MECCANISMI NEUROADATTATIVI E REGOLAZIONE DELLA SFERA COGNITIVA

MECHANISMS OF NEUROADAPTATION AND REGULATION OF COGNITION

DOTTORANDA
Dott.ssa Isabella Panaccione

TUTOR:
Prof. Ferdinando Nicoletti

CO -TUTOR:
Prof.ssa Agata Copani

EXTERNAL SUPERVISOR
Prof. Zafar I. Bashir
MRC Centre for synaptic plasticity
University of Bristol, Bristol

COORDINATORE
Prof. Roberto Avola

UNIVERSITÀ DEGLI STUDI DI CATANIA
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MECHANISMS OF NEUROADAPTATION AND REGULATION OF COGNITION

Isabella Panaccione

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University of Catania
University of Rome “Sapienza”
MRC centre for Synaptic Plasticity, University of Bristol, UK
Abstract

Perirhinal cortex plays a key role in processing recognition memory. Evidences that repeated exposure to familiar objects produces a decremental response in perirhinal neurones led to the proposal that recognition memory depends on long-term depression. However, long-term potentiation is also expressed in perirhinal cortex. Long-term potentiation is thought to be involved in many form of synaptic plasticity, especially learning and memory. Nevertheless, not much is known on mechanisms maintaining late-phases of long-term depression in perirhinal cortex.

This study shows that LTP in adult perirhinal cortex is maintained by the persistent activity of Protein Kinase Mζ. The inhibition of PKMζ, in fact, completely reverts an established potentiation. This work also focuses on mechanisms that could regulate the persistent activation of PKMζ in perirhinal cortex. The results of the experiments show that synaptic depotentiation appear to down-regulate the activity of PKMζ. Also, the role of PDK1 in regulating the activity of PKMζ is studied. The experiments run provide evidences that the inhibition of PDK1 leads to a decrease of the activity of PKMζ.

This work also explores the mechanisms of synaptic plasticity occurring in perirhinal cortex early in the development. Starting from the observation that it’s not possible to induce LTP in P14 animals, and the only form of potentiation obtainable in P14 perirhinal cortex is de-depression, several experiments have been run to investigate the possible mechanisms underlying this “high levels” of basal synaptic transmission at this stage. PKMζ maintains long-term synaptic potentiation; in P14 perirhinal cortex, the application of the selective PKMζ inhibitor ZIP decreases basal synaptic transmission, but has no effect once LTD has been induced. Moreover, ZIP decreases synaptic transmission in a previously de-depressed pathway,
providing evidences that in P14 perirhinal cortex LTP mechanisms are present but already saturated in a PKMζ-dependent way. This potentiation of the basal synaptic transmission is lost later during the neurodevelopment (i.e. at PND35); at this stage it is possible to induce LTP in perirhinal cortex, and the inhibition of PKMζ completely reverts the potentiation.

Mechanisms regulating the sustained activity of PKMζ in P14 perirhinal cortex are also examined in this work. New PKMζ is synthesized following the induction of LTP via intracellular mechanisms involving different kinases (i.e. PI3K) and ultimately mTOR-dependent translation. The inhibition of PI3K and mTOR in P14 perirhinal cortex produces a PKMζ-dependent decrease in the basal synaptic response. Therefore, our results suggest that synaptic transmission in immature connections in perirhinal cortex relies on PI3K-, mTOR- and PKMζ-dependent mechanisms. Further experiments show that these processes could be regulated by a continuous activity of Group I mGluRs.

Taken together, these results highlight the crucial role of PKMζ in the synaptic potentiation, and suggest that its sustained activity is required to stabilize young synapses during the neurodevelopment.
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ABBREVIATIONS

aCSF, artificial cerebrospinal fluid
AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
AMPAR, AMPA receptor
CaMKII, calcium/calmodulin dependent protein kinase II
CREB, cyclic adenosine monophosphate response element
DA, Dark Agouti
D-AP5, D-2-amino-5-phosphonopentanoate
DAG, diacyl glycerol
DMS, delayed matching to sample task
DMSO, Dimethyl sulfoxide
DNA, deoxyribonucleic acid
DNMS, delayed non-matching to sample task
EC, entorhinal cortex
EPSP, excitatory postsynaptic potential
fEPSP, field EPSP
GABA, γ-amino-butyric acid
GABAR, GABA receptor
GluR, glutamate receptor subunit
GPCR, G-protein coupled receptor
HAA, 3-Hydroxyanthranilic acid
HCl, Hydrochloric acid
HFS, high frequency stimulation
Hz, hertz
iGluR, ionotropic glutamate receptor
IP$_3$, inositol (1,4,5)triphosphate
KA, kainate
KAR, kainate receptor
LFS, low frequency stimulation
LTD, long-term depression
LTP, long-term potentiation
mAChR, muscarinic acetylcholine receptor
MAPK, mitogen-activated protein kinase
mGluR, metabotropic glutamate receptor
MPEP 2-Methyl-6-(phenylethynyl)pyridine hydrochloride.
mRNA, messenger ribonucleic acid
MTL, medial temporal lobe
NMDA, N-methyl-D-aspartate
NMDAR, NMDA receptor
NSF, N-ethylmaleimide sensitive factor
N-terminus, amino-terminus
P14, Postnatal Day 14
P35, Postnatal Day 35
PDK1, 3-phosphoinositide dependent protein kinase-1
PI3K, phosphatidylinositol 3-kinase
Pin1 protein interacting with NIMA 1
PICK1, protein interacting with C kinase (PKC)
PIP2, phosphatidylinositol (4,5)–bisphosphate
PKA, cAMP-dependent protein kinase
PKC, protein kinase C
PKM, protein kinase M
PLC, phospholipase C
PP1, protein phosphatase 1
PP2A, protein phosphatase 2A
PPR, paired-pulse ratio
PSD, postsynaptic density
PSD-95, postsynaptic density protein 95
PTK, protein tyrosine kinase
STP, short-term potentiation
TE, temporal association cortex
TM, transmembrane
ZIP, PKMζ inhibitory peptide
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1 GENERAL INTRODUCTION
1.1 Introduction

The brain has the ability to acquire novel information as the process of learning and to store and retrieve information as the process of memory. Memory is one of the most fascinating processes happening in the brain, and is crucial in everyday life. Therefore, fully understanding the mechanisms for learning and memory remains an ultimate goal of neuroscience, also to be able to provide treatments and therapies for many people, for example amnesiacs and patients with neurodegenerative diseases, such as Alzheimer’s disease.

Memory can be compartmentalised into a different subtypes, involving specific structural components (Eichenbaum 2002). The two main divisions of memory, declarative and non-declarative, are commonly recognised (Mesulam 1998). Declarative memory includes episodic and semantic memories. Episodic memory refers to the explicit recall of personal experiences, whilst semantic memory refers to the explicit recall of general facts related to the world around us (Tulving, Schacter et al. 1988). Non-declarative memory concerns the unconscious memory for learning skills and procedures and also emotional responses (Mesulam 1998).

