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Synaptic plasticity between amygdala and perirhinal cortex

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1 Abstract

The central aim of the present research has been to investigate the cellular and molecular mechanisms by which emotions can influence recognition memory. I have been characterising synaptic transmission and plasticity mechanisms between amygdala (important for consolidation of memory for emotionally salient stimuli) and perirhinal cortex (crucial for recognition memory) using extracellular field potential recordings in rat brain slices that include these two areas. Initially, I have described for the first time the basic properties of synaptic plasticity at amygdala/perirhinal synapses, and I have found molecular pathways involved in long-term potentiation at this pathway. Afterward, I studied the interaction between amygdala and perirhinal cortex, namely how synaptic plasticity in the amygdala influences synaptic plasticity within perirhinal cortex. Results from this work have provided a functional model to study the mechanisms by which emotionally salient visual experiences are better remembered than neutral ones.

2 General Introduction

2.1 Where is the memory trace?

One of the most important function of our brain is the ability to form and store memories, and one of the most challenging goal of is to understand the cellular and molecular mechanisms that underlie information storage, learning and memory. Experiences and interaction with the environment leave “traces” in our brain that influence our subsequent behaviour. If we assume that these traces represent learning and memory, it has to be elucidated which mechanisms make these traces last. Some studies have provided evidences of possible mechanisms for consolidation of the memory traces:

1. *functional mechanisms*: functional modifications of existing synapses and neurons, changes in neuron excitability and in synaptic strength, i.e. long-term potentiation (LTP) and long-term depression (LTD) (Griffiths et al., 2008; Sah et al., 2008);
2. *structural changes* or rewiring: synaptogenesis and outgrowth of axons and dendrites, formation or elimination of new dendritic spines and synapses (Chklovskii et al., 2004; Barnes et al., 2009; Hofer et al., 2010);
3. *molecular changes*: include the activation of synaptic proteins, i.e. PKMζ (Shema et al., 2007; Serrano et al., 2008) or synaptic tagging (Redondo et al., 2011)

An example of structural changes is shown in Fig. 2-1, representing the growth of new synapses after learning. I will describe functional changes in details in the following chapters.

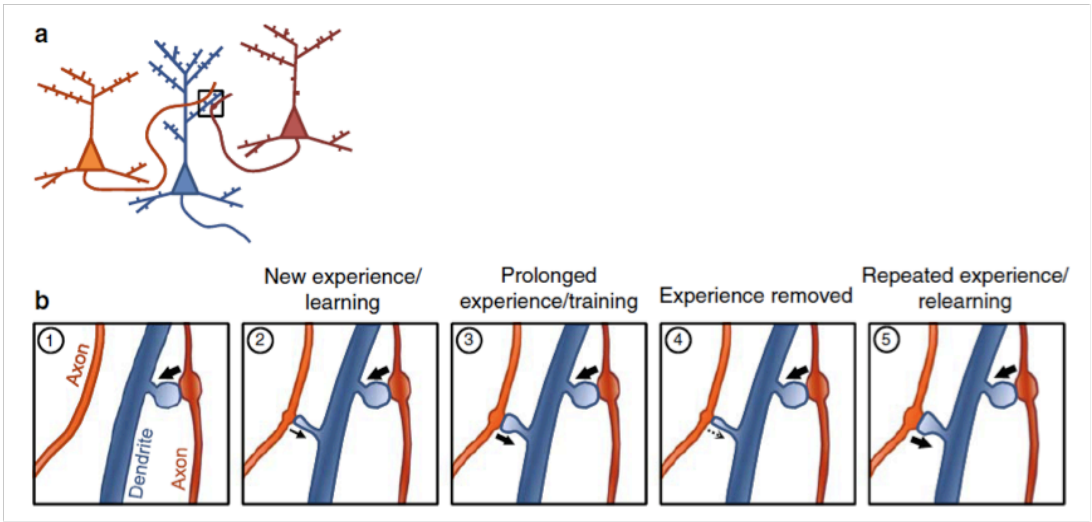

Fig. 2-1 Model for long-term information storage in neuronal circuits. a. Schematic of simplistic neuronal circuit. **b.** Magnified region of the circuit in a, including presynaptic boutons on axons and postsynaptic spines on the dendrite. During a novel experience or during learning of a task, new synapses are formed (growth of new spine and bouton depicted in panel 2), which are stabilised and strengthened with ongoing experience or prolonged training (panel 3). After cessation of experience, new synapses might become weakened or inactivated, while their structural basis remains (panel 4). With repeated experience, synapses can quickly become re-activated, leading to faster adaptation or learning (panel 5). Thickness of black arrows indicates strength of information transmission at synapses (from Hofer et al., 2010)

2.2 The synaptic plasticity and memory theory

The role of activity -dependent synaptic plasticity in learning and memory is a central issue in neuroscience. Most of the research work has been focused on the link between long -term synaptic changes and long -term memories. But how does LTP or LTD equal memory? An accredited hypothesis is the synaptic plasticity and memory SPM theory (Martin et al., 2000). This theory states that:

"Activity-dependent synaptic plasticity is induced
at appropriate synapses during memory formation,
and is both necessary and sufficient for the information storage
underlying the type of memory mediated
by the brain area in which that plasticity is observed"

Martin and colleagues (Martin et al., 2000) outlined a series of criteria that the SPM theory has to fulfill in order to explain how synaptic plasticity is related to memory:

- *detectability: if an animal displays memory of some previous experience, a change in synaptic efficacy should be detectable somewhere in its nervous system;*
- *mimicry: if it were possible to induce the same spatial pattern of synaptic weight changes artificially, the animal should display 'apparent' memory for some past experience which did not in practice occur;*
- *anterograde alteration: interventions that prevent the induction of synaptic weight changes during a learning experience should impair the animal's memory of that experience;*
- *retrograde alteration: interventions that alter the spatial distribution of synaptic weights induced by a prior learning experience (see detectability) should alter the animal's memory of that experience.*

Study of synaptic plasticity in the amygdala have fulfilled most of the criteria of the SPM theory. Detectability has been met, since it has been shown that fear conditioning learning induces LTP in the lateral nucleus of the amygdala (LA) (Rogan et al. 1997, Fanselow et al., 1999, Blair et al., 2001; Maren et al., 2004; Sah et al., 2008). Anterograde alteration has also been met in pharmacological studies. It has been found, for example, that infusion of the L-type voltage dependent calcium channels blocker verapamil into LA during training blocks the acquisiton of fear conditioning, but does not impair the expression of previously learned conditioned fear responses (Blair et al., 2001). A number of behavioural studies have shown that infusion of the N-Methyl-D-Aspartate (NMDA) antagonist into LA and the adjacent basal nucleus impairs acquisition of fear learning in a variety of tasks, including contextual fear conditioning (Fanselow and Kim 1994; Maren et al. 1996). Although retrograde alteration and mimicry criteria are difficult to meet especially in other brain regions, recent data in the amygdala have provided interesting evidences in favor of these two critera. In fact, it has been demonstrated that inhibition of PKMzeta in the basolateral amygdala disrupts previously established contextual fear conditioning (Serrano et al. 2008), according to the retrograde alteration criteria. Recent advanced techniques using optogenetics showed that activation of specific LA pyramidal cells as an unconditioned stimulus (US), in the absence of a peripheral shock US, produced fear conditioning (Johansen et al., 2010).

The recent lines of evidences are promising in explaining the connections between synaptic plasticity and memory in a more direct and predictable way. I will further discuss fear conditioning and synaptic plasticity in the amygdala in the following chapter.

3 The rat perirhinal cortex

3.1 Anatomy of the rat perirhinal cortex

Perihinal cortex is part of the hippocampal system that comprises the hippocampal formation (dentate gyrus, CA fields, and subiculum) and the parahippocampal region (the perirhinal PRh, postrhinal POR, and entorhinal ENT cortices together with the presubiculum and the parasubiculum) (Furtak et al., 2007). The hippocampal system is involved in various memory functions. It is located dorsal to the ENT and rostral to the POR, it can be differentiated from the ENT by the presence of large, heart-shaped pyramidal cells in layer V. The rostral boundary of the PRh is marked by the subcortical posterior limit of the claustrum. At its dorsal limit, the PRh is bordered by the ventral temporal cortex (TEv)(Amaral, 1998; Shi and Cassell, 1999).

Using Broadman's classification of the brain areas, we refer to rat perirhinal cortex as to area 35 and area 36, respectively located above and below the rhinal sulcus (see Fig. 3-1). They differentiate for a number of cytoarchitectural characteristics, for example, layer thickness and cell density (Burwell et al., 1995; Burwell, 2001). Area 35 is a narrow strip of cortex that primarily occupies the ventral bank of the fundus of the rhinal sulcus. It is an agranular cortex characterized by a broad layer I, a layer II populated by small round cells and layer V characterized by the small cells located superficially and progressively larger cells located more deeply in the layer. Area 36 is a broader, more dorsally situated strip of cortex that includes much of the dorsal bank of the rhinal sulcus as well as a portion of the dorsally adjacent cortex. It is characterised by a prominent layer II containing mostly round cells larger and often darker than those seen in area 35. The granular layer is very weak and contains granule cells and cells that constitute layers III and V. Layer IV becomes more prominent in the dorsal portion of the field. (See Fig. 3-1 B) (Burwell and Amaral, 1998b).

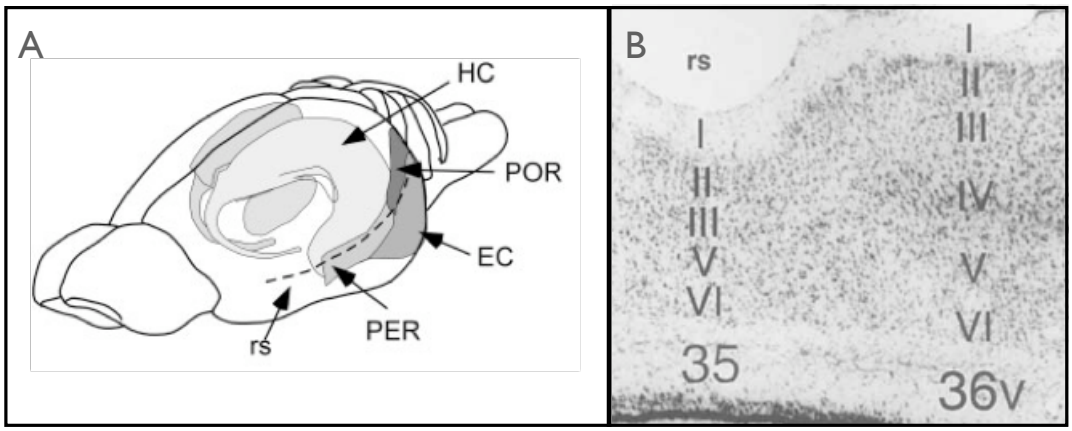

Fig. 3-1 A) The hippocampal system: perirhinal cortex (PER), entorhinal cortex (EC), postrhinal coretx (POR) and the hippocampus (HC) B) Nissl-stained standard coronal sections through the perirhinal cortex of layers of area 35 and ventral area 36 (B) (adapted from Furtak et al., 2007 and Burwell et al., 1995).

3.2 Connections of the rat perirhinal cortex

Perirhinal cortex is an associative cortex that receives and project to different brain areas (Fig. 3-2). Based on the percentage of retrogradely-labeled cells of a study published in 2007 by Furtak and Burwell, following are the major connections of perirhinal cortex (Furtak et al., 2007).

3.2.1 Cortical connections

About half of all afferent connections to area 36 of the PRh originate from cortical structures. The temporal cortex (especially its ventral portion) provides the heaviest input to area 36 (about half of the cortical input) conveying auditory, olfactory, and visual sensory information (Burwell and Amaral, 1998a). Area 36 also receives input from primary and secondary auditory regions within the temporal cortex. Nearly one third of all cortical input to caudal area 36 are provided by the occipital regions, with a large portion of the projections originating in the visual association regions. Area 36 send projections to the parietal, temporal, and frontal areas. The strongest efferent projection from area the rostral part of area 36 arises terminates in somatosensory cortex. The mid-

rostrocaudal and caudal levels of area 36 strongly project to temporal regions and the piriform cortex, respectively. Projections to the cingulate and occipital regions are weak. (Furtak et al., 2007).

About half of all afferent connections to area 35 of the PRh originate from cortical structures. Area 35 receives strong afferent projections from both the piriform cortex and insular regions, (Burwell and Amaral, 1998a). It receives fewer cortical afferents from the temporal cortex as compared to area 36 and they mainly terminate in the mid-rostrocaudal level of area 35. Projections from area 35 to cortical structures are weaker than those of area 36. The strongest projection terminates in frontal areas and in the insular areas. Projections arising in area 35 terminate also in the supplementary and primary motor regions. Area 35 also has a moderate efferent connection to the parietal cortex.

3.2.2 Subcortical connections

Subcortical structures contribute roughly one-third of the total afferent connections of area 36 and the principal subcortical afferent from the amygdala and contributes to roughly half of the subcortical inputs. In particular, the lateral nucleus of the amygdala contributes most heavily to this strong projection, which terminates largely in rostral area 36. Inputs originating from the thalamus (especially its dorsal part) strongly innervate area 36. The septal nuclei, the basal ganglia and the hypothalamus provide weak input. Area 36 of PRh cortex provides widespread inputs to subcortical structures. Most are modest but a very heavy projection targets the caudate putamen. In addition, there is a strong efferent projection that terminates throughout the amygdala nuclei. There are some strong projections from the mid-rostrocaudal division of area 36 to the olfactory area. Moderate projections are sent to the thalamus. Based on the percentage of retrogradely-labeled cells, subcortical area 35 of the PRh. The strongest projection to area 35 originates in the olfactory areas, followed closely by the amygdala and the claustrum. The amygdala, the lateral nucleus in particular, strongly projects to caudal area 35, while the claustrum contributes input to all rostrocaudal levels of area 35. Area 35 receives a moderate (and

little) projection respectively from the dorsal thalamus, the ventral thalamus along with the septal nuclei, the basal ganglia, and the hypothalamus.

Overall, the subcortical efferents of area 35 are weak. The exception is the very strong projection that terminates in the basal ganglia, The next largest projection is to olfactory areas. Moderate projections are sent to the basomedial nucleus of the amygdala, weak projections to the claustrum, thalamus and hypothalamus.

3.2.3 Hippocampal connections

Overall, the hippocampal system projects weakly to area 36, the POR and ENT provide the majority of the parahippocampal input to area 36. less than one fifth of area 36 input arises from the ventral hippocampus. This projection originates primarily in field CA1. Area 36 provides only modest input to the hippocampal formation (CA1 and the Subiculum) and provides very strong input to the ENT. Moderate input terminate in the POR. Input to area 35 originates in the parahippocampal region. In particular, ENT accounts for roughly three-quarters of the hippocampal system input. In addition, caudal area 35 receives a modest input from the POR. Area 35 receives modest input from the dorsal and ventral HPC. Heavy efferent projections from area 35 terminate almost exclusively in the ENT. The POR, in contrast, receives a moderate projection that originates in caudal area 35.

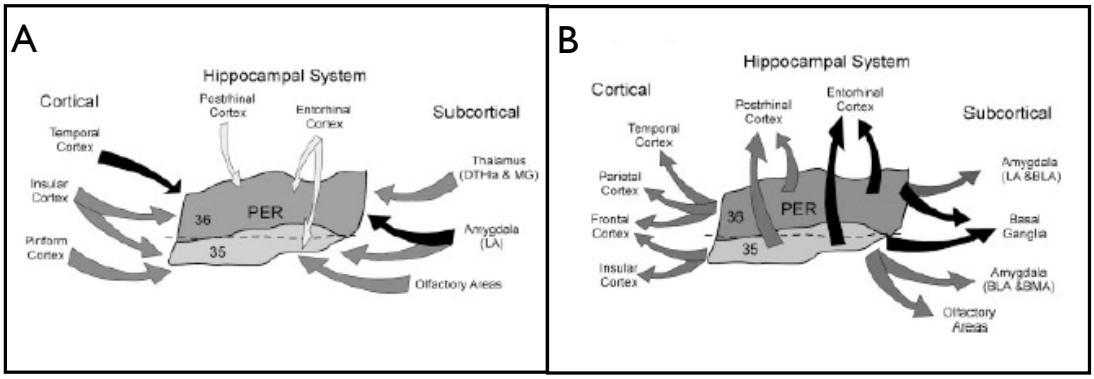

Fig. 3-2 Connections of the rat perirhinal cortex (PER), area 35 and 36. Projection to PER (A) and from PER (B) with cortical and subcortical structures. (Adapted from Furtak et al., 2007).

3.3 The role of perirhinal cortex in learning and memory

One of the most important functions of perirhinal cortex regards a form of declarative memory known as recognition memory. Recognition memory consists in the ability of recognising as familiar something that has been previously encountered. This form of memory is very important in everyday life. Two tests can be easily used to study the formation of recognition memory in laboratory: the delay-non-matching-to-sample (DNMS) task and the novel object preference (NOP) test. The classic DNMS consists in training the animal to displace an object to obtain a food reward. A sample object is presented to the subject and after a delay, the sample is presented again, along with a new stimulus. The subject is rewarded for selecting the new stimulus. Unlike the DNMS, the NOP does not require long training sessions, it is easy to learn and quicker to perform. It exploits the natural tendency of the animal to explore novel objects over familiar objects. It consists of two phases. During the sample phase the animal is presented with two identical objects to be explored, after a delay, test starts and the animal is presented with a novel object and an object identical to the sample objects. If the animal recognise the object seen during the sample phase as familiar, he will spend more time exploring the new object.
The use of these tasks in combination with drug infusions or *in vivo* recording studies has brought to a better understanding of the mechanisms underlying recognition memory. Normal glutamatergic transmission in rat perirhinal cortex appears to be essential to recognition memory performance. NMDA or metabotropic glutamate receptors produce impairments at long (24 h), though not shorter (20 min) delays (Barker et al., 2006a,b). Intriguingly, blockade of kainate glutamatergic receptors produces the opposite pattern of impairment: a deficit after a delay of 20 min but not after a delay of 24 h (Barker et al., 2006b). Infusion of the L-type voltage-dependent calcium channel blocker impairs perirhinal long -term recognition memory (Seoane et al., 2009). Again, long-term (24 h) recognition memory is impaired by interference with the actions of phosphorylated CAMKII (calcium calmodulin kinase, 2) (Tinsley et al., 2010), BDNF (brain-derived neurotrophic factor) (Seoane et al., 2010), or PKMzeta

(Outram et al., 2010). Infusions of the muscarinic receptor antagonist scopolamine into monkey PRh disrupts DNMS object recognition, a result that is consistent with the finding that PRh ACh release increases significantly in monkeys performing the DNMS task (Tang and Aigner, 1996; Tang et al., 1997). One recent study has demonstrated the importance of CREB protein phosphorylation in PRh long-term object recognition memory (Warburton et al., 2005). In this study, CREB inhibition within rat PRh impaired NOP performance with a long (24-h) but not a short (15-min) retention delay and also disrupted the normal decremental response of PRh neurons to familiar versus novel pictures. Recording studies *in vivo* support a role for the perirhinal cortex in visual recognition memory. The responses of a subset of neurones in primate perirhinal cortex have been shown to be repetition -sensitive in response to visual stimuli, their response is maximal to the first stimulus and significantly reduced to repeated presentations (Xiang and Brown, 1998). These neurones can be classified into *novelty*, *recency* or *familiarity* neurones according to the circumstances in which a decrement is seen. In the case of a *novelty neurons* the decrement is only seen the first time that the stimulus is repeated and not in subsequent repetitions (i.e. when the stimulus becomes familiar). Furthermore, when the stimulus becomes familiar, the response becomes much briefer upon first and repeat viewings. *Recency neurons* show a decrement in response to repeat stimuli whether or not it is already familiar to the animal. These neurones therefore only detect whether or not the stimulus has been seen in the recent past. *Familiarity neurons* show no decrement between the initial first and second presentations of novel stimuli but do show a decrement during first and repeat presentations of familiar stimuli. Approximately 25 % of visually responsive neurones in the perirhinal cortex change their response with stimulus repetition (Xiang and Brown, 1998; Brown and Aggleton, 2001). The remaining ~75 % of visually responsive neurones are thought to encode information pertaining to the physical characteristics of the stimulus.

Different studies have also reported an involvement of perirhinal cortex in forms of memory other than recognition memory. Evidences have shown that PRh lesions made before or after training produce deficits in contextual fear conditioning (Corodimas & LeDoux, 1995; Bucci et al., 2000; Burwell et al.,

2004) and fear conditioning to discontinuous but not continuous auditory cues (Kholodar-Smith et al., 2008; Lindquist et al., 2004).

4 The rat amygdala

4.1 Anatomy of the rat amygdala

The amygdala originates its name from the Greek term for almond because in the early 19th century, when it was first described as an independent brain region, it comprised the only almond -shaped basolateral nucleus and not the whole structure. Subsequently, a large number of structures that surround the basolateral complex have been identified in many species and constitute what is now known as the amygdaloid complex. The amygdala is located in the temporal lobe and there has been much debate about how the amygdala should be classified on the basis of histological criteria such as density, configuration, shape, size of the cells or trajectory of the fibers. The most traditional view is that amygdala consists of an evolutionary primitive division associated with the olfactory system (the corticomedial region: cortical, medial, central nuclei) and and evolutionary newer division associated with the neocortex (the basolateral region: lateral, basal and accessory basal nuclei). A more recent hypothesis suggests that the amygdala is neither a structural nor a functional unit, but a region that belongs to other regions or system of the brain. According to this scheme, for example, the lateral and basal nuclei are seen as extensions of the cortex, while the central and medial nucleus are viewed as ventral extensions of the striatum (LeDoux, 2007). Independently of which hypothesis one could support, the functions performed by the aforementioned nuclei still remain the same. We will review the connections and the functions of the amygdala following the classification used by the first hypothesis in order to make the understanding easier. Moreover, because this study is conduced in the rat and given the large number of studies conduced on the rat amygdala in the literature, we will review the rat amygdala according to the classification done by Price and colleagues (Price et al.1987). Many other studies are present in other species such as monkeys or cats (Amaral et al., 1992; Price et al., 1987).

The amygdaloid complex is composed by three groups of neurons: the

basolateral group that includes the lateral nucleus, the basal nucleus, and accessory basal nucleus; the *cortical group* which includes the cortical nucleus and the nucleus of the olfactory tract and the *centromedial group* consisting of the medial and central nuclei. Two more structures are considered part of the amygdaloid complex but are not included in any of the previous groups: the intercaleted cell masses and the amygdalohippocampal area.

The basolateral nuclei

The lateral nucleus (LA) is located dorsally in the amygdala and it is bordered laterally by the external capsule (ec) and medially by the central nucleus (Ce). It has three subdivisions: the smaller celled dorsolateral subdivision, the larger celled ventrolateral subdivision, and the medial subdivision. The basal nucleus (BA) is located ventral to the LA and is subdivided into the rostral magnocellular subdivision and the more caudal intermediate and parvicellular subdivisions. The accessory basal nucleus (AB) is found ventral to the basal nucleus and lies adjacent to the amygdalohippocampal area (AHA). It is comprised of the magnocellular subdivision, the intermediate subdivision, and the parvicellular subdivision (Sah et al., 2003).

Cortical nuclei

They comprise the nucleus of the lateral olfactory tract (lOT), the bed nucleus of the accessory olfactory tract (BAOT), the anterior and posterior cortical nucleus (CoA and CoP, respectively), and the periamygdaloid cortex (PAC). The BAOT is at the very rostral part of the amygdala where it is bordered laterally by the CoA. The CoA is a layered structure located lateral to the NLOT. The CoP is also three layered and is located in the most caudal parts of the amygdala where it borders the AHA dorsally and the PAC laterally. The PAC is found ventral to the basal nucleus and is subdivided into three subdivisions: the periamygdaloid cortex, the medial division, and the sulcal division (Sah et al., 2003).

