a stereotaxic atlas of the rat brain (Paxinos and Watson, 1998).

After a survival period of 10-12 days, the animals were deeply anesthetized with sodium pentobarbital (50 mg/kg; i.p.) and perfused through the heart with 300 ml of saline (0.9%), followed by 500 ml of a fixative containing 1% glutaraldehyde and 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4). Then, the brains were extracted from the skull and post-fixed in the same fixative overnight. Finally, the brains were sectioned at 60 µm in PB using a vibrating microtome and the sections were collected in PBS.

2.2.2 **Immunocytochemistry for light and electron microscopy**

After sectioning, PHAL was detected by immunoperoxidase. Observations at the light microscopic level were made in order to determine the location of the PHAL injection site and the distribution of the anterograde labeling. For these observations, all
the sections were incubated in 1% sodium borohydride for 30 min, rinsed repeatedly in PB and 0.1M tris buffered saline (TBS, pH 7.4), and incubated for 30 min in a blocking solution. The blocking solution contained 1% BSA, 3% normal goat serum (NGS), and 0.04% Triton X-100 in TBS. The sections were then incubated overnight in rabbit anti-PHAL (1:1000, Vector Labs, Burlingame, CA). The next day, the sections were rinsed in TBS, incubated for 30 min in biotinylated goat anti-rabbit IgG (1:400, Jackson Immuno Research, West Grove, PA), and rinsed repeatedly in TBS. Finally, the sections were incubated for 30 min in the avidin-biotin peroxidase complex (ABC). The peroxidase was revealed using a solution of 0.022% diaminobenzidine (DAB) and 0.003% hydrogen peroxide in TBS. Then the sections were rinsed in TBS and 0.01 M PBS (pH 7.4). After rinsing, the sections were incubated for 30 min in a buffer containing 0.8% BSA, 0.1% gelatin, and 3% NGS in PBS.

At the end of the incubations, all the sections were mounted onto glass slides, air-dried, and dehydrated in a graded series of alcohol. Finally the glass slides were coverslipped in Permount mounting medium (Fisher Scientific, Pittsburgh, PA) for light microscopic observations.

For electron microscopy, up to this point, the sections were processed as above with the exception that Triton X-100 was not used in the blocking solution. Next, these sections were rinsed in PB and post-fixed in osmium tetroxide (2% in PB) for 1 hour. After post-fixing with osmium tetroxide, the sections were rinsed several times in PB. Then, the sections were dehydrated in alcohol and propylene oxide. Finally, they were embedded in Durcupan resin (Fluka, Gymea, Australia) and baked for 48 hours at 60°C. Then, the sections were transferred from resin blocks to glass slides, coverslipped, and
left in the oven at 60°C for 24 hours. Once the regions of interest were cut and removed from the glass, they were trimmed in trapezoidal shape, and placed in cylinder-shaped resin blocks for cutting with an ultramicrotome (Ultracut-E, Leica Microsystems, Wetzlar, Germany). These blocks were sectioned at 60-70 nm with a 45°-diamond knife. Ultrathin sections were then collected onto copper mesh grids. Finally, the grids were counterstained with 5% uranyl acetate and Reynold’s lead citrate, air-dried, and stored in grid boxes for analyzing with the electron microscope.

2.2.3 Data analysis

First, slides prepared for light microscopy were examined in order to determine the location of the PHAL injection in each animal. Only tissue from animals with injection sites restricted to the targeted locations were included in the data analysis: the PRC or adjacent temporal neocortex, with no diffusion to nearby regions.

Each grid was searched for clearly visible synapses with PHAL-labeled terminals. One (electron) micrograph was taken per synapse. For each animal, a total of 100 synapses were recorded from two locations in the PRC: half of the micrographs were taken close to the injection site (≤1mm), and the other half were taken from distant sites (>1mm). In PRC injections, a section was considered to be a ‘close site’ if PHAL-labeled somata (cell bodies) could be detected within 1mm in the AP axis. However, the sections were taken ≥ 200µ away from sections with labeled somata, to be sure that they were outside the injection site. In neocortical injections, micrographs taken from PRC levels in transverse register with the injection site were considered to be close site. To be considered ‘distant site’, micrographs had to be at least 1mm away from the perirhinal
region in transverse register with the neocortical injection site. For cases with rostral injection sites (AP, -3.3), the distant sites were taken from more caudal levels; and for cases with caudal injection sites (AP, -5.2), the distant sites were taken from more rostral levels.

Prior to electron microscopic observations of the tissue, the trapezoids in each grid were drawn at low magnification onto a grid notebook. PHAL-labeled profiles were searched with the electron microscope and micrographs were taken when PHAL was detected in presynaptic elements. PHAL-labeled terminals were identified by the presence of flocculent deposits that resulted from the DAB reaction. Micrographs were taken only when the PHAL-labeled presynaptic element was forming a clearly visible synapse. For each micrograph, the type of synapse (symmetric or asymmetric) and the target of the synaptic contact (dendrite or dendritic spine) were recorded. The observer was blind to the provenance of the tissue while taking the micrographs and recording the type of the synapse and the synaptic contact.

Synapses and the postsynaptic targets were identified according to previously defined criteria (Peters et al., 1991). Contacts were classified as synapses only when the synaptic cleft and postsynaptic density were clearly visible. The presence of synaptic vesicles in the presynaptic element was also required.

Three main criteria were used to identify dendritic spines: they had to be relatively smaller; the spine apparatus, or part of it had to be clearly visible; and they had to lack microtubules and mitochondria. In addition, a presynaptic element was identified as a spine when it was clearly emerging from a nearby dendrite and had the typical shape of a spine (see Figs. 5.5A, 5.7A). In contrast, dendritic shafts had to be relatively larger
and include either microtubules, mitochondria, endoplasmic reticulum, or a combination of these structures.

Once the micrographs were taken, they were transferred to Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA) for adjusting brightness and contrast. Adjustments were applied to the entire image.
CHAPTER III

(Aim 1)

Neocortical Input Patterns that Differentially Recruit Intrinsic Perirhinal Connections Lead to Contrasting Forms of Synaptic Plasticity
3.1 Rationale

Previous *in vitro* studies showed that the PRC can undergo activity-dependent LTP or LTD like many cortical structures. Modifying the frequency of tetanus of inputs has been a typical strategy to induce activity-dependent long-term synaptic plasticity in the PRC. High-frequency tetanic stimulation (100 Hz) leads to perirhinal LTP (Bilkey, 1996; Ziakopoulos et al., 1999; Seoane et al., 2009). This is not an artifact of the slice preparation as LTP of hippocampal-evoked responses could also be induced *in vivo* following theta-burst stimulation in field CA1 (Cousens and Otto, 1998). Furthermore, 200-900 low-frequency stimuli at 1 Hz (Cho et al., 2001) or 3000 stimuli at 5 Hz (Seoane et al., 2009) produce LTD in the PRC. In addition to input parameters, the holding potential of the perirhinal cells is crucial in determining the polarity of long-term plasticity (Cho et al., 2001).

The important question here is whether altering the frequency of neocortical inputs to PRC is the most realistic way to simulate activity-dependent perirhinal plasticity observed in object recognition and associative memory tasks. Is it possible to induce LTD in one condition and LTP in another condition by merely arranging the spatiotemporal organization of neocortical inputs without changing the intensity or frequency of stimuli?

Previous *in vitro* physiological studies have revealed that neocortical stimuli elicit neuronal responses that propagate through the entire rostrocaudal extent of the PRC (Biella et al., 2001; Martina et al., 2001). However, the effect of neocortical stimuli depends on the distance between the neocortical stimulation site and the recorded perirhinal neuron (Biella et al., 2001; Martina et al., 2001). Neocortical stimulation sites located at the same rostrocaudal level as the recorded cells evoke large biphasic IPSPs
that curtail the initial EPSP, possibly due to GABAergic interneurons activated by neocortical inputs in a feedforward manner (Pinto et al., 2006). On the other hand, rostrocaudally distant sites evoke apparently pure excitatory responses because longitudinal perirhinal pathways do not appear to engage feedforward interneurons (Biella et al., 2001; Martina et al., 2001).

In light of these in vitro data, it is justified to consider the intrinsic rostrocaudal network of the PRC as a candidate to shift the polarity of long-term perirhinal plasticity. Therefore, we hypothesized that the polarity of the long-term plasticity in PRC depends on the spatiotemporal organization of neocortical inputs, and to what extent these inputs recruit the intrinsic perirhinal network.

### 3.2 Methods

In the whole brain kept in vitro preparation, as shown in figure 2.2A, three tungsten recording microelectrodes were placed at different rostrocaudal levels of perirhinal area 36 at a depth of approximately 300 µm, corresponding to layer III (Fig. 2.2A2, empty circles), and three pairs of stimulating electrodes (tip separation: 200 µm) were inserted at the same depth in the laterally adjacent neocortex (Fig. 2.2A2, dots), in rostrocaudal register with the recording area 36 electrodes.

TFS was utilized to induce activity-dependent long-term plasticity. TFS consisted of thirty 1-sec trains of neocortical stimuli (8 Hz), with a 0.5 sec stimulus-free period after each 8 stimuli. In the control phase, 10 electrical stimuli (300 µsec, 0.5 mA; 0.1 Hz) were applied at each neocortical site and evoked responses were averaged separately for each stimulation site. Then, in the induction phase, TFS was applied at either one
randomly chosen site or at two sites. Five and thirty minutes after TFS, single shocks were applied at each stimulation site as in the control phase. When two neocortical sites were stimulated during induction, the stimuli were either applied simultaneously or, as a control, with a delay of 62.5 ms. In other control experiments, the stimulation intensity was reduced by decreasing shock durations from 300 to 100 µsec during TFS.

In addition to field potential recordings, the voltage-sensitive dye di-2-ANEPEQ was used (Invitrogen, Eugene, OR; 1.5 µg dissolved in 100 ml of perfusate). When perfused through the vascular system of the brain, this dye is immediately taken up by neurons to signal changes in their membrane potential by changes in fluorescence intensity. The optical signal was observed through a Leica epifluorescence microscope positioned above the perirhinal region (field of view: 10 by 7.6 mm) and collected with a CCD camera (MiCAM02, SciMedia, Costa Mesa, CA) at a frame rate of 200 Hz and the data is expressed as delta F/F.

Field potential and optical recordings of responses evoked from the temporal neocortex were obtained 5 and 30 min after TFS induction and compared to the measurements taken in the control phase.

### 3.3 Results

#### 3.3.1 Field potentials and optical signals evoked by neocortical stimuli in area 36

In the whole brain in vitro preparation, neocortical stimuli evoked biphasic field potential responses in layer III of area 36, consistent with earlier observations (Biella et al., 2002). These responses consisted of a negative component followed by a positive component (Fig. 3.1A2, B2). These perirhinal field potentials seemed to depend on
propagation in the perirhinal network itself, since the latency of responses increased with distance between the stimulation and recording sites (Fig. 3.1A1-2, B1-2; red dots show stimulation site). In addition, compared to field potential responses seen at the perirhinal site in transverse register with the stimulation site, response amplitudes decreased 17 ± 8 and 57 ± 7% at the intermediate and most distant recording sites, respectively (t-tests, p = 0.006 and 0.00005, n = 30).

Fig. 3.1 Area 36 responses to neocortical stimulation as measured with field potentials and optical measurements of voltage-sensitive signals. Panels A and B show area 36 responses to electrical stimuli delivered at the most anterior or posterior neocortical stimulation sites, respectively. The schemes in panel 1 show the stimulation (red) and recording sites. These neocortical stimuli elicited field potential (2) and optical responses (3-4). Panel 3 plots the fluorescence in all pixels included in the 3 square regions shown in panel 4 as a function of time. Arrowheads indicate when the stimulus was applied. The dashed vertical lines labeled T1-3 in 2 and 3 coincide in time. Panel 4 shows time lapsed pictures of optical signals elicited by neocortical stimuli at the times indicated by the dashed vertical lines T1-3.

Optical signals followed a similar time course as the field potentials (Fig 3.1A3-4, B3-4). Similar to the field potentials, spatial and temporal spread of the optical signal
showed that the neocortical input propagated longitudinally in the PRC, not in the adjacent neocortex. For instance, when the neocortical stimulus was applied rostrally, the signal appeared first rostrally in the PRC and then propagated caudally (Fig 3.1A4). Furthermore, no optical signal was observed in the caudal parts of temporal neocortex (Fig 3.1A4). The reverse pattern was seen when electrical stimuli were delivered caudally in the neocortex (Fig 3.1B4).