Research using amnesiac patients has greatly aided the elucidation of the systems and structures involved in memory in the human brain. The classic example is patient H.M., described in a pioneering paper by Scoville and Milner in 1957, reviewed by Burwell and Amaral (Burwell and Amaral, 1998a, b). H.M. underwent a bilateral resection of the medial temporal lobe (MTL) to relieve severe epilepsy. As a result he sustained severe global anterograde amnesia, manifest as an inability to form new memories. In addition, he suffered some retrograde amnesia covering the decade prior to his surgery (Scoville and Milner 1957). This and subsequent studies have established the vital role of the MTL in the acquisition of declarative memory. The anterograde amnesia suffered by H.M included a loss of recognition memory, which requires a capacity for both identification and judgement of the prior occurrence of what has been identified (Brown and Aggleton 2001).
Earlier work suggested that the contributions of the hippocampus and amygdala were important in recognition memory (Eichenbaum 1999). However, other areas of the brain are now considered to be important, namely the cortical areas surrounding the hippocampus such as the perirhinal cortex and parahippocampal and entorhinal cortices (see Brown and Aggleton, 2001 for review).

1.1.1 The perirhinal cortex

The perirhinal cortex in the rat comprises two narrow strips of cortex (Brodmann’s areas 35, more ventral, and 36, broader and dorsal) located above and below the rhinal sulcus (Burwell et al., 1995; Burwell, 2001). The anterior inferior temporal association cortex (TE) is located dorsorostrally to the perirhinal cortex and the entorhinal cortex is located ventrocaudally. The region is also bordered rostrally by the insular cortex and by the postrhinal cortex caudally, which bears similarities to the parahippocampal cortex in primates (Burwell, Witter et al. 1995). Rat perirhinal cortex has been shown to be highly analogous to primate perirhinal cortex in comparisons carried out by Burwell et al (1995).

The cytoarchitecture of the perirhinal cortex has been thoroughly characterised by Burwell (2001). Areas 35 and 36 were subdivided along differences in cytoarchitectonics into five further areas: dorsal, ventral and posterior (35d, 35v, 36d, 36v and 36p). However, there is no sharp delineation between sub-regions (Burwell 2001). Area 35 is agranular cortex, meaning it lacks a granular layer IV, whilst area 36 is dysgranular cortex, meaning that it has a sparse layer IV. Layer II of area 36 is less dense than layer III and is characterised by aggregates of medium sized round or polygonal cells. Small pyramidal cells are mixed with the round cells and become more numerous as one proceeds
caudally. Layer V cells also form a size gradient such that cells are smaller superficially than at deeper levels.

![Figure 1.1.2 Photomicrographs of Nissl stained sections of area TE and perirhinal cortex. Sections from cortical layers of areas (A) TEv, (B) 36d, (C) 36v, (D) 36p, (E) 35d and (F) 35v are shown. The relative size of cortical layers are illustrated in the different sections, with a relatively smaller layer IV in area 36 compared to an absence in area 35. 'ic', internal capsule. Panels A-C correspond to −3.80mm and D and F to −6.72mm relative to Bregma. From Burwell (2001).](image)

### 1.1.2 Connections of the perirhinal cortex

The perirhinal cortex can be considered as a polymodal associational cortex. It receives input from all of the sensory modalities, in addition to input from other polymodal associational areas such as the prefrontal cortex and the entorhinal
There have been three major studies (Deacon, Eichenbaum et al. 1983; Burwell and Amaral 1998; Burwell and Amaral 1998) demonstrating that afferents to area 36 of the perirhinal cortex arise from postrhinal cortex, entorhinal cortex, temporal association cortex (TE) and from area 35. Area 35 receives afferents from postrhinal cortex, agranular insular cortex, entorhinal cortex and area 36. Area 35 receives its predominant cortical input from polymodal association cortex, particularly from olfactory areas, whilst area 36 receives relatively more of higher cortical input than area 35. Although direct projections to the perirhinal cortex from the visual cortex are generally weak, connectivity is represented to a greater degree between these cortices via the postrhinal cortex. The somatosensory and visuo-spatial cortices are connected to the perirhinal cortex via projections to the postrhinal cortex. This connectivity results in relatively even contributions to the perirhinal cortex from olfactory, auditory, visual and visuo-spatial regions (Suzuki and Amaral 1994; Suzuki and Amaral 1994). The perirhinal cortex sends large projections to the entorhinal cortex, which sends reciprocal connections back to perirhinal cortex. The entorhinal cortex provides the major cortical input to the hippocampus (Burwell and Amaral 1998). The hippocampus projects efferents back to the entorhinal cortex, providing a connection back to the rhinal cortex.

**Figure 1.1.3 Schematic diagram illustrating the connectivity of the rat perirhinal cortex.** The diagram shows parallel routes by which sensory information reaches the perirhinal cortex and from there the hippocampus. The thickness of the lines indicates the relative size of the projections. Modified from Brown and Aggleton (2001).
1.2 The involvement of the perirhinal cortex in learning and memory

Numerous studies have attempted to dissect the role of components of the medial temporal lobe (MTL) in learning and memory. The following section will concentrate upon the components involved in object recognition memory, as well as upon the behavioural tests most commonly used to assess this form of memory.

1.2.1 Tests of memory function in laboratory animals

There is a wide range of tests that have been developed to explore different aspects of memory in laboratory animals. However, as this thesis concentrates upon object recognition memory, only tests that rely upon judging the relative familiarity of presented stimuli will be focussed upon. The recognition tasks employed are most commonly variants of delayed matching (DMS) or non-matching (DNMS) to sample tasks with trial unique stimuli, which were originally introduced for monkeys in the 1970s (Gaffan 1974; Mishkin and Delacour 1975). The tests consist of three phases, a sample phase, a delay phase and finally a test phase. In the sample phase the subject is presented with an object, which it can displace to obtain a reward. The delay phase can be variable in length and is followed by the test phase. In this phase the subject is presented with a copy of the object presented in the sample phase which can be considered to be the ‘familiar’ object, and also with a second, ‘novel’ object. A DMS task demands the subject to select the familiar object to receive a reward, whilst a DNMS task demands the subject to select the novel object to receive a reward. A DNMS task provides a better test of recognition memory as, by rewarding selection of the familiar object, a DMS task involves both reward association learning and recognition memory. DNMS tasks only reward objects in the test phase that have not been previously associated with reward in the sample phase and so involves only recognition memory.

The spontaneous test of object recognition (Ennaceur and Delacour 1988) is a one-trial DNMS task and relies on the natural preference of rats to explore
novel objects over familiar objects. During the task, rather than a reward being obtained for exploration of an object, the rat’s spontaneous exploration of the objects is recorded. In addition to eliminating reward association learning, this task does not require extensive pre-training that is required for many other standard DMS/DNMS tasks, such as the Y-maze (Aggleton, Hunt et al. 1986). The delay period within this test can be lengthened to increase the difficulty level and can be run with unique objects each time. In addition, variations of this test can be created to test memory for place (recognition that an object is in a location where previously there had been no object) and memory for object in place (recognition that a specific object has changed position with another object) (Dix and Aggleton 1999).