Centromedial nuclei

The centromedial group is in the dorsomedial portion of the amygdaloid complex and consists of the central (CeA), medial (M), and the amygdaloid part of the bed nucleus of stria terminalis (BNST). The CeA is located dorsomedially in the rostral part of the amygdala, bordered laterally by the basolateral complex, dorsally by the globus pallidus, and medially by the stria terminalis. The CeA has four divisions: the capsular subdivision (CeC), lateral subdivision (CeL), intermediate subdivision (CeI), and medial subdivision (CeM) (McDonald et al., 1992). The medial nucleus is found near the surface bounded medially by the optic tract. It begins at the level of the NLOT and extends caudally. It has four subdivisions: rostral, central (dorsal and ventral), and caudal (Sah et al., 2003).

4.2 Connections of the rat amygdaloid complex

4.2.1 Afferents to the amygdaloid complex

Based on the information content of the afferents, inputs to the amygdala can be separated into those arising in cortical and thalamic structures and those arising in the hypothalamus or brain stem. Cortical and thalamic inputs supply information from sensory areas and structures related with memory systems. Hypothalamic and brain stem inputs arise from regions involved in behaviour and autonomic systems. The major source of sensory information to the amygdala is the cerebral cortex. These projections are glutamatergic, predominantly arising from layer V pyramidal neurons. The majority are ipsilateral and enter the amygdala via the external capsule. Most cortical projections originate in association areas and transmit processed information by a series of cortico -cortical connections originating in the primary sensory cortex. These inputs can be divided into those that relay modality-specific sensory information, those that are polymodal, and those arising in the medial temporal lobe memory system (Sah et al., 2003).

The amygdala receives inputs from all modalities: olfactory, somatosensory, gustatory and visceral, auditory, and visual.

All regions of the olfactory stream have projections to the amygdaloid complex. Olfactory projections arise from the main and accessory olfactory bulbs as well as the primary olfactory cortex. The main olfactory bulb projects mainly to the nucleus of the lateral olfactory tract, anterior cortical nucleus, whereas the accessory olfactory bulb projects to the bed nucleus of the accessory olfactory tract, the medial nucleus, and posterior cortical amygdala (Scalia et al., 1975). The piriform cortex and anterior olfactory nucleus have projections to the lateral amygdala, basal, and accessory basal nuclei.

For somatosensory inputs, few projections arise directly from primary somatosensory areas. Most afferents reach the amygdala via the insular cortex and target the lateral, basal, and central nucleus (Shi and Cassell, 1998a/b). Somatosensory information also reaches the amygdala by projections from the parabrachial nucleus and thalamic nuclei, the medial geniculate and the posterior internuclear nucleus (PIN), which have been suggested to be involved in the transmission of nociceptive information. Inputs arising in the PIN target all subdivisions of the LA, but also innervate the accessory basal nucleus and the medial subdivision of the central nucleus (Linke et al., 2000).

Auditory and visual information also reach the amygdala from association areas rather than primary cortex. These pathways are thought to be particularly relevant during fear conditioning. For auditory information, area Te1, the primary auditory cortex in rat, has no direct projections to the amygdala (Shi and Cassell, 1997). Injections of anterograde tracers in Te3 show fibers in the LA, with the dorsolateral subdivision being the most common target. Retrograde tracing studies have shown that these projections arise from cortical layers II and IV (LeDoux et al., 1991). Subcortical acoustic inputs arise from the thalamic medial geniculate nucleus and target the same areas of the LA (Ledoux et al., 1990). As with acoustic inputs, visual cortical projections to the amygdala also originate both from thalamic and high-order visual areas. Cortical projections from these areas follow a cascade to the amygdala in large part via Te2 (Shi and Cassell, 2001). These fibers terminate in the dorsal subdivision of the LA, the CeL, and some in the magnocellular basal nucleus.

There are several sources of polymodal sensory information to the amygdala

originating in areas related to long -term declarative memories, including prefrontal cortex, perirhinal cortex, the entorhinal cortex and the hippocampus. Projections between the amygdala and these structures are reciprocal and strong (Pitkänen, 2000). The prefrontal cortex projects mostly to the basal nucleus but afferents to the LA as well as accessory basal, central, and medial nuclei have also been described (McDonald et al., 1996). The perirhinal cortex instead sends its projections mostly to the medial portion of the LA although projections to basal and cortical nuclei have also been described (Shi and Cassell, 1999). The entorhinal cortex in comparison appears to project to most amygdalar nuclei. Inputs from hippocampus targets mainly the basal nucleus.

4.2.2 Efferents from the amygdaloid complex

The amygdaloid nuclei have widespread projections to cortical, hypothalamic, and brain stem regions. In general, projections from the amygdala to cortical sensory areas are light and originate in cortical and basolateral areas of the amygdala. The perirhinal area, along with other areas in the frontal cortex that project to the amygdala, receive reciprocal connections from the LA, BA, AB, M, and periamygdaloid cortex (Pitkänen et al., 2000). The cortical nuclei that receive olfactory projections all send substantial reciprocal projections back to the olfactory cortex.

The basolateral complex (LA, B, AB) has a substantial projection to the medial temporal lobe memory system with afferents to hippocampus and perirhinal cortex (Petrovich et al., 2001). A large projection is also found to the nucleus accumbens (McDonald et al., 1991). Similar to the LA, the basal nucleus also has substantial projections to hippocampus, but in addition has a major projection to prefrontal cortex, nucleus accumbens, and the thalamus. Efferents from the basolateral complex arise from pyramidal-like neurons and are thought to be glutamatergic (Parè et al., 1995). Activation of the central nucleus induces this autonomic response by stimulating groups of neurons in the brain stem that control the autonomic system, or alternatively by stimulating hypothalamic nuclei that modulate these centres (LeDoux et al., 1988). In agreement with these behavioural responses, the medial subdivision of the central nucleus has

substantial projections to the hypothalamus, bed nucleus of the stria terminalis, and several nuclei in the midbrain, pons, and medulla both CeA and BNST have strong projections to ascending monoaminergic and cholinergic neuron groups. These include the noradrenergic locus coeruleus, the dopaminergic substantia nigra and ventral tegmental area, the serotonergic raphae, and the cholinergic nucleus basalis (Amaral et al., 1992; Davis et al., 2001). These systems innervate large regions of the forebrain and temporal lobe memory systems. Large numbers of neurons in the medial subdivision of the central nucleus and medial nucleus are GABAergic, and these projections from the central nucleus have been suggested to be inhibitory (Pitkänen et al., 1994; Saha et al., 2000). Functionally, activation of CeA neurons in the rat results in rises in blood pressure and heart rate. A GABAergic projection from the CeA suggests these fibers are likely to innervate local inhibitory cells in brain stem nuclei.

4.2.3 Intra-amygdaloid connections

The lateral nucleus gives rise to the most extensive set of intra-amygdaloid connections. It sends projections to the central nucleus, the medial nucleus, the basal nucleus and the accessory basal nucleus. The AB projects to the LA, the central nucleus and to the medial nucleus. The basal nucleus is reciprocally connected with LA and sends projections to the Ce. The medial nucleus instead project to the LA, the CeA and the AB (Fig. 4-1)

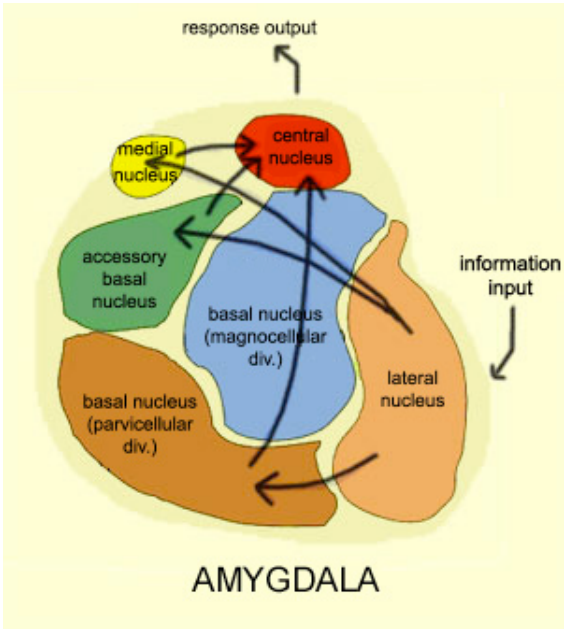

Fig. 4-1 Intra-amygdaloid connections (from Aggleton, 1992).

The hierarchical organization of the intra-amygdaloid connections explains what happens after the information enters the amygdaloid complex. When the information enters, local filtering mechanisms within the amygdala circuitries might determine whether incoming neuronal activity will evoke a response. If a response is evoked, neuronal activity spreads within the division or becomes distributed to the other divisions or to the other amygdaloid nuclei in point-to-point manner. As a consequence, representations of the input information are established in parallel in different locations of the amygdaloid complex, with each location receiving input from other selective areas of the brain. After information becomes associated with or modulated by information from the other functional systems processed in parallel in different locations of the amygdala, it enters the output regions of the amygdala, particularly the central nucleus and the amygdalo–hippocampal area. The convergence of inputs in these areas might serve to gather the modulated stimulus representations and to bring them together finally to elicit appropriate behavioural responses. Studies of internal circuitries show that the amygdala has a clear and precise organization that is tailored to the computational functions it performs (for a review Pitkänen et al., 1997).

4.3 The role of the amygdala in learning and memory

The amygdala is involved in a wide range of behavioural functions and a malfunction or changes in its structures have been linked with different psychiatric disorders in humans, including anxiety disorders (the post -traumatic stress disorder, phobia and panic state), schizophrenia, depression and autism. (Kucharska-Pietura et al., 2003; Munson et al., 2006; Koenings et al., 2009; Townsand et al., 2010). The first study that suggests a role of the amygdala in processing emotions was conduced in the 1930s from Klüver and Bucy. The authors reported a change in the emotional behaviour of monkeys after bilateral ablation of a portion of the medial temporal lobe, that later on was recognized as a bilateral amygdala ablation. These changes resulted in: 1) “psychic blindness,” or the inability to recognize the emotional significance of objects; 2) hypersexuality, often directed indiscriminately; 3) altered emotional behaviour, particularly placidity; 4) hyperorality and the ingestion of inappropriate objects (pica); 5) “hypermetamorphosis,” or the tendency to react to every visual stimulus; and 6) memory deficits. This finding suggests for the first time that the amygdala could play an important role in emotional behaviour and in modulating memory. Since the first studies, a wide range of findings have reported a role of the amygdala in processing emotions, in particular fear, and its fundamental role in conditioning learning (LeDoux, 1992) and in modulating memory (McGaugh, 2002) . The test that has been widely used to study fear in rodents and in humans with some adaptations (Orr et al., 2000) is the fear conditioning test. This test is a type of Pavlovian learning task in which animals are presented with a neutral conditioning stimulus (CS) that is paired with an aversive unconditioned stimulus (US). The animals learn that the CS predicts the US and will exhibit specific behavioural responses such as freezing when the CS is presented alone. Additionally subjects also learn to associate the environment in which the CS-US pairings take place with the US, and will exhibit specific behavioural responses when in the environment in the absence of the CS. It is a simple and quick way to examine associative learning that is long lasting. This task can be designed to assess many types of conditioning sensitive to either the hippocampal system, the amygdalar system, or both. When a single or discrete CS, such as a tone, is associated with the US, this

type of conditioning is dependent upon the amygdala (Fanselow and LeDoux, 1999; LeDoux, 2000; Maren, 2001). In a recent study it has been shown that specific activation of LA pyramidal cells as an US, in the absence of a peripheral shock US, produced fear conditioning, confirming that this nucleus is fundamental for associative conditioning learning (Johansen et al., 2010). A large number of studies have provided convincing evidences that associative plasticity in the LA contributes to fear memory formation (Sah et al., 2008; Maren et al., 2004; Blair et al., 2001). It is widely believed that LA plasticity underlying fear learning occurs as a result of a Hebbian mechanism whereby the shock US directly depolarizes LA pyramidal cells that are concurrently activated by weaker CS inputs, resulting in potentiation of the CS input synapses (Paré, 2002; Rogan et al., 2000). It has been proposed and now widely accepted that auditory information representing the CS and somatosensory information representing the US reach the lateral nucleus of the amygdala (LA) from both thalamic and cortical sources. Within the LA, individual neurons respond to both auditory and somatosensory stimuli suggesting convergence of CS and US inputs at the cellular level. Sensory information from the LA is then relayed to the central nucleus, both directly and indirectly via the basal, accessory basal, and intercalated nuclei. The central nucleus, in turn, projects to areas of the brainstem and hypothalamus that control the expression of defensive behaviours, hormonal secretions and autonomic responses (Sigurdsson et al., 2007) (Fig 4-2).

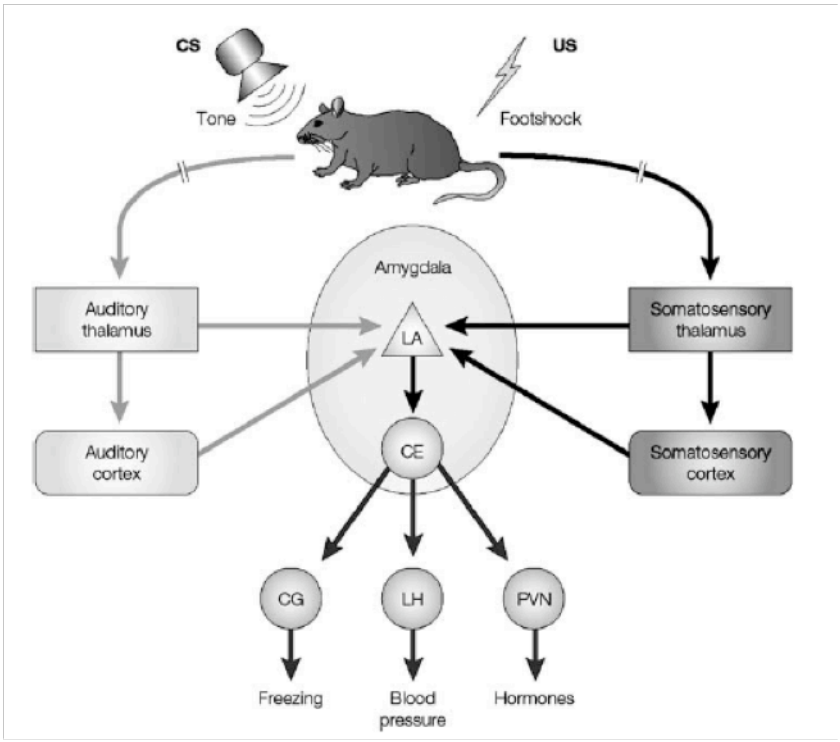

Fig. 4-2 Neural circuits underlying auditory fear conditioning (adapted from Sigurdsson et al., 2007)

The amygdala also plays a critical role in modulating memory consolidation, especially mediating the effects of acute stress on learning and memory (for a review Roozendaal et al., 2009). Acute and chronic stress exposure can induce functional and morphological changes as well as neuronal remodelling in the amygdala (Vyas et al., 2002 and 2004; for a review Roozendaal et al., 2009). Studies have reported for example that noradrenergic activation of the basolateral amygdala modulates consolidation of object recognition memory in rats (Roozendaal et al., 2008) and also inhibitory avoidance task (Ferry et al., 1999), contextual fear conditioning (Huff et al., 2005) or water -maze spatial training (Hatfield et al., 1999).

5 Excitatory synaptic transmission in the central nervous system

Communication between neurons happen when an action potential reaches the terminal of a presynaptic neuron and voltage-dependent calcium (Ca^{2+}) channels located in the presynaptic membrane open allowing Ca^{2+} to enter the cell. This influx of calcium ions triggers a series of events, which ultimately results in the release of the neurotransmitter from a synaptic vesicles into the synaptic cleft. Neurotransmitters drift across the synaptic space and bind special proteins located on the postsynaptic membrane called receptors. Neurotransmitters can initiate a process of excitatory synaptic transmission or inhibitory synaptic transmission, depending on which neurotransmitter is released and whether it causes a depolarization or a hyperpolarisation of the postsynaptic neurons.

The amino acid glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS) and it exerts its effects by binding to glutamate receptors. It binds to two categories of receptors: ionotropic glutamate receptors (iGlurs) and metabotropic glutamate receptors (mGluRs) (Fig. 5-1). The iGluRs are the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, N-methyl-D-aspartate (NMDA) receptors and kainate (KA) receptors. (AMPA, Kainate, NMDA). For the purpose of this manuscript I will give only a brief description of mGluRs and a more detailed description of iGluRs.

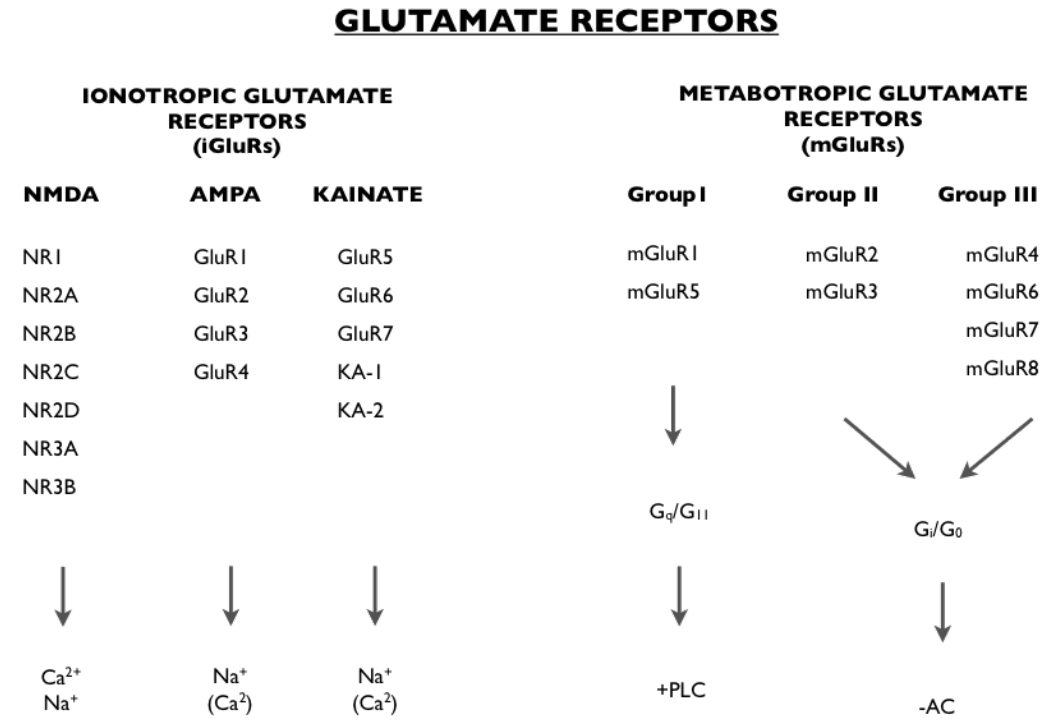

Fig. 5-1 Glutamete Receptors and their constitute subunits. iGluRs can be subdivided based on sequence homology and pharmacology, and are tetrameric complexes that allow the conductance of cations such as Ca2+ and Na+. mGluRs are G-protein coupled receptors and can be subdivided based on their intracellular signalling mechanisms. Group I mGluRs are positively coupled to phospholipase C (PLC), and group II and group III mGluRs are negatively coupled to adenylyl cyclise (AC). (Image adapted from Kew and Kemp, 2005).

5.1 Ionotropic glutamate receptors (iGluRs)

The ionotropic receptors are ligand gated ion channels and they are activated in response to the binding of a ligand molecule such as glutamate. Once the ligand is bound to the receptor, the receptor opens and positive charged ions such as Na^+ and Ca^{2+} pass through the channel located in the centre of the receptor complex. This flow of ions results in a depolarisation of the plasma membrane and the generation of an electrical current that is propagated down the processes (dendrites and axons) of the neuron to the next in line. The structure of ionotropic glutamate receptors is shown in Fig. 5-2. They are formed by a large extracellular N-terminal that cointains the ligand binding domain, three transmembrane regions and the intracellular C-terminal domain.

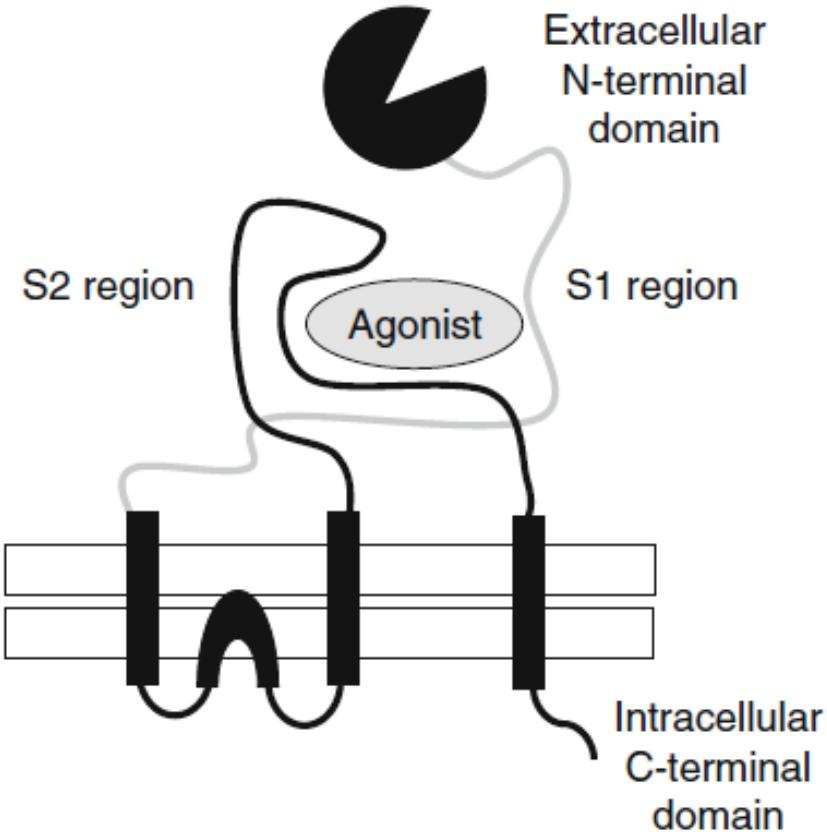

Fig. 5-2 Schematic representation of a ionotropic glutamate receptors (adapted from Kew and Kemp, 2005).

5.1.1 AMPA Receptors

AMPA receptors are responsible for the fast synaptic transmission in the CNS. They are permeable to Na^+ and when glutamate binds to this receptor, the influx of Na^+ ions results in neuronal depolarisation and the generation of an excitatory postsynaptic potential (EPSP) with the resultant generation of an action potential if threshold is reached creating a depolarization of the postsynaptic neuron.

AMPAR receptors are hetero -oligomeric proteins, four different genes (GluR1-4) encode AMPAR subunits (Hollmann and Heinemann, 1994). The extracellular and transmembrane regions of AMPAR subunits are very similar but vary in their intracellular cytoplasmic tails: GluR1, GluR4 and the long splice form of GluR2 have long cytoplasmatic carboxy -terminal tail (c-tail), while

GluR2, GluR3 and a short splice form of GluR4 have short and similar c -tails. Alternative splicing of the C -terminal domains determines the binding of the subunits to specific interacting proteins as well as the models of regulation of the receptors by protein phosphorylation. All four AMPAR subunits also occur in two alternatively spliced versions, Flip and Flop (Sommer et al., 1990) and form part of the extracellular ligand-binding domain. Flip variants are predominant prenatally, whereas Flop variants become expressed postnatally and reach levels equivalent to those of Flip in the adult. The Flip and Flop splice variants have effects on the rate and extent of desensitisation of heteromeric AMPA receptors and also influence their sensitivity to allosteric modulators (Kew and Kemp, 2005) (Fig. 5-3).