Comparing the time course of field potentials and optical signals shows that these two measures overlap at their peak points. This can be seen by comparing the three dashed lines in figures 3.1A2-3 and 3.1B2-3, which show three time points corresponding in the field potentials and optical signals.

In addition to the close parallel between the timing of field potential and optical responses, the origin of the field potential responses was indicated by the fact that neocortical stimuli could often be seen to evoke orthodromic discharges in layer III area 36 neurons. As shown in figure 3.2, these orthodromic responses typically coincided with the rising phase of the positive component of the field potentials recorded at the same site by the same microelectrode (see inset in Fig. 3.2B for population analysis of latencies). However, it should be noted that in the experiments described below, we purposefully avoided recording unit activity because they distorted the field potentials, complicating the analysis of activity-dependent changes in neocortically-evoked field responses.
Fig. 3.2 Neocortical stimuli elicit orthodromic responses in area 36 neurons. A and B show representative examples of single-unit orthodromic responses elicited in area 36 neurons by neocortical stimuli. Each panel shows 8 superimposed responses. The evoked signal was not filtered to allow examination of the temporal relationship between the evoked field potentials and unit activity. The recording and stimulation sites are indicated in the scheme shown as an inset in panel A. Inset in panel B shows the frequency distribution of median orthodromic response latencies in 102 area 36 neurons. To correct for changes in response latencies related to the varying distance between neocortical stimulation sites and perirhinal recording sites, all data was aligned in time to the negative peak of the evoked field potentials.

3.3.2 Effect of theta-frequency stimulation at a single neocortical stimulation site in area 36

To study activity-dependent changes produced by neocortical inputs in area 36, we first tested the effects of TFS at a single neocortical stimulation site on field potentials and optical signals (n=12). Across these experiments, each neocortical stimulation site served as an induction site the same number of times. These experiments consisted of three phases: (1) a control phase where stimuli were delivered 10 times at the three neocortical sites independently at a low frequency (0.1 Hz) and evoked responses averaged; (2) an induction phase where a randomly selected neocortical stimulation site received TFS while the other two sites were not stimulated; and (3) two post-induction phases where 5 and 30 minutes later, stimuli were delivered independently at the three neocortical sites at 0.1 Hz as in the control phase.

Irrespective of the induction sites, TFS caused a marked and long-lasting depression of evoked responses at all recording sites as seen in both the field potential
recordings and optical signals. This depression was specific to the induction site, leaving the responses evoked by the two control sites unchanged. Figure 3.3 shows a representative example of this phenomenon, where the most rostral and caudal neocortical stimulation sites (panels 1 and 3 of Fig. 3.3A-C) served as control sites whereas the middle site (panels 2 of Fig. 3.3A-C) received TFS. Both optical signals (Fig. 3.3A2, B2) and field potentials (Fig. 3.3C2) evoked from the induction site were significantly reduced (30 min test: field potentials reduced to $82.0 \pm 0.3\%$ of baseline, t-tests, $p < 0.05$).

Across experiments, a repeated measures ANOVA on the field potentials revealed a significant effect of time ($F_{2,58} = 12.37$, $p < 0.0001$) and stimulation sites ($F_{1,58} = 15.521$, $p = 0.0005$) plus a significant interaction between the two ($F_{2,58} = 5.388$, $p = 0.007$). Similar results were obtained with the optical signals (time, $F = 10.092$, $p = 0.0002$; stimulation site, $F = 10.43$, $p = 0.002$; interaction, $F = 9.52$, $p = 0.003$). Bonferroni-corrected post-hoc tests showed that the field potentials elicited from the induction sites were significantly reduced compared to control sites (t-tests, $p = 0.0003$, $n=12$) to $65.8 \pm 6.3\%$ and $68.7 \pm 7.1\%$ of baseline at the 5 and 30 min tests, respectively (Fig. 3.5A1, squares). Similar results were obtained for optical responses (t-tests, $p < 0.0001$): reduction to $56.8 \pm 8.8\%$ and $73.1 \pm 9.9\%$ of baseline at the 5 and 30 min tests, respectively (Fig. 3.5B1, squares). Optical and field potential responses evoked from the control sites were unchanged ($p > 0.2$) at both the 5 min ($103.1 \pm 5.4\%$ and $91.1 \pm 4.5\%$) and 30 min test phases ($94.8 \pm 4.6\%$ and $95.1 \pm 5.2\%$; Fig. 3.5A2 and B2, squares).
It should be noted that neocortically-evoked responses in the PRC were found to be much lower (57 ± 7%) when the neocortical stimulation site was far away in the rostrocaudal axis vs. in transverse register with the recording site. This suggests that the number of activated perirhinal synapses at distant levels is insufficient to shift the polarity of responses from depression to potentiation. For this reason, TFS at a single neocortical site does not lead to LTP at distant perirhinal levels even if these long-range pathways do not engage inhibitory interneurons.
Fig. 3.3 TFS at a single neocortical stimulation site produces a long-lasting and site-specific depression of evoked responses. (A1-3) Electrical stimuli were delivered in the temporal neocortex at the sites indicated by red circles in the schemes (top). In the control phase (Baseline), single shocks were delivered at each site independently. During the induction phase, one of the sites (A2) received TFS (8 Hz stimulation) while the others were not stimulated. Five and thirty min later, single shocks were delivered at all sites independently. The pictures in A show optical signals generated by the 3 neocortical stimuli (20 ms after the stimuli) during the control phases (top) as well as 5 (middle) and 30 min (bottom) after TFS. B plots the
fluorescence in all pixels included in the 3 square regions shown in the pictures of panel A. Arrowheads indicate when the stimulus was applied. C shows the field potential responses elicited by the neocortical stimuli. In B and C, the black, green, and red lines indicate activity obtained during the control phase, as well as 5 and 30 min after TFS, respectively.

3.3.3 Effect of paired theta-frequency stimulation in area 36

To test whether the perirhinal network allows for associative plasticity, we tested the effects of TFS simultaneously applied at two distant neocortical sites on field potentials and optical signals. The control and post-induction phases were exactly same as before. However, TFS was applied at two stimulating electrodes while the third one was left unstimulated and served as a control.

Irrespective of the particular combination of stimulating electrodes used, the theta-frequency induction paradigm led to a long-lasting potentiation of responses evoked from the paired stimulation sites, whereas no change in responses was observed in test phases when the previously unstimulated control site was stimulated (n=9). As for the response depression described above, this potentiation was global in that it affected all recording sites and could be detected in both the field potentials and optical responses.

Figure 3.4 illustrates an example. In this case, the most rostral and caudal stimulation sites (panels 1 and 3 of Fig. 3.4) received the simultaneous paired TFS while the third site (panels 2 of Fig. 3.4) was left unstimulated during the induction phase. Both the optical signals (Fig. 3.4A1,3, B1,3) and field potentials (Fig. 3.4C1,3) evoked from the induction sites were significantly enhanced (30 min test: field potential enhanced to 330 ± 2% and 211 ± 3% of baseline for the rostral and caudal sites, respectively, t-tests, p < 0.05).

A repeated measures ANOVA was run across experiments for the optical signals, revealing a significant effect of time (F_{2,86} = 6.42, p = 0.002) and stimulation sites (F_{1,86} =
14.29, p = 0.0005) in addition to a significant interaction between the two (F_{2,86} = 10.2, p = 0.0001). Bonferroni-corrected post-hoc tests showed that optical responses elicited from the induction sites were significantly enhanced relative to those evoked from control sites (p < 0.0001, n=9) to 146.4 ± 7.7% and 131.1 ± 8.7% of baseline at the 5 and 30 min tests, respectively (Fig. 3.5B1, circles). Paired TFS caused a persistent and significant increase in field potential responses evoked from the induction sites as well (142.6 ± 13.5% of baseline at the 30 min test; paired t-test, p = 0.021; Fig. 3.5B1, circles). On the other hand, optical and field potential responses evoked from the control site did not change (t-tests, p > 0.11 for all tests) at both the 5 min (99.0 ± 4.2% and 99.5 ± 3.5%) and 30 min tests (93.5 ± 5.2% and 91.8 ± 8.0%; Fig. 3.5A2,B2, circles).
Fig. 3.4 TFS at 2 neocortical stimulation sites produces a long-lasting potentiation of responses evoked by the paired sites. (A1-3) Electrical stimuli were delivered in the temporal neocortex at the sites indicated by red circles in the schemes (top). In the control phase (Baseline), single shocks were delivered at each site independently. During the induction phase, 2 of the sites (A1, A3) received TFS (8 Hz stimulation) while the third (A2) was not stimulated. Five and 30 min later, single shocks were delivered at all sites independently. The pictures in A show optical signals generated by the 3 neocortical stimuli (20 ms after the stimuli) during the control phases (top) as well as 5 (middle) and 30 min (bottom) after TFS. B plots the fluorescence in all pixels included in the 3 square regions shown in the pictures of panel A. Arrowheads indicate when the stimulus was applied. C shows the field potential responses elicited by the neocortical stimuli. In B and C, the black, green, and red lines indicate activity obtained during the control phase, as well as 5 and 30 min after TFS, respectively.
3.3.4 Impact of stimulation intensity on the effect of paired theta-frequency stimulation

In the above experiments, it could be argued that the contrasting effects of TFS applied at one versus two neocortical sites might only reflect differences in the intensity of stimulation during induction. To assess this possibility, we repeated the paired TFS experiments except that during induction, the stimulation intensity was lowered by reducing the stimulus duration to ≈33% of those used in the previous set of experiments. In other words, all aspects of the protocol were unchanged except for a drastically lower stimulation intensity during the induction phase. In separate experiments, we determined that lowering the neocortical stimulus duration caused a large and significant reduction in the amplitude of evoked field potential responses in the perirhinal cortex (-58 ± 12%, n=19; paired t-test, p = 0.0001).

Remarkably, qualitatively identical results were obtained with the lower stimulation intensity (Fig. 3.5, empty triangles). That is, the responses evoked from the induction sites showed as much potentiation as with the higher stimulation intensity relative to those evoked from the unstimulated sites (n=9). Across experiments, a repeated measure ANOVA on the optical signals revealed a significant effect of time ($F_{2,68} = 3.82, p = 0.027$) and stimulation sites ($F_{1,68} = 6.54, p = 0.015$) plus a significant interaction between the two ($F_{2,68} = 4.65, p = 0.012$). Bonferroni-corrected post-hoc t-tests confirmed that optical responses elicited from the induction sites were significantly enhanced relative to those evoked from control sites ($p < 0.0017, n=9$) to 149.8 ± 13.9% and 138.9 ± 13.3% of baseline at the 5 and 30 min tests, respectively (Fig. 3.5B1, empty triangles). Similarly, paired low-intensity TFS caused a persistent and significant increase in field potential responses evoked from the induction sites (143.3 ± 18.2% of
baseline at the 30 min test; paired t-test, p = 0.036; Fig. 3.5A1, empty triangles). In contrast, optical and field potential responses evoked from the control site were unchanged (t-tests, p > 0.1 for all tests) at both the 5 min (99.2 ± 7.2% and 107.9 ± 8.4%) and 30 min tests (94.4 ± 45.1% and 110.3 ± 6.7%; Fig. 3.5A2,B2, empty triangles).

**Fig. 3.5** Site-specific effects of TFS in the various conditions tested. Percent of baseline field potential (A) and optical (B) response amplitudes (y-axis, normalized to baseline) seen at the sites that received TFS (1) or acted as controls (2), 5 and 30 min (x-axis) after induction across all experiments. Dashed lines indicate standard error of the baseline condition where the greatest variability was observed. Different symbols are used to represent data obtained in the different conditions investigated: empty squares, TFS applied at a single neocortical site; empty circles, TFS simultaneously applied at 2 neocortical sites; empty triangles, low-intensity TFS applied simultaneously at 2 neocortical stimulation sites; solid triangles, TFS applied asynchronously (62.5 ms delay) at 2 neocortical stimulation sites. (C) Correlation between changes in response amplitudes seen in the various conditions investigated in this study, as measured with local field potentials (y-axis) and optical signals (x-axis).

### 3.3.5 Impact of input timing on the effect of paired theta-frequency stimulation

In the above experiments, it is unclear whether the response potentiation caused by paired TFS required strict coincidence of the stimuli during induction. Thus to address this question, we next examined whether delaying one of the two inputs by half a theta cycle affected the response potentiation at the induction sites compared to those
evoked from the control sites (n=12). Note that the duration of this offset (62.5 ms) is roughly 4-5 times longer than the time required for neocortical inputs to propagate through the full rostrocaudal extent of the PRC.