1.2.2 Lesion and cannulation studies of recognition memory

Lesion studies essentially involve the surgical removal of part of the MTL system and subsequent monitoring of performance on certain recognition memory tasks. A number of studies have examined the effect of lesions in monkeys and rats upon recognition memory for individual objects in DNMS tasks (Meunier, Bachevalier et al. 1993; Suzuki, Zola-Morgan et al. 1993; Mumby and Pinel 1994; Ennaceur, Neave et al. 1996; Meunier, Hadfield et al. 1996; Nemanic, Alvarado et al. 2004; Buckley 2005). In primates, the differential contribution to recognition memory has been demonstrated by the components of the rhinal cortex, namely the entorhinal, perirhinal and parahippocampal cortices. Selective lesions of different areas of the rhinal cortex have revealed that this region does not play a uniform role in recognition memory. Lesions of the perirhinal cortex have been shown to produce severe deficits in DNMS tasks (Meunier et al., 1993; Meunier et al., 1996). Lesions of the entorhinal cortex produced only a mild or transient deficit (Meunier, Bachevalier et al. 1993), whilst parahippocampal lesions did not produce any deficits in DNMS tasks (Meunier, Hadfield et al. 1996). This effect was also shown in rats, with lesions of either the whole rhinal cortex (Mumby and Pinel 1994) or of the perirhinal cortex only (Wiig and Bilkey 1994) resulting in severe deficits in DNMS performance, including the spontaneous test of object recognition memory (Ennaceur, Neave et al. 1996; Aggleton, Keen et al. 1997;
Ennaceur and Aggleton 1997; Bussey, Muir et al. 1999; Nemanic, Alvarado et al. 2004; Winters, Forwood et al. 2004; Buckley 2005). It has been shown that the deficit in recognition memory is not isolated to the visual modality. Lesions of the perirhinal cortex have been shown to impair tactile, olfactory and appetitive recognition memory (Otto and Eichenbaum 1992; Suzuki, Zola-Morgan et al. 1993; Corodimas and LeDoux 1995; Buffalo, Ramus et al. 1999; Fortin, Wright et al. 2004).

Recognition memory may be supported by two independent types of retrieval: the recollection of a specific experience and a sense of familiarity gained from previous exposure to particular stimuli (Brown and Aggleton 2001; Aggleton and Brown 2006). There is considerable debate regarding the exact roles of the hippocampus and perirhinal cortex in object recognition memory; however, perirhinal damage is far more disruptive than hippocampal damage for recognition memory, and hippocampal lesions often have no effect on recognition tests (Aggleton, Hunt et al. 1986; Nemanic, Alvarado et al. 2004; Buckley 2005). On the other hand, hippocampal damage is more disruptive on tests of spatial memory in rats than perirhinal damage (Ennaceur et al., 1996; Glenn and Mumby, 1998; Murray et al., 1998; Aggleton et al., 2004; Winters et al., 2004; Murray et al., 2005). These findings indicate that the role of the perirhinal cortex and hippocampus in recognition memory can be doubly-dissociated. In other words, perirhinal cortex is important for object recognition memory, whereas the hippocampus is crucial for spatial memory. It has been demonstrated that in tasks that require the use of both spatial and object recognition memory, such as the object-in-place task (Dix and Aggleton 1999), lesions of either the hippocampus or the perirhinal cortex affect performance (Gaffan and Parker 1996; Bussey, Duck et al. 2000). Therefore, these structure seem to interact as part of a memory system.

Cannulation studies in the perirhinal cortex have aided the understanding of the mechanisms that are involved in recognition memory. The direct application of pharmacological agents to the perirhinal cortex in vivo via a cannula means that the role of the perirhinal cortex in recognition memory can be examined, rather than a potential global effect of applying antagonists systemically. Studies have ascertained the role for different metabotropic and ionotropic glutamate
receptors (Winters and Bussey 2005; Barker, Bashir et al. 2006; Barker, Warburton et al. 2006) and also the role of cholinergic and GABAergic transmission in recognition memory (Warburton, Koder et al. 2003; Wan, Warburton et al. 2004).

1.2.3 In vivo electrophysiological studies of recognition memory

Recording studies in vivo support a role for the perirhinal cortex in visual recognition memory (Aggleton and Brown 1999). The responses of a subset of neurones in primate perirhinal cortex have been shown to be repetition-sensitive in response to visual stimuli. In optimal recording conditions (i.e. high stimulus repetition frequency, short delay interval) roughly 50% of neurones studied are repetition-sensitive. The response of such neurones is typically maximal to the first stimulus and significantly reduced to repeat presentations (Xiang and Brown 1998). The observed neuronal response reduction has been proposed as a potential neural substrate for familiarity discrimination, with a long-term depression (LTD)-like process at the synaptic level underlying this decremental response. These response reductions are long lasting (at least 24 hours) (Fahy et al., 1993; Xiang and Brown, 1998) and are still seen when stimuli are repeated at very short delays (750ms) (Xiang and Brown 1998). Also, the responses are highly stimulus specific (a neurone that has responded weakly to a stimulus that has been seen before still responds strongly to a novel stimulus (Xiang and Brown 1998) and the response reductions are automatic and do not require any pre-training (Xiang and Brown 1998). This response change to individual stimuli is not seen in the hippocampus (Brown and Aggleton 2001).

It has also been shown in monkeys that these decremental neurones can be further sub-classified into novelty, recency or familiarity neurones according to the circumstances in which a decrement is seen (Brown and Aggleton 2001). In the case of a novelty neurone 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 does become familiar, the
response becomes much briefer upon first and repeat viewings. *Recency neurones* 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 neurones* 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 (Fahy, Riches et al. 1993). 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.

1.2.4 Immunohistochemical studies of neuronal activation in the perirhinal cortex

The expression of the immediate early gene c-fos provides a potential marker for changes in neuronal activation (Herrera and Robertson 1996). The counting of Fos stained nuclei (Fos is the protein product of the c-fos gene) in different brain regions after exposure to novel and familiar stimuli has been used to examine the activity of these brain areas in relation to familiarity discrimination (Zhu, Brown et al. 1995). In rat perirhinal cortex, but not in hippocampus, the activation of c-fos is greater when novel objects are seen than when familiar objects are seen (Zhu, Brown et al. 1995). The paired viewing procedure (Zhu, McCabe et al. 1996) is a within subject design where, for each rat, one eye is exposed to a novel object and the other is simultaneously exposed to a familiar object, so the difference in c-fos expression between each hemisphere of a rat can be compared. In a study by Wan et al (1999), neurones in the perirhinal cortex and area TE of the temporal lobe showed significantly greater c-fos expression in response to novel images than familiar images of individual objects. The evidence from these Immunohistochemical studies supports the data from lesion and electrophysiological studies that the perirhinal cortex plays a critical role in familiarity discrimination of individual items. The hippocampus is relatively uninvolved in familiarity discrimination unless spatial factors are involved in the judgment of prior occurrence.
1.2.5 Viral transduction as a technique to study memory

The development of viral vectors to mediate long-term transgene expression in specific cell types has been a relatively recent tool used in studies of memory, behaviour and neuronal gene function (Warburton 1999). Lentiviral vectors have been shown to be highly efficient for in vivo gene delivery and have achieved stable long-term expression in terminally differentiated neurones for up to 16 months (Naldini, Blomer et al. 1996; Dull, Zufferey et al. 1998; Bienemann, Martin-Rendon et al. 2003; Wong, Goodhead et al. 2006). In the perirhinal cortex, an adenovirus has been used to disrupt binding of the transcription factor CREB (cyclic adenosine monophosphate response element) through the expression of a dominant negative form of CREB (Warburton, Glover et al. 2005). Adenoviral transduction resulted in a block of long-term recognition memory at a behavioural level, a block of LTP at a plasticity level and also a block in the differential neuronal activation at a cellular level (Warburton, Glover et al. 2005).