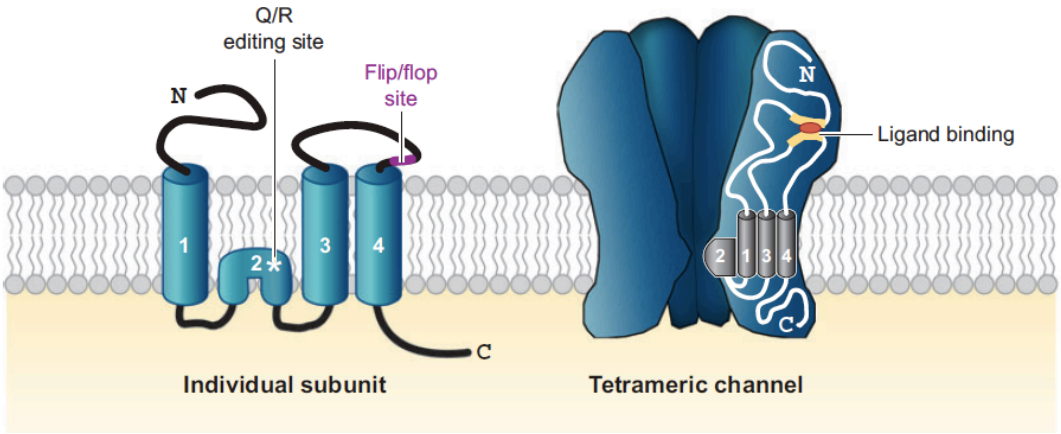

Fig. 5-3 Structure of the AMPAR subunits and the tetrameric channel. The individual subunits are composed of four transmembrane domains, and the channel consists of four subunits, which are usually two dimers. The dimers are usually two different subunits, such as GluR1 and -2 or GluR2 and -3 (Shepherd and Huganir, 2007)

Native AMPA receptor channels are impermeable to calcium, a function controlled by the GluR2 subunit. The calcium permeability of the GluR2 subunit is determined by the post-transcriptional editing of the GluR2 mRNA, which changes a single amino-acid in the TMII region from glutamine (Q) to arginine (R). This is the so called Q/R editing site -GluR2(Q) is calcium permeable whilst GluR2(R) is not. Almost all the GluR2 protein expressed in the CNS is in the GluR2(R) form, giving rise to calcium impermeable AMPA receptors.

The C-terminus of the GluR2 subunit contains binding sites for a large number of interacting proteins such as NSF, AP2, as well as a terminal PDZ domain that binds PICK1 and GRIP, while the GluR1 subunit interacts with SAP97. These interactions are crucial for understanding the role of these receptors in synaptic plasticity (see next chapter).

5.1.2 NMDA Receptors

NMDA receptors mediate postsynaptic current that has a slow rise time and decay time. NMDAR has some basic properties: it is an ion channel sensitive to Na^+, K^+ and Ca^{2+}, it is voltage-dependent because it needs a strong depolarization of the postsynaptic cell for its activation and it requires the binding of both glutamate and glycine to open. At resting membrane potentials, NMDARs are closed and ions cannot flow through the receptor channel due to a block by the Mg^{2+} ion. This is because at resting membrane potential, the driving force for Mg^{2+}, which is concentrated extracellularly, to enter the cell is high. The Mg^{2+} therefore competes with Na^+ and Ca^{2+} for access to the cell. However, as Mg^{2+} ions are too large to pass through the pore, the channel becomes effectively blocked. If the cell is depolarised then the Mg^{2+} block is removed and the current can flow (Dingledine et al., 1999). For these properties, NMDARs are known as “coincidence detectors” for postsynaptic depolarization and presynaptic release of glutamate.

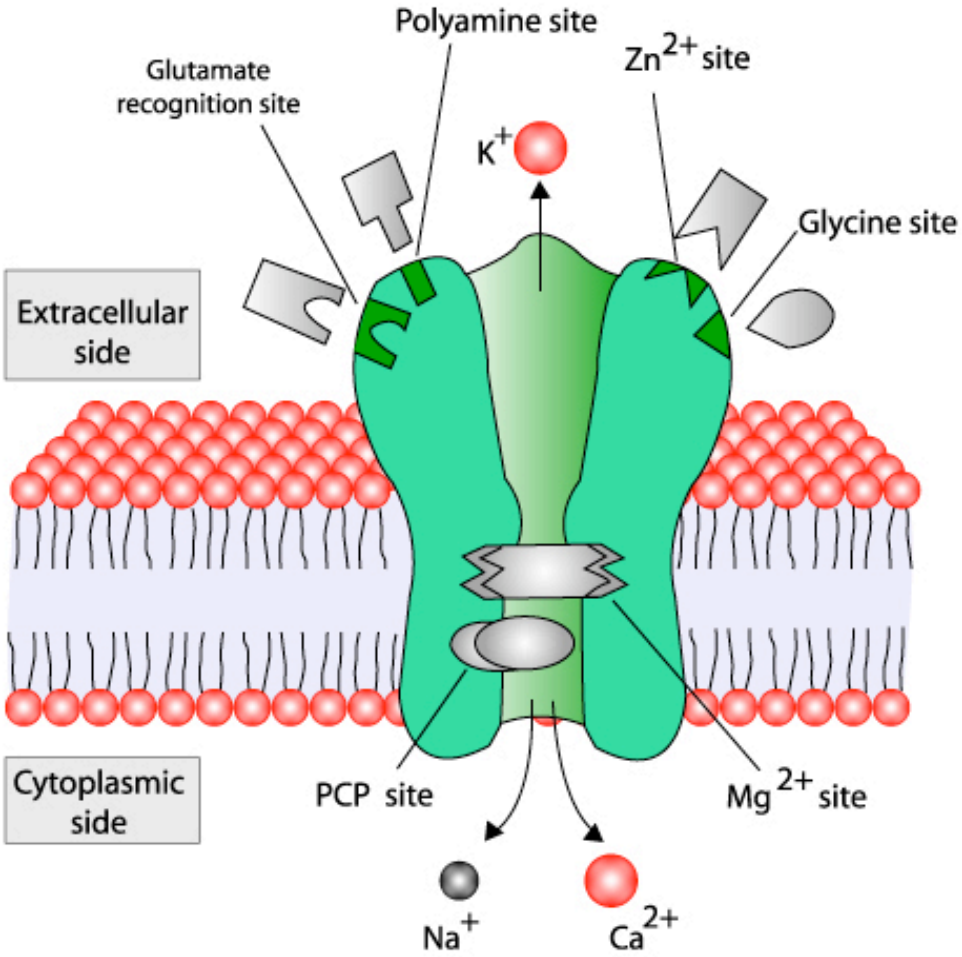

Fig. 5-4 Schematic representation of the structure of NMDA receptor complex (adapted from: http://www.frca.co.uk/article.aspx?articleid=100515).

NMDARs are heteromeric assemblies of NR1 (GluN1, with 8 different splice variants), NR2 (GluN2A, GluN2B, GluN2C and GluN2D) and NR3 (GluN3A and GluN3B) subunits that form ligand-gated channels with various cellular, biophysical and pharmacological properties depending on the composition of subunits and splice variants. Structurally they have the same membrane topology of ionotropic glutamate receptors. The c -terminal of both NR1 and NR2 interacts with several intracellular scaffolding proteins, it contains many serine/thereonine phosphorylation sites for proteins such as the cAMP -dependent protein kinase A (PKA), protein kinase C (PKC) and CaMKII (Chen and Roche, 2007), it is also involved in the regulation of receptor trafficking and function (Groc et al., 2009). Glutamate binds to NR2 subunits while the co-agonist glycine binds to the NR1 subunit. The NMDAR -dependent rise in

postsynaptic Ca^{2+} is important because it activates kinases (CAMKII, PKA, PKC and mitogen-activated protein kinase MAPK), and protein phosphatases, which ultimately results in an increase or decrease of AMPAR density and/or conductance relevant for long -term synaptic plasticity processes (Kerchner & Nicoll, 2008; Newpher & Ehlers, 2009) (Fig. 5-4).

5.1.3 Kainate Receptors

The last class of ionotropic glutamate receptors, the Kainate Receptors (KARs), have not been studied extensively so far, due to the lack of specific pharmacological antagonists. KARs are tetrameric receptor complexes composed of combinations of GluR5-7, KA1 and KA2, and have similar topology to AMPAR and NMDAR complexes. For the purpose of this manuscript I will not describe them in details.

5.2 Metabotropic glutamate receptors (mGluRs)

Metabotropic glutamate (mGlu) receptors are G-protein coupled receptors (GPCRs) that have been subdivided into three groups: mGluR1, mGluRII and mGluRIII, each group cointains different subunits for a total of eight sunbunits: mGlur1-8. The mGluRs bind glutamate within a large extracellular domain and transmit signals through the receptor protein to intracellular signalling partners. Group I mGlu receptors (mGlu1 and mGlu5) are positively coupled to PLC and intracellular calcium signalling, while group II (mGlu2 and mGlu3) and group III receptors (mGlu4**,** mGlu6**,** mGlu7 and mGlu8) are negatively coupled to adenylyl cyclase. The widespread expression of these receptors makes their study particularly interesting for drug targeting in neurological and psychiatric disorders such as Alzheimer's disease, parkinson's disease, anxiety, depression and schizophrenia.

5.3 Voltage -dependent calcium channels (VDCCs)

Voltage -dependent calcium channels are the best source of Ca^{2+} influx into neurons, muscles cells, endocrine cells and sensory cells. They activate in response to membrane depolarization. Electrophysiological studies have identified different forms of VDCCs, depending on their threshold of activation in response to depolarization. The high-voltage activated (HVA) channels require a strong depolarization and the low-voltage activated (LVA) channels that activate in response to a weak depolarization. The HVA are L -type, N -type, P/Q -type and R -type. The LVA comprises the T -type VDCC (see Fig. 5-5)

Channel	Current	Localisation	Specific antagonists	Cellular functions
$Ca_v1.1$	L	skeletal muscle transverse tubules	dihydropyridines, phenylalkylamines, benzothiazepines	excitation–contraction coupling
$Ca_v1.2$	L	cardiac myocytes, endocrine cells, neuronal cell bodies and proximal dendrites	dihydropyridines, phenylalkylamines, benzothiazepines	excitation–contraction coupling, hormone release, regulation of transcription, synaptic integration
$Ca_v1.3$	L	endocrine cells, neuronal cell bodies and dendrites	dihydropyridines, phenylalkylamines, benzothiazepines	hormone release, regulation of transcription, synaptic integration
$Ca_v1.4$	L	retina	not established	neurotransmitter release from rods and bipolar cells
$Ca_v2.1$	P/Q	nerve terminals and dendrites	ω-agatoxin IVA	neurotransmitter release, dendritic Ca^{2+} transients
$Ca_v2.2$	N	nerve terminals and dendrites	ω-CTx-GVIA	neurotransmitter release, dendritic Ca^{2+} transients
$Ca_v2.3$	R	neuronal cell bodies and dendrites	SNX-482	repetitive firing
$Ca_v3.1$	T	neuronal cell bodies and dendrites, cardiac myocytes	none	pacemaking, repetitive firing
$Ca_v3.2$	T	neuronal cell bodies and dendrites, cardiac myocytes	none	pacemaking, repetitive firing
Cav3.3	T	neuronal cell bodies and dendrites	none	pacemaking, repetitive firing

Fig. 5-5 Classification of the voltage dependent calcium -channels (adapted from International Union of Pharmacology)

All VDCCs contain a pore -forming subunit, the α_1 subunit that determines the main biophysical and pharmacological properties of the different forms of VDCCs. There are three families of the α_1 subunit: the Ca_v1 (that encodes the L-type VDCC); the Ca_v2 (that encodes the P/Q, the R -and the N -type VDCCs)

and the Ca_v3 (that encodes the LVA T -type VDCC). The LVA channels are thought to be formed by the only α_1 subunit (Catterall W., 2000).

The HVA are heteromultimeres formed by the α_1 subunit associated to three auxiliary subunits: $Ca_v\beta$, $\alpha_2\Delta$ and γ^2. The α_1 subunit of all channels ($Ca_v\alpha1$) is composed by four transmembrane domains, each consisting of six transmembrane helices (S1-S6). The segment four, S4, functions as the voltage sensor while S5 and S6 constitute the pore of the channels. The cytoplasmatic region of the α_1 subunit is important for phosphorylation and interaction with regulatory proteins, the intracellular c -terminal contains a binding site for calmodoulin CaM which mediates Ca^{2+} -triggered inactivation of the channels upon prolonged membrane depolarization (Catterall W., 2000). The intracellular subunit $Ca_v\beta$ has α helices but no transmembrane segments; the γ^2 is a glycoprotein with four transmembrane segments and α_2 is an extracellular, extrinsic membrane protein (Fig. 5-6).

Calcium currents recorded in different cell types have shown various physiological and pharmacological properties. For example, the L-type channels require a strong depolarization for activation, they activate and inactivate slowly and are blocked by the organic calcium channels antagonists (i.e. dihydropyridines, phenylalkylamines and benzothiazepines). The other HVA channels (P/Q-,N-,R -type) also require a strong depolarization and are blocked respectively by agatoxin, conotoxin and SNX -482.

Voltage-gated Ca^{2+} channels are critical for signalling, plasticity, and injury in the nervous system. Studies in knockout or natural mutant mice indicate that many of these channels provide a target for pharmacologic treatment of absence epilepsy, cerebellar ataxia, and neuropathic pain (for a review see Bennaroch, 2007).

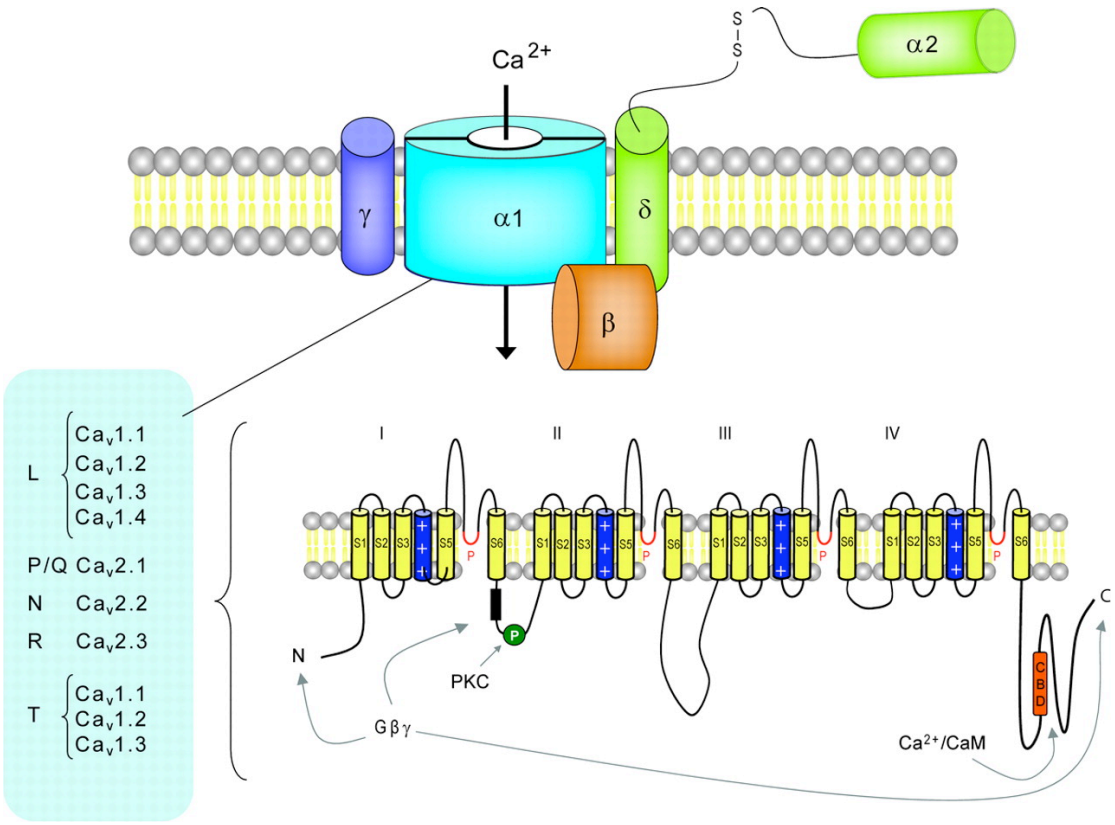

Fig. 5-6 General structure of voltage -gated calcium channels (VDCCs) (Bennaroch, 2007).

5.4 Cyclic Adenosine 3',5'-Monophosphate -dependent Protein Kinase A (cAMP -PKA)

The cAMP-dependent protein kinase, PKA is a second messenger -dependent enzyme (Smith et al., 1993) that has been linked to a wide range of cellular processes, including transcription, metabolism and apoptosis (Huggenvik et al., 1991, Hubbard et al., 1993, Matten et al., 1994).

PKA is composed of two regulatory (R) and two catalytic (C) subunits. The R subunits exist in two forms (RI and RII). Four genes encode the R subunits (RI-Alpha, RI-Beta, RII-Alpha and RII-Beta), and three encode the C subunits (C-Alpha, C-Beta and C-Gamma). Depending upon the associated RI and RII, PKA can be classified as Type I (predominantly located in the cytoplasm) and Type II (anchored to specific locations within the cell by A Kinase-Anchoring Protein, AKAP). Anchored PKA modulates the activity of various cellular proteins, including AMPA/Kainate channels, Glutamate receptor-gated ion channels, L-

type Ca^{2+} channels in skeletal muscle, hormone-mediated Insulin secretion in clonal beta cells, Vasopressin-mediated translocation of Aquaporin-2 into the cell membrane of renal principal cells, motility of mammalian sperm and the sperm Acrosome reaction (Dodge -Kafka and Kapiloff, 2006; Trewhella, 2006). Regulation of PKA in the cell is related primarily to modulation of its phosphotransferase activity. The holoenzyme contains two C subunits bound to homo -or heterodimers of either RI or RII subunits. The C subunits do not interact with one another. The R subunits each have an N-terminal dimerization domain and two cAMP binding sites.

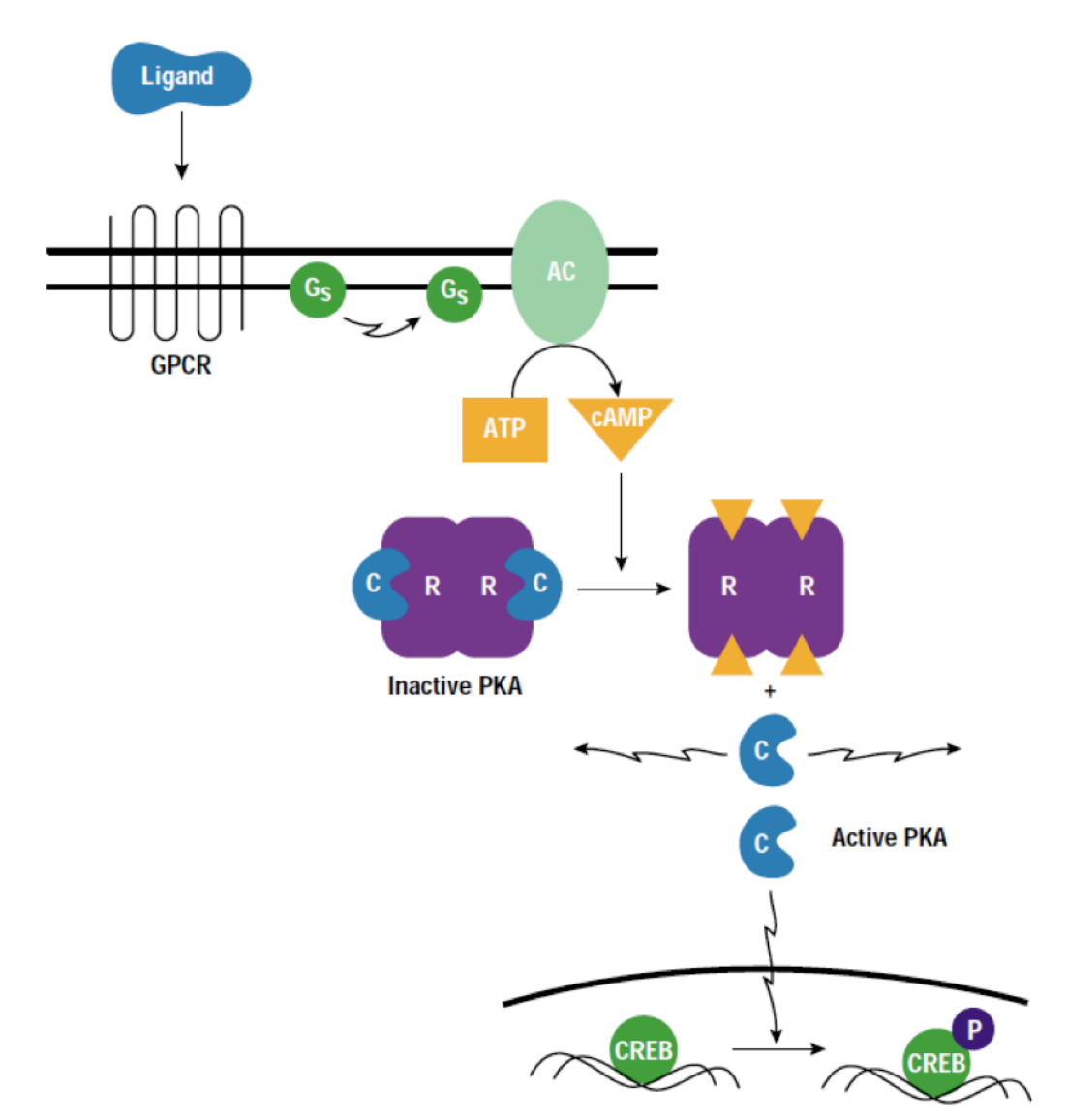

Fig. 5-7 Activation of PKA (from: http://homepages.strath.ac.uk/~dfs99109/BB329/MCSlect6.html).

Activation proceeds by the cooperative binding of two molecules of cAMP to each R subunit, which causes the dissociation and subsequent activation of each C subunit from the R subunit dimer (Fig. 5-7). cAMP is a cyclic nucleotide that serves as an intracellular and extracellular “second messenger” mediating the action of many peptide or amine hormones. The level of intracellular cAMP is regulated by the balance between the activity of two types of enzyme: AC (Adenylyl Cyclase) and the cyclic nucleotide PDE (Phosphodiesterase). Several receptors are responsible for the activation of cAMP-PKA pathway, GPCRs (G-Protein Coupled Receptors) being the most common receptors. When cyclic AMP levels are low, catalytic subunits are bound to a regulatory subunit dimer and are inactive. As the concentration of cyclic AMP increases, it binds to the regulatory subunits, leading to an allosteric change conformation which causes unleashing of the catalytic subunits. Free catalytic subunits are active and begin to phosphorylate their targets.

It has been shown that at the mossy fibers synapses, presynaptic LTP induction involves the cAMP/PKA pathway (Weisskopf et al., 1994). A similar sequence of events has been demonstrated to underlie presynaptic LTP induction at cerebellar parallel fiber synapse (Salin et al.,1996) corticothalamic synapses (Castro -Alamancos et al., 1999) and at cortico-lateral amygdala synapses (Fourcaudot et al., 2008).