Whether we considered optical or field potential responses, asynchronous TFS caused no change in response amplitudes at the induction and the control stimulation sites. This was true for comparisons of field potential responses at the induction (baseline vs. 30 min post induction: paired t-test, p = 0.23) and control stimulation sites (baseline vs. 30 min post-induction: paired t-test, p = 0.35) as well as for optical signals (baseline vs. 30 min post-induction: induction sites, p = 0.13; control sites, p = 0.49). The average data obtained in these experiments is shown in figure 3.5A,B (solid triangles).

Across the various conditions investigated above, a near perfect correlation was found between the activity-dependent shifts in response amplitudes as assessed with field potentials and optical signals (r = 0.939; Fig. 3.5C).

3.4 Discussion

Aim 1 was undertaken to assess whether neocortical input patterns that differentially recruit intrinsic longitudinal perirhinal connections lead to contrasting forms of synaptic plasticity. We tested whether the perirhinal network allows for associative synaptic plasticity between coincident but spatially distributed neocortical activation patterns, as presumably occurs during associative memory formation in vivo. The results show that the polarity of neocortically-induced plasticity (depression vs. potentiation) in the PRC depends on the spatiotemporal distribution of neocortical inputs: TFS at a single neocortical site leads to a global perirhinal LTD; whereas simultaneous
TFS at two distant neocortical sites results in LTP at all rostrocaudal levels of the PRC. This illustrates the ability of perirhinal network to associate spatially distributed neocortical inputs.

Earlier work on the PRC revealed that it plays a critical role in high-order perceptual functions as well as in recognition and associative memory in various sensory modalities. (Zola-Morgan et al., 1989, 1993; Bunsey and Eichenbaum, 1993; Suzuki et al., 1993; Meunier et al., 1993, 1996; Mumby and Pinel, 1994; Eacott et al., 1994; Higuchi and Miyashita, 1996; Herzog and Otto, 1997; Buckley and Gaffan, 1998). Some lesion experiments suggest that PRC can perform at least some of these functions independent of the hippocampus.

It is already known that in horizontal slices of the PRC, as well as in the whole brain kept in vitro, neocortical stimuli elicit synaptic responses that propagate through the full rostrocaudal extent of the perirhinal cortex (Biella et al., 2001, 2010; Martina et al., 2001). However, the nature of the evoked responses depends on the distance to the neocortical stimulation site in the rostrocaudal axis: a single neocortical stimulus leads to a brief monosynaptic excitation that is followed by a biphasic synaptic inhibitory potential in area 36 in transverse register to the neocortical stimulation site. This suggests the involvement of inhibitory interneurons, thus a feedforward inhibitory mechanism, present in direct neocortical inputs to the perirhinal neurons in the vicinity. On the other hand, perirhinal recordings made at a distance from the neocortical stimulation site revealed apparently pure excitatory responses, which indicates the lack or scarcity of inhibition in the intrinsic rostrocaudal perirhinal circuits (Biella et al., 2001; Martina et al., 2001). Consistent with these findings, we found that application of TFS at a single
neocortical site results in a prolonged and input specific depression of evoked responses at all recording sites; whereas concurrent activation of two distant neocortical inputs causes a persistent and global potentiation of responses triggered by both inputs, leaving those elicited from control sites unchanged.

If the long-range pathways conveying distant neocortical inputs do not contact inhibitory interneurons, as suggested by previous physiological studies, why is it that TFS at a single site does not lead to LTP at rostrocaudally distant perirhinal levels? The answer likely resides in the contrasting number of synapses activated by neocortical stimuli at perirhinal levels in transverse register vs. far from the stimulation site. Indeed, in baseline conditions, perirhinal response amplitudes were markedly lower (by around 60%) at a distance compared to close to the neocortical stimulation site. This suggests that much fewer synapses are involved in the long-range propagation of neocortical signals than recruited at perirhinal levels in transverse register with the neocortical stimulation site. Presumably, at distant perirhinal levels, the number of activated synapses is insufficient to bring about the critical level of depolarization required for the induction of LTP. Below is a discussion of these observations for recognition and associative memory functions of the PRC.

Recent studies on PRC mostly focused on LTD because of its suspected involvement in the familiarity-induced response depressions seen in studies of recognition memory. Indeed, single-unit recording studies in rats and monkeys have shown that a proportion of perirhinal neurons display reduced responses to visual stimuli that have been presented previously, whether the animals were required to use the information to guide their behavior or not (Brown et al., 1987; Fahy et al., 1993; Li et al.,
1993; Miller et al., 1993; Sobotka and Ringo, 1993). Such cells are more frequently encountered in the PRC (25% of cells) than in the hippocampus (1%) (Rolls et al., 1993; Riches et al., 1991; Colombo and Gross, 1994; Eichenbaum et al., 1996; Xiang and Brown, 1998).

The relationship between perirhinal LTD \textit{in vitro} and decreased responsiveness of perirhinal neurons observed \textit{in vivo} during recognition memory tasks was strengthened by reports showing that drugs impairing recognition memory also interfere with perirhinal LTD induction \textit{in vitro}. For instance, administration of the muscarinic antagonist scopolamine impaired recognition memory, reduced the familiarity-induced suppression of perirhinal firing, and blocked the induction of LTD in perirhinal slices (Warburton et al., 2003). Another example is the administration of benzodiazepines which impaired both recognition memory and perirhinal LTD observed \textit{in vitro} (Wan et al., 2004).

In addition to studies focusing perirhinal LTD \textit{in vitro} and the role of PRC in recognition memory, other \textit{in vivo} single-unit studies point to a different behavior of perirhinal neurons (Messinger et al., 2001; Naya et al., 2003a) during associative memory tasks. In these experiments, monkeys are trained to form associations between two arbitrary visual stimuli. In this experimental paradigm, many neurons in area 36 and the adjacent temporal neocortex (area TE) initially showed selective responses to some stimuli. However, as a result of training, many of the same neurons also became preferentially activated by the paired associate stimulus (pair-coding). The incidence of pair-coding neurons is much higher in area 36 (33%) than in the adjacent temporal neocortex (5%) (Naya et al., 2003a).
At first glance, the familiarity-induced response depressions and the emergence of pair-coding in perirhinal neurons seems contradictory, since both result from repeated presentations of the same stimuli. However, the data presented here illustrate how the two phenomena can coexist and emerge from the network properties of the perirhinal cortex. Basically, the extent to which the stimulation conditions recruit the intrinsic longitudinal perirhinal connections has a decisive influence: repeated activation of one set of neocortical inputs, as is expected to occur when a single visual stimulus is presented, should cause a reduction of evoked responses because such stimuli would strongly excite local inhibitory interneurons with limited involvement of longitudinal connections. In contrast, activation of distributed neocortical inputs, as when different sensory stimuli have to be associated, would recruit longitudinal perirhinal connections to a higher extent and ultimately lead to a potentiation of responses evoked by the paired stimuli. Moreover, since the pathways linking different transverse perirhinal levels are reciprocal (Witter et al., 1986), subsequent activation of one of the two sets of neocortical inputs might be sufficient to reactivate the entire distributed pattern.

One apparent problem with this model is that pair-coding was observed even when the stimuli to be associated were presented with a delay of 1-2 sec between them (for instance see Naya et al., 2003a). This is in contradiction with our observation that paired TFS did not cause LTP when one of the two inputs was delayed by half a theta cycle (62.5 ms). The likely solution to this timing problem comes from earlier single-unit studies where it was observed that perirhinal neurons display delay activity in such tasks (Colombo and Gross, 1994; Sobotka, 2000; Naya et al., 2003b). In addition, the PRC possesses “persistent-firing” neurons that discharge for seconds to minutes after the
termination of the original spike-initiating stimulus (Bang and Brown, 2009; Navaroli et al., 2012). Recent studies suggest that muscarinic-dependent persistent-firing neurons can form transient memories to associate stimuli that are separated in time (Egorov et al., 2002; Fransen et al., 2006; Hasselmo, 2006; Bang and Brown, 2009; Esclassan et al., 2009; Navaroli et al., 2012). Thus, it is very likely that the delay activity and persistent-firing of perirhinal neurons bridge the temporal gap between the two inputs and allow for associative plasticity to take place.
CHAPTER IV

(Aim 2)

Induction and Expression Mechanisms of TFS-Dependent
Perirhinal LTD and LTP
4.1 Rationale

The results presented in Chapter III suggest that convergence of pathways conveying distributed neocortical inputs to the PRC during TFS is crucial for induction of activity-dependent LTP. However, the trajectory of these pathways is still unknown. One important question is whether distant sites in PRC are interconnected via axons coursing in the perirhinal cortex and/or temporal neocortex. An earlier study performed on horizontal slices showed that perirhinal cuts abolished the longitudinal propagation of neocortical inputs whereas cuts in the adjacent neocortex did not. This suggests that distant sites in PRC are connected by axons that course in the PRC itself, not the neocortex (Martina et al., 2001). However the situation could be different in the in whole brain in vitro preparation where all the cortical connections are preserved. Specifically, if neocortical axons routing the PRC curve out of the plane of the horizontal slice, knife cuts would have no effect since the axons are already cut during slice preparation. In contrast, in the intact network of the whole brain preparation the contribution of all neocortical axons can be tested. Inactivating the mid-rostrocaudal levels of the PRC during TFS will reveal the involvement of longitudinal perirhinal connections in long-term plasticity.

The above considerations are based on the hypothesis that the longitudinal perirhinal network is required for LTP, whereas LTD is induced locally, presumably by activation of perirhinal feedforward inhibitory interneurons. Since the induction of LTD and LTP rely on the activation of different cortical connections, different types of receptors might underlie these processes. Earlier in vitro studies have implicated NMDARs and group I mGluRs in the induction of perirhinal LTP and LTD (Bilkey,
1996; Ziakopoulos et al., 1999; Cho et al., 2000, 2001; Jo et al., 2008). It was first reported that bursts of afferent stimulation at 100Hz could produce NMDAR-dependent LTP of evoked responses in perirhinal slices kept in vitro (Bilkey, 1996; Ziakopoulos et al., 1999). The involvement of NMDARs in LTP has been shown numerous times (see Malenka and Bear, 2004 for review). First observation of LTP (in the hippocampus) was also NMDAR-dependent (Bliss and Lømo, 1973). As in other brain structures, NMDAR activation in the PRC is known to be dependent on the amount of depolarization produced by the stimulation conditions. In addition, during perirhinal LTD, intracellular calcium levels are also influenced by a different family of glutamate receptor, the mGluRs (Cho et al., 2001). Consistent with this, the mGluRs, have been implicated in the induction of perirhinal LTD (Cho et al., 2000; Jo et al., 2008). The role of mGluRs in LTD is not restricted to PRC. In fact, it has been shown that cerebellar LTD is also group I mGluR-dependent (Aiba et al., 1994). In pyramidal cells of CA1, group I mGluR-dependent LTD coexist with NMDAR-dependent LTD (Oliet et al., 1997).

Therefore, in light of earlier in vitro data, we hypothesized that TFS at a single neocortical site only results in a group I mGluR-dependent and input-specific LTD of evoked responses at all perirhinal levels. In these circumstances number of activated synapses is insufficient to depolarize the longitudinal axis and remove the Mg$^{2+}$ block of the NMDARs. On the other hand, concurrent TFS of two rostrocaudally distant neocortical sites recruits the intrinsic perirhinal connections by causing sufficient depolarization to overcome feedforward inhibition and remove the Mg$^{2+}$ block of NMDARs. If this is correct, the LTP should be abolished when the longitudinal intrinsic perirhinal connections are inactivated.
4.2 Methods

4.2.1 Inactivation with lidocaine microinjection

In order to transiently and selectively inactivate limited portions of the PRC and the adjacent neocortex, we used the voltage-gated sodium (Na+) channel blocker lidocaine (Sigma-Aldrich, St. Louis, MO) in the whole brain in vitro preparation. Three kinds of experiments were performed to detect the effects of lidocaine and the trajectory of cortical pathways, where lidocaine or vehicle (saline) was infused but no TFS was applied. At the end, a fourth experiment was carried out with TFS as in the experiments of aim 1.