1.3 Excitatory neurotransmission in the CNS

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. These can be divided into two distinct categories, the ionotropic glutamate receptors (iGluRs) (reviewed by Dingledine et al., 1999) and the metabotropic glutamate receptors (mGluRs), which mediate their effects via coupling to G-protein-coupled second messenger systems (Conn and Pin 1997). The iGluRs can be further subdivided into three categories based upon their pharmacology (reviewed by Dingledine et al., 1999; Mayer and Armstrong, 2004). These are the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, N-methyl-D-aspartate (NMDA) receptors and kainate (KA) receptors.

1.3.1 AMPA receptors

In the CNS, AMPARs mediate the majority of fast synaptic transmission and gate Na⁺, K⁺ and Ca²⁺ upon ligand binding. 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 (Michaelis, 1998; Dingledine et al., 1999).

### 1.3.1.1 AMPAR subunit structure

Endogenous receptors are believed to be tetrameric heteromers of subunits (GluR1-4 subunits) and these consist of the extracellular N-terminal and ligand binding domains, transmembrane region and the intracellular C-terminal domain (reviewed by Dingledine et al., 1999; Palmer et al., 2005a).

**Figure 1.3.1. The topology of AMPAR subunit.** Each subunit consists of an extracellular N-terminal domain, four hydrophobic regions (TM1–4), and an intracellular C-terminal domain. The ligand-binding site is a conserved amino acid pocket formed from a conformational association between the N terminus and the loop linking TM3 and TM4. A flip/flop alternative splice region and R/G RNA editing site are also present within the TM3/TM4 loop. TM2 forms an intracellular re-entrant hairpin loop which contributes to the cation pore channel. The Q/R RNA editing site is present within the TM1/TM2 loop. The intracellular C terminus contains phosphorylation sites and conserved sequences that have been shown to interact with a number of intracellular proteins. Adapted from Palmer et al., (2005a).

Although in NMDA receptors (NMDARs) there is Zn$^{2+}$ modulation at a similar site, no endogenous ligands have been found to bind at the AMPAR N-terminal domain (Mayer and Armstrong 2004).
Transmembrane (TM) regions 1, 3 and 4 all span the cell membrane but TM2 forms a re-enterant loop on the intracellular side of the cell. The re-enterant loop is thought to contribute to the pore channel, which is permeable to Ca\(^{2+}\), K\(^+\) and Na\(^+\) (Michaelis, 1998; Dingledine et al., 1999; Palmer et al., 2005a), however the edited form of the GluR2 subunit is Ca\(^{2+}\)-impermeable. The orientation of the transmembrane regions was determined by using proteolytic sites, N-glycosylation patterns and specific antibodies (Molnar, McIlhinney et al. 1994; Wo, Bian et al. 1995). The intracellular C-terminal domain of all AMPAR subunits is an interaction site for numerous proteins that are involved in receptor trafficking and synaptic plasticity (reviewed in Song and Huganir, 2002; Malinow and Malenka, 2002; Henley, 2003; Bredt and Nicholl, 2003; Collingridge et al., 2004; Malenka and Bear, 2004; Palmer et al., 2005a), such as PDZ domain-containing proteins, ABP/GRIP, PICK-1, PSD-95 and NFS.

1.3.1.2 Post-transcriptional modification

Functional diversity in AMPARs is largely determined by the expression of the genes that encode the different AMPAR subunits. There is approximately 70% sequence homology between genes encoding each subunit (Hollmann and Heinemann 1994) although further diversity is generated by post-transcriptional modifications. Alternative splicing can occur in the extracellular region of the fourth transmembrane domain to create ‘flip’ and ‘flop’ splice variants (Sommer, Keinanen et al. 1990). Flip variants dominate before birth, whereas flop variants are in low abundance before the eighth postnatal day and are up-regulated to about the same level as the flip forms in adult animals. The flip forms of subunits desensitize four times more slowly than the flop forms (Dingledine et al., 1999; Palmer et al., 2005a). GluR2 and 4 subunits also undergo alternative splicing in the C-terminus to give long and short isoforms, with the short isoforms of GluR2 accounting for 90% of total GluR2 (Kohler, Kornau et al. 1994). The long form of GluR4 is predominant and is largely expressed in the cerebellum (Gallo, Upson et al. 1992).

An additional post-transcriptional modification process is RNA editing. This leads to a single amino acid change at residue 607 in GluR2 subunits from
glutamine (Q) to arginine (R). In the adult rat 99% of GluR2 subunits are the R form and this residue is located in the channel-forming segment of the subunit (Dingledine, Borges et al. 1999). As a result, the edited subunits are Ca^{2+}-impermeable, because the size and charge of the amino acid side chain in the R form prevents the passage of Ca^{2+} ions through the channel. Changes in the amount of edited GluR2 subunits have been implicated in a number of diseases due to a link between Ca^{2+} permeability and excitotoxicity. These include Alzheimer’s disease, Huntington’s disease, schizophrenia, amyotrophic lateral sclerosis (ALS) and epilepsy (Akbarian, Smith et al. 1995; Brusa, Zimmermann et al. 1995; Tanaka, Grooms et al. 2000; Kwak and Kawahara 2005).

1.3.1.3 Post-translational modification of subunit isoforms
The primary methods of post-translational modification of AMPAR subunits are glycosylation and phosphorylation. It has been proposed that N-glycosylation is involved in the maturation and transport of the receptor or could protect AMPARs from proteolytic degradation (Standley and Baudry 2000).

The regulated phosphorylation of AMPARs adds yet another level of modulation to an already complex scenario. Phosphorylation can regulate intermolecular interactions, channel properties and trafficking and is intricately linked with synaptic plasticity (reviewed by Smart, 1997; Palmer et al., 2005a). There appears to be a general role for developmental regulation of AMPAR properties by phosphorylation. I.e., an increase in PKC phosphorylation of AMPARs, primarily at S831 on GluR1, in striatal spiny neurones may play a role in the early stages of Parkinson’s disease (Oh, Geller et al. 2003).

1.3.1.4 Subunit composition of AMPARs
The subunit composition of AMPARs is critical in determining the functional and trafficking properties of resulting channels (Malinow and Malenka 2002). AMPARs that lack edited GluR2 are Ca^{2+}-permeable and have an inwardly rectifying current/voltage (IV) relationship, so that at positive membrane potentials there is a voltage-dependent block of the pore channel by polyamines. As opposite, AMPARs that contain edited GluR2 are relatively Ca^{2+}-impermeable (Bowie and Mayer 1995; Dingledine, Borges et al. 1999; Malinow and Malenka 2002). In hippocampal neurones AMPARs comprise
mainly GluR2 with GluR1 (GluR1/GluR2) or with GluR3 (GluR2/GluR3) (Wenthold, Petralia et al. 1996). There have been numerous studies made that examine the changes in AMPARs with different subunit compositions during and following synaptic plasticity (Shi et al., 2001; Lee et al., 2004; Holman et al., 2006; Plant et al., 2006). A general and simplified proposal is that GluR1/2 complexes are driven into the synapse during hippocampal LTP, and are subsequently replaced by GluR2/GluR3 complexes through constitutive recycling (Shi et al., 2001). Relatively little is known about the roles of individual subunits in the removal of AMPARs from synapses but progress is being made in understanding this complex process (Lee et al., 2004; Holman et al., 2006; McCormack et al., 2006).