6 Synaptic Plasticity

Synaptic plasticity is the ability of our neurons to change the efficacy or the strength of synaptic communication. These changes consist in either an enhancement or a depression of synaptic transmission at these synapses and they can be short- or long-lasting.

6.1 Short -term plasticity

Transient forms of synaptic plasticity have been associated with short-term adaptations to sensory inputs, transient changes in behavioural states and short-lasting forms of memory. Two forms of short-term plasticity are post-tetanic potentiation and post-tetanic depression (see Zucker and Regehr, 2002; Shepherd, 1998 for review).

Post-tetanic potentiation is a transient increase in the amplitude of a synaptic response that is seen after a brief train of stimuli. If a pair of stimuli is delivered and a potentiation of the second EPSP is observed, the phenomenon is defined 'paired-pulse facilitation' (PPF). This type of plasticity is largely believed to be pre-synaptic in origin (Bear et al., 1994). The first pulse leads to depolarisation of the presynaptic terminal and to an increase in intracellular Ca^{2+} that results in neurotransmitter release. If an optimal interval occurs between the first and second pulse, residual Ca^{2+} from the first pulse, plus the influx of Ca^{2+} due to the second pulse results in a greater increase in presynaptic Ca^{2+}. This increases the probability of glutamate release from a given synapse, which results in a global increase in the amount of transmitter released and therefore a subsequent greater postsynaptic response to the second pulse (Sheperd, 1998; Zucker et al., 2002).

Post-tetanic depression is also thought to rely primarily on presynaptic mechanisms (Zucker et al., 2002). Depression of a synaptic response can occur

if there is a repetitive activation of a synapse that leads to a transient depletion of the presynaptic pool of neurotransmitter, or by the action of an inhibitory neurotransmitter such as GABA. Depression may also result from desensitisation of postsynaptic receptors after repeated binding of neurotransmitter (Zucker et al., 2002). If a pair of stimuli is delivered and a depression of the second EPSP is observed, the phenomenon is defined 'paired-pulse depression' (PPD).

6.2 Long-term plasticity

Lasting changes of synaptic transmission are thought to play an important role in the construction of neural circuits during development, and in the formation of long-term memories in the mature nervous system. Long-term plasticity consists in an enhancement (LTP) or depression (LTD) of synaptic strength, that may persist for many hours, weeks or more.

6.2.1 Long-term potentiation

In the early 1970s Bliss and Lomo found that repetitive stimulation of the excitatory synapses in the hippocampus causes an increase in synaptic strength that could last for hours or even days (Bliss and Lomo, 1973); this mechanisms is now known as long-term potentiation. Since then this phenomenon has been widely studied in different brain regions and it became the best candidate for understanding the cellular and molecular mechanism by which memories are formed and stored.

The classical model of long-term potentiation is the one studied at excitatory synapses between Schaffer collaterals and commissural axons and the apical dendrites of CA1 pyramidal cells of the hippocampus. The basic properties of this form of LTP that occurs in the CA1 region of the hippocampus are: it is *input-specific,* it is elicited at the synapses that are activated by afferent activity and not at adjacent synapses on the same postsynaptic cell, it *is associative,* a

strong activation of a group of synapses can enhance the synaptic strength at adjacent synapses on the same cell if they are both activated within a small temporal window. Moreover it is triggered rapidly but can last for long periods of time. Three phases characterise the development of LTP: induction, expression and mantenance.

LTP induction

When a weak stimulation is applied to the presynaptic cell, its depolarization allows the release of neurotransmitter from the presynaptic vesicles. In excitatory synapses this neurotransmitter is usually glutamate and it binds to the postsynaptic AMPA receptors. The AMPA receptor is one of the main excitatory receptors in the brain, and is responsible for most of its rapid, moment-to-moment excitatory activity. Glutamate binding to the AMPA receptor triggers the influx of positively charged sodium ions (Na^{2+}) into the postsynaptic cell, causing a short depolarization called the excitatory postsynaptic potential (EPSP). NMDARs are present at postsynaptic membranes but at resting potential they are blocked by magnesium that does not allow calcium to enter the cell.

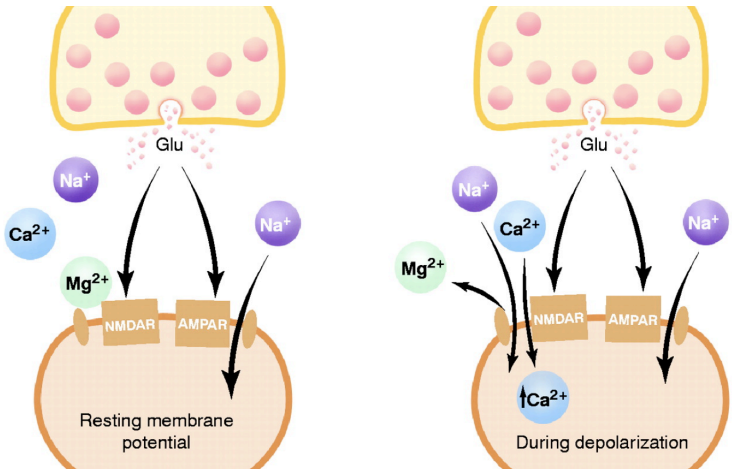

Figure 6-1 Model of the induction of LTP. During normal synaptic transmission, Glu is released from the presynaptic bouton and acts on both AMPARs and NMDARs. However, Na^+ flows only through AMPAR because the NMDAR is blocked by Mg^+ (left). Depolarization of the postsynaptic cell relieves the Mg^+ block of the NMDAR channel, allowing Na^+ and Ca^{2+} to flow into the dendritic spine by means of the NMDAR (right). The increase of calcium influx is the crucial trigger for LTP (Malenka et al., 1999).

When a set of repeated stimuli is given at high frequency (usually at 100 Hz), the postsynaptic cell is progressively depolarized. In synapses that exhibit NMDA receptor-dependent LTP, sufficient depolarization unblocks NMDARs from the magnesium ion block allowing Ca^{2+} flow into the cell. The rapid rise in intracellular calcium concentration triggers a series of processes that mediate the early phase of LTP (see Fig. 6-1). For example, the transient rise of calcium activates the calcium/ calmodulin-dependent protein kinase (CaMKII), present at high concentration in the postsynaptic density (PSD), that in turn activates different downstream signalling cascade that are important for the later expression of LTP (Fukunaga et al., 1993; Lee et al., 2009). Moreover, protein kinase A (PKA) can be activated during this early phase of long -term potentiation, especially during early postnatal development when CaMKII expression is low (Malinow et al., 1993; Yasuda et al., 2003). Protein kinase C (PKC) has also been found to be important for LTP induction (Hu et al., 1987; Bliss and Collingridge, 1993). Lately also the mitogen-activated protein kinase (MAPK) cascade that activates extracellular signal-regulated kinases (ERKs) (Sweatt, 2004), the phosphatidylinositol 3-kinase (PI3 kinase) (Man et al., 2003) and the tyrosine kinase Src (Salter and Kalia, 2004) have been shown to play a role in triggering LTP.

LTP expression

The expression of LTP is a phenomenon that can depend either on presynaptic or postsynaptic mechanisms, or both. When LTP is expressed postsynaptically (for example, in most of NMDAR -dependent LTP) two major postsynaptic mechanisms are involved: the increase in the number of AMPARs at the synapse via trafficking, and the modification of AMPARs via the phosphorylation of the GluR1 subunit (Malenka et al., 1999; Malinow et al., 2002; Song et al., 2002; Bredt et al., 2003; Lee et al., 2003; Malenka et al., 2004). In other cases, although LTP is triggered postsynaptically, it is expressed presynaptically through the activation of a retrograde messenger (e.g. nitric oxide) to communicate from the postsynaptic cell back to the presynaptic terminal (Malenka et al., 1999 and 2004). The identification of this last form of LTP still remains elusive. However, the coexistence of both forms of long -term potentiation has been shown at glutamatergic synapses in the lateral nucleus of the amygdala (LA). In fact, conventional pairing-induced LTP and spike timing-

dependent LTP in thalamic projections to the LA are expressed postsynaptically and may implicate trafficking of AMPA receptors at stimulated synapses (Humeau et al., 2005; Rumpel et al., 2005), whereas LTP in cortical input to the LA is expressed presynaptically, resulting from an increase in the probability of neurotransmitter release (Tsvetkov et al., 2002).

LTP maintainance

One of the most exciting feature of LTP is its long-lasting property. It has been shown both *in vitro* and *in vivo* that LTP can last for hours or even days and years (Abraham et al., 2002). The processes that permit long-term potentiation to be long-lasting depend on proteins synthesis and gene expressions. Signalling molecules that are thought to link LTP induction to changes in gene transcription include calmodulin-dependent protein kinase IV (CaMKIV), mitogen activated protein kinase (MAPK) and PKA, which act downstream to phosphorylate the transcription factor CREB and zif268 (Lynch, 2004b; Warburton et al., 2005; Miyamoto 2006; Reymann et al., 2007).

A key role is played by PKMζ, an isoform of PKC believed to be critical for the maintenance of LTP. PKMζ becomes upregulated and activated approximately ten minutes following tetanic stimulation via an unknown mechanism. Unlike the kinases involved in LTP induction (PKA, PKC, MAPK, CaMKII), PKMζz lacks a regulatory domain and therefore may remain 'persistently' active, sustaining AMPA receptor phosphorylation (Osten et al., 1996). The role of PKMζ has been extensively studied *in vitro* in the hippocampus (Sacktor et al., 1996) and *in vivo* in the neocortex (Shema et al., 2006) and in relation with specific behaviours such as fear conditioning (Serrano et al., 2008).

Recent lines of research show that LTP can be maintained in the absence of new protein synthesis, albeit under defined experimental conditions. For example, it has been shown that application of mature brain-derived neurotrophic factor (BDNF) stabilizes LTP for at least several hours, even during application of the protein synthesis inhibitor anisomycin (Pang et al., 2994; Santi et al., 2006). Thus, in the presence of sufficient BDNF, LTP can become protein synthesis-independent.

Although the NMDARs-dependent is the most common form of LTP, there are

evidences of LTP whose induction and expression do not require activation of NMDARs, such as the LTP at mossy fibers-CA3 synapses in the hippocampus (Nicoll and Malenka, 1995; Bortolotto et al., 2003). Another example is the LTP occurring at glutamatergic synapses between the neocortex and the lateral nucleus of the amygdala, that requires activation of presynaptic L -type voltage dependent calcium channels for its induction and expression (Fourcaudot et al., 2009).

6.2.2 Long -term depression

The first evidence for homosynaptic long-term depression (LTD) resulting from a low frequency stimulation (LFS), without prior induction of LTP, was demonstrated in the CA1 region *in vitro* (Dudek and Bear, 1992, Mulkey and Malenka, 1992). Since then, homosynaptic LTD has been studied in many brain areas: i.e. in the cerebellum, in the visual cortex, in the striatum, in the perirhinal cortex, in the amygdala and prefrontal cortex (see Kemp and Bashir, 2001 for a review).

The induction of NMDARs -dependent LTD occurs as for the induction of LTP: Ca^{2+} enters through NMDARs when the neuron is depolarised and the Mg^{2+} block is relieved. What differentiates the direction of synaptic plasticity is the quantity of calcium that is required, and the temporal course of calcium elevation. It has been proposed that LTP induction involves a marked elevation in Ca^{2+} concentration compared to a moderate rise for LTD (Ismailov et al., 2004). As a consequence of this model, moderate increase in Ca2+ favours phosphatase activation whilst a large increase favours kinase activation which, in turn, inhibits phosphatase activity. The resultant changes in synaptic efficacy are, therefore, opposite in direction. It also appears that a prolonged elevation of Ca^{2+} is crucial for the induction of LTD, and if the elevation is brief, at an equivalent Ca^{2+} concentration LTD will not be induced (Mizuno et al., 2001).

As for LTP, there is a developmental change in the efficiency of LFS protocols to induce LTD. However, the typical protocol for the induction of LTD is a prolonged repetitive stimulation at 0.5-5 Hz, and a robust change usually occurs after many stimuli, e.g. 900. Expression of LTD is mediated by two main

processes: the post-translational modification of AMPARs by dephosphorylation, and the physical loss of AMPARs from the synapse. The requirement for protein synthesis for a stable expression has been shown also for LTD, but in contrast to LTP it can not be blocked by inhibitors of mRNA transcription, but only by inhibitors of mRNA translation (Manahan -Vaughan et al., 2000; Sajikumar et al., 2003). Some forms of LTD are not dependent on NMDARs; one example is the mGluR-dependent LTD that has been observed in several brain regions (Kemp et al., 2001).

6.3 Synaptic plasticity in the Perirhinal cortex

The first study showing that synaptic plasticity in perirhinal cortex could be induced *in vitro* using evoked field recordings, dates back to the late 90s and demonstrated that LTP in PRh is input specific and NMDARs -dependent (Bilkey, 1996). Following this evidence, another study demonstrated that plasticity in the perirhinal cortex was both input- and layer-dependent (Ziakopoulos et al., 1999). NMDAR-dependent LTP could only be induced in intermediate (layer II/III) pathways and not in superficial (layer I) pathways, with an increase in magnitude of potentiation in the temporal vs. the entorhinal side. There are also differences in GABAergic transmission between temporal and entorhinal inputs, with differential regulation of inhibitory synaptic transmission (Garden et al., 2002), which are important in the control of neuronal activity in the perirhinal cortex (Wan et al., 2004). Long-term potentiation in PRh cortex has been shown to be NMDARs-dependent. In details, the application of the selective antagonist NVP-AAM077 demonstrated that induction of LTP requires NR2A-containing NMDARs (Massey et al., 2004). Conversely, application of L -type voltage dependent calcium channels antagonist does not block LTP in this brain region, while it impairs both LTD and depotentiation (Seoane et al., 2010). A study conduced *in vivo* and *in vitro* reported the importance of CREB phosphorylation for LTP in perirhinal cortex: in particular LTP maintenance was impaired after interference with CREB phosphorylation (Warburton et al., 2005). Also endogenous BDNF secretion is critical for synaptic plasticity in PRh cortex (Aicardi et al., 2004). This study reported that stimulations inducing LTP lasting

more than 180 min trigger a rapid, large increase in BDNF secretion that persists for 5-12 min. In contrast, stimulation procedures that induce only the initial phase of LTP lead to a very short-lasting (1 min) increase in BDNF secretion of much smaller magnitude. Moreover, LTP was prevented by BDNF sequestration by TrkB-IgG. These observations suggest that BDNF is required for LTP occurrence, and that prolonged duration of BDNF secretion at high levels is needed for LTP maintenance. Unlike LTP, they showed that LTD is accompanied by a reduction in endogenous BDNF secretion below the basal level. They found that a stimulation (5 Hz) inducing a LTD lasting more than 180 min causes a decrease in BDNF secretion during the period of stimulation. Unlike for LTP, the maintenance of LTD does not seem to require a more prolonged change in BDNF secretion (Aicardi et al., 2004).

Long-term depression in perirhinal cortex was first reported by Ziakopoulos and colleagues in 1999: a low -frequency stimulation at 1Hz induced NMDA -dependent LTD in the temporal intermediate pathway, but not in the other pathways (Ziakopoulos et al., 1999). The role of mGlus in LTD in the PRh cortex has been widely studied (McCaffery et al., 1999; Cho et al., 2000, 2002). Harris et al. (2004) have shown in cultured perirhinal neurones that mGluR2 activation evokes a reduction in basal cAMP levels, which could lead to increased mGluR5 function via reduced PKA mediated phosphorylation and decreased desensitisation of mGluR5 (Harris et al., 2004). It has recently been reported that a developmental change in plasticity mechanisms occurs in the perirhinal cortex (Jo et al., 2006). LTD was shown to switch in a visual-experience manner from an mGluR5 -to a mAChR-dependent form. This study suggests that the early experience-independent mGluR5 form of LTD is important in early processes of synaptic circuitry development before eye opening. A recent study demonstrated that NMDAR -LTD in PRh cortex requires the calcium sensor calmodulin, while mGluR-LTD depends specifically on NCS-1, the prototypic member of the NCS family, that binds directly to the BAR domain of PICK1 in a Ca^{2+}-dependent manner and that the association between these two proteins is enhanced following stimulation of mGluRs (Jo et al., 2008). Since the role of acetylcholine has been found to play a crucial role in learning and memory in the perirhinal cortex (Massey et al., 2001; Warburton et al., 2003), other studies *in vitro* have been carried; in particular, it has been

shown that pharmacological activation of muscarinic acetylcholine receptors using the agonist carbachol results in long-lasting depression that can be blocked by the M1 receptor antagonist pirenzipene; the induction was independent of NMDARs (Massey et al., 2001). The application of the muscarinic receptor antagonist scopolamine blocked LTD *in vitro* and recognition memory *in vivo*, but LTP was unaffected (Warburton et al., 2003). This suggests that cholinergic mechanisms in the perirhinal cortex play an important role in synaptic plastic mechanisms.. Recently, a study reported a form of LTD that is not dependent upon NMDARs; it appears that Ca^{2+} influx through the KARs themselves, which then triggers Ca^{2+} release from internal stores, is sufficient to induce LTD. However, this form of LTD is only present in young animals, cannot be induced in adolescent animals (Park et al., 2006). Recently it has been suggested that LTD could underlie visual recognition memory in perirhinal cortex (Griffiths et al., 2008). LTD relies on internalization of AMPA receptors through interaction between their GluR2 subunits and AP2, the clathrin adaptor protein required for endocytosis. In this study the authors show that the blockade of GluR2-AP2 interaction blocks LTD in perirhinal cortex *in vitro* and impairs visual recognition memory *in vivo*. This effect was explained by a specific block of AMPAR endocytosis, resulting in preventing the expression of NMDAR-dependent LTD. Furthermore, in perirhinal cortex slices from virally transduced, recognition memory-deficient animals, there was a deficit in LTD but not in LTP. These results suggest that internalization of AMPA receptors, a process critical for the expression of LTD in perirhinal cortex, underlies visual recognition memory.

6.4 Synaptic plasticity in the amygdala

It has been widely recognized that long-term potentiation in the amygdala underlies fear conditioning learning. According to this cellular hypothesis, fear conditioning is mediated by an increase in the strength of synapses that transmit CS information to principal neurons in the LA (Rogan et al., 1995; Rogan et al., 1997, 2001; LeDoux, 2000; Blair et al., 2001; Maren, 2001). This model states that prior to conditioning, the CS inputs are relatively weak and

therefore the CS is unable to elicit fear responses. In contrast, the US inputs are stronger and capable of eliciting robust responses in LA neurons. Because CS and US inputs converge onto LA neurons (Romanski et al., 1993b), during fear conditioning the CS inputs are active during strong postsynaptic depolarization caused by the US. As a result, the CS inputs become stronger, making the CS more effective at driving LA neurons, which in turn can drive downstream structures that control fear responses such as the central nucleus (Blair et al., 2001). To study the mechanism of fear conditioning *in vitro*, two inputs are stimulated: the internal capsule from which thalamic auditory inputs originate and the external capsule, source of cortical auditory inputs to the LA (Chapman and Bellavance, 1992; Huang and Kandel, 1998; Weisskopf et al., 1999b). When LTP is induced stimulating the internal capsule, two different pharmacological forms of post-synaptically induced and expressed LTP (Humeau et al., 2005) are observed, depending on the protocol of stimulation used. Using a pairing protocol, in which weak presynaptic stimulation of thalamic afferents is paired with a brief depolarization of the post -synaptic cell, results in a form of LTP that is VDCCs -dependent and NMDARs -independent (Weisskopf et al., 1999b). Using a tetanus that produced prolonged depolarizations of the pos -synaptic cell, results in a VDCCs -independent LTP that was blocked by NMDARs antagonist (Bauer et al., 2002). When LTP in the amygdala is induced by stimulating the cortical afferents, the result is a post -synaptic induced NMDARs -dependent LTP that involves PKA activation (Huang and Kandel, 1998). The authors demonstrated that this form of LTP was blocked by the cAMP-PKA antagonist RpcAMP and they also showed that application of an activator of the cAMP-PKA pathway (forskolyn) resulted in a potentiation that occlueds LTP induced by tetanus. Another form of LTP, probably the most important for its implication in fear conditioning, is the associative LTP induced by the co -activation of the thalamic and the cortical inputs onto principal neurons in the LA. The authors have reported that this form of LTP is induced and expressed presynaptically. Repeated stimulation of thalamic afferents transiently increases the probability of release at cortical afferents in a NMDARs -dependent manner and that expression of LTP might involve a more persistent increase in the probability of release at cortical synapses. The expression of this form of LTP depends on the persistent

enhancement of presynaptic L-VDCCs efficacy and involves PKA -dependent mechanisms (Humeau et al., 2003; Fourcaudot et al., 2008, 2009).

Although many studies have described LTP in the amygdala, only a few studies have indicated that long-term depression occurs in neurons within the lateral amygdala. Only recently, a study demonstrated that homosynaptic LTD could be induced at the lateral -basolateral amygdalar (LA -BLA) synapses by prolonged low frequency stimulation (1Hz, 15 min) (Wang and Gean, 1999). This form of LTD requires the activation of NMDARs, an increase in postsynaptic calcium influx and phosphatase activity, thus it seems to be postsynaptically induced. A following study has reported the role of presynaptic group II metabotropic glutamate receptors (mGluRII) in the induction of LTD at LA-BLA synapses. Further studying investigated long -term depression in the LA and showed that L-type calcium channels (Tchekalarova and Albrecht, 2007) and mGluRII are involved in induction of LA-LTD, and that both NR2A - and NR2B -subunits of NMDARs are crucial for the induction of LTD in the LA (Müller et al., 2009; Kaschel et al., 2004).

7 Aims

A common feature of memory is that emotionally salient events are better remembered than neutral ones. This property is of great benefit in every day life but when it deteriorates, it can lead to a series of neuropsychological conditions, for example post -traumatic stress disorder (PTSD). Given that synaptic plasticity in the amygdala is important for consolidation of memory for emotional salient stimuli (Bauer et al., 2002), while synaptic plasticity in the perirhinal cortex is crucial for recognition memory (Griffiths et al., 2008), it is interesting to study how amygdala influences perirhinal cortex. There is a large body of evidence for the existence of anatomical connections between amygdala and perirhinal cortex while much less is known about the functional interaction between these two areas. For example, a study has demonstrated that activation of the noradrenergic system in the basolateral amygdala influences recognition memory (Roozendaal et al., 2008). In fact, norepinephrine administered after 3 min of object recognition training produced dose-dependent enhancement of 24-h object recognition memory whereas propranolol administered after 10 min of training produced dose-dependent impairment of memory (Roozendaal et al., 2008). These findings provide evidence that post-training noradrenergic activation of the BLA enhances memory of a low-arousing training experience that would otherwise not induce long-term memory. Other studies investigating functional connections between amygdala and PRh have reported that *in vitro* single stimulation of either the perirhinal cortex or amygdala did not result in sufficient neural activation of the deep layers of areas 35 to provoke activity propagation into the entorhinal cortex. However, the deep layers of area 35 were depolarized much more strongly when the two stimuli were applied simultaneously, resulting in spreading activation into the entorhinal cortex. These observations suggest that a functional neural basis for the association of higher order sensory inputs and emotion-related inputs exists in the perirhinal cortex and that transfer of sensory information to the entorhinal-hippocampal circuitry might be affected by the

association of that information with incoming information from the amygdala (Kajiwara et al., 2003). Moreover, it has been proposed that amygdala inputs can facilitate the responsiveness of perirhinal cells to neocortical inputs (Pelletier et al., 2005) and that, during an appetitive trace -conditioning task, activity of the basolateral amygdala increased impulse transmission from perirhinal to entorhinal neurons. This effect disappeared once the animal learned to anticipate the reward by learning the association between conditioned stimuli and rewards (Paz et al., 2006).