First, in order to determine a volume and concentration of lidocaine that is sufficient to interfere with propagation while having only local effects (little spread from the infusion site), we performed control experiments where we examined how various amounts of lidocaine infused in the perirhinal cortex affected responses elicited by direct stimulation of the PRC itself. Lidocaine was infused at mid-rostrocaudal levels of the PRC with two concentric electrodes on either side of the infusion site, as shown in figure 4.1A. These electrodes could be used to both record and stimulate the perirhinal cortex. By trial and error, and guided by earlier studies on lidocaine diffusion in cerebral cortex (Tehovnik and Sommer, 1997; Broadbent et al., 2006; Fujita et al., 2010), we determined that a total volume of 0.8 µl of lidocaine (4%) divided in multiple small infusions along two microsyringe penetrations (Fig. 4.1A, red asterisks) could produce a significant reduction of longitudinal propagation with little spread from the infusion site. One of the microsyringes was placed at perirhinal area 35 while the other was aimed at area 36. We found that, with this volume and concentration of lidocaine, the effects of lidocaine
dissipated within 60 min.

Next, we examined how the same amount of lidocaine infused in the temporal neocortex (Fig. 4.2A) affected the longitudinal propagation of neocortical inputs in the perirhinal cortex. Two concentric electrodes, one rostral and the other caudal to the lidocaine infusion site, were used to stimulate the neocortex and three electrodes were used to record in area 36 (Fig. 4.2A).

Last, we repeated the same experiments with the exception that lidocaine was microinjected into the PRC (Fig. 4.3A). Four recording electrodes were used this time: two of them were placed rostral to, and the other two caudal to, the lidocaine injection site.

For all three experiments described above, 10 electrical stimuli (100 μs, 0.5 mA; 0.1 Hz) were applied at stimulation sites of interest in the control phase. Then the infusions were performed. Five, thirty, and sixty min after concluding the infusions, we again obtained independent averages of the responses elicited by the same stimulation sites as in the control phase.

Finally, we tested the effects of perirhinal infusion of lidocaine on the activity-dependent plasticity produced by TFS at one (Fig. 4.4A1) versus two distant neocortical stimulation sites (Fig. 4.4B1). Two recording electrodes were aimed at perirhinal area 36, one rostral and one caudal to the lidocaine infusion site. In all cases, TFS was applied 5 min after finishing the lidocaine microinjection and the impact of this manipulation was assessed 60 min later, when the lidocaine effects had dissipated, as revealed by our control experiments.
4.2.2 Arterial perfusion of glutamate receptor antagonists

The NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (AP5) was obtained from Sigma-Aldrich (St. Louis, MO), whereas the group I metabotropic glutamate receptor (mGluR) antagonist 1-aminoindan-1,5-dicarboxylic acid (AIDA) was obtained from Tocris Bioscience (Ellisville, MO). All drugs were dissolved in artificial cerebrospinal fluid. AP5 (100 µM) or AIDA (100 µM) was applied via the arterial perfusate for 12 min in the absence of stimulation. We focused on the role of NMDA and group I metabotropic receptors because earlier in vitro studies on activity-dependent plasticity in the perirhinal cortex have implicated these receptors in the induction of perirhinal LTP and LTD (Bilkey, 1996; Ziaiopoulos et al., 1999; Cho et al., 2000, 2001; Jo et al., 2008). As usual, in experiments where TFS was utilized, recordings were made before the TFS as well as 5 and 30 minutes after. Control experiments were done to determine whether AP5 or AIDA application affected response amplitudes in the absence of TFS.

4.3 Results

4.3.1 Network mechanisms underlying the contrasting effects of TFS at one vs. two distant sites

Lidocaine microinjection experiments in the absence of TFS showed that signal propagation occurred in the rostrocaudal axis of the PRC, but not the adjacent neocortex (Figs. 4.1 - 4.3). Figure 4.1B shows a representative experiment where mid-rostrocaudal level of PRC is inactivated. In this experiment, the responses evoked and recorded from perirhinal sites either rostral or caudal to the lidocaine infusion site (close sites) vs. cases
where the infusion site was in between the stimulation and recording sites (distant sites) are contrasted. Prior to the infusion, when perirhinal electrode 4 was stimulated, field responses were observed at recording sites 1-3 (baseline, black traces). Thirty minutes after the lidocaine infusion (red traces), responses recorded at the close site (3) were unchanged. In contrast, at distant sites (electrodes 1 and 2), a large response reduction was observed. One hour after the lidocaine infusion, all responses returned to baseline level.

Fig. 4.1 Differential impact of local lidocaine infusions in area 36 during perirhinal stimulation. (A) The position of concentric electrodes (filled circles) and lidocaine infusions (red asterisks). In this case, I stimulated and recorded in the PRC. (B) Field responses elicited at sites 3, 2, and 1 by electrical stimuli delivered through perirhinal electrode 4 (black, baseline; red and blue, 30 and 60 min post-lidocaine infusion, respectively). (C) Average of results obtained in 8 lidocaine and 8 vehicle (saline) experiments.
As shown in figure 4.1C, these effects were consistent across multiple experiments. Here the normalized fluctuations in response amplitude as a function of time from lidocaine infusion (empty symbols, n=8) or vehicle (filled symbols, n=8) at close (circles) and distant (triangles) sites are shown. Compared to the vehicle experiments, the only significant reduction caused by perirhinal lidocaine infusions were seen at distant sites, when the lidocaine was infused in between the stimulation and recording sites. This effect was seen 5 and 30 min post-infusion (5 min, 61.4 ± 3.6% of baseline, t-test, p < 0.001; 30 min, 66.1 ± 4.2%, t-test, p < 0.001) and had vanished 60 min post-infusion (90.5 ± 0.8%, t-test, p = 0.32).

Next, we examined how the same amount of lidocaine infused in the temporal neocortex (Fig. 4.2) or adjacent perirhinal cortex (Fig. 4.3) affected the longitudinal propagation of neocortical inputs in the PRC. With neocortical infusions of lidocaine (Fig. 4.2A), no reduction in longitudinal propagation was observed. That is, perirhinal field potential responses at sites rostrocaudally distant from the neocortical stimulation sites were not significantly reduced (n=8; 5 min, t-test, p = 0.188; 30 min, t-test, p = 0.491; 60 min, t-test, p = 0.737).
Fig. 4.2 Differential impact of local lidocaine infusions in neocortex during neocortical stimulation. (A) The position of concentric electrodes (filled circles) and lidocaine infusions (red asterisks). In this case, I stimulated the neocortex and recorded in the PRC. (B) Field responses elicited at sites 1 and 3 by electrical stimuli delivered through neocortical electrode 4 (black, baseline; red and blue, 30 and 60 min post-lidocaine infusion, respectively). (C) Average of results obtained in 8 experiments.

In contrast with neocortical lidocaine infusion, when lidocaine was infused at the same rostrocaudal level but in the PRC (Fig. 4.3A), a large and significant reduction in response amplitude was seen at perirhinal sites rostrocaudally distant from the neocortical stimulation site (Fig. 4.3C, triangles) with no difference in response amplitudes at the close perirhinal sites (Fig. 4.3C, circles). Similar to perirhinal stimulations, this effect was seen 5 and 30 min post-infusion (n=8; 5 min, 63.1 ± 4.6% of baseline, t-test, p = 0.008; 30 min, 54.7 ± 2.9%, t-test, p = 0.001) and had vanished 60 min post-infusion (t-test, p = 0.943). These contrasting effects of neocortical and perirhinal infusions of lidocaine strongly suggest that a large portion of the axons conveying long-range neocortical influences course in the perirhinal cortex itself, not in the adjacent temporal neocortex.
Fig. 4.3 Differential impact of local lidocaine infusions in area 36 during neocortical stimulation. (A) The position of concentric electrodes (filled circles) and lidocaine infusions (red asterisks). In this case, I stimulated the neocortex and recorded in the PRC. (B) Field responses elicited at sites 1 and 4 by electrical stimuli delivered through neocortical electrode 5 (black, baseline; red and blue, 30 and 60 min post-lidocaine infusion, respectively). (C) Average of results obtained in 8 experiments.

Finally, we tested the effects of lidocaine with TFS. With TFS application at a single neocortical stimulation site, contrasting results were obtained depending on the position of the recording site with respect to the lidocaine infusion site (Fig. 4.4A1). At close recording sites, a -48.4 ± 12.2% reduction in response amplitude was observed, statistically indistinguishable from the depression seen in controls (-51.1 ± 21.9%, t-test, p = 0.92). When lidocaine was infused in between the recording and stimulation sites (Fig. 4.4A2, Distant site), once again, TFS at one neocortical site produced no significant change in response amplitude (-8.2 ± 6.7%), compared to the reduction in controls (-23.9 ± 10.2%, t-test, p = 0.39).
When TFS was applied simultaneously at two distant neocortical sites after perirhinal infusions of lidocaine, compared to the ubiquitous potentiation observed in control cases (Fig. 4.4B2, empty bars), we observed a significant response depression at close sites (-26.1 ± 5.9%, t-test, p < 0.001) and the same trend at distant sites (-17.7 ± 5.9%). Overall, these results point to a critical role of longitudinal perirhinal pathways in the induction of activity-dependent plasticity of neocortical inputs in the perirhinal cortex.

**Fig. 4.4** Impact of lidocaine infusion in the PRC on activity-dependent modification of neocortical responses induced by TFS applied at 1 (A) or 2 (B) neocortical stimulation sites. The schemes in the top panels illustrate the relative position of neocortical stimulating (St.) and area 36 recording electrodes (dots) located at proximity (C for close) or at a distance (D) from the stimulation site. Red asterisks mark the lidocaine infusion sites in area 36. (A1) TFS at 1 neocortical stimulation site. (B1) TFS at 2 neocortical stimulation sites. In this case, depending on the stimulating electrode considered, the same area 36 recording site was termed a close or distant site, hence the absence of C and D labels. After measurement of baseline response amplitudes, the same amount of lidocaine used in control experiments was infused at mid-rostrocaudal perirhinal levels (red asterisks). Five minutes after concluding the infusion, TFS was
applied at 1 (A) or 2 (B) neocortical stimulation sites. Panel 2 shows impact of neocortical TFS was then assessed after the lidocaine effect had dissipated (60-min post-infusion). Filled and empty bars show the results obtained in lidocaine and control experiments, at perirhinal recording sites close to (i.e., in rostrocaudal register) the neocortical induction sites or at distant sites (n = 8).

4.3.2 *NMDAR dependence of activity-dependent plasticity*

Since previous work implicated NMDA receptors in the induction of LTP and LTD in perirhinal slices (Bilkey, 1996; Ziaxopoulos et al., 1999; Cho et al., 2000, 2001; Barker et al., 2006), we examined whether activation of NMDARs during TFS was required for induction of the long-lasting potentiation or depression of neocortically-evoked field potential responses described above.

Figure 4.5 illustrates the changes in response amplitudes elicited from the induction sites when TFS was applied in the absence (white bars) or presence of AP5 (gray bars). Here it should be noted that when AP5 was applied for an identical duration, but in the absence of TFS, baseline response amplitudes were not affected by AP5 (Fig. 4.5A, No Stim., gray bar; -3.6 ± 3.2% change from baseline, n=5, paired t-test, p > 0.45).

When TFS was applied at only one neocortical stimulation site (Fig. 4.5B), no difference was seen between the control (white bars) and AP5 (gray bars) conditions. Just like the control condition, TFS at one neocortical site evoked a significant depression of responses (-30.8 ± 5.4% change from baseline, n=15, t-test, p = 0.0003). The magnitude of this depression was not significantly different in the control and AP5 conditions (t-test, p = 0.95). Again, this response depression was seen at all perirhinal recording sites. As for the control experiments, responses evoked from the control neocortical stimulation sites were unchanged (0.6 ± 11.2% change from baseline, t-test, p = 0.54).
Surprisingly, while simultaneous TFS at two neocortical sites elicited a potentiation of responses evoked from the induction sites with the control perfusate (Fig. 4.5C, Two sites, white bar), in the presence of AP5 the same protocol reversed this effect and evoked a response depression (gray bar; -24 ± 6.4% change from baseline, n=10), as measured 30 min post induction. The difference between the two conditions was statistically significant (t-test, p = 0.0016). This depression of responses elicited from the induction sites was global so that they could be observed at all perirhinal recording sites. In contrast, responses elicited from the control neocortical stimulation sites did not change (22.8 ± 16.7% change from baseline, t-test, p = 0.3).

4.3.3 Group I mGluR dependence of activity-dependent plasticity

As mentioned above, since NMDAR inactivation did not affect the activity-dependent depression of perirhinal responses evoked with single-site TFS, we next examined whether metabotropic glutamate receptors were also involved in neocortically-evoked response changes in field potential responses due to TFS.