1.3.1.5 Expression pattern of AMPAR subunits in the brain

Numerous studies have demonstrated the widespread and varied distribution of AMPAR subunits in the brain (reviewed in Hollmann and Heinemann, 1994) using immunocytochemistry, receptor autoradiography and in situ hybridisation studies (Keinanen, Wisden et al. 1990; Petralia and Wenthold 1992; Martin, Blackstone et al. 1993; Beneyto and Meador-Woodruff 2004). The distribution of GluR1, GluR2 and GluR3 are heterogeneous, with differential regional distribution and different levels of expression throughout numerous structures in the brain, with GluR1 being the most ubiquitous subunit (Beneyto and Meador-Woodruff, 2004). GluR4 is enriched in the cerebellum with generally low levels in the rest of the CNS (Petralia and Wenthold, 1992; Martin et al., 1993). Specifically, within the neocortex, (which includes the perirhinal cortex) GluR1-3 are present in all cortical layers except layer I, with GluR1 enriched in layers V and VI. GluR2 has high expression in layers II-III but much less in layer V, whilst GluR3 is highest in layer IV of the neocortex (Xu, Tanigawa et al. 2003; Beneyto and Meador-Woodruff 2004). Within the hippocampal formation there is high expression of GluR1-3 in all areas (Beneyto and Meador-Woodruff, 2004). There are similar developmental changes in regional expression of subunits in both rat and human (Talos, Fishman et al. 2006; Talos, Follett et al. 2006). Rodent cortical pyramidal neurones exhibit a developmental lag in GluR2 and GluR3 expression relative to GluR1, the expression of which is
higher than in adult throughout development and peaks at P10-12 (Talos et al., 2006b). GluR2-4 expression levels progresses with age but at P21 are still lower than adult levels (Talos et al., 2006). This has a clinical significance as the relatively low ratio of the Ca\(^{2+}\)-impermeable GluR2 receptors to non-GluR2 receptors in the second postnatal week can lead to greater susceptibility to ischaemic injury. Indeed an equivalent pattern has been found in humans that could potentially lead to a targeted therapeutic strategy (Talos et al., 2006a)

1.3.1.6 AMPA receptor trafficking

The regulation of trafficking of AMPA receptor is of great interest, since it is involved in many aspects of neuronal plasticity. Experience-dependent strengthening of neocortical excitatory synapses in vivo is associated with the delivery of GluR2-lacking AMPARs to the synapse (Clem and Barth 2006). Interestingly, the increase in GluR2-lacking AMPARs after LTP induction is transient and after around 25 minutes they are replaced by GluR2-containing AMPARs during the maintenance phase of LTP (Plant, Pelkey et al. 2006). In addition, the association between LTP and the insertion of AMPARs at the synapse has been demonstrated in vivo. In the barrel cortex, experience drives the delivery of GluR1 subunits into the synapse, shown by an increase in rectification and sensitivity to joro spider toxin, which is selective for GluR2-lacking AMPARs (Clem and Barth 2006). Auditory fear conditioning in the amygdala also drives the trafficking of GluR1-containing AMPARs into the synapse (Rumpel et al., 2005). When trafficking of GluR1 was blocked, short-term and long-term memory of the fear conditioning was disrupted (Rumpel, LeDoux et al. 2005). It appears therefore there is a consensus that during LTP a multi-step process is required for the trafficking of AMPARs to the synapse.

The endocytosis of AMPARs in response to stimulation occurs initially from extrasynaptic sites and this is then followed by a decrease in synaptic AMPARs. In contrast to LTP, where the GluR1 subunit appears to be crucially involved in exocytosis, in LTD the GluR2 subunit appears to have the dominant role since it has been shown to directly interact with adaptor protein 2 (AP2) (Lee, Liu et al. 2002). This protein couples to clathrin, and along with dynamin, plays a pivotal role in clathrin-mediated endocytosis at synapses (Carroll, Beattie et al. 1999;
Wang and Linden 2000). In addition, it has been demonstrated that in the absence of plasticity-inducing stimuli, AMPARs undergo constitutive cycling (Shi et al., 2001). A recent study by McCormack et al (2006) has proposed that there are activity-independent trafficking pathways that serve to maintain the capacity for bidirectional plasticity in neurones (McCormack, Stornetta et al. 2006). Synaptic AMPAR exchange is slow, with a rate constant of around 17hrs and involves the removal of GluR1 and GluR4 subunits and the addition of GluR2 subunits, which restores the ability for new LTP or LTD (McCormack, Stornetta et al. 2006). In GluR2 knockout mice there is a failure of synaptic AMPAR exchange, but not in GluR1 knockout mice; therefore GluR2 is found to be essential for this process.

It’s interesting to note that PKMζ seems to be involved in the maintenance of LTP through the regulation of NSF/Glu2-dependent AMPA receptor trafficking (Yao, Kelly et al. 2008). Consistently with these remarks, it has been proposed that PKMζ blocks the internalization of AMPA receptor, rather than facilitating their insertion in the membrane through a mechanism depending once again on its interaction with the mGlu2 subunit (Migues, Hardt et al. 2010).

1.3.2 Kainate receptors

The kainate subfamily of iGluRs consists of five subunits, GluR5-7 and KA1 and KA2 (Chittajallu, Braithwaite et al. 1999) The GluR5-7 receptors have a relatively low affinity for kainate and can form homomeric receptors. In comparison, KA1 and KA2 subunits have a higher affinity for kainate and form heteromeric receptors with GluR5-7 subunits (Chittajallu, Braithwaite et al. 1999). KA receptors (KARs) are considered to have a similar transmembrane topology to AMPAR and NMDARs (Michaelis, 1998; Dingledine et al., 1999; Kew and Kemp, 2005). Similar to AMPAR subunits, GluR5 and GluR6 subunits contain the Q/R editing site, the R form of which is impermeable to Ca\textsuperscript{2+} ions. The lack of specific antibodies has thus far hindered understanding of the exact subunit composition of native KARs and their synaptic localisation, however there is a differential distribution of the mRNA of KA subunits throughout the brain (Chittajallu et al., 1999; Isaac et al., 2004).
The relatively recent development of specific agonists and antagonists to kainate receptors has greatly aided the elucidation of KA receptors’ physiological function (Paternain, Morales et al. 1995; Wilding and Huettner 1995; Clarke, Ballyk et al. 1997; Bleakman and Lodge 1998; More, Nistico et al. 2004). It is now understood that KARs, like AMPARs, mediate fast excitatory transmission and some forms of synaptic plasticity (Isaac, Mellor et al. 2004). Also, KARs can act to depress excitatory transmission in the Schaffer collateral-commissural pathway (Clarke, Ballyk et al. 1997; Clarke and Collingridge 2002) and seem to be involved in the induction and expression of LTD of KAR-mediated synaptic transmission in layer II/III of the perirhinal cortex via a mechanism involving mGluR5, PKC and PICK1 (Park, Jo et al. 2006). KARs have also been shown to play a role in object recognition memory within the perirhinal cortex (Barker et al., 2006b).