So far, some evidence for a functional interaction between these two areas has been proposed but the molecular and cellular mechanisms underlying this interaction are still unknown and no research has investigated yet the impact that amygdala activity has on the synaptic plasticity processes that underlie learning in perirhinal cortex.

The aim of this study is to define for the first time the basic properties of synaptic transmission and the mechanisms of plasticity at the amygdala - perirhinal cortex synapses and also to describe how activity at the amygdala - perirhinal cortex synapses influences synaptic plasticity within the perirhinal cortex. Results from this study will provide a model of the mechanisms by which emotions influence recognition memory.

8 Materials and Methods

8.1 Materials

8.1.1 The rig

Electrophysiological recordings *in vitro* require basic elements: a recording chamber, a recording electrode, a stimulation electrode, an amplifier and a stimulation box, and a microscope. Constant flow (flow rate set to 2ml/min) of oxygenated artificial Cerebral Spinal Fluid, aCSF (95%O_2/ 5%CO_2) at 28-30 ^{o}C is pumped by a peristaltic pump (Watson-Marlow Ltd, UK), though polythene tubing (ID 1.4 mm, OD 1.9mm, Portex Ltd, UK) up to a 2 ml syringe where aCSF is again bubbled with oxygen and carbon dioxide to ensure saturation. The aCSF was further heated (30-32 °C) by a heating system that was connected with the chamber. This allows to better control the temperature of the chamber and to keep it constant within and between experiments avoiding the formation of air bubbles inside the recording chamber. Waste aCSF was removed by suction from the recording chamber (Charles Austen Pumps Ltd, UK) via a plastic pipette. Slices were maintained in a submerged recording chamber (containing about 1.5ml of solution) and held in place by a nylon mesh stretched over a U-shaped piece of twisted temper annealed silver wire (0.55 mm diameter, Advent Research Materials, Oxon, UK) affixed to a nylon mesh (Fig. 8-1). A Nikon microscope (x40 magnification) was positioned directly above the recording chamber. Stimulation electrodes were positioned either side of the recording chamber while the recording electrode was placed right in the front. The three electrodes were mounted on magnetic stands (Narishige, Japan) onto an anti-vibration metal platform to minimise vibrations and prevent drift of equipment. All electrical equipment was earthed.

Recording and bath electrodes

Recording pipettes were made from fine borosilicate glass capillaries (1.5mm external diameter, 0.86mm internal diameter; Harvard Apparatus, UK). An electrode puller (PC-10 micropipette vertical puller, Narishige, Japan) was used to produce micropipettes with a resistance of 2-5 MΩ. Micropipettes were filled with aCSF and placed in an electrode holder (Axon Instruments, Union City, USA), containing the chloride silver wire recording electrode. The electrode holder was connected to a headstage (CV-4, Axon Instruments, USA) mounted on a micromanipulator (MWS-32, Narishige, Japan). Silver wire (0.25 mm diameter, Advent Research Materials Ltd, UK) was used to make both recording and reference bath electrodes. The wire was chlorided in bleach overnight. This helps to reduce noise and DC drift during recording. Chloride ions can be soluble in solution and this allows current to flow in both directions through the electrode. The recording electrode was attached to the headstage and the reference bath electrode was fixed around the recording chamber and grounded to the headstage.

Stimulation electrodes and protocol

Bipolar stimulating electrodes were made by twisting together two strands of insulated 0.05 mm nickel-chromium wire (Advent Research Materials Ltd, UK). The strands were passed through a glass micropipette for protection and held in place with Blue Tack™. The electrodes were mounted onto a lightweight manipulator stand (Narishige, Japan) and individually connected to stimulus isolation units (Digitimer Ltd, UK) (S. Griffiths, PhD Thesis, University of Bristol, 2007). Analogue signals from the headstage were low-pass filtered (cut off: 5kHz) before being amplified by an Axopatch amplifier (200B) and converted into digital data using an analogue-digital (A/D) data board (Digidata 1200, Axon Instruments, USA). Digital data were recorded on a PC using the software package Ltp230d (Anderson and Collingridge, 2001) with a sampling frequency of 10kHz.

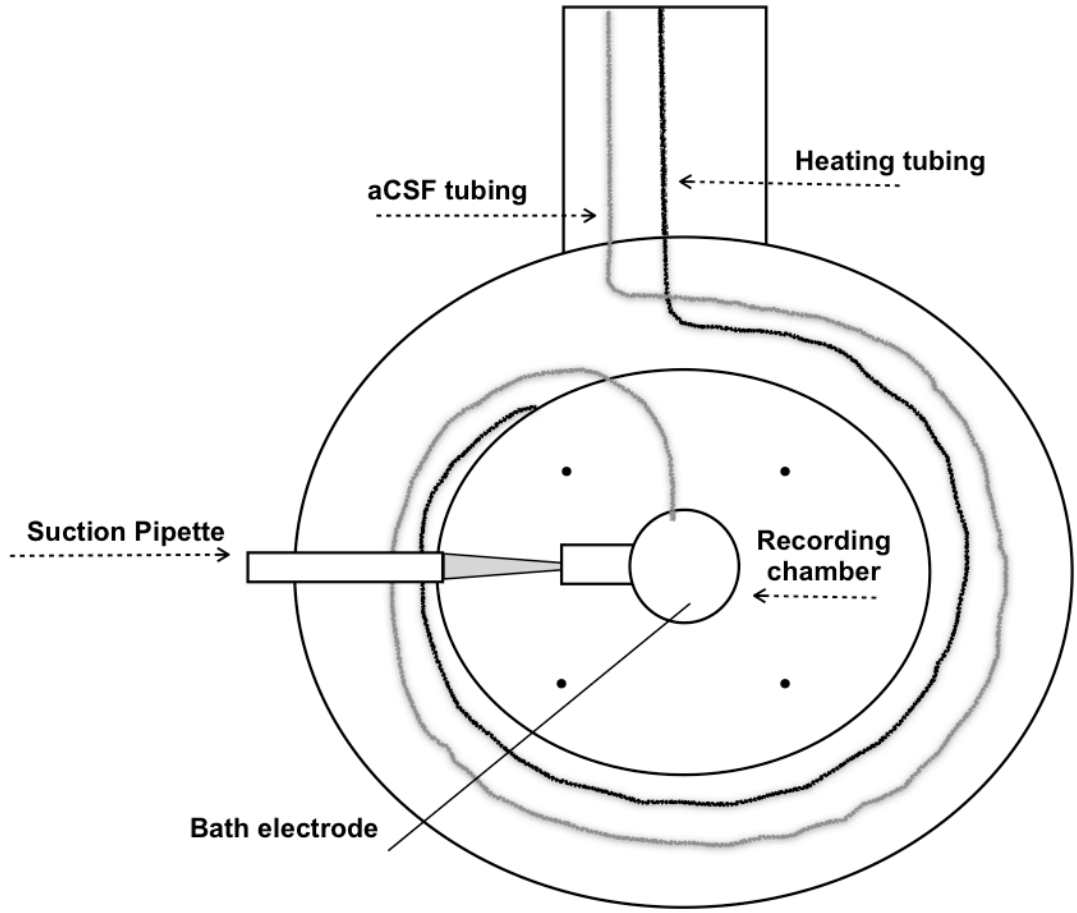

Fig. 8-1 The recording system (adapted from S. Griffiths, PhD Thesis, University of Bristol, 2007)

8.2 Method

8.2.1 Animals

Juvenile male pigmented Dark Agouti rats between 28 -35 days of age (55-90g) supplied by Harlan Laboratories were maintained on a 12 h light/12 h dark cycle (dark phase during normal daylight). All efforts were made to minimise animal suffering, and experiments were performed in accordance with the UK Animals (Scientific Procedures) Act (1986) and had approval from the University of Bristol Ethics Committee.

8.2.2 Preparation of slices

Every effort was made to minimise the number of animals used and reduce suffering and pain. Animals were anaesthetised with isoflurane and medical oxygen until the pedal withdrawal reflex and blinking reflex had ceased. A guillotine was used to decapitate the animal in accordance with the UK Animals (Scientific Procedures) Act 1986 (ASPA revised 1997). The brain was rapidly removed and submerged in ice-cold aCSF (aCSF; bubbled with 95% O_2/5% CO_2, pH 7.4) which comprised the following (in mM): 124 NaCl, 3 KCl; 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgSO_4$, and 10 D-glucose. The olfactory bulb, the cerebellum and the brain stem were removed. The brain was divided at the midline and each hemisphere was positioned with the medial face down. Then, the dorsal side of the brain was cut along a plane orthogonal to the sagittal plane, which was tilted at a 10° posterosuperior-anteroinferior angle of a line passing between the lateral olfactory tract and the base of the brain stem (Stoop and Pralong, 2000; Fig. 8-2). The brain was glued on its dorsal side to a vibroslice stage (Campden Instruments, Sileby UK) using cyanoacrylate adhesive. Three ventral slices (400 µm thick) between ~7.6 and 7.10 mm from Bregma as described by Paxinos and Watson rat brain atlas were obtained from the cut of a single hemisphere, each containing area 35 of perirhinal cortex and the lateral nucleus of the amygdala, the most dorsal slice contained also a small portion of area 36. The two hemispheres were cut separately, in a randomized order. After cutting, slices were stored submerged in aCSF (20–25°C) for 1–6 h before transferring to the recording chamber. A single slice was placed in a submerged recording chamber (30-32°C; flow rate ~2 ml/min). Slices were stored submerged in aCSF (20–25°C) for 1–6 h before transferring to the recording chamber. A single slice was placed in a submerged recording chamber (28–30°C; flow rate ~2 ml/min) when required. This was made to allow to recover and equilibrate for at least one hour before recording commenced.

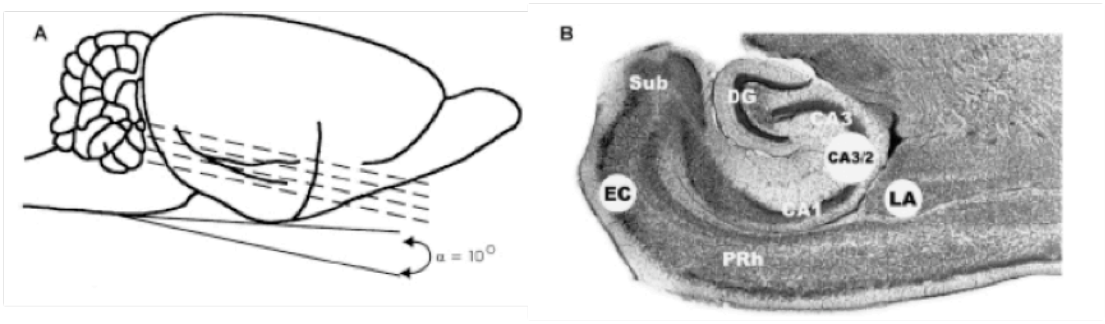

Fig. 8-2 Preparation of the slice. A) Part of the right hemisphere with the surface exposed that will be glued to the cutting block at a 10° angle to the plane touching the lateral olfactory tract and the base of the brain stem. Dashed lines indicate the positions along which the near horizontal slices of 400um thickness were obtained. B) Horizontal slice stained with cresyl violet, including the hippocampus, the entorhinal cortex, the perirhinal cortex and the amygdala (Adapted from Stoop and Pralong, 2000). Abbreviations. Sub, subiculum; DG, dentate gyrus; EC, entorhinal cortex; PRh, Perirhinal cortex; CA1, CA3 of the hippocampus; LA, lateral amygdala.

8.2.3 Data analysis

Data was reanalysed using the Ltp230d reanalysis program, single sweeps were averaged offline every 4 sweeps, except during trains when sweeps were averaged every 50 sweeps. Reanalysed data was imported into Sigmaplot (Jandel Scientific, Germany) for analysis and for pooling purposes. For experiments involving a baseline, data for the whole experiment was normalised to the mean of the points that comprised the baseline. For experiments involving drug application, the baseline was used only if there was no significant difference between baseline and perfusion, otherwise 10 minutes of perfusion were considered to be the baseline for data normalisation. The significance of plasticity changes from baseline was established using either parametric (paired or unpaired *t* tests or ANOVAs with repeated measures) or non -parametric (Mann-Whitney t-test or Kruskal -Wallis or Friedman's ANOVA) as appropriate. A significance level of $p<0.05$ was used. Non-parametric tests were used when the normality test (Shapiro-Wilk) failed ($p<0.05$).

8.2.4 Extracellular field recordings

In vitro extracellular field recordings were made from the perirhinal cortex (Ziakopoulos et al., 1999; Massey et al., 2004). Evoked field EPSPs (fEPSPs) were recorded with a microelectrode (glass micropipette filled with aCSF, 2-5 MΩ) placed in layers II/III of area 35. One stimulating electrode was placed on one side (~0.5 mm) of the recording electrode and designated the intracortical input (PRh/PRh). A second stimulating electrode was placed in the lateral nucleus of the amygdala, just below the external capsule (ExtC) and designated the subcortical input (LA/PRh) (see Fig. 8-3) Stimuli (current costant) were delivered alternately to the two bipolar stimulating electrodes (each electrode 0.033 Hz). Input/output curves were produced by stimulating PRh/PRh input initially at "minimal" intensity (sufficient to produce a fEPSP discernable from the noise), then intensity was increased in 3 V steps until a maximal fEPSP was achieved. The minimal intensity across experiments ranged between 0.2 and 0.4 mV. fEPSPs were reduced to 60% of maximum amplitude to achieve a baseline of synaptic transmission before induction of synaptic plasticity. In the LA/PRh, input/output curves were not obtained since the amplitude of the maximal fEPSPs obtained ranged between 0.03 and 0.1 mV. For this reason, fEPSPs were maintained at their maximum amplitude. GABAergic inhibitors were not used for these experiments. A period of stability lasting for 30 minutes or more was required to establish a baseline before high-frequency stimulation was delivered to induce LTP. The protocols used are 1) 4 trains, each of 100 Hz, 1 s, every 15 s (4HFS); 2) 45 pulses at 100 Hz (underthreshold HFS); and 3) paired HFS in which the underthreshold HFS was delivered to PRh/PRh the 4HFS to LA/PRh, after the first two trains (PHFS). Field potentials were recorded and reanalysed off-line (Anderson and Collingridge, 2001). The peak amplitude of evoked fEPSPs was measured and expressed relative to the preconditioning baseline. LTP was measured at 60 or 90 min after induction. The amplitude of fEPSPs were measured rather than the initial slope, since in the perirhinal cortex the initial slope of the response is generally obscured by non-synaptic potentials. At the end of the experiment, Ca^{2+}-free aCSF was bath applied. This eliminated the synaptic component of the response and allowed

the subsequent exclusion of the non-synaptic component when reanalysing the peak amplitude.

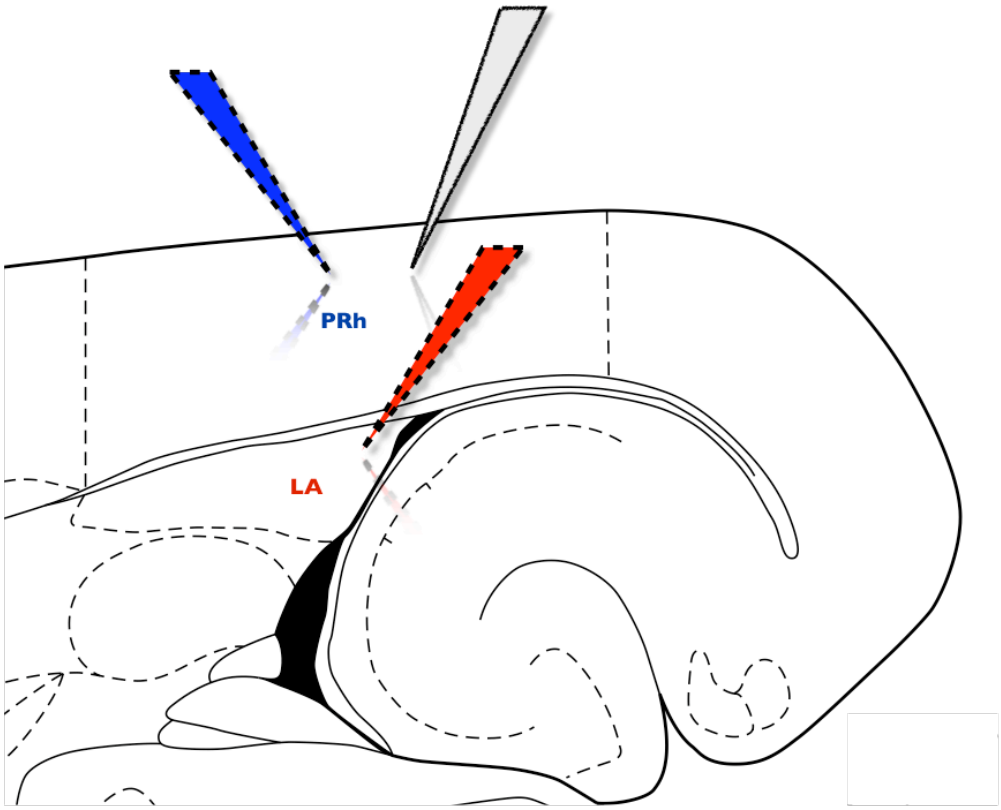

Fig. 8-3 Schematic representation of the approximate position of the recording electrode (grey) and the two stimulating electrodes (blue and red).

8.2.5 Synaptic Responses

Synaptic Responses evoked by the two stimulating electrodes differ in terms of latency and amplitude. The response obtained from PRh/PRh neurons is bigger and has a shorter latency (~0.5 mV; 5 -8 ms) compared to the LA/PRh response (~0.05 mV; 7 -25 ms).

8.3 Pharmacological agents

Pharmacological compounds were bath applied as appropriate at the following concentrations:

100uM D-AP5 (D-2-amino-5-phosphonopentanoate) (Ascent Scientific, UK)

20uM or 50 uM verapamil (5-[*N*-(3,4-Dimethoxyphenylethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile hydrochloride) (Sigma Aldrich, UK)

50 uM forskolyn (3*R*,4a*R*,5*S*,6*S*,6a*S*,10*S*,10a*R*,10b*S*)-6,10,10b-trihydroxy-3,4a,7,7,10a-pentamethyl-1-oxo-3-vinyldodecahydro-1*H*-benzo[*f*]chromen-5-ylacetate) (Tocris Bioscience, Bristol, UK)

10uM H89 (*N*-[2-[[3-(4-Bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride) (Tocris Bioscience, Bristol, UK).

Stock solutions were made by dissolving drugs into ddH_20 or Dimethyl sulfoxide (DMSO) and stored at -20 ^oC. All stock solutions were made ≥ 100x the concentration required. Pharmacological compounds were bath applied as appropriate in different experiments.

9 Results

9.1 Results: Long-term potentiation at PRh/PRh and LA/PRh synapses

EPSPs were recorded from layer II/III of PRh cortex when stimulating neurons in the perirhinal cortex and neurons in the lateral nucleus of the amygdala. The aim of this section is to investigate the basic properties of long-term potentiation at LA/PRh neurons.

9.1.1 Long-term potentiation (LTP) at PRh/PRh synapses

Previously published works have shown that tetanic stimulation (four trains, each of 100 Hz, 1 second, every 15 seconds: 4HFS) delivered to PRh neurons induces LTP in PRh cortex of adult Dark Agouti (DA) rats (Ziakopolous, et al. 1999). We confirmed that the same protocol of stimulation induces LTP in perirhinal cortex of young DA rats (postnatal day 30) as shown in Fig. 9-1. Paired t-test between fPESPs during the last ten minutes of baseline (1) and the last ten minutes of LTP (2) shows a statistical significant difference between 1 and 2 ($p<0.01$).

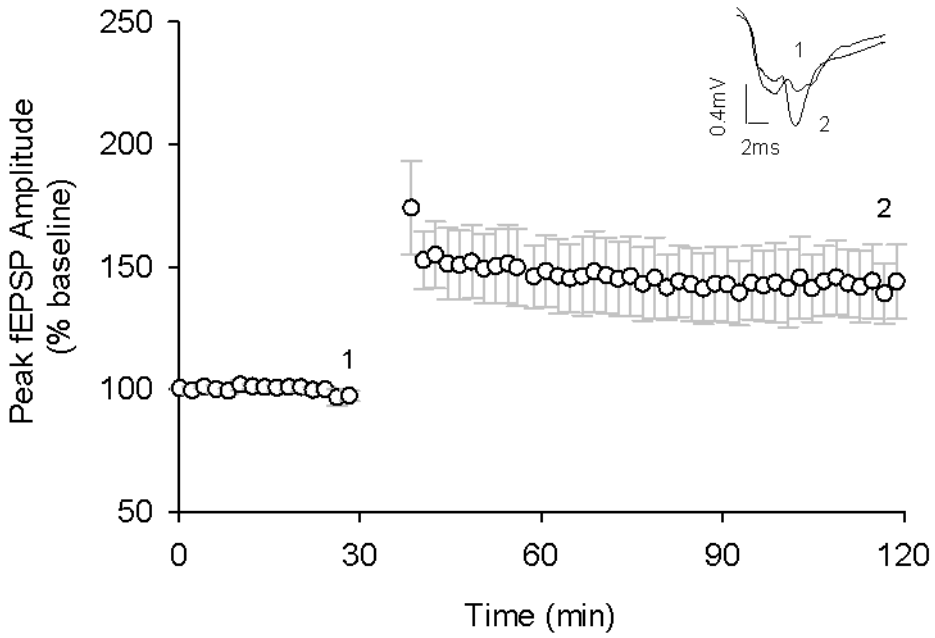

Fig. 9-1 LTP at PRh/PRh synapses. Pooled data n=6. The peak amplitude of fEPSPs at 50-60 minutes after the tetanic stimulation (2) is significantly higher (~45%) compared to the last ten minutes of baseline (1). 2 vs 1= $p<0.01$(**). Top right traces show the peak amplitude of the last point of baseline and the last point of LTP.

9.1.2 Long-term potentiation (LTP) at LA/PRh synapses

The following graph (Fig. 9-2) shows that tetanic stimulation (4HFS) of LA fibers induces LTP in perirhinal cortex. LTP was measured, compared to baseline, one hour after the induction protocol to the tested pathway. In this set of experiments PRh/PRh pathway served as a control to show that the recording was stable. The normality test used (Shapiro-Wilk) failed ($p<0.05$) with these data set, therefore we used the Friedman repeated measures analysis of variance on ranks which revealed a statistically significant interaction between time (baseline, 1 and LTP, 2) and stimulating pathway (control pathway, PRhPRh and experimental pathway, LA/PRh). Post hoc analysis with the Dunn's multiple comparisons test revealed that within the control pathway no significant difference was seen between time points 1 and 2 demonstrating that the recording was stable, while at LA/PRh synapses the peak amplitude of

fEPSP at 80-90 minutes (2) was significantly different than the fEPSP at 20-30 minutes (1) (2 vs 1 $p<0.01$).