Figure 4.5 shows the changes in response amplitudes elicited from the induction sites when TFS was applied in the absence (white bars) or presence of the group I mGluR selective antagonist AIDA (black bars). Once again, merely applying AIDA without TFS had no significant effect on perirhinal responses 5 minutes after perfusion (Fig. 4.5A, No Stim., black bar; 4.4 ± 3.7% change from baseline, n=5, paired t-test, p > 0.45).

As described above, with the control perfusate, TFS at a single neocortical site elicited a depression of responses evoked from the induction site (Fig. 4.5B, One site, black bar). However, in the presence of AIDA, the impact of TFS at a single site was
reversed at 30 min, and a response potentiation was observed globally at all perirhinal recording electrodes (45.8 ± 21.8% change from baseline, n=15, t-test, p = 0.0002). This potentiation was statistically significant compared to the control condition (t-test, p = 0.019). In contrast, responses elicited from the control neocortical stimulation sites showed no change (1.8 ± 11.3% change from baseline, t-test, p = 0.38).

On the other hand, when TFS was applied concurrently at two neocortical stimulation sites (Fig. 4.5C, Two sites, white and black bars), no difference was seen between the control and AIDA conditions. Just as in the control condition, simultaneous TFS of two distant neocortical sites evoked a significant potentiation of responses (51.4 ± 12.8% change from baseline, n=10, t-test, p = 0.0002). The magnitude of this potentiation was not significantly different in the control and AIDA conditions (t-test, p = 0.62). This response potentiation was seen at all perirhinal recording sites. Responses evoked from the control neocortical stimulation sites, where no TFS was applied, were unchanged (4.8 ± 7.5% change from baseline, t-test, p = 0.41).

In additional control experiments, AP5 and AIDA were perfused together. As hypothesized, no significant change in the response amplitudes was observed when TFS was applied at a single neocortical site (0.9 ± 11.1% change from baseline, n=5, t-test, p = 0.84). Similarly, no change was observed with paired-site stimulation (2.7 ± 7.6% change from baseline, n=5, t-test, p = 0.76). These results implicate that the NMDA and group I mGlu receptors have competing influences, so that when AP5 and AIDA are perfused together, their effects counterbalance each other.
Fig. 4.5 Induction mechanisms of LTD and LTP. Percent change in field potential response amplitude (y-axis; normalized to baseline) with no TFS (A), with TFS applied at one neocortical site (B), or at 2 neocortical sites simultaneously (C). White, gray, and black bars indicate data obtained with control perfusate, AP5, or AIDA, respectively.

4.4 Discussion

Experiments in aim 2 were undertaken to shed light on mechanisms underlying the effects of TFS depending on the spatial distribution of activated inputs. The results show that, in agreement with previous studies (see Biella et al., 2001, 2010; Martina et al., 2001), neocortical signals propagate through the full rostrocaudal extent of the perirhinal cortex and that activation of intrinsic perirhinal pathways is required for associating distributed neocortical inputs, as is the case in pair-coding. Moreover, we show that the induction of perirhinal LTD and LTP depends on the competing influence of group I mGlu and NMDA receptors, respectively.

Previously, interruption of intrinsic perirhinal versus neocortical pathways with restricted knife cuts revealed that the propagation of neocortical activity does not occur in
the neocortex but depends on longitudinal axonal pathways coursing in the PRC (Martina et al., 2001). This finding was corroborated by observing the propagation of fluorescent voltage-dependent signals in the experiments of aim 1. The signal propagated in the rostrocaudal axis almost exclusively via the PRC, but not the adjacent neocortex. Results depicted in figures 4.1 - 4.3 provide support for this conclusion. In addition, we showed that the intrinsic perirhinal pathway is required for the induction of activity-dependent LTP of neocortical inputs in the PRC. These results suggest that the longitudinal perirhinal pathways conveying long-range neocortical influences contact principal cells but not inhibitory interneurons (Biella et al., 2001; Martina et al., 2001). These intrinsic perirhinal pathways consist of the longitudinal axons of both the perirhinal and neocortical neurons (Deacon et al., 1983; Room and Groenewegen, 1986; Witter et al., 1986; Lavenex et al., 2004). In fact, the presence of a prominent system of rostrocaudally oriented intrinsic connections within the PRC is a general property that characterizes rats (Deacon et al., 1983), cats (Witter et al., 1986), and monkeys (Lavenex et al., 2004), suggesting that our findings might apply to other species.

It should be noted that our simplified model (Fig. 1.2) omits many details regarding the intrinsic perirhinal connections as well as neocortical inputs to the PRC. Indeed, a recent trend in systems neuroscience favors investigation of interactions between different cortical layers and their role in behavior (see Suh et al., 2011; Wester and Contreras, 2012). However, by focusing on the network properties instead of the connectome, above experiments were designed to test for the role of longitudinal connections of the perirhinal network as a whole.
Our experiments involving arterial perfusion of AP5 and AIDA revealed that there are two separate and competing receptor-level mechanisms affecting activity dependent plasticity in the PRC. We found that perirhinal LTP requires the activation of NMDARs, as reported previously (Bilkey, 1996; Ziakopoulos et al., 1999; Cho et al., 2000). In the absence of NMDAR activation, repetitive activation of neocortical inputs, irrespective of how much depolarization it produces, leads to a depression of synaptic efficacy. When rostrocaudally distant neocortical inputs are paired, the balance shifts toward excitation because long-range intrinsic pathways do not engage inhibitory interneurons. Presumably, increased excitation in the intrinsic perirhinal pathways removes the Mg$^{2+}$ block of NMDARs, further increasing the intracellular calcium levels, which would in turn lead to addition of new AMPARs (Durand et al., 1996; Shi et al., 1999) and/or the phosphorylation of existing AMPAR subunits (Barria et al., 1997). Since the longitudinal perirhinal connections are required for the observed perirhinal LTP, we believe that these changes mostly take place in the synapses between principal perirhinal cells along the rostrocaudal axis.

The fact that blocking NMDARs transformed the response potentiation produced by paired activation of two neocortical stimulation sites into a response depression suggests that synaptic efficacy in the PRC is subjected to at least two competing mechanisms of regulation.

Consistent with previous *in vitro* (Cho et al., 2000; Jo et al., 2008) and *in vivo* (Barker et al., 2006) experiments implicating metabotropic glutamate receptors in perirhinal LTD, our results show that the other mechanism is group I mGluR activation. Arterial perfusion of AIDA led to LTP of neocortical inputs to the PRC in both the single
and paired site TFS conditions. This finding suggests that the local inhibitory influences on direct neocortical projections to a restricted portion of the PRC in transverse register are modulated by the activation of group I mGluRs.

Metabotropic glutamate receptors, consisting of 8 types grouped into 3 families, are localized both pre- and post-synaptically and have diverse functions. It is already known that group I mGluRs are usually postsynaptic and they affect neuronal excitability of in a number of ways (see Manahan-Vaughan, 1997; Bortolotto et al., 1999; Swanson et al., 2005). In addition to their role in perirhinal LTD (Cho et al., 2000; Barker et al., 2006; Jo et al., 2008), group I mGluRs have been implicated in some forms of hippocampal LTP (Behnisch and Reymann, 1993; Wilsch et al., 1998). Furthermore, group I mGluRs, specifically the mGluR5, can enhance NMDAR-mediated activity (Ugolini et al., 1999). Having stated these effects of group I mGluRs, how can arterial perfusion of AIDA resulted in LTP irrespective of the type of TFS used? The likely answer to this dilemma is the relationship of group I mGluRs to the GABAergic interneurons activated by direct neocortical inputs. It has been shown in thalamus that group I mGluRs enhances IPSC frequency and tonic GABA_A current (Errington et al., 2011). Perhaps a similar mechanism is underlying the role of group I mGluRs in evoked perirhinal LTD. Whether group I mGluRs are located on GABAergic interneurons or principal perirhinal neurons, it is very likely that their activation increases the inhibitory influence on the principal perirhinal neurons. As a result, the threshold to activate the intrinsic perirhinal circuit is not met and LTP is not achieved. It has been shown in the dorsal lateral geniculate nucleus of thalamus that group I mGluRs are localized in GABAergic interneurons that form dendrodendritic synapses. Their activity is regulated
by L-type voltage-dependent calcium channels (VDCC) and their activation enhances the feedforward inhibition on thalamocortical cells (Errington et al., 2011).

Several factors suggest that a similar mechanism might exist for neocortical inputs to the PRC. First, similar to thalamocortical cells, principal perirhinal neurons are subjected to feedforward inhibition in response to neocortical input (Pinto et al., 2006). Second, when TFS is applied at a single site with AIDA perfusion, it reverses the neocortically-evoked LTD into LTP, pointing the role of group I mGluRs in inhibiting principal perirhinal neurons. Third, verapamil, an L-type VDCC antagonist, blocks the LTD and depotentiation in slices of PRC; and impairs acquisition and retrieval of long-term recognition memory in vivo (Seoane et al., 2009). These L-type VDCCs could be located on feedforward interneurons, as is the case in the dorsal lateral geniculate nucleus of thalamus. As in the visual thalamus (Errington et al., 2011), group I mGluRs are localized on inhibitory interneurons of the entorhinal cortex (Deng et al., 2010), hippocampus (Le Duigou and Kullmann, 2011), and cerebellum (Karakossian and Otis, 2004); and group I mGluR activation excites these interneurons, which in turn leads to stronger inhibition of the postsynaptic targets, namely the principal neurons. In light of these studies and the findings of this chapter, it is justified to propose a similar model for the PRC; where the feedforward inhibition of the principal perirhinal neurons are regulated by the activation of group I mGluRs localized on the interneurons.
CHAPTER V

(Aim 3)

Proportion of Inhibitory Synapses Formed by Neocortical Axons vs. Intrinsic Perirhinal Axons with Perirhinal Neurons
5.1 Rationale

The experiments described in Chapter III revealed that single-site TFS of neocortical inputs results in activity-dependent perirhinal LTD, whereas simultaneous paired-site TFS results in perirhinal LTP. Based on earlier electrophysiological studies (Biella et al., 2001; Martina et al., 2001), we hypothesized that the contrasting effects of single vs. paired-site TFS results from the fact that direct neocortical inputs and short-range perirhinal connections recruit significantly more inhibition in the PRC than the long-range axons of perirhinal and neocortical neurons coursing in the rostrocaudal axis of the PRC. However, there is no direct evidence that this assumption is valid. Using anterograde tract-tracing at the light and electron microscopic level, the experiments described in this chapter aim to test this idea.

It should be noted that when PHAL was injected in the temporal neocortex and sections were analyzed in perirhinal regions in rostrocaudal register with the injection site, the neocortical projections were called “direct neocortical inputs”. “Short-range perirhinal connections” refer to synapses observed at nearby sites when PHAL was injected in the PRC. On the other hand, “long-range perirhinal connections” refer to synapses found at distant levels. Thus, these are the connections that constitute the intrinsic perirhinal network discussed in this thesis.

So that the readers can appreciate the significance of our observations, I will first review salient aspects of synaptic ultrastructure in the cerebral cortex. These general principles, gained over nearly 60 years of research, will guide the interpretation of our data.
5.1.1 Ultrastructural properties of excitatory and inhibitory synapses

In 1897, Sir Charles Sherrington coined the term “synapse” in order to describe the junction between two neurons (see Foster and Sherrington, 1897). Anatomical recognition of this junction, the synapse, dates back to the classical studies of Ramón y Cajal (1911, 1934) and other prominent neuroanatomists (Bartelemez and Hoerr, 1933; Bodian, 1937, 1940). Later, Gray (1959) became the first researcher to classify chemical synapses of the central nervous system into two categories based on their ultrastructure: Type I and Type II. According to Gray, Type I synapses possess a narrow and often discontinuous presynaptic density with a thicker postsynaptic density. These synapses typically occur onto outer segments of dendrites and dendritic spines. On the other hand, Type II synapses are characterized by a thin, symmetrical and usually discontinuous electron density on both the presynaptic and postsynaptic membranes; and these synapses are found on dendritic shafts and somata (Gray, 1959). Later on, Colonnier (1968) suggested a new terminology for these two types of synapses primarily based on the thickness of their postsynaptic density and vesicle profiles. He called Gray’s Type I synapse “asymmetric”, and Type II synapse “symmetric”.