1.3.3 NMDARs

In contrast to AMPARs, NMDARs mediate postsynaptic current that has a much slower rise time and decay time. The activation of NMDARs is dependent upon both agonist binding and membrane depolarisation for receptor channel opening. At resting membrane potential, ions cannot flow through the channel due to a block by the Mg^{2+} ion, rendering NMDARs voltage-dependent. If the cell is depolarised then the Mg^{2+} block is removed and the current can flow (Dingledine, Borges et al. 1999). NMDARs also require glycine as a co-agonist, so both glutamate and glycine have to be bound before the channel will open. Recently, D-serine has been shown to act as a co-agonist, which is released by astrocytes (Panatier, Theodosis et al. 2006). It is thought that glycine is present at a sufficient concentration *in vivo* and *in vitro* to bind all NMDARs (Bashir, Tam et al. 1990; Dingledine, Borges et al. 1999; Wenthold, Prybylowski et al. 2003). NMDARs therefore act as ‘coincidence detectors’ for postsynaptic depolarisation and presynaptic release of glutamate.

An important feature of NMDAR function lies in its permeability to Ca^{2+} as well as Na^{+} and K^{+}. Entry of Ca^{2+} into the cell via NMDARs not only further depolarises the cell, but can also activate many Ca^{2+} sensitive enzymes. Around 7-18% of inward current through NMDARs is carried by Ca^{2+} ions.
The influx of Ca$^{2+}$ has been shown to be very important in the induction of long-term plasticity (Dingledine, Borges et al. 1999).

Structurally, NMDAR subunits have the same membrane topology as AMPAR and KAR subunits, with three transmembrane domains and a re-entrant loop, an intracellular C-terminus and a large extracellular N-terminus that contains a ligand binding domain (Stephenson 2001). A variety of NMDAR subunits have been identified (NR1-4) (Cull-Candy, Brickley et al. 2001). NR1 subunits have eight splice variants and contain the glycine binding site. Four genes encode NR2 subunits (NR2A-D) and the glutamate binding site is found in these subunits. A third subunit exists, NR3, which has two isoforms NR3A and NR3B (Stephenson 2001). Native NMDARs are believed to be tetrameric heteromers of NR1 and NR2 subunits with a stoichiometry believed to be a dimer of dimers, NR1-NR1-NR2-NR2. In receptors containing the NR3 subunit, it is seems likely that a NR3 subunit substitutes for one of the NR2 subunits (Dingledine et al., 1999; Cull-Candy et al., 2001; Kew and Kemp, 2005). These receptors function as Ca$^{2+}$-impermeable excitatory glycine receptors that respond to agonist application with low efficacy (Chatterton, Awobuluyi et al. 2002).
NR1 transcripts are expressed in nearly all neurones, while NR2 subunits are expressed more discretely. The NR2A and NR2B subunits are the major and most widespread NR2 subunits, with NR2C largely restricted to the cerebellum and NR2D most heavily expressed early in development (Monyer, Burnashev et al. 1994; Stephenson 2001; Wenthold, Prybylowski et al. 2003). The NR2B subunit dominates early in development and gradually decreases postnatally and is predominately expressed in the forebrain. NR2C subunits are restricted to the cerebellum and NR2D subunits are expressed prenatally and restricted to the diencephalon and brain stem (Lynch and Guttmann 2001; Stephenson 2001; Molnar and Isaac 2002).

There is evidence that suggests that in adult cortex NR2A subunits are preferentially localised to synaptic sites and NR2B subunits are localised extrasynaptically (Stocca and Vicini 1998; Rumbaugh and Vicini 1999). A study by Massey et al. (2004) has demonstrated that in the perirhinal cortex the
subunit composition and postsynaptic localisation of NMDARs are critical
determinants of their roles in synaptic plasticity (Massey, Johnson et al. 2004).
NR2A-containing NMDARs are required for LTP induction and depotentiation
and NR2B-containing NMDARs are required for de novo LTD (Massey,
Johnson et al. 2004). A similar result was also shown in the hippocampus (Liu,
Wong et al. 2004)

Numerous studies have been conducted that examine the role of NMDARs in
learning and memory. Morris et al. (1990) demonstrated that infusion of the
NMDAR antagonist D-AP5 into the hippocampus blocked the acquisition of
spatial memory tested by the Morris water-maze (Morris, Davis et al. 1990).
Moreover, in perirhinal cortex, the antagonism of NMDARs by D-AP5 impaired
the acquisition of recognition memory after a long but not a short delay.
However, recognition memory after a 24 hour delay was impaired only when
NR2A and NR2B antagonists were infused together, not when either was
infused separately (Barker et al., 2006b). This suggests that there could be two
independent mechanisms that underlie long-term recognition memory; one
dependent on a process used in LTP/depotentiation (requiring NR2A subunits)
and another dependent on a process used in LTD (requiring NR2B subunits),
either being capable of supporting familiarity discrimination at long delays.

Many diseases are proposed to involve excitotoxicity, such as stroke, epilepsy,
hypoxic injury and also neurological disorders such as Alzheimer’s disease,
Huntington’s disease and Parkinson’s disease (Lynch and Guttmann 2001).
The NR2B antagonist ifenprodil administered to a rat model of Parkinson’s
disease led to a significant improvement in locomotor activity (Loftis and
Janowsky 2003).

1.3.4 Metabotropic glutamate receptors
Metabotropic glutamate receptors (mGluRs) were discovered in 1987
(Sugiyama, Ito et al. 1987) and to date there are a total of eight mGluR
subunits, named mGluR1-8. These are classified into three groups based on
their amino acid sequence identity and signal transduction coupling; mGluR1
and mGluR5 belong to group I; mGluR2 and mGluR3 belong to group II and
mGluR4 and mGluR6-8 belong to group III (for a review see Conn and Pin,
Group I mGluRs couple to phospholipase C (PLC), stimulating the hydrolysis of phosphatidylinositol (4,5) -bisphosphate (PIP$_2$) into diacyl glycerol (DAG) and inositol (1,4,5)-triphosphate (IP$_3$). This results in the activation of PKC and the release of Ca$^{2+}$ from intracellular stores. On the other hand, group II and group III mGluRs are negatively coupled to adenylate cyclase (AC), resulting in a reduction in intracellular levels of cyclic adenosine monophosphate (cAMP) (Conn and Pin 1997; Michaelis 1998; Pin, Galvez et al. 2003). mGluRs of the same group show approximately 70% sequence homology, whereas between groups homology is approximately 45%.

mGluRs form homodimers composed of two mGluR subunits. Each subunit has a large extracellular N-terminal domain, seven transmembrane domains linked by relatively short loops and an intracellular C-terminus of varying length. The glutamate binding site is proposed to exist between two globular extracellular domains with a hinge region. The C-terminus is likely to be involved in the targeting and tethering of mGluRs to specific neuronal compartments and possibly also interaction with the respective G-protein. G-protein coupling is also thought to be made through the intracellular transmembrane loops (Conn and Pin, 1997; Michaelis, 1998; Pin et al., 2003; Kew and Kemp, 2005).