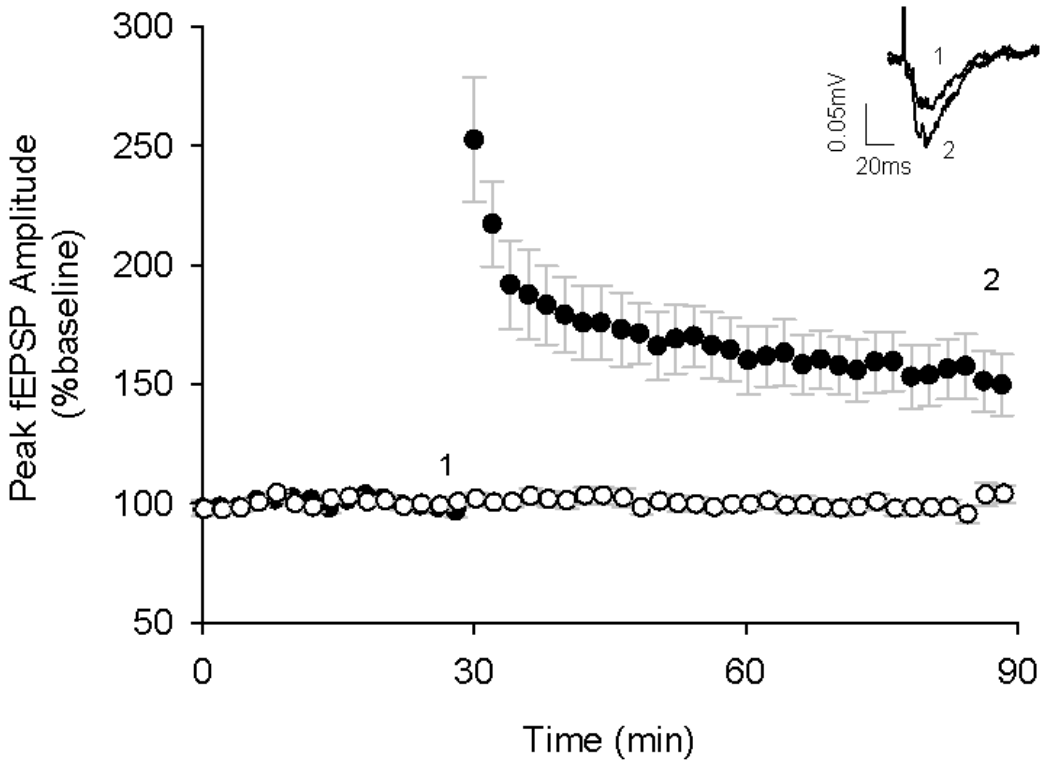

Fig. 9-2 LTP at LA/PRh synapses. Pooled data n=8. The peak amplitude of fEPSPs at 50-60 minutes after the tetanic stimulation (2) is significantly higher (~55 %) than the last ten minutes of baseline (1). At LA/PRh synapses (filled circles) 2 vs 1, $p<0.01$(**). Open circles= PRh/PRh pathway (control pathway). Top right traces show the peak amplitude of the last point of baseline and the last point of LTP.

9.1.3 Effects of D-AP5 upon LTP at PRh/PRh synapses and LA/PRh synapses

In another set of experiments we recorded a stable baseline (1) and then we bath applied 100μM D-AP5 (2) from 30 minutes before the 4HFS up to four minutes after the protocol of stimulation. Perfusion with D-AP5 did not affect the induction of LTP (3) at LA/PRh synapses. Two way repeated measures ANOVA revealed a significant difference between conditions (control pathway, PRh/PRh and experimental pathway, LA/PRh) along time ($p<0.01$). Post hoc analysis (with Bonferroni correction) revealed that within the control pathway no

significant difference was present between time points 1, 2 and 3 demonstrating that the recording was stable. While at LA/PRh synapses the peak amplitude of fEPSP at 110-120 min (LTP) (3) was significantly different than the fEPSP at 20-30 min (baseline) (1) and at the 50-60 min (drug application) (2). (3 vs 1 and 2 p<0.001***) (Fig. 9-3). In a set of experiments we showed opposite effects of 100 μM of D-AP5 on LTP when comparing LTP at both pathways in the same group of slices: at LA/PRh synapses perfusion was not significantly different from baseline, and LTP induced in the presence of D-AP5 (3) and LTP induced after the drug washout (4) were not statistically different. LTP (3 and 4) were different from perfusion, but not from baseline (4 vs 2 p=0.008**; 3 vs 2 p<0.02*). Perfusion with D-AP5 instead blocked LTP at PRh/PRh cortex, that was stably induced after the drug washout (4 vs 1 p=0.002**, 4 vs 2 p<0.001***, 4 vs 3 p=0.002**) (Fig. 9-4). We therefore demonstrated that LTP at LA/PRh synapses is NMDAR-independent while we showed that LTP at PRh/PRh synapses is NMDAR-dependent in young animals, similar to what was reported in coronal slices of adult animals (Ziakopolous et al., 1999).

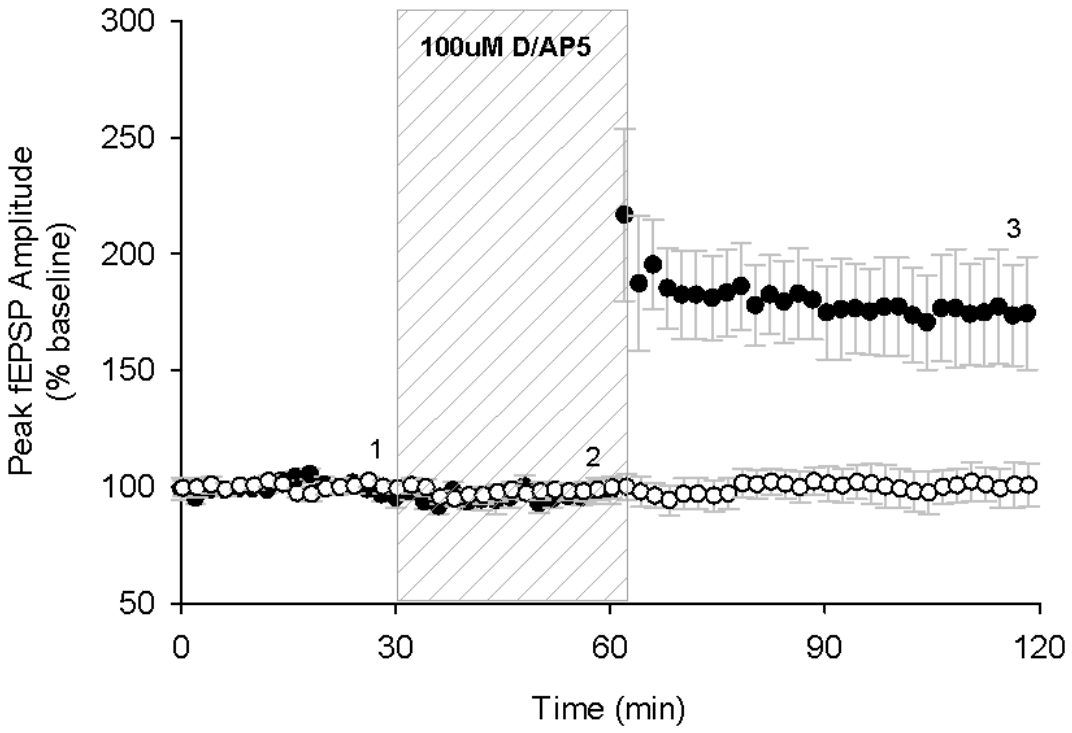

Fig. 9-3 Effects of 100 μM D-AP5 upon LTP at LA/PRh synapses. Pooled data n=5. After a stable baseline (1), 100 μM D-AP5 (2) is bath applied before the 4HFS is delivered to LA/PRh synapses (filled

circles). The Peak amplitude of fEPSPs at the last ten minutes of LTP (3) is significantly higher (~75%) compared to 1 and 2 (3 vs 1 and 2 p<0.001***). Open circles= Prh/PRh, control pathway.

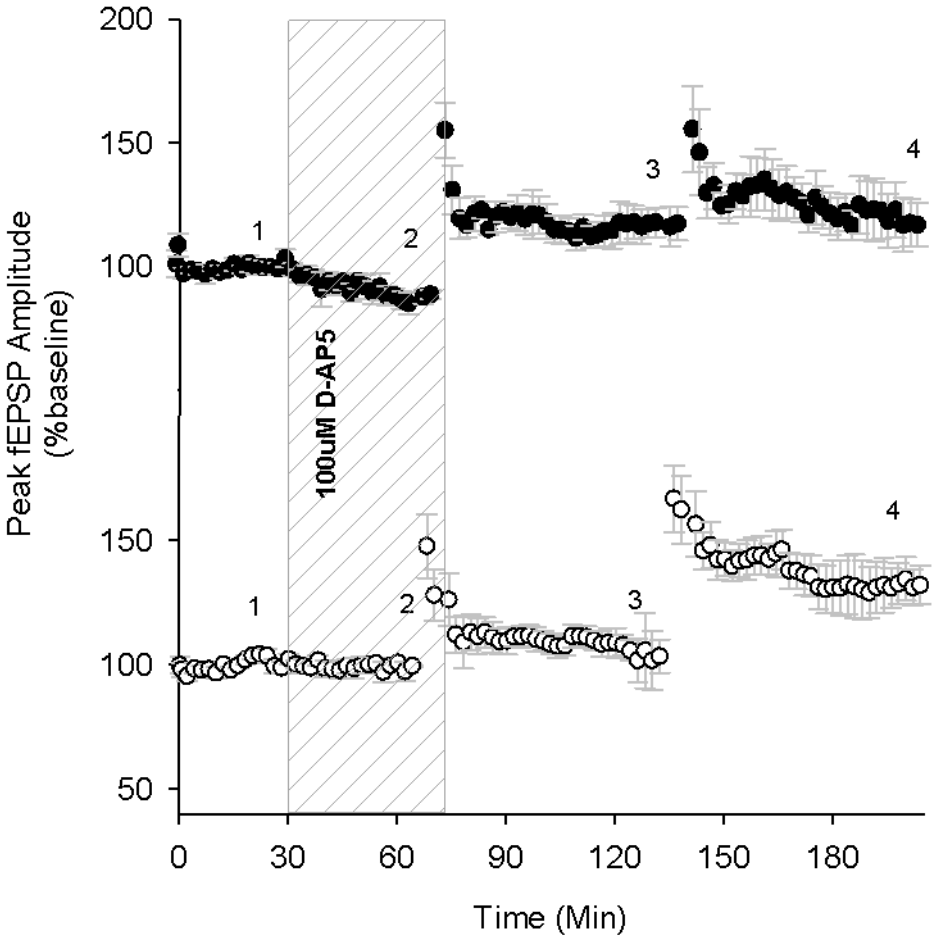

Fig. 9-4 Effects of 100 μM D-AP5 upon LTP at LA/PRh synapses (filled circles) and PRh/PRh synapses (open circles). Pooled data n=5. Application of 100 μM D-AP5 (2) blocks LTP at PRh/PRh synapses **(4 vs 1 p=0.002**, 4 vs 2 p<0.001***, 4 vs 3 p=0.002**)** but does not block LTP at LA/PRh (4, 3 vs 2 p<0.05*) in the same slices.

9.1.4 Effects of verapamil upon LTP at PRh/PRh and LA/PRh synapses

The L-type voltage-dependent calcium channel blocker verapamil was bath applied at a concentration of 20 μM, 20 minutes before, during the protocol of stimulation and 2 minutes after the 4HFS was delivered to LA neurons. This blocked the induction of LTP at LA/PRh synapses (Fig. 9-5) as revealed by a one way repeated measures analysis of variance.

In a set of experiments (Fig. 9-6) we bath applied verapamil (2) before the tetanic stimulation to LA fibers and after one hour verapamil was applied again (3) before giving the stimulation to PRh/PRh synapses. These set of experiments were done to evaluate in the same slices the effects of the drug upon LTP at the both pathways. As shown in Fig. 9-5, verapamil blocked LTP (3) at the extracortical input but did not affect LTP induction (4) at the intracortical input, as shown in previous studies in adult rats (Seoane et al., 2009). A two way repeated measures ANOVA and post hoc analyses with Bonferroni correction revealed that at LA/PRh synapses no difference was found between baseline, verapamil perfusion and LTP; while at PRh/PRh synapses LTP was stably induced after verapamil perfusion (4 vs 3 $p<0.003^{**}$, 4 vs 1 and 2 $p<0.002^{**}$).

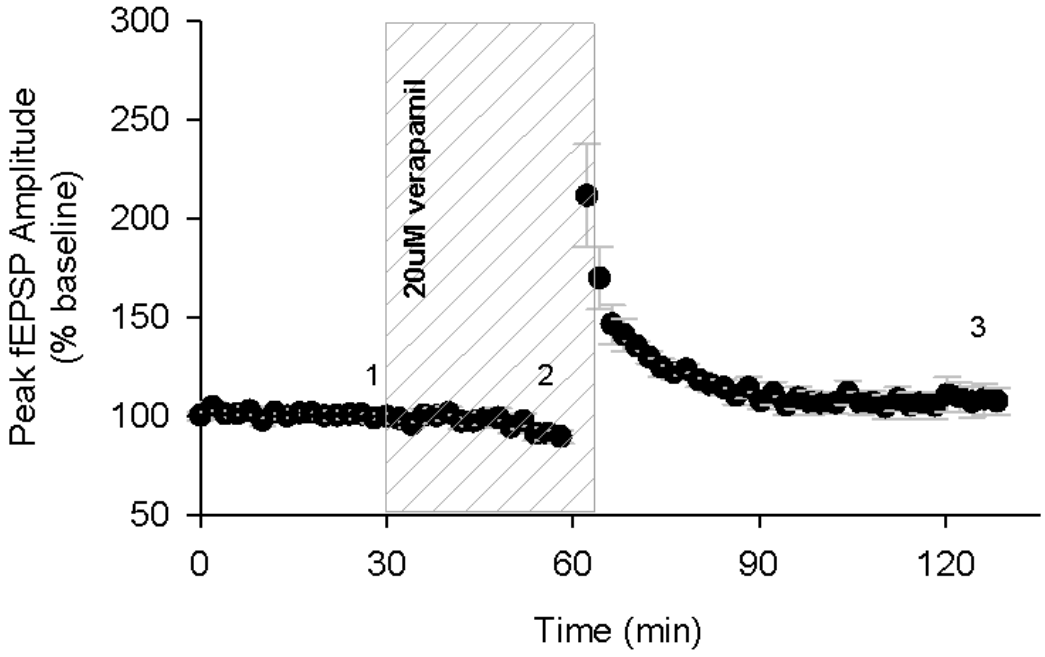

Fig. 9-5 Effects of 20 μM verapamil upon LTP at LA/PRh synapses. Pooled data n=8. Bath application of 20 μM verapamil blocks LTP at LA/PRh synapses. There is no significant difference between 1,2 and 3 $p>0.05$.

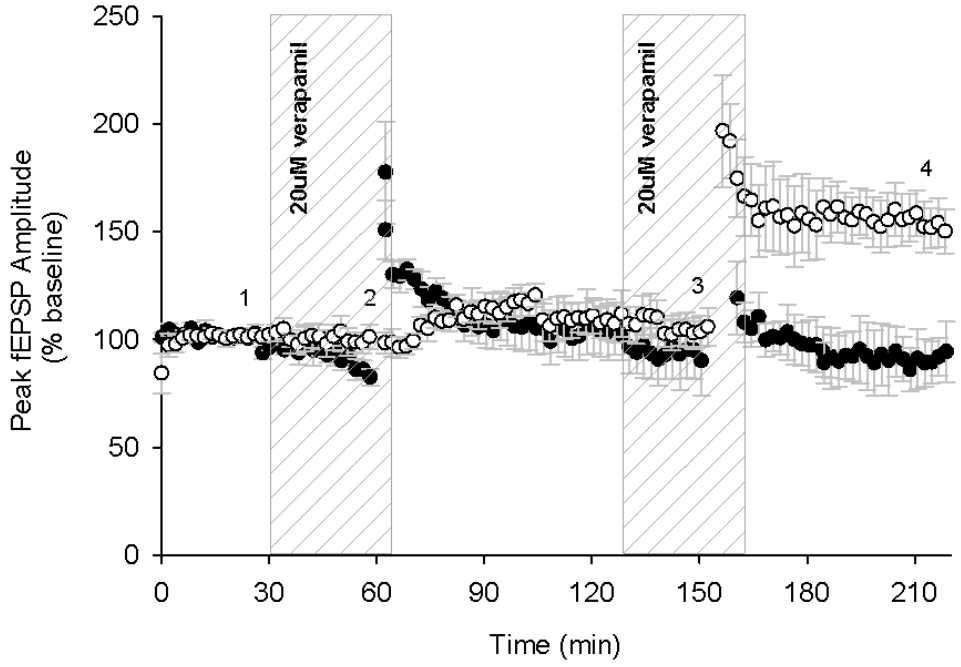

Fig. 9-6 Effects of 20 μM verapamil upon LTP at LA/PRh synapses (filled circles) and PRh/PRh synapses (open circles). Pooled data n=4. Perfusion with verapamil blocks LTP at LA/PRh synapses but does not affect LTP (~50%) at Prh/PRh synapses (4 vs 3 p<0.003**, 4 vs 2, 1 p<0.002**).

9.1.5 Effects of forskolin upon basal transmission at PRh/PRh and LA/PRh synapses

The cAMP/PKA activator forskol-n was bath applied during recording of basal transmission for 20 minutes. As shown in Fig. 9-7 50 μM of forskolin enhanced synaptic transmission of both inputs. Two way repeated measures analysis of variance and post hoc analyses with Bonferroni correction have been used to compare the peak amplitude of fEPSPs at 110-120 minutes of recording (2) and during the last ten minutes of baseline (1). At LA/PRh synapses 2 vs 1 p<0.002**; at PRh/PRh synapses 2 vs 1 p<0.004**.

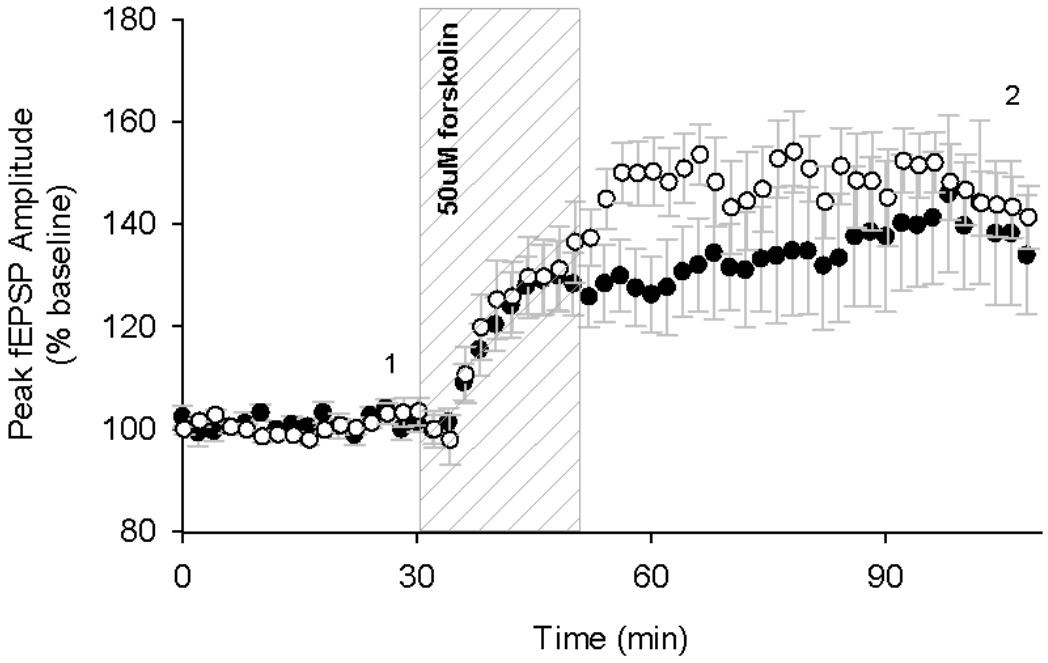

Fig. 9-7 Effects of 50 μM forskolyn upon synaptic transmission at LA/PRh (filled circles) and Prh/PRh synapses (open circles). Pooled data n=8. At LA/PRh synapses 2 vs 1 p<0.002**; at PRh/PRh 2 vs 1 p<0.004**. The averaged peak amplitude of the fEPSPs during the last ten minutes of recording resulted potentiated of ~40 % at LA/PRh and ~45 % at PRh/PRh synapses.

9.1.6 Effects of H89 upon LTP at PRh/PRh and LA/PRh synapses

Application of 10 μM H89 20 minutes before and up to 4 min after 4HFS, did not have any effects on LTP at PRh/PRh neurons (Fig. 9-8). One way ANOVA and post hoc analyses with Bonferroni correction reported a difference between LTP (3), drug application (2) and baseline (1) at PRh/PRh: 3 vs 1 p<0.001***, 3 vs 2 p<0.001***. Application of H89 instead reduces LTP at LA/PRh neurons (Fig. 9-9) (3 vs 1 p<0.01**; 3 vs 2 p<0.04*). Comparisons between LTP (2 hours after 4HFS) with H89 (gray circles) or without H89 (black circles) were made at LA/PRh synapses (Fig. 9-10). Two-way repeated measures ANOVA and post hoc analyses with Bonferroni correction reported a significant difference between LTP and H89 LTP (p<0.004**), and a significant difference between LTP and baseline in the control LTP without H89 (p<0.02**).

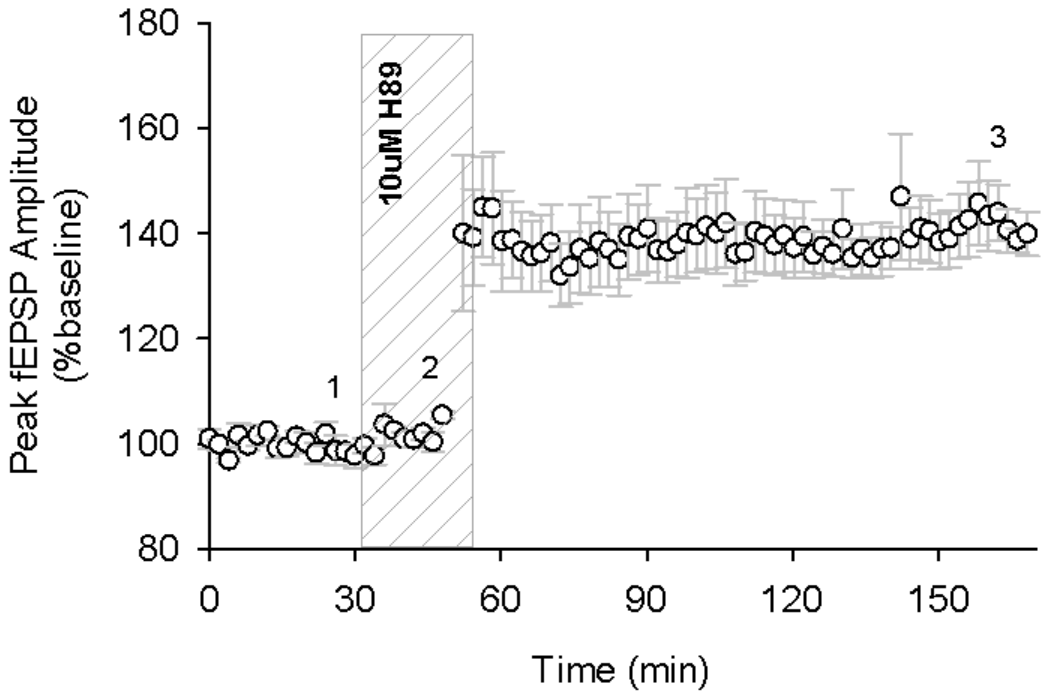

Fig. 9-8 Effects of 10 μM H89 upon LTP at PRh/PRh synapses. Pooled data n=4. Peak amplitude of fEPSPs were enhanced of ~ 40 % during the last ten minutes of LTP. 3 vs 1 p<0.008**, 3 vs 2 p<0.01**.