Following this classification, many researchers have tried to correlate these two types of synapses with function (Andersen et al., 1963; Eccles, 1964; Andersen and Eccles, 1965). The initial hypothesis stated that asymmetric synapses should be excitatory, because they are found on the outer segments of dendrites and dendritic spines, where electrical stimulation leads to EPSPs; whereas symmetric synapses should be inhibitory, because they are found on somata and dendritic shafts, where electrical stimulation leads to IPSPs (Palay, 1967). This hypothesis worked well in most cases, like
the synapses between pyramidal cells of hippocampus, which are found to be excitatory and always bear the characteristics of a Type I (asymmetric) synapse (Andersen and Eccles, 1965; Hamlyn, 1962); or the Type II (symmetric) synapses between basket cells and Purkinje cells of cerebellum, which are always inhibitory (Andersen et al., 1963; Eccles, 1964; Andersen and Eccles, 1965).

However, significant deviations from this general rule were noted. For instance, the synapses formed by climbing fibers with the dendrites of Purkinje cells are symmetric but excitatory (Eccles et al., 1966). In addition, the spinal cord and some subcortical structures like the superior colliculus possess asymmetric synapses with inhibitory functions (see Peters et al., 1991). Nonetheless, the correlation between asymmetric synapses and excitation vs. symmetric synapses and inhibition is, to the best of my knowledge, perfect in the cerebral cortex. Pyramidal cells of the cerebral cortex exclusively form asymmetric synapses (Peters et al., 1991). As a result, asymmetric synapses presented in this chapter refer to excitatory connections; whereas symmetric synapses are inhibitory and most likely are synapses where GABA is the neurotransmitter used. In support of this, numerous studies using pre- or post-embedding glutamate and/or GABA immunocytochemistry have revealed that, in the cerebral cortex, the axon terminals forming symmetric synapses are typically immunopositive for GABA, but not glutamate (see Smith and Paré, 1994; Peters and Palay, 1996; DeFelipe, 1997; Apergis-Schoute et al., 2006; Pinto et al., 2006; McDonald et al., 2011). Conversely, the axon terminals forming asymmetric synapses are typically enriched in glutamate and immunonegative for GABA (see Peters et al., 1991; Smith and Paré, 1994; Peters and Palay, 1996; DeFelipe, 1997; Apergis-Schoute et al., 2006; Pinto et al., 2006; Muller et
al., 2007, 2011; Hur et al., 2009; Coleman et al., 2010).

5.1.2 Ultrastructural identification of postsynaptic elements

The type of postsynaptic element (dendritic shaft vs. dendritic spine) is widely used in the cortical literature to infer the identity of the target neurons (principal or local-circuit neuron) (Colonnier, 1968; Peters and Palay, 1996; Apergis-Schoute et al., 2006; Pinto et al., 2006; Muller et al., 2007, 2011; Hur et al., 2009; Coleman et al., 2010; McDonald et al., 2011). In particular, it is commonly assumed that dendritic spines belong to principal (pyramidal or stellate) cells because other cell types are aspiny or sparsely spiny in the cerebral cortex (Peters et al., 1991; DeFelipe and Farinas, 1992). For this reason, asymmetric synapses onto spines are invariably interpreted as representing excitatory inputs onto principal cells.

On the other hand, not all synapses onto dendritic shafts are formed with local-circuit cells. However, since the vast majority of excitatory inputs to principal cortical cells end on dendritic spines, asymmetric synapses onto dendritic shafts are typically interpreted as excitatory synapses onto local-circuit cells. In contrast, determining the identity of the cells contacted by inhibitory inputs is problematic. Indeed, inhibitory synapses onto principal cells and local-circuit neurons have the same ultrastructural correlates: symmetric synapses with dendritic shafts or somatic profiles. However, previous ultrastructural studies on the PRC found little evidence of inhibitory synapses onto local-circuit cells (Apergis-Schoute et al., 2006; Pinto et al., 2006). In these studies, symmetric synapses onto dendritic shafts consistently involved GABA-immunopositive axon terminals contacting GABA-immunonegative dendritic shafts.
In light of the above, I assume the following when interpreting my electron microscopic observations. Asymmetric synapses onto dendritic spines indicate excitatory principal cell to principal cell connections. Asymmetric synapses onto dendrites are more likely to represent excitatory synapses onto inhibitory neurons. However, in a very low proportion of these, the postsynaptic target might be a principal perirhinal cell. Symmetric synapses onto dendrites are interpreted as inhibitory synapses formed by local-circuit cells onto principal perirhinal cells. Finally, in the rare cases of symmetric axospinous synapses, it is assumed that a perirhinal local-circuit cell or a GABAergic neocortical cell is contacting a principal perirhinal cell.

5.1.3 Different forms of inhibition

Based on these considerations, inhibition in the intrinsic perirhinal circuit can take two forms. First, PHAL-positive excitatory (glutamatergic) axon terminals can form synapses onto inhibitory (GABAergic) neurons that then inhibit principal perirhinal neurons. The second form of inhibition is characterized by PHAL-positive inhibitory (GABAergic) axon terminals contacting principal (glutamatergic) perirhinal neurons.

According to our hypothesis, both forms of inhibition are present between direct neocortical inputs and perirhinal neurons, whereas most of the inhibition in short-range perirhinal connections arises from inhibitory (GABAergic) perirhinal synapses onto principal (glutamatergic) perirhinal neurons. This is because the perirhinal local-circuit neurons are primarily excited by neocortical principal cells, described as the first form of inhibition. On the other hand, both the neocortical and perirhinal neurons projecting to rostrocaudally distant levels of the PRC should form mostly asymmetric synapses with
principal perirhinal cells, thus lack inhibitory influences.

In this chapter (aim 3), these possibilities were tested using anterograde tracing with PHAL and by comparing the proportion of asymmetric (excitatory) and symmetric (inhibitory) synapses formed by neocortical axons vs. intrinsic perirhinal axons with perirhinal neurons at the electron microscopic level.

5.2 Methods

In each animal, the anterograde tracer PHAL was injected either into the PRC or adjacent temporal neocortex. Since the PRC and temporal neocortex have connections with their contralateral counterparts (Agster and Burwell, 2009), PHAL injections were made unilaterally. Furthermore, the injection site was either in the rostral or caudal portions of the targeted structure. Rostral neocortical PHAL injections were performed in the secondary auditory cortex (AuV; Paxinos and Watson, 1998). Caudal neocortical injections were performed in the temporal association cortex (TeA; Paxinos and Watson, 1998).

To facilitate and increase the yield of the electron microscopic observations, we aimed to obtain relatively large PHAL injection sites. Accordingly, PRC injections involved both areas 36 and 35. To this end, the tip of the injection pipette was positioned at the border between areas 36 and 35 (DV, -4.6 for rostral and -4.1 for caudal sites; Fig. 5.1A1). Similarly, in neocortical injections, PHAL was injected to cover a large area of the adjacent temporal neocortex (Fig. 5.1B1). In order to determine the extent of the PHAL injections, we searched for PHAL-labeled somata at the light microscopic level (see inset in Fig. 5.1B1). Only cases where PHAL-labeled somata were confined to the
targeted regions are considered below. For all cases, we analyzed sections taken from perirhinal sites rostrocaudally adjacent to (within 1mm) or distant from (>1mm) the PHAL injection sites. Sections from distant sites were collected at least 1mm away from the PHAL injection site, sometimes as far as 2.5mm.

In order to detect inhibitory synapses, the ultrastructure of the synapse was analyzed. Synapses were classified as symmetric or asymmetric (Colonnier, 1968), and postsynaptic elements were identified either as dendrites or dendritic spines. Classical criteria (detailed in Chapter II) were used to identify the types of synapses and postsynaptic elements. For a detailed description of the anterograde tracing and electron microscopy techniques refer to Chapter II (General Methods).

5.3 Results

As mentioned in the methods section of this chapter, to facilitate the electron microscopic observations, we aimed to obtain relatively large PHAL injections, which involved both the superficial and deep layers. PHAL injections were confined to the targeted regions in 8 animals: 2 rostral perirhinal, 2 caudal perirhinal, 2 rostral neocortical, and 2 caudal neocortical injections. The following is based on the analysis of these 8 cases. Figure 5.1 shows dark-field photomicrographs of representative perirhinal (Fig. 5.1A1) and neocortical (Fig. 5.1B1) injection sites and of the resulting anterograde labeling at distant sites in the PRC (Fig. 5.1A2, B2). Following PHAL injections in the temporal neocortex and PRC, labeled axons contributed numerous en passant and terminal varicosities in all the target regions examined (Fig. 5.1A3, B3).
**Fig. 5.1** Dark-field photomicrographs showing iontophoretic injection of PHAL in the PRC (A1) and adjacent temporal neocortex (B1). **Inset** in B1 shows labeled somata at the PHAL injection site. Low- (2) and high-power (3) photomicrographs of anterograde labeling in area 35 of PRC (A) following the PHAL injection shown in A1, and in area 36 of PRC (B) following the PHAL injection shown in B1. Arrows in A3 and B3 point to axonal varicosities. Scale bars in A1, A2 and A3 apply to B1, B2 and B3, respectively. Hpc, hippocampus; rh, rhinal sulcus; Str, striatum.
5.3.1 Distribution of anterogradely labeled axons (light microscopic observations)

The pattern of anterograde labeling seen after PHAL injections in the PRC and temporal neocortex conformed to earlier descriptions. Following perirhinal injections, massive anterograde labeling was seen in the amygdala, particularly in the lateral nucleus (Witter and Groenewegen, 1986b; Insausti et al., 1987; McIntyre et al., 1996). Moderate to dense labeling was also observed in the adjacent rim of temporal neocortex and in the lateral entorhinal cortex (Witter and Groenewegen, 1986a; McIntyre et al., 1996; Burwell and Amaral, 1998a,b; Pinto et al., 2006). Light labeling was seen in distributed regions of the striatum (Witter and Groenewegen, 1986b; McIntyre et al., 1996). Note that the mappings provided in figure 5.2 do not include the extra-cortical projections.

The rostrocaudal extent of longitudinal perirhinal connections was also consistent with earlier descriptions (Deacon et al., 1983; Room and Groenewegen, 1986; Witter et al., 1986; Lavenex et al., 2004; Pinto et al., 2006). In contrast to the labeling seen in the lateral entorhinal cortex, which decayed steeply with rostrocaudal distance from the perirhinal injection site, that seen in the PRC showed much less attenuation. This can be seen in figure 5.2 that depicts the distribution of PHAL-labeled axons in perirhinal regions adjacent to (Fig. 5.2A) vs. distant from (Fig. 5.2B) the PHAL injection site.

PHAL-positive intrinsic perirhinal axons were seen to terminate in all perirhinal layers with slight density variations depending on rostrocaudal distance from the injection site. This contrasts with some earlier descriptions where intrinsic perirhinal axons were reported to mostly target superficial layers (Deacon et al., 1983; Room and Groenewegen, 1986; Burwell and Amaral, 1998a,b). However, we note that other studies,
relying on larger PHAL injections as used here, also reported dense labeling of deep perirhinal layers (Apergis-Schoute et al., 2006; Pinto et al., 2006).

Fig. 5.2 Distribution of anterogradely labeled axons produced by PHAL injection in the PRC. Schemes depict coronal sections close to (within 1mm) (A) and rostrocaudally distant from (B) the PHAL injection site. At caudal levels (B), much labeling was observed in amygdala and some in the striatum, but this is not depicted here. Amy, amygdala; Hpc, hippocampus; rh, rhinal sulcus; Str, striatum.

Following PHAL injections in the temporal neocortex, the densest anterograde labeling was observed in neocortical areas surrounding the injection site and in perirhinal area 36, in transverse register with the injection site. Moderate labeling was also observed in area 35 and light labeling in the lateral entorhinal cortex. As shown in the mappings of Fig. 5.3, the density of anterogradely labeled axons in the PRC decreased dramatically with distance from the injection site (compare Figs 5.3A and 5.3B). This shows that only
a minority of neocortical neurons project to rostrocaudally distant levels of the PRC, and contrasts sharply with the prominent longitudinal connections of perirhinal neurons (Fig. 5.2). PHAL-positive neocortical axons were observed to terminate in all perirhinal layers with marked density variations depending on the rostrocaudal level of the PRC. At distant perirhinal levels, where labeling becomes very light in general, labeled profiles were usually observed in Layer I and deep layers (Fig. 5.3B).