**Figure 1.3.3 Schematic representation of the mGluR subunit structure.** As is characteristic of metabotropic receptors, mGluRs have seven transmembrane domains. The intracellular loop between transmembrane regions III and IV is important for coupling to G-proteins. Modified from Conn and Pin (1997).
Although mGluR family members can mediate synaptic transmission via activation of slow excitatory postsynaptic potentials, they generally exert a more modulatory role, regulating neuronal excitability, synaptic transmission and plasticity (Kew and Kemp 2005). Group I mGluRs are typically localised postsynaptically in somatodendritic domains, whereas group II and III receptors are predominantly presynaptic, localised in axonal domains and axon terminals (Kew and Kemp 2005). Electrophysiological evidence suggests that mGluRs are located postsynaptically in the perirhinal cortex, though it is not known if presynaptic mGluRs are present (Cho et al., 2000; Cho et al., 2002). The activation of NMDARs and group I mGluRs is necessary for LTD induction (Cho et al., 2000). In the hippocampus, presynaptic mGluRs have been shown to reduce GABA release thereby reducing inhibitory transmission (Conn and Pin 1997).

1.4 Synaptic plasticity

Synapses can be considered dynamic structures that possess the property of being able to change their structure and/or efficiency according to what input they receive. At a basic level, synaptic plasticity can be split into potentiation and depression of synaptic transmission. These are generally defined as changes in the amplitude of postsynaptic potentials that are dependent upon the prior activity of the synapse. Plasticity can last over a period of milliseconds to minutes (short-term) or for hours or days (long-term). Long-term plasticities have attracted great interest as they have been implicated in underlying the brain’s ability to learn and store memories (Bliss and Lomo 1973; Bliss and Collingridge 1993; Malenka and Bear 2004).

1.4.1 Short-term plasticity

The short-term plasticities include facilitation, post-tetanic potentiation and post-tetanic depression (see Zucker and Regehr, 2002; Shepherd, 1998 for review). Facilitation is usually referred to as ‘paired-pulse facilitation’ (PPF) because it is studied by giving a pair of stimuli to a synaptic pathway and comparing the
amplitude of the second EPSP to the first. This type of plasticity is largely believed to be pre-synaptic in origin (Bear and Malenka 1994). The first pulse leads to depolarisation of the presynaptic terminal and an increase in intracellular Ca\(^{2+}\) that ultimately results in neurotransmitter release. If an optimal interval of ~50ms occurs between the first and second pulse, residual Ca\(^{2+}\) lingering from the first pulse, plus the influx of Ca\(^{2+}\) from 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 amount of transmitter released and therefore a subsequent greater postsynaptic response to the second pulse (Shepherd 1998; Zucker and Regehr 2002).

Post-tetanic potentiation is a transient increase in the amplitude of a synaptic response that is seen after a brief train of stimuli. Post-tetanic potentiation, like PPF, is also reliant upon increases in the probability of transmitter release resulting from increases in residual calcium in the presynaptic terminal. Post-tetanic depression is also thought to rely primarily on presynaptic mechanisms (Zucker and Regehr 2002). If pairs of stimuli are delivered, around 50 milliseconds apart, then a depression of the second EPSP can be observed in hippocampal neurones in a phenomenon known as ‘paired-pulse depression’ (PPD). 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 and Regehr 2002).

### 1.4.2 Long-term plasticity

LTP is characterised by a long-lasting increase in synaptic efficacy induced typically by a 100 Hz high frequency stimulation (HFS) protocol and is thought to underlie the changes that occur in the brain during learning (Bliss and Lomo, 1973; Bliss and Collingridge, 1993). LTP has been most extensively studied in the CA1 region of the hippocampus and there have been a vast number of studies into the mechanisms of this phenomenon at different synapses and the circuits which operate in the mammalian brain (Malenka and Bear 2004). LTD is
in essence opposite to LTP, in that it is characterised by a long-lasting decrease in synaptic efficacy. It is induced by applying low frequency stimulation (LFS) to a synaptic pathway. Similar to LTD is the process of depotentiation, whereby LFS is given to a pathway that has already been potentiated and is expressing LTP and this increase in synaptic strength is subsequently reversed. *De novo* LTD itself can be reversed by HFS in the process of de-depression (Kemp and Bashir, 2001; Collingridge et al., 2004). These ‘bi-directional and reversible alterations in synaptic efficiency make possible the dynamic storage of vast amounts of neurally encoded information’ (Collingridge, Isaac et al. 2004).
1.4.2.1 NMDAR-dependent LTP

1.4.2.1.1 LTP induction

Since the important discovery that antagonism of the NMDAR by D-AP5 blocked LTP induction in the CA1 region of the hippocampus (Collingridge, Kehl et al. 1983), there has been a plethora of primary research papers and reviews that aim to elucidate the mechanisms underlying NMDAR-dependent LTP (Bliss and Collingridge 1993; Bear and Malenka 1994; Malenka and Nicoll 1999; Lisman, Schulman et al. 2002; Malinow and Malenka 2002; Lisman 2003; Malenka 2003; Collingridge, Isaac et al. 2004; Malenka and Bear 2004). For the induction of LTP (and LTD) to occur a rise in intracellular calcium ($Ca^{2+}$) must take place, brought about by NMDAR activation. At resting membrane potentials NMDARs are inactive, due to the Mg$^{2+}$ block of the channel. But, when a neuron becomes depolarised, typically following the activation of AMPARs, the Mg$^{2+}$ block of the NMDA channel is relieved. This allows Na$^+$ and Ca$^{2+}$ to enter the neuron, creating an intracellular rise in Ca$^{2+}$ (Malenka and Bear 2004). Regardless of how the LTP is induced, there is compelling evidence to indicate that calcium/calmodulin dependent protein kinase II (CaMKII) is required as a mediator for NMDAR-dependent LTP (see Lisman et al., 2002: Lisman, 2003 for an extensive review). During synaptic activity, the activated kinase translocates from the cytoplasm and binds to the NMDAR, where it can sense the very high Ca$^{2+}$ levels, resulting in the downstream activation of signalling cascades that are involved in LTP expression. Other kinases have been implicated in playing key roles in LTP, although whether they act as mediators or modulators of LTP often remains contentious. There are a large number of protein phosphatase complexes, such as PP1, which have roles in the modulation of CaMKII-dependent signalling, potentially enabling a subtle and diverse modulation of synaptic transmission in the hippocampus, although their exact roles in LTP are not yet fully established (Colbran 2004).
1.4.2.1.2 LTP Expression

There appear to be two major post-synaptic mechanisms that are involved in the expression of NMDAR-dependent LTP. Namely these include 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 and Nicoll 1999; Malinow and Malenka 2002; Song and Huganir 2002; Bredt and Nicoll 2003; Lee, Takamiya et al. 2003; Malenka and Bear 2004).

The phosphorylation of AMPARs primarily occurs at various sites on the GluR1 subunit by CaMKII and PKC, which results in an increase in single channel conductance of the AMPAR with homomeric GluR1 subunits (Benke, Luthi et al. 1998). A recent study by Boehm et al., (2006) in the hippocampus has identified another PKC phosphorylation site on the GluR1 subunit at serine 818 (S818) (Boehm, Kang et al. 2006). The phosphorylation state of this site controls stable incorporation of GluR1 into the synapse. LTP-inducing stimuli phosphorylate this site, and its phosphorylation is important for the establishment of LTP and they believe this is likely to act by facilitating an interaction with a delivery and/or stabilising protein (Boehm, Kang et al. 2006). This further elucidates the link between the modification by phosphorylation of AMPARs and their trafficking to the synapse during LTP.