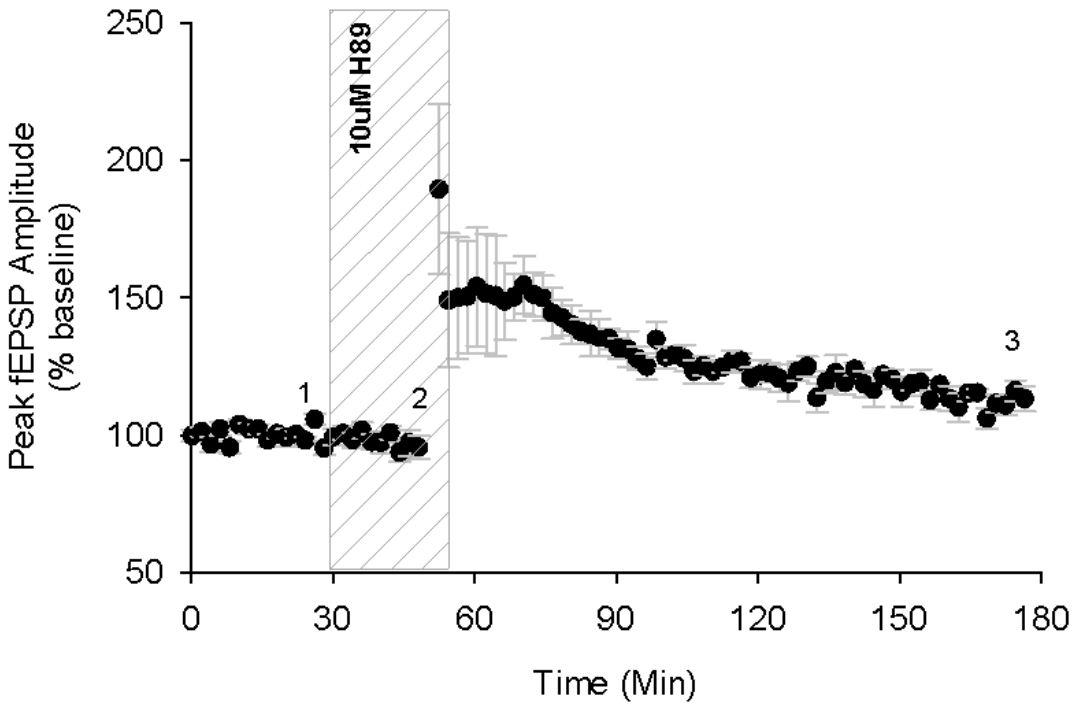

Fig. 9-9 Effects of 10 p<0.02 M H89 upon LTP at LA/PRh synapses.** Pooled data n=5**.** 3 vs 1 p<0.008**, 3 vs 2 p<0.01**. Application of H89 results in a reduction of the extent of LTP (~15 %).

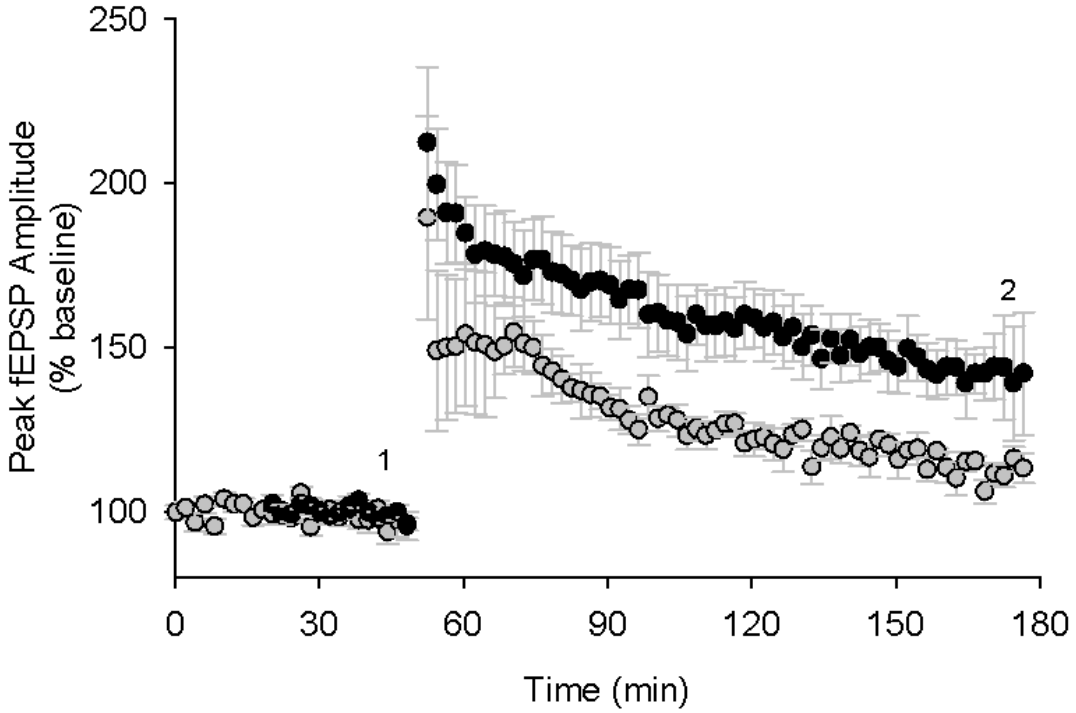

Fig. 9-10 Comparisons between LTP in the presence of H89 (gray circles, n=5) and control LTP (black circles, n=7). Control LTP is bigger compared to H89 LTP (p<0.004**). Within control LTP (2 vs 1 p<0.02**). No differences were found between 2 and 1 at H89 LTP.

9.1.7 Comparisons between control LTP and drug applications within PRh/PRh

Fig. 9-11 shows the comparison between drug treatments and control LTP at PRh/PRh synapses. One way repeated measures ANOVA revealed a significant difference between treatments and Dunnett's multiple comparisons test indicated that only application of D-AP5 has effects on PRh/PRh LTP (D-AP5 LTP vs control LTP, $p<0.05$*).

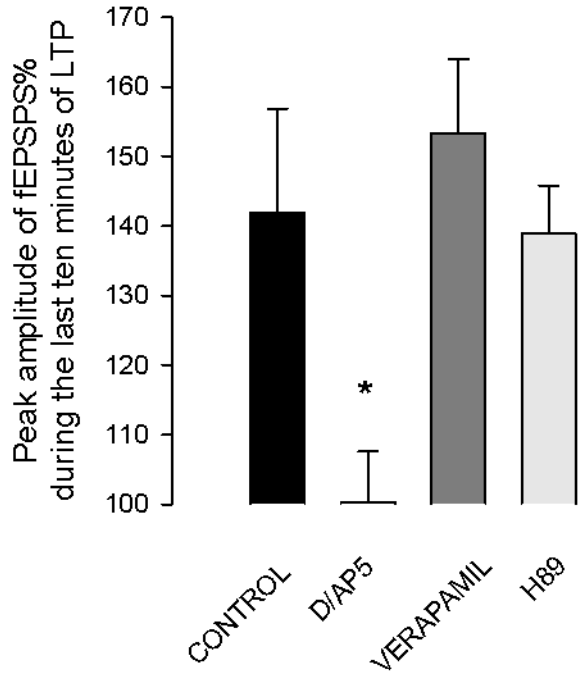

Fig. 9-11 LTP at PRh/PRh synapses and drug applications. D-AP5 blocks LTP (~1 %) at the intracortical input.

9.1.8 Comparisons between control LTP and drug applications within LA/PRh

Fig. 9-12 shows the comparison between drug treatments and control LTP at LA/PRh synapses. One way repeated measures ANOVA revealed a significant difference and Dunnett's multiple comparisons test indicated that application of verapamil and H89 affected LA/PRh LTP (treatments LTP vs control LTP, $p<0.05$*).

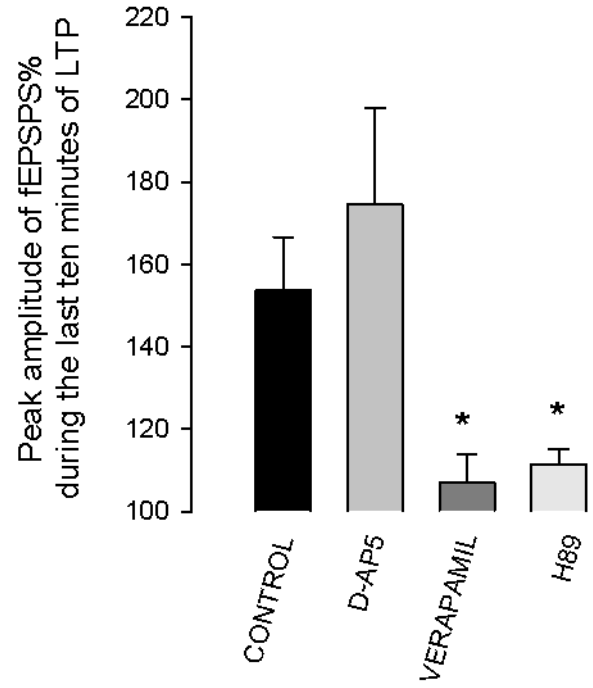

Fig. 9-12 LTP at LA/PRh synapses and drug applications. Verapamil (LTP fEPSP ~7 % compare to baseline fEPSP) and H89 (LTP fEPSP ~11 % compare to baseline fEPSP) affects LTP at LA/PRh synapses.

9.2 Results: associative long -term potentiation in perirhinal cortex

The first part of this chapter showed the basic properties of synaptic plasticity at LA/PRH synapses and at PRh/PRh synapses. The aim of this second section is to investigate whether synaptic plasticity at LA/PRh influences synaptic plasticity at PRh/PRh synapses.

9.2.1 High Frequency Stimulation (HFS) of 45 pulses at 100 Hz to PRh/PRh neurons

HFS of 45 pulses at 100 Hz delivered to PRh neurons failed to induce LTP at PRh/PRh synapses (Fig. 9-13). Paired t-test between baseline and post HFS was used to exclude the presence of a statistical significance.

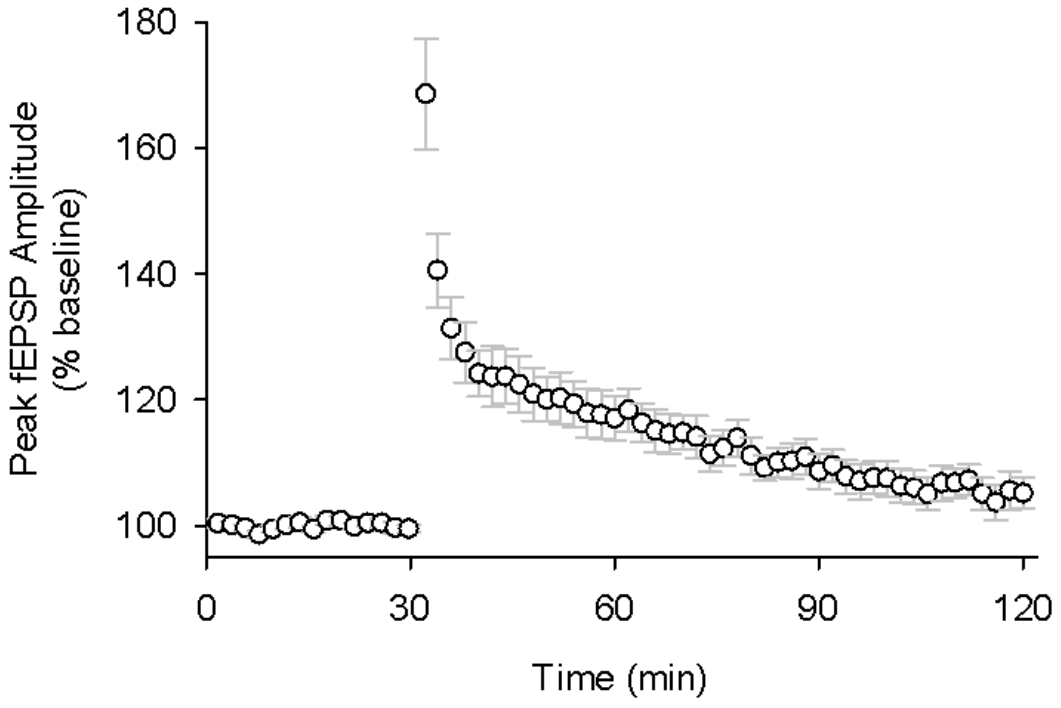

Fig. 9-13 HFS of 45 pulses at 100 Hz did not induce LTP at PRh/PRh synapses. Pooled data n=14.

9.2.2 HFS of 45 pulses at 100Hz to PRh/PRh neurons paired with 4HFS to LA/PRh synapses.

When HFS stimulation of 45 pulses at 100 Hz to PRh/PRh was given after the first two trains and before the last two trains of the 4HFS delivered to LA/PRh, it was capable to induce a long -term potentiation of PRh neurons (Fig. 9-14). The Friedman repeated measures analysis of variance on ranks was used, since normality test (Shapiro-Wilk) failed ($p<0.05$), and a significant difference ($p<0.001$***) was found. All pairwise multiple comparisons procedure with the Dunn's test revealed that LTP (2) at PRh/PRh synapses is significantly different from baseline (1) in the paired condition ($p<0.05$*) as well as at LA/PRh synapses ($p<0.001$***). Fig. 9-15 shows the difference between the "HFS alone" (open circles) and the "paired HFS", (filled circles) at PRh/PRh synapses. Paired t-test reported that the "paired HFS" LTP is greater than "HFS alone" LTP ($p<0.008$**).

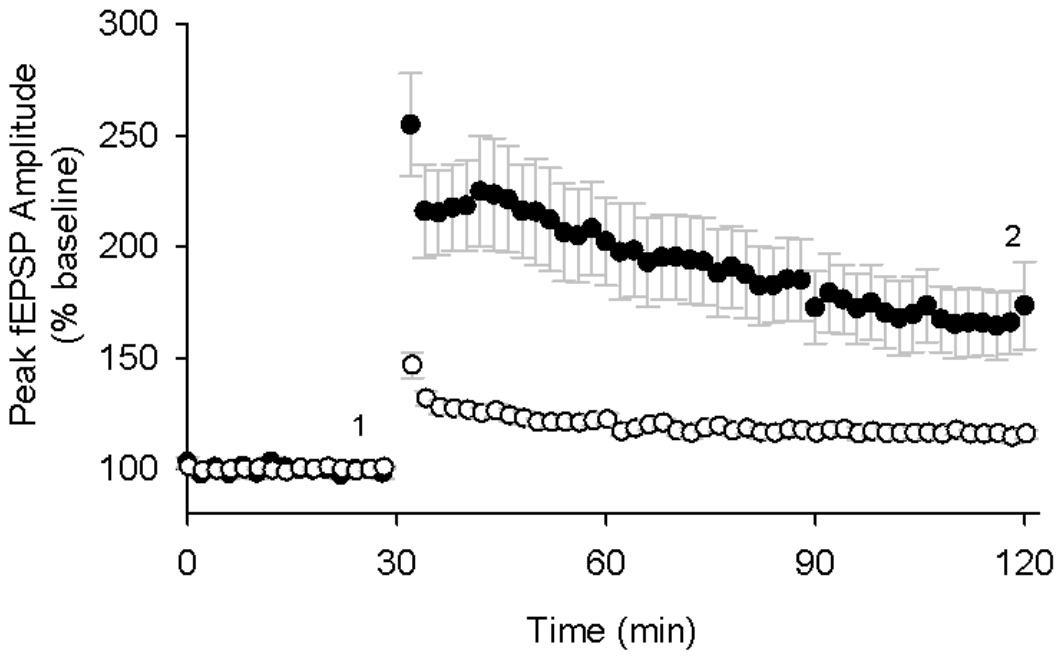

Fig. 9-14 HFS of 45 pulses at 100 Hz to PRh/PRh neurons (open circles) paired with 4HFS to LA/PRh synapses (filled circles). Pooled data n=11. Peak amplitude of fEPSPS during the last ten minutes of LTP is ~70 % bigger than baseline at LA/PRh (2 vs 1, $p<0.001$***) and of ~20 % at PRh/PRh input (2 vs 1, $p<0.008$**).

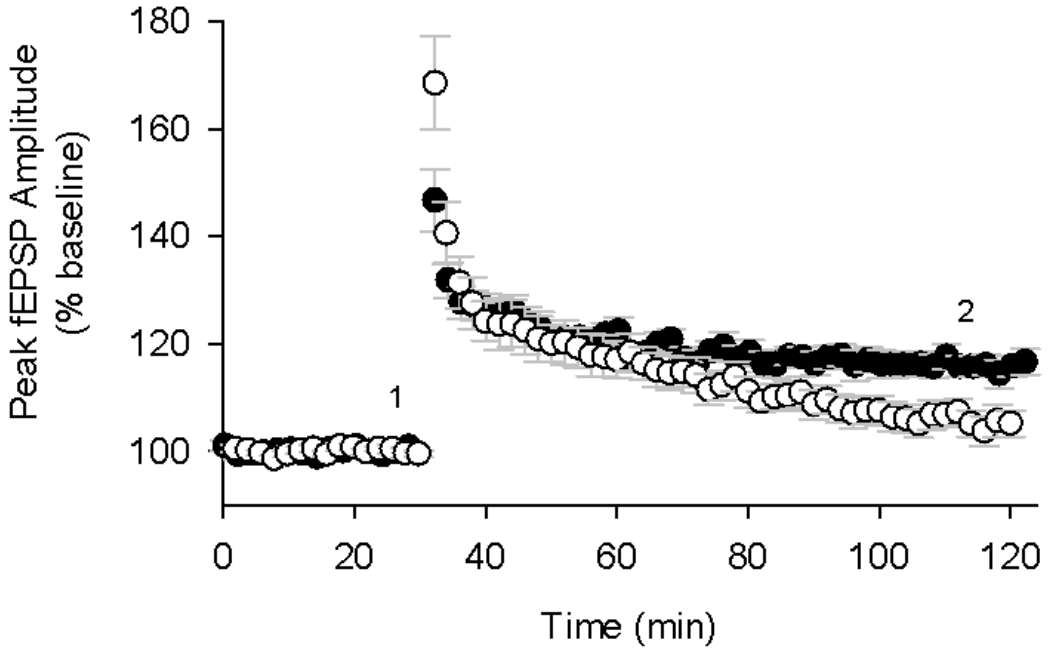

Fig. 9-15 Comparison between HFS alone (open circles) and PHFS (black circles) to PRh/PRh synapses. Paired HFS LTP vs HFS alone LTP, p<0.008**.

9.2.3 Effects of D-AP5 upon associative LTP.

Bath application of 100 μM of D-AP5 before the paired protocol of stimulation did not affect the associative LTP at either input (Fig. 9-16 A). A two way repeated measures ANOVA revealed a difference between time and stimulators. The three time points were baseline (1), D-AP5 perfusion (2) and LTP (3). Post hoc analyses with Bonferroni correction indicated a difference between 3 vs 1 and 2 (p<0.001***) at the LA/PRh synapses while at PRh/PRh synapses 3 was different compared to only 2 (p<0.03*), even if baseline is not different from perfusion as shown in Fig. 9-16 B.

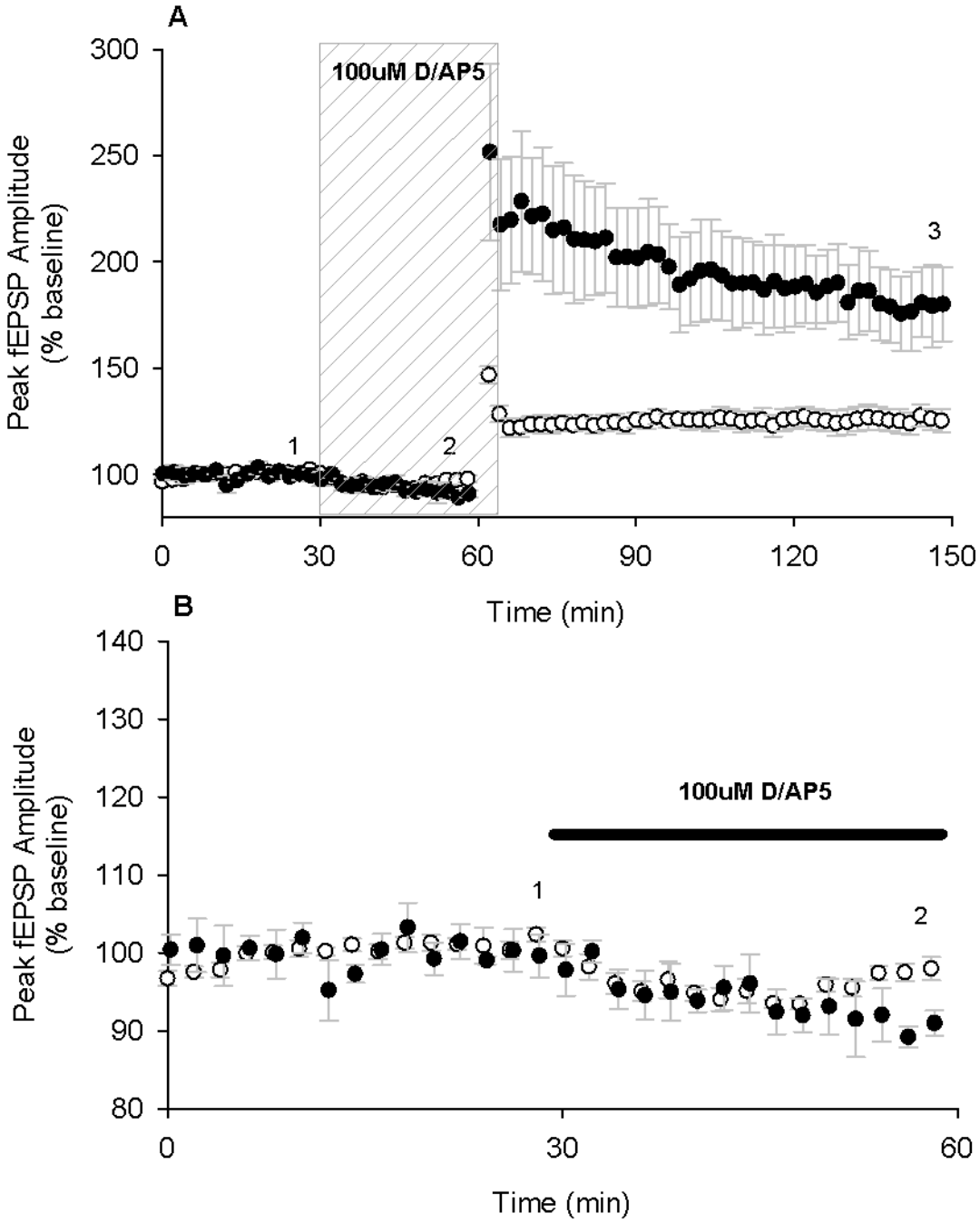

Fig. 9-16 Effects of 100 μM D-AP5 upon associative LTP at LA/PRh (filled circles) and PRh/PRh (open circles). Pooled data n=6. A) D-AP5 did not affect associative LTP. The peak amplitude of fEPSPs increased of ~25 % at PRh/PRh input and of ~75 % at LA/PRh input. B) Baseline versus D-AP5 perfusion. Two-way repeated measures ANOVA with factors for time and stimulating pathway failed to report a significant difference between baseline and perfusion.

9.2.4 Effects of verapamil upon associative LTP

Bath application of 20 μM of verapamil did not affect paired LTP at either input (Fig. 9-17). Two way analysis of variance and post hoc analyses with Bonferroni correction revealed a significant difference between baseline/perfusion and LTP at LA/PRh ($p<0.001$**) and at PRh/PRh synapses ($p<0.05$*).