**Fig. 5.3** Distribution of anterogradely labeled axons produced by PHAL injection in the temporal neocortex. Schemes depict coronal sections close to (within 1mm) (A) and rostrocaudally distant from (B) the PHAL injection site. At caudal levels (B), much labeling was observed in amygdala and some in the striatum, but this is not depicted here. Amy, amygdala; Hpc, hippocampus; rh, rhinal sulcus; Str, striatum.
5.3.2 *Electron microscopic observations*

The sections prepared for electron microscopic observations were first examined in the light microscope for areas containing dense anterograde labeling. In the electron microscope, the sections were scanned for the presence of PHAL-immunoreactive axon terminals. PHAL-labeled structures were easy to differentiate from unlabeled elements because of the electron-dense amorphous DAB reaction product associated with them. In the PRC, the DAB reaction product occurred in non-myelinated axons, varicosities and presynaptic boutons where it was associated with the external surface of microtubules, electron-lucent vesicles and mitochondria as well as with the internal surface of the plasmalemma.

Each time a PHAL-positive axon terminal was seen to form a synapse, it was photographed. For each PHAL injection, we documented two separate sets of 50 such synapses in target perirhinal regions: 50 from perirhinal sites adjacent to, and 50 from perirhinal sites rostrocaudally distant from, the PHAL injection site.

Whenever possible, all the 50 synapses were obtained from the same grid. However, in some cases, especially at perirhinal sites distant from the neocortical injections, several grids were required to collect 50 synapses due to the lower density of the projection. Overall, the following account is based on the analysis of 800 synapses distributed equally across the 8 injection sites. Figure 5.4 summarizes the distribution of synapses we observed. Figures 5.5 - 5.8 show representative examples of these synapses.

To lighten the description of our ultrastructural findings, we provide proportions of synapses. However, the raw numbers of synapses used to compute these proportions are provided in figure 5.4.
Fig. 5.4 Proportion of asymmetric vs. symmetric synapses and postsynaptic targets of PHAL-labeled axon terminals. Bar graph shows percentage of asymmetric and symmetric synapses for perirhinal (first 4 bars) and neocortical (last 4 bars) injections of PHAL. The first and last two bars respectively represent data obtained at perirhinal sites adjacent to and distant from the PHAL injection site. Numbers on bars refer to actual number of synapses analyzed in each condition. Each pair of bars, depicting asymmetric and symmetric synapses in a given site and injection group, includes 200 micrographs in total.

5.3.2.1 Synaptic articulation of short- and long-range intrinsic perirhinal axons

Our electron microscopic observations revealed similarities as well as interesting differences between the synaptic articulation of short- and long-range perirhinal axons. In both cases, a majority PHAL-positive axons formed asymmetric synapses (66.5% and 86% for short- and long-range projections, respectively). Another similarity resided in the prevalent postsynaptic target identified in these asymmetric synapses: a majority were dendritic spines (82% and 94.2% for short- and long-range projections, respectively). Examples of such axospinous synapses are illustrated in figure 5.5. A more surprising similarity between short- and long-range perirhinal projections resided in the relatively
high proportion of PHAL-positive axon terminals forming symmetric synapses (33.5% and 14% for short- and long-range projections, respectively), most with dendritic profiles (97% and 85.7% for short- and long-range projections, respectively). Examples of such symmetric synapses are provided in figure 5.6.

However, despite these similarities, clear differences emerged. First, as shown in figure 5.4, short-range projections formed a significantly higher proportion of asymmetric synapses with dendritic profiles (18.1% and 5.8% for short- and long-range projections, respectively; $\chi^2$-test, $p = 0.0008$). Second, the proportion of symmetric synapses found in short-range projections was more than double that seen in the long-range projections (33.5% and 14% for short- and long-range projections, respectively; $\chi^2$-test, $p < 0.0001$).
Fig. 5.5 Two examples of asymmetric synapses (arrowheads) formed by labeled perirhinal axon terminals (PHAL-t) at rostrocaudally distant perirhinal levels. In both cases an asymmetric synapse is made onto a dendritic spine of a perirhinal neuron. d, dendrite; s, spine.
Fig. 5.6 Two examples of symmetric synapses (arrowheads) formed by labeled perirhinal axon terminals (PHAL-t) at close to PHAL injection site. In both cases a symmetric synapse is made onto a dendrite of a perirhinal neuron. d, dendrite.
5.3.2.2 Synaptic articulation of short- and long-range neocortical projections to the perirhinal cortex

The pattern of results obtained in neocortical projections closely paralleled that described above for intrinsic perirhinal connections. As was seen in intrinsic perirhinal connections, the majority short- and long-range PHAL-positive neocortical axons formed asymmetric synapses (76% and 96%, respectively). The prevalent postsynaptic target identified in these asymmetric synapses were dendritic spines (77.6 and 87.5% for short- and long-range projections, respectively), as for intrinsic perirhinal axons. Examples of axospinous synapses formed by temporal neocortical projections in the PRC are illustrated in figure 5.7. Also reminiscent of the results obtained with intrinsic perirhinal projections, a relatively high proportion of PHAL-positive neocortical axon terminals formed symmetric synapses at close sites (24%), typically with dendritic profiles (95.8%). At rostrocaudally distant sites, 4% of the observed synapses were symmetric and all of them were onto dendrites. Examples of such symmetric synapses are provided in figure 5.8.

The differences identified between the short- and long-range intrinsic perirhinal axons were also manifest in neocortical projections. First, neocortical axons projecting to nearby perirhinal sites formed a significantly higher proportion of asymmetric synapses with dendritic profiles (22.4%) than those projecting to distant sites (12.5%; \( \chi^2 \)-test, \( p = 0.016 \); Fig. 5.4). Second, as was seen in intrinsic perirhinal connections, the proportion of symmetric synapses formed by neocortical axon terminals at nearby perirhinal sites was much higher than at distant sites (24% and 4% for short- and long-range projections, respectively; \( \chi^2 \)-test, \( p < 0.0001 \); Fig. 5.4). The only qualitative
difference seen between neocortical vs. perirhinal connections resided in the much lower incidence of PHAL-positive neocortical axon terminals at longitudinally distant perirhinal sites. This aspect is consistent with our light microscopic observations that revealed a steep reduction in the density of PHAL-positive axons with longitudinal distance from the injection sites (Fig. 5.3).
Fig. 5.7 Two examples of asymmetric synapses (arrowheads) formed by labeled neocortical axon terminals (PHAL-t) at rostrocaudally distant perirhinal levels. In both cases an asymmetric synapse is made onto a dendritic spine of a perirhinal neuron. d, dendrite; s, spine.
Fig. 5.8 Two examples of symmetric synapses (arrowheads) formed by labeled neocortical axon terminals (PHAL-t) onto dendrites of perirhinal neurons in transverse register to neocortical PHAL injection site. d, dendrite.
To conclude, irrespective of the PHAL injection site (perirhinal cortex vs. temporal neocortex), at rostrocaudally distant perirhinal levels we observed mostly asymmetric synapses, typically onto dendritic spines. Most synapses were asymmetric at close sites as well. However, the proportion of symmetric synapses in close sites was substantially higher compared to distant sites. This difference was sixfold in the temporal neocortex injections (Fig. 5.4). Another striking difference was found between short- and long-range axons forming asymmetric synapses: the short-range axons of both perirhinal and neocortical neurons formed a significantly higher proportion of asymmetric synapses with dendritic profiles compared to long-range projections (Fig. 5.4). It is also important to note that no significant difference was observed between the proportions of symmetric synapses formed at close vs. distant sites following PHAL injections in rostral vs. caudal levels of the PRC ($\chi^2$-test, $p = 0.27$) or temporal neocortex ($\chi^2$-test, $p = 0.21$).

5.4 Discussion

The results described in Chapters III and IV, together with previous physiological studies (Biella et al., 2001; Martina et al., 2001), suggested a lack, or lower level of, inhibitory influences in long-range connections of the perirhinal network. The experiments detailed in this chapter were undertaken to test this assumption. Anterograde tracing coupled to electron microscopic observations were used to determine whether the level of inhibition is similar or different in short- vs. long-range neocortical and intrinsic perirhinal projections. Ultrastructural analyses revealed that there is significantly more inhibition in short- compared to long-range neocortical and perirhinal projections.
Impulse transmission in the PRC is known to be subjected to potent inhibition (de Curtis et al., 1999; Biella et al., 2001, 2002, 2003, 2010; Martina et al., 2001; Pelletier, 2004, 2005; reviewed in de Curtis and Paré, 2004). For instance, neocortical stimuli elicit large, biphasic IPSPs in perirhinal neurons located at the same rostrocaudal level as the neocortical stimulation site (Biella et al., 2001; Martina et al., 2001). A prior tracing study in guinea pigs examined the synaptic articulation of neocortical inputs with perirhinal neurons located in transverse register with target perirhinal neurons (Pinto et al., 2006). This study revealed that the inhibition of principal perirhinal cells seen following neocortical stimuli arises from two sources: from perirhinal GABAergic interneurons activated by neocortical inputs and from GABAergic neocortical neurons that project to principal perirhinal cells (Pinto et al., 2006). The latter conclusion was supported by a different study that relied on electrophysiological methods coupled to retrograde tracing and GABA immunohistochemistry (Apergis-Schoute et al., 2007).

The present study confirms and extends these observations by comparing the termination pattern of short- and long-range neocortical inputs, in a different species (rats), and by also considering the synaptic articulation of intrinsic perirhinal connections. With respect to short-range neocortical inputs, consistent with the conclusions of Pinto et al. (2006), the present study revealed two potential substrates for neocortically-evoked inhibition: excitatory inputs to local-circuit inhibitory neurons of the perirhinal cortex (asymmetric synapses onto dendritic profiles) as well as direct neocortical inhibitory inputs to perirhinal cells (symmetric synapses onto dendritic profiles).

Interestingly, the present study indicates that both forms of inhibition are not only prevalent in short-range neocortical inputs, but also in short-range perirhinal projections.
In contrast, significantly less inhibitory influences were observed in long-range neocortical and perirhinal projections. Indeed, irrespective of the PHAL injection site (perirhinal cortex vs. temporal neocortex), we found two major differences between short- and long-range projections. First, neocortical axons projecting to nearby perirhinal sites as well as short-range perirhinal connections formed a significantly higher proportion of asymmetric synapses with dendritic profiles compared to long-range projections. As explained in the Rationale section of this chapter, in the cerebral cortex, the vast majority of excitatory inputs to principal cells end on dendritic spines. Therefore asymmetric synapses onto dendritic shafts are typically interpreted as excitatory synapses onto local-circuit cells. Excitation of perirhinal local-circuit neurons is the first step in feedforward inhibition of principal perirhinal cells; once activated, these local-circuit neurons will most likely inhibit nearby principal perirhinal cells.

Second, whether we examined neocortical or perirhinal inputs, the proportion of symmetric synapses formed at nearby perirhinal sites was much higher than at rostrocaudally distant perirhinal levels. As mentioned in the Rationale section, it is not possible to identify with certainty the postsynaptic targets (principal cells vs. local-circuit neurons) of inhibitory synapses, because the postsynaptic elements are the same in both cases: a symmetric synapse is observed either onto a dendritic shaft or a somatic profile. However, previous ultrastructural studies on the PRC found little evidence of inhibitory synapses onto GABA-immunopositive local-circuit cells (Apergis-Schoute et al., 2006; Pinto et al., 2006). For that reason, the symmetric synapses observed in this chapter are tentatively interpreted as inhibitory synapses formed with principal perirhinal cells. Such inhibitory synapses are encountered much more frequently in short- than long-range
perirhinal and neocortical projections. Instead, long-range perirhinal and neocortical projections are dominated by asymmetric axospinous synapses, which represent excitatory inputs to principal perirhinal cells.

These two ultrastructural differences clearly demonstrate that while short-range neocortical and perirhinal projections are characterized by potent inhibitory influences, perirhinal and neocortical axons projecting to rostrocaudally distant perirhinal levels involve much lower levels of inhibition. As described in the General Discussion, this anatomical organization may underlie the differential effects of single- vs. paired-site TFS observed in vitro.
CHAPTER VI

General Discussion
The perirhinal cortex does not passively relay processed sensory information from the neocortex to the hippocampus. Instead, it is the cortical region primarily responsible for recognition and associative memory (Zola-Morgan et al., 1989; Buckley and Gaffan, 1998; Parker and Gaffan, 1998; Gaffan and Murray, 1992; Meunier et al., 1993, 1996; Murray et al., 1993; Suzuki et al., 1993; Goulet and Murray, 2001). Single-unit recording studies showed that a depression of perirhinal responses to familiar stimuli underlies recognition memory (Brown et al., 1987; Fahy et al., 1993; Miller et al., 1993; Rolls et al., 1993; Sobotka and Ringo, 1993; Waburton et al., 2003; Eichenbaum et al., 1996) and that the opposite pattern, a potentiation of perirhinal responses to repeatedly presented pairs of stimuli, underlies associative memory (Messinger et al., 2001; Naya et al., 2003a). The main difference between recognition and associative memory tasks is that while a single stimulus is repeatedly presented to assess recognition memory, two stimuli are presented contiguously in associative memory tasks.