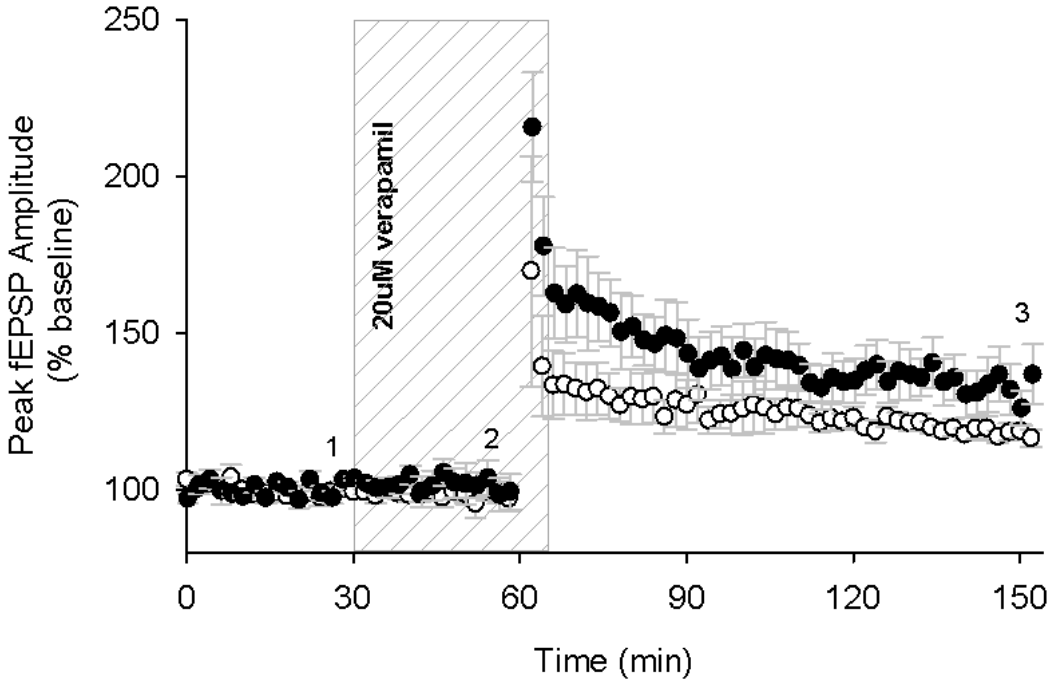

Fig. 9-17 Effects of 20 μM verapamil upon associative LTP at LA/PRh (filled circles) and PRh/PRh (open circles). Pooled data n=6. Verapamil did not affect associative LTP. The peak amplitude of fEPSPs increased of ~20 % at PRh/PRh synapses and of ~40 % at LA/PRh synapses.

9.2.5 Effects of D-AP5 and verapamil upon paired HFS

Bath application of 100 μM of D-AP5 and 20 μM verapamil blocked the associative LTP at both inputs (Fig. 9-18). Two way repeated measures ANOVA showed that LTP was not induced at either input. The analysis did not report a difference between baseline and perfusion, but LTP at LA/PRh is different from perfusion ($p<0.05$) but not from baseline.

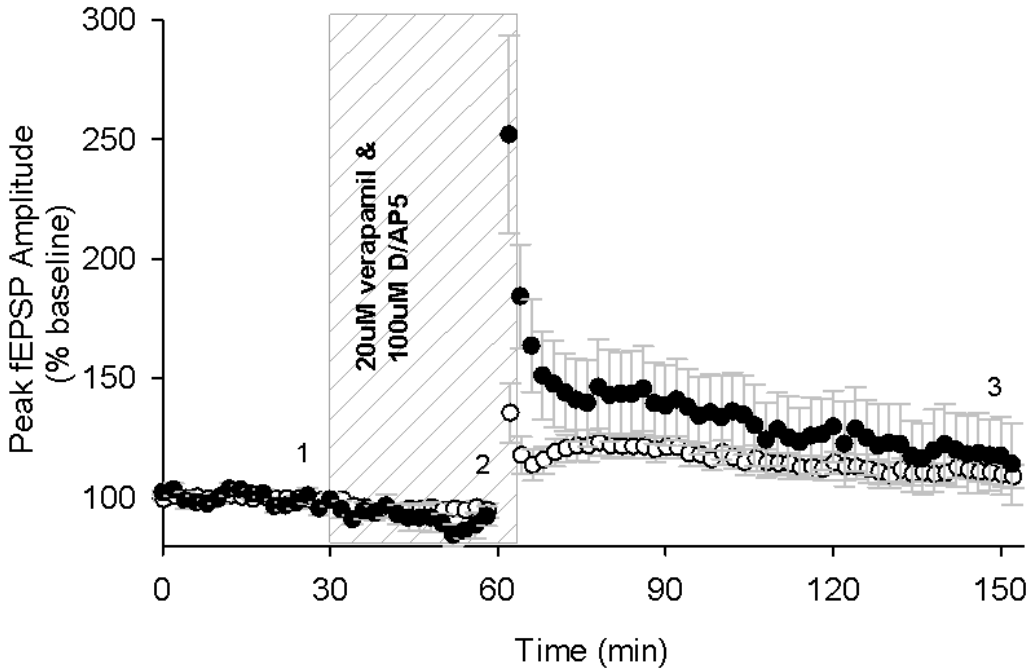

Fig. 9-18 Effects of 100 μM D-AP5 and 20uM verapamil upon associative LTP at LA/PRh (filled circles) and PRh/PRh (open circles). Pooled data n=5. Combined perfusion with D-AP5 and verapamil blocked associative LTP at both inputs.

9.2.6 Comparisons between paired HFS and drug application within PRh/PRh synapses

In Fig. 9-19 we show the difference between the "HFS alone" that does not induce LTP and the other four conditions: Paired HFS, PHFS and D-AP5, PHFS and verapamil and PHFS and D-AP5/verapamil within PRh/PRh synapses. We used the Dunnett's method to analyse the difference of the different conditions to the "HFS alone". The statistical significant differences we obtained are the following: between "HFS alone" and PHFS ($p<0.05$*), "HFS alone" and PHFS D-AP5 ($p<0.05$*), "HFS alone" and PHFS verapamil ($p<0.05$*). In the presence of both D-AP5 and verapamil, PHFS was not induced and was not significantly different from the "HFS alone".

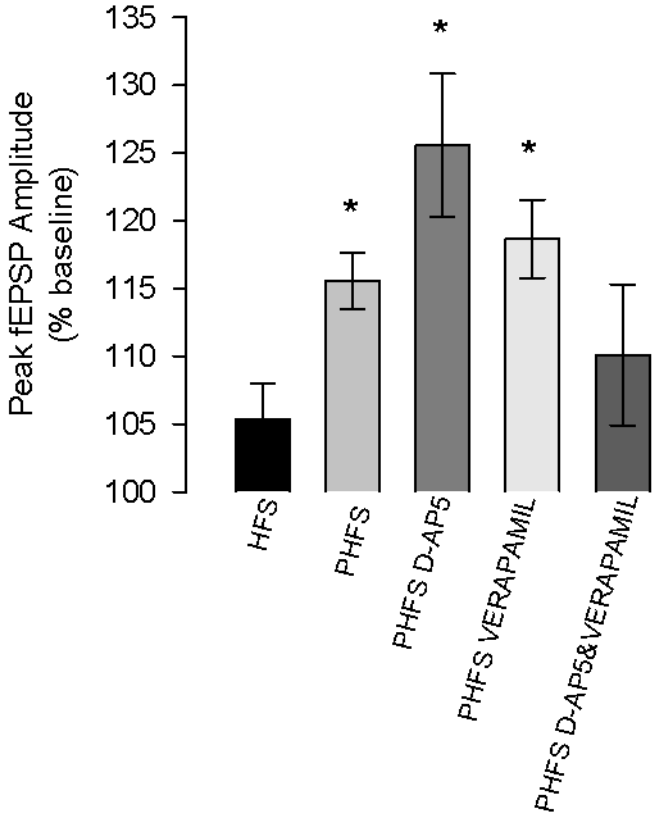

Fig. 9-19 Differences between "HFS alone" and the other conditions (PRh/PRh). PHFS/PHFS D-AP5 and PHFS verapamil are statistically different from "HFS alone" ($p<0.05$*).

10 Discussion

There is evidence showing that synaptic plasticity in the amygdala is important for consolidation of memory for emotionally salient stimuli (Bauer et al., 2002), while synaptic plasticity in the perirhinal cortex is crucial for recognition memory (Griffiths et al., 2008). A large number of studies demonstrate the existence of anatomical connections between amygdala and perirhinal cortex (Pitkänen et al., 2000; Furtak et al., 2007) but less is known about the functional interaction between amygdala and perirhinal cortex (Pelletier et al., 2005; Paz et al., 2006), which may be important for understanding how amygdala influences cortical processes, in particular how emotions can modulate forms of declarative memories.

This thesis investigates for the first time the basic properties of synaptic plasticity between the lateral nucleus of the amygdala and perirhinal cortex and provides a functional model for understanding how emotions influence recognition memory.

Before discussing the main findings of this study, I shall first make some statements about the methodological approach. Most of the studies in literature have used coronal slices to investigate synaptic plasticity at thalamo-amygdala or cortex-amygdala synapses (Fourcaudot et al., 2008; Huang et al., 1999) and also within perirhinal cortex (Ziakopolous et al., 1999; Griffiths et al., 2008). In our study we used the preparation proposed by Stoop and Pralong (Stoop et al., 2000) that consists of a modified horizontal brain slice that maintains intact the connections between amygdala, hippocampus, perirhinal cortex and entorhinal cortex. This preparation allows us to easily locate the lateral nucleus of the amygdala (that looks like a pale triangle), the perirhinal cortex with its laminar structure, the external capsule (a thin white stripe that separates the two areas) and the hippocampus. Moreover this cut allows us to distinguish better area 35 (from which we stimulated and recorded) from area 36 of

perirhinal cortex, making it easier to maintain stimulation and the recording constant across experiments. Two stimulators were used to alternately activate fibers in the perirhinal cortex and fibers in the lateral nucleus of the amygdala. The use of two stimulators makes it possible to compare the different mechanisms of synaptic plasticity of the two separate presynaptic inputs terminating onto the same group of neurons. Moreover, in some experiments, when the manipulation occurred only in one pathway, the other served as a control to show that the recording was stable and any changes in the other pathway was a consequence of experimental manipulations. Because blocking GABAergic transmission can cause seizures in perirhinal cortex, we did not use any GABA antagonists, making our recording more similar to physiological conditions but resulted in obtaining very small synaptic responses from the LA/PRh input (with the maximal amplitude between ~0.03 and 0.1 mV); therefore even a very small variation of the peak amplitude was detectable. To make sure that the responses were stable, we recorded at least one hour of stable baseline for each experiment but here we show only the last thirty minutes. However, in some experiments the baseline of LA/PRh pathway decreased during drug perfusion (i.e. Fig 9-4, 9-6, 9-16 and 9-18) but did not differ significantly from baseline.

The first set of experiments shows that using this preparation with young animals of about 30 days of age, it is possible to induce long-term potentiation at the intracortical input. The results here confirmed published work using the same protocol of stimulation on adult animals (Ziakopolous et al., 1999) or with different strains (Aicardi et al., 2004). We demonstrated that it is possible to induce a long and stable potentiation also at LA/PRh synapses when using the same protocol of stimulation. This result is the first showing that synaptic transmission at LA/PRh synapses can be strengthened. Since the direct high frequency stimulation of the amygdala neurons generates long-term potentiation of perirhinal neurons, we investigated the basic properties of this synaptic plasticity. The most studied form of long-term potentiation in the brain is the one that requires the postsynaptic activation of NMDARs. When a set of repeated stimuli is given at a high frequency, the postsynaptic cell is progressively depolarized; sufficient depolarization unblocks NMDARs from the magnesium ion block allowing Ca^{2+} flow into the cell. Given that in PRh cortex

LTP depends on the activation of NMDARs (Bilkey, 1996; Ziakopolous et al.,1999), we tested the involvement of these receptors in long-term potentiation at LA/PRh synapses. Surprisingly, LTP at LA/PRh synapses is not blocked in the presence of the NMDAR antagonist, D-AP5, suggesting that NMDARs are not required for LTP at this input. Moreover, in a set of experiments conducted on the same group of slices, we examined the involvement of NMDARs on LTP at both inputs and the results confirmed that LTP at extracortical input (LA/PRh) does not require NMDARs while at the intracortical input (PRh/PRh) blocking NMDARs also blocks LTP, that is stably induced in the same pathway after the drug washout. These experiments show for the first time that LA/PRh LTP is NMDAR-independent.

As neurotransmitter release is a Ca^{2+}-dependent process, long-term modifications in presynaptic Ca^{2+} influx or sensing could possibly result in an increase in release probability. Action-potential driven presynaptic Ca^{2+} influx is mediated by voltage-dependent Ca^{2+} channels (VDCCs), a different family of molecularl and pharmacologically distinct ion channels underlying various forms of synaptic plasticity. Data present in literature have demonstrated that LTP in the amygdala can require either NMDARs or L- type VDCCs, depending on the protocol of stimulation or on the fibers stimulated (Huang et al., 1998; Wiesskopf et al., 1999b; Bauer et al., 2002; Fourcaudot et al., 2008, 2009). We wanted to investigate the role of L-VDCCs in this form of NMDARs - independent LTP. Interestingly, application of verapamil, an L-VDCC antagonist, blocked LTP at LA/PRh synapses. This result was confirmed by another set of experiments conducted in the same group of slices, in which we showed that application of verapamil blocked LA/PRh LTP but not LTP at PRh/PRh. A reason for this could be that the L-VDCCs are high voltage activated channels and the strong protocol of stimulation used is so effective at raising calcium levels that the need for calcium through NMDARs is bypassed (Weisskopf et al., 1999). The finding that L-VDCCs are not involved in LTP in perirhinal cortex has already been reported in a recent paper that demonstrated the involvement of L-VDCCs in the induction of LTD but not LTP in perirhinal cortex in adult rats (Seoane et al., 2009). We confirmed this L-VDCCs - independent LTP in PRh LTP in younger animals.

A wide range of studies has demonstrated the role of PKA in various forms of long-term potentiation. The induction of LTP mobilizes the cAMP/PKA - dependent cascade either in the presynaptic terminals to directly enhance presynaptic functions (Huang et al., 1998; Bayazitov et al., 2007; Fourcaudot et al., 2008) or in the postsynaptic neurons to promote production of retrograde messengers (Arancio et al., 1996; Futai et al., 2007). Here, we investigated the involvement of cAMP/PKA and found that enhancing the level of endogenous cAMP via the adenylyl cyclase activator forskolyn resulted in an enhancement of basal transmission at both inputs. When cAMP/PKA pathway is blocked, LTP induction is significantly reduced at LA/PRh synapses but not at the intracortical input. Similar results of an involvement of cAMP/PKA in the amygdala have been reported (Huang and Kandel, 1998b and Fourcaudot et al., 2008). In particular, studies have demonstrated a role of PKA in presynaptic LTP trough Rab3A interacting molecule (RIM1) phosphorylation at cerebellar parallel fiber synapses (Lonart et al., 2003), at hippocampal mossy fiber synapses (Castillo et al., 2002) and at cortico-amygdala synapses (Fourcaudot et al., 2008). RIM1 is a multimodal scaffolding protein that interacts with a number of active-zone proteins involved in neurotransmitter release, such as presynaptic VDCCs. The interaction between RIM1 and β subunits of VDCCs facilitates the anchoring of neurotransmitter-containing vesicles in the proximity of VDCCs and enhances neurotransmitter release. At cortico-amygdala synapses, LTP requires the activation of presynaptic L-VDCCs (Fourcaudot et al., 2009) and the activation of cAMP/PKA (that downstream targets the presynaptic RIM1α) is important for maintenance of Ca^{2+} influx (Fourcaudot et al., 2008). Given these findings in literature and given the dependence of LA/PRh LTP on both cAMP/PKA and VDCCs reported in this Thesis, it is likely that the mechanisms of action in our case are similar to the one proposed by Fourcaudot and colleagues (2008) at cortico-amygdala synapses. In the intracortical input, instead LTP depends on the activation of post-synaptic NMDARs and does not require cAMP/PKA for its induction, suggesting the need of other proteins.

The first part of this study has defined the basic properties of synaptic plasticity at amygdala-perirhinal synapses. The second part of this project has

investigated how synaptic plasticity at LA/PRh synapses can influence synaptic plasticity at PRh/PRh cortex.

We know that an event with a low emotional salience is not able to induce long-term memory, but when this event is accompanied by an emotional meaning, it is able to induce long-term memory. In other words, we remember better emotional salient stimuli than neutral ones. In order to recreate this "model" *in vitro*, we found a weak protocol of stimulation that was not sufficient to induce LTP in perirhinal cortex when given alone, but when paired with a strong stimulation of the LA fibers, it was capable of inducing LTP at PRh/PRh synapses. The explanation is fascinating. The weak stimulation (45 pulses at 100Hz) applied to the PRh/PRh fibers allows the release of neurotransmitter from the presynaptic vesicles. The glutamate released binds to the postsynaptic AMPA receptors triggering the influx of positively charged sodium ions (Na^{2+}) into the postsynaptic cell, causing a short depolarization (the excitatory postsynaptic potential, EPSP). This small and brief EPSP though is not effective in activating NMDARs or L-VDCCs that are responsible for the influx of Ca^{2+} into the cell and in the absence of Ca2+, LTP cannot be induced. When the weak stimulation to PRh/PRh neurons is paired with a strong stimulation (4 trains at 100Hz, 4HFS) to LA/PRh neurons, it is now able to induce LTP at PRh/PRh synapses. According to the Hebbian theory of plasticity, induction of associative LTP requires the occurrence of two simultaneous events: the presynaptic release of neurotrasmitter and the postsynaptic depolarization. The strong 4HFS stimulation induces LTP at LA/PRh pathway through the activation of L-VDCCs that allows a sufficient quantity of Ca^{2+} to enter the postsynaptic cell and strongly depolarize it. Since the two inputs (LA/PRh and PRh/PRh) converge onto the same perirhinal neurons, the glutamate released from the presynaptic PRh fibers stimulated at a weak intensity, together with the depolarization of the postsynaptic cell induced by the strong stimulation of the LA fibers, constitute the events necessary for the induction of associative LTP at PRh/PRh synapses. In this condition, PRh/PRh LTP requires the simultaneous activation of the LA neurons. When LA/PRh LTP is blocked, PRh/PRh LTP is prevented. According to this, we found that when NMDARs are blocked, LTP at LA/PRh synapses is still induced and therefore LTP at PRh/PRh is also induced by the paired stimulation. Blocking L-VDCCs still

induces LTP. We showed that L-VDCCs are necessary and sufficient to induce LTP at LA/PRh but this does not exclude that a small NMDARs component is involved in this form of LTP. In fact, even if L-VDCCs are blocked, when stimulating both pathways a higher quantity of glutamate is released from the two presynaptic terminals and more AMPARs are activated resulting in a bigger depolarization of the postsynaptic cell compared to stimulating only one pathway. This depolarization is necessary to unblock the NMDARs. We showed that it is possible to prevent this associative LTP when simultaneously blocking both sources of calcium: the L-VDCCs and the NMDA receptors.

These results provide the first evidence for a possible mechanism by which emotions influence recognition memory: irrelevant events (reflected by the weak stimulation used here) may be remembered for much longer when they occur in the circumstances for other emotionally significant events (reflected by the strong stimulation used here).

What implication could this form of plasticity have in terms of behaviour? Which role does it have in learning and memory?

It is evolutionary adaptive to recognize events with a negative salience, in order to avoid them. During fear conditioning learning, the lateral nucleus of the amygdala receives information regarding the conditioned and the unconditioned stimulus from the thalamus and the cortex, then LA activates the central nucleus of the amygdala which in turns controls the expression of a behavioural response (Sigurdsson et al., 2007). In this study we activate fibers originating in the lateral nucleus of the amygdala that have been shown to make synapses onto pyramidal cells of perirhinal cortex (Pitkänen, 2000; Furtak et al., 2007). We assume that these fibers convey information of the emotional learning that has just occurred, from the LA (site of fear conditioning learning) to the perirhinal cortex, which is required for recognition memory and that contains neurons that respond strongly to novelty (Xiang et al., 1998; Brown et al., 2001). Therefore, if the emotional learning occurring in the LA leads to a behavioural response (through the activation of the central nucleus of the amygdala), LA output might concomitantly strengthen the memory associated with that learning event via perirhinal cortex activation. The demonstration that LA/PRh pathway modifies synaptic plasticity of PRh/PRh pathway is likely to represent the mechanism by which emotions reinforce recognition memory.

This reinforcement of recognition memory can be useful; for example to avoid unpleasant situations, events, or stimuli, but can also produce maladaptive behaviours because remembering a traumatic event can have devastating effects on the individual, such as happens during posttraumatic stress disorder (PTSD) that is marked by intrusive, chronic, and distressing memories of highly emotional events.

Evidences from different studies have demonstrated an interaction between cAMP-PKA, L-VDCCs and adrenoreceptors. Activation of β-adrenoceptors affects adenylate cyclase and stimulates the cAMP/PKA signal transduction pathway (Perkins & Moore, 1973). The β-adrenergic receptor is coupled to the G proteins, which are composed of a $G\alpha_s$ and a $G\beta_y$ subunit. $G\alpha_s$ has long been known to play a central role in responses evoked with β-adrenergic receptor stimulation because of its ability to activate adenylyl cyclase leading to the stimulation of PKA and phosphorylation of various proteins. In cardiac muscle, β-adrenergic receptor stimulation has long been known to lead to the activation of L-VDCCs and the primary mediator of this effect has generally been assumed to be the $G\alpha_s$ adenylyl cyclase–PKA pathway (McDonald et al. 1994; Xiong et al., 1995; Zhong et al., 2001). Given that LTP at LA/PRh synapses requires the activation of the cAMP/PKA pathway and of L-VDCCs, it would be interesting to study whether this mechanism is also mediated by the noradrenergic system. Furthermore, extensive evidence indicates that adrenal stress hormones, namely epinephrine and glucocorticoids, modulate consolidation of emotionally motivated memory in animals and human subjects (McGaugh and Roozendaal, 2002; McGaugh, 2004). It has also been shown that systemic administration of epinephrine in rats can facilitate consolidation of object recognition memory with a little arousal component through a mechanism dependent on activation of β-adrenoceptors (Roozendaal et al., 2008; Dornelles et al., 2007). Given that noradrenergic activation of the basolateral amygdala modulates recognition memory in rats, we would like to study the involvement of the noradrenergic system in this form of associative plasticity we have showed here. Coactivation of LA/PRh pathway induces synaptic plasticity in the perirhinal cortex, that otherwise would have not occurred, probably by adding an emotional value to the event or to the stimulus to be remembered. We have

assumed that LA/PRh pathway transports information related to this emotional value; this information then reaches the perirhinal cortex and modulates (strengthens) recognition memory. Therefore, LA/PRh stimulation might influence recognition memory probably by stimulating the noradrenergic system. If this is the case, we expect that the activation of the noradrenergic system replaces the LA/PRh stimulation. For example, we can test whether bath application of a beta adrenergic agonist such as isoprenaline, before and during the weak stimulation to the intracortical input still induces LTP at PRh/PRh synapses in the absence of the strong 4HFS to the LA; or we can test whether inhibition of the noradrenergic system through the application of an antagonist such as propanolol before the paired stimulation prevents expression of associative LTP. These results would strongly support the hypothesis that associative LTP between LA and perirhinal cortex requires the activation of the noradrenergic system, providing an elegant neural mechanism to study how we remember emotionally salient stimuli better than neutral ones.

Results from this study describe for the first time the basic mechanisms underlying synaptic plasticity between the lateral nucleus of the amygdala and the perirhinal cortex and show how activation of the amygdala modulates synaptic plasticity within perirhinal cortex. These results are necessary because they lay the foundations for more sophisticated studies involving new techniques of investigation, to further develop the knowledge about the interaction between these two areas.

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