How can repeated presentations of the same stimulus/stimuli (repeated activation of the same neocortical inputs) lead to two opposite forms of plasticity, namely depression in recognition memory and potentiation in associative memory? The experiments described in this thesis were undertaken to shed light on this question.

6.1 Summary of background and general hypothesis

As reviewed in Chapter I, the PRC receives inputs from high-order associative cortical areas, mainly from an elongated strip of cortex that borders the PRC laterally (Deacon et al., 1983; Room and Groenewegen, 1986; Suzuki and Amaral, 1994; Burwell and Amaral, 1998a). These neocortical inputs are organized topographically with rostral
areas targeting anterior perirhinal levels and posterior ones focusing on caudal perirhinal sectors. In addition, the PRC is endowed with a prominent system of intrinsic longitudinal connections that can support the propagation of neocortical influences rostrocaudally (Witter et al., 1986; Lavenex et al., 2004). However, earlier electrophysiological studies had revealed that neocortical inputs strongly recruit perirhinal interneurons located at the same transverse level, limiting the depolarization of principal cells (Biella et al., 2001; Martina et al., 2001). In contrast, at a distance, it was observed that neocortical stimuli seemed to only evoke excitation, possibly because longitudinal perirhinal pathways do not engage interneurons (Biella et al., 2001; Martina et al., 2001).

These physiological findings suggested that via longitudinal connections, distributed neocortical activation patterns can propagate rostrocaudally and converge on subsets of perirhinal cells. Also, neocortical inputs recruit perirhinal inhibitory interneurons at the corresponding transverse level, limiting the depolarization of principal cells. However, because longitudinal axons within the PRC do not engage feedforward interneurons, simultaneous activation of two distant neocortical sites should shift the balance toward excitation in perirhinal cells receiving direct neocortical inputs. Given the role of coincident neuronal activity in synaptic plasticity, we reasoned that this process might allow the perirhinal network to associate coincident but spatially distributed neocortical inputs.
6.2 *Approaches used and summary of results*

To study the role of intrinsic perirhinal network in activity-dependent plasticity, we used field potential recordings and functional imaging with voltage-sensitive dyes in the whole-brain *in vitro* preparation. As described in Chapter III, we first tested whether neocortical input patterns that differentially recruit intrinsic perirhinal connections lead to contrasting forms of synaptic plasticity. We found that the polarity of neocortically-induced perirhinal plasticity (depression vs. potentiation) depends on the spatiotemporal distribution of neocortical inputs. TFS applied at a single neocortical site resulted in LTD whereas TFS applied simultaneously at two distant neocortical sites lead to LTP. Next, in Chapter IV, we aimed to shed light on the mechanisms underlying the differential effects of restricted vs. spatially distributed neocortical input patterns using pharmacological agents and local lidocaine infusions. We found that neocortically-induced LTD did not require the intrinsic perirhinal network and depended on the activation of group I mGluRs. In contrast, the LTP induced by paired neocortical stimulation was found to depend on the recruitment of longitudinal perirhinal pathways and on the activation of NMDARs. Overall the results obtained in Chapters III and IV, together with previous physiological studies (Biella et al., 2001; Martina et al., 2001), suggested that the reason why focused vs. distributed neocortical activity patterns had different effects on perirhinal plasticity was the lack (or lower level) of inhibition in long-range connections of the perirhinal network as compared to short-range neocortical inputs. Chapter V tested this assumption using anterograde tracing with PHAL coupled to electron microscopic observations. These anatomical experiments revealed that there is significantly more inhibition in short- compared to long-range neocortical and perirhinal projections.
6.3 *Hypothesized mechanisms of activity-dependent perirhinal LTD and LTP*

Combining the results described in Chapters III-V, I propose the following explanation for the mechanisms underlying neocortically-evoked perirhinal LTD and LTP. The reader is referred to figure 6.1 to facilitate understanding of these ideas. When TFS is applied at a single neocortical site (Fig. 6.1A), perirhinal cells in transverse register with the neocortical stimulation site are excited by glutamatergic inputs. However, these neocortical inputs also recruit inhibitory interneurons, limiting the depolarization of principal cells (Fig. 6.1A, Close). Based on our ultrastructural findings, this inhibition likely arises from multiple sources. These include neocortical inhibitory neurons projecting to the PRC as well as perirhinal inhibitory neurons recruited by neocortical afferents. In addition, if some principal perirhinal cells manage to escape from this inhibition and fire, they will recruit feedback interneurons, further enhancing inhibition at perirhinal sites adjacent to the stimulated region. Indeed, when PHAL injections were made in the PRC, 18% of the asymmetric synapses observed at nearby perirhinal sites were axodendritic. Since neocortically elicited EPSPs are curtailed by strong feedforward inhibition, each time neocortical inputs arrive at a nearby perirhinal region during TFS, orthodromic firing of recipient perirhinal cells is severely limited. As a result of these multiple inhibitory mechanisms, principal neocortical neurons cannot reliably activate principal perirhinal cells.

Although our results indicate that group I mGluRs are necessary for LTD induction, the exact mechanisms are unclear. Most importantly, the location of the critical group I mGluRs (principal cells vs. interneurons) has not been determined. Previous work in the PRC has revealed that postsynaptically located group I mGluRs exert their effects
by increasing phosphorylation of CREB via IP$_3$-dependent mobilization of Ca$^{2+}$ from intracellular stores (Cho et al., 2001; Kemp and Bashir, 2001; Harris et al., 2004; Swanson et al., 2005; Jo et al., 2008). Based on work done in the hippocampus, the end result would likely be internalization of AMPARs and modification of AMPAR- and NMDAR-mediated transmission (Snyder et al., 2001) via mRNA translation (Huber et al., 2000). However, prior investigations in other brain structures, such as the dorsal thalamus, suggest that group I mGluRs might also be located presynaptically, on inhibitory interneurons of the PRC. In dorsal lateral geniculate nucleus for instance, group I mGluRs are localized on the axon terminals of GABAergic interneurons and their activation enhances the feedforward inhibition of thalamocortical neurons (Errington et al., 2011). As discussed in Chapter IV, a similar mechanism might exist in the PRC. In both scenarios, the excitability of principal cells would be significantly reduced, preventing the neocortically-evoked depolarization from recruiting the intrinsic perirhinal circuit. Therefore, at distant perirhinal sites, although there is less feedforward inhibition in longitudinal axons, relatively few glutamatergic synapses would be activated (Fig. 6.1A Induction), and the threshold for NMDAR-dependent LTP would not be exceeded. What would remain is a locally induced LTD and consequent depression of perirhinal responses at all rostrocaudal levels (Fig. 6.1A Overlay).

When TFS is applied at two neocortical sites simultaneously (Fig. 6.1B), the local inhibitory influences described above do not change. Strong feedforward inhibition and group I mGluR activation occurs separately at the two perirhinal regions adjacent to the stimulated neocortical sites. However, in this condition, the convergence of inputs arising from long-range neocortical and perirhinal neurons overwhelms the local inhibitory
pressures triggered at the level of the stimulation sites (Fig. 6.1B Induction). Indeed, as revealed in the ultrastructural analyses of Chapter V, long-range horizontal connections arising in neocortical and perirhinal neurons, mostly form asymmetric axospinous synapses and engage substantially fewer inhibitory interneurons than short-range projections. Thus, these longitudinal pathways form a Hebbian-circuit. By removing the Mg$^{2+}$ block of NMDARs localized on principal perirhinal cells, this increased depolarization would initiate a robust Ca$^{2+}$ influx, overcome local group I mGluR-dependent inhibitory influences, and lead to LTP induction at all perirhinal levels (Fig. 6.1B Overlay).

The shift from LTD to LTP requires the activation of the intrinsic perirhinal network. Neocortical inputs that sufficiently recruit the longitudinal perirhinal connections meet the threshold for perirhinal LTP, suppressing the LTD. However, this does not mean that when perirhinal LTD is observed, there is no LTP going on in the network, or vice-versa. On the other hand, it is very likely that both LTD and LTP would be found in distinct perirhinal synapses at all times. In fact, it has been shown that total synaptic weight in a given network is conserved by balanced synaptic depression and potentiation (Royer and Paré, 2003). What underlies the shift of activity-dependent perirhinal depression to potentiation is not the elimination of the LTD, but a change in the relative power of LTP as the number and/or efficacy of synapses that undergo LTP increase.
Fig. 6.1 Hypothesized mechanisms of activity-dependent LTD and LTP induced by TFS applied at one (A) or two neocortical sites (B). Simulated synaptic responses (recorded intracellularly) elicited by neocortical stimuli are shown (Pre, Post, Overlay) for perirhinal sites in rostrocaudal proximity to (Close), versus distant (Distant) from, the neocortical stimulation site(s).

6.4 Behavioral implications

Considering the huge gap that exists between our studies on perirhinal plasticity in vitro and memory formation in conscious animals, only speculations can be offered regarding the behavioral implications of our findings. These speculations rely on the notion that single site TFS resembles the in vivo situation where a single stimulus is presented repeatedly, as in object recognition tests, while paired TFS of two neocortical
sites models associative memory formation, where two separate stimuli are integrated in the PRC. Taking a Gestaltist stand on perception, the notion that single site TFS mimics the conditions seen during recognition memory formation does not seem too far-fetched. Indeed, a particular stimulus is likely to be represented by clustered neurons in a spatially restricted circuit. For instance, circumscribed damage to different regions of the temporal and parietal lobes lead to selective loss in the ability of humans to identify particular classes of entities such as tools, animals, and persons (Tranel et al., 1997; Damasio et al., 2004). However, the neocortical representation of two stimuli will not always be more distributed.

While the spatial distribution of neocortical inputs representing two objects to be associated is difficult to define, the temporal relationship between them can be assessed precisely. In our in vitro experiments, only concurrent TFS induced perirhinal LTP, which was not observed when one of the inputs was delayed by half a theta cycle (62.5 ms). Yet, it remains that in normal conditions, stimuli to be associated do not always appear exactly at the same time. Indeed, it has been shown that associative memory formation and pair-coding behavior can occur when there is a 2-sec delay between the two stimuli to be associated (see Naya et al., 2003a). However, as argued in the Discussion of Chapter III, this lag in timing could be overcome by perirhinal neurons that display delay activity in associative memory tasks (Colombo and Gross, 1994; Sobotka, 2000; Naya et al., 2003b).

In any case, trying to link object identification to single site TFS and pair coding to paired TFS would be too audacious. For this reason, one might still ask: what would be the behavioral implications of the present thesis? In particular, what is the longitudinal
perirhinal network doing for memory? This thesis combines descriptive studies showing that the perirhinal cortex, by virtue of its intrinsic network, can support two distinct long-term plasticity mechanisms, each suggested as underlying one kind of perirhinal-dependent memory. Thus, it presents one possible mechanism whereby the same cortical area can support two different functions, which are very likely to underlie two separate behaviors.

6.5 Final Conclusion

The experiments presented in this thesis were undertaken to shed light on the mechanisms underlying the associative memory functions of the PRC. In this context, the intrinsic perirhinal network was shown to be critical for associating spatially distributed neocortical inputs. We found that the spatiotemporal organization of neocortical inputs and in particular to what extent these inputs recruit the intrinsic perirhinal network, determines the polarity of perirhinal long-term plasticity: LTD vs. LTP. When spatially distributed neocortical inputs are simultaneously activated (refer to Chapter III), rostrocaudally distant perirhinal levels are first excited separately. Then, due to lack of inhibitory influences on long-range axons (refer to Chapter V), the excitation spreads throughout the rostrocaudal extent of the PRC and recruits many initially silent principal cells in the intrinsic perirhinal circuit. Ultimately, the intrinsic perirhinal circuit exceeds the threshold for NMDAR activation and LTP induction (refer to Chapter IV). We propose that this form of perirhinal LTP underlies the associative functions of the PRC. An important challenge for future studies will be to test this idea in vivo.
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