1.4.2.1.3 Maintenance of LTP

Much of the work on NMDAR-dependent LTP has focussed upon the mechanisms responsible for the initial increase in synaptic strength lasting 30-60 minutes, although arguably of greater interest and importance are the mechanisms that allow LTP to last for hours, days or even weeks (Malenka and Bear 2004). It is well established that the longer lasting components of LTP require new protein synthesis and gene transcription (Abraham and Williams 2003; Lynch 2004; Miyamoto 2006; Reymann and Frey 2007). As will be described more extensively in following chapters, PI3K and the mammalian target for rapamycin (mTOR) seem to be involved in the maintenance of LTP via protein synthesis and translation. 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 (Lynch, 2004b; Warburton et al., 2005; Miyamoto, 2006; Reymann and Frey, 2007). CREB phosphorylation can lead to the activation of the immediate early gene c-fos and zif268 (Christy and Nathans 1989; Ahn, Olive et al. 1998). The expression product of c-fos is Fos, which can act as an accurate marker for recognition memory processes (Zhu et al., 1996; Wan et al., 1999; Warburton et al., 2003; Wan et al., 2004; Warburton et al., 2005). Inhibition of CREB phosphorylation in the perirhinal cortex (caused by the transduction of a dominant-negative inhibitor of CREB, which prevented the ability of CREB to bind to DNA) blocked LTP and also long-term recognition memory (Warburton, Glover et al. 2005). Other studies have demonstrated that there is a link between the CREB phosphorylation and the maintenance of LTP with memory in other parts of the brain, such as the hippocampus (Pittenger, Huang et al. 2002; Nguyen and Woo 2003; Reymann and Frey 2007).

The proposed link between LTP and other memory systems, such as spatial learning in the hippocampus and fear conditioning in the lateral amygdala has been also extensively studied (Martin and Morris 2002; Morris 2003; Sigurdsson, Doyere et al. 2007). One of the early classic experiments utilised the water maze to establish that spatial memory and LTP in the hippocampus are both NMDAR-dependent (Morris, Anderson et al. 1986). A recent study has used GFP-tagged GluR1 viral constructs to demonstrate that fear conditioning drives synaptic incorporation of GluR1 receptors in the lateral amygdala (Rumpel et al., 2005). Their results indicate that blocking GluR1-receptor trafficking in ~10-20% of neurones undergoing plasticity is sufficient to impair memory formation. This is an elegant set of experiments as it demonstrates a clear link between in vitro plasticity mechanisms, i.e. trafficking of GluR1 to the synapse during LTP and in vivo memory processes during learning.

The PKC isozyme, protein kinase M zeta (PKMζ) and phosphatidylinositol 3-kinase (PI3K) have been implicated in having roles in the delivery of GluR1-containing AMPARs to synapses that have undergone LTP and in LTP maintenance (Ling, Benardo et al. 2002; Sanna, Cammalleri et al. 2002).
Injection of a peptide inhibitor of PKM\textsubscript{\varepsilon} into the dentate gyrus does not block LTP induction; it reverses established LTP when applied up to 5 hours post-tetanisation (Serrano, Yao et al. 2005). The peptide inhibitor has recently been shown to reverse LTP in the hippocampus \textit{in vivo} and results in a loss of recent spatial learning, indicating that LTP maintenance can sustain spatial memory (Pastalkova, Serrano et al. 2006).

Morphological changes in the structure of the synapse occur during LTP, including the growth of new dendritic spines, enlargement of pre-existing dendritic spines and their associated postsynaptic densities (PSDs) (Abraham and Williams 2003; Matsuzaki, Honkura et al. 2004). Some studies have shown that the polymerisation of the actin cytoskeleton in the spines is important for LTP, with \textit{in vitro} and \textit{in vivo} studies showing that there is inhibition in the spine of actin depolymerisation following LTP (Kim and Lisman 2001; Fukazawa, Saitoh et al. 2003).

\textbf{1.4.2.2 NMDAR-dependent LTD}

\textbf{1.4.2.2.1 Induction of LTD}

NMDAR-dependent homosynaptic \textit{de novo} LTD was first demonstrated in the CA1 region of the hippocampus, where \textit{in vitro} LFS was effective at inducing LTD without the requirement for the prior induction of LTP (Dudek and Bear 1992; Mulkey and Malenka 1992). The ability of synapses to undergo homosynaptic LTD (depression only in the pathway receiving the induction protocol) has been established in brain regions other than the hippocampus, including the perirhinal cortex (Kemp and Bashir 2001). The induction of NMDAR-dependent LTD has been shown to be dependent upon a rise in postsynaptic Ca\textsuperscript{2+} levels (Dudek and Bear, 1992; Mulkey and Malenka, 1992). As in the induction of LTP, Ca\textsuperscript{2+} enters through NMDARs when the neurone is depolarised and the Mg\textsuperscript{2+} block is relieved. The quantitative characteristics of the postsynaptic Ca\textsuperscript{2+} signal that is required to trigger LTD remain to be fully elucidated, although it has been proposed that LTP induction involves a marked elevation in Ca\textsuperscript{2+} concentration compared to a moderate rise for LTD (Ismailov, Kalikulov et al. 2004). According to this model, there is a difference in the Ca2+-
dependencies of the kinases and phosphatases; a 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 has been proposed that a temporal factor must also be considered in the elevation of Ca2+ concentration that determines LTP or LTD induction (Mizuno, Kanazawa et al. 2001). It appears that a prolonged elevation of Ca2+ is crucial for the induction of LTD, and if the elevation is brief, at an equivalent Ca2+ concentration LTD will not be induced. In addition to Ca2+ entering through NMDARs, a role for Ca2+ release from intracellular stores has been proposed for LTD, although it is of little contribution when NMDARs are optimally activated (Nakano, Yamada et al. 2004).

The typical protocol for the induction of LTD is a prolonged repetitive stimulation at 0.5-5Hz and a robust change usually occurs after many stimuli e.g. 900. However, there is a developmental down-regulation of LFS-LTD such that by ~35 days, LFS is less effective at inducing LTD and by adulthood, LFS results in no LTD in evoked-field recordings (Dudek and Bear 1993; Staubli and Ji 1996; Kemp, McQueen et al. 2000). This change is not due to a loss of the ability of synaptic transmission to depress, but more conditions or different protocols are required in order for the synapse to express LTD (Kemp and Bashir 1997; Kemp, McQueen et al. 2000; Massey, Johnson et al. 2004). Interestingly, it is possible to induce LTD with LFS at at 5Hz in adult tissue in whole-cell recordings, although it is unclear as to why a different recording method makes this is possible (Cho et al., 2000; Park et al., 2006). In addition, it appears that LFS can induce LTD in aged animals (over 20 months) via Ca2+ entry through L-type Ca2+ channels (Norris, Halpain et al. 1998) and also in animals that have been exposed to stress (Bartanusz et al., 1995; Yang et al., 2005). The facilitation of LTD induction by stress has been proposed to be due to the blockade of glutamate uptake and the activation of extrasynaptic NR2B subunits (Yang, Huang et al. 2005), and the increase in N2RB mRNA in the hippocampus (Bartanusz, Aubry et al. 1995).
1.4.2.2 Expression and maintenance of LTD

The posttranslational modification of AMPARs by dephosphorylation and the physical loss of AMPARs from the synapse are the two main modes by which LTD is expressed (Song and Huganir 2002; Malenka and Bear 2004). A moderate increase in Ca\textsuperscript{2+} associated with LTD induction could activate protein phosphatase 2B/calcineurin (PP2B) via the calcium–calmodulin complex. This in turn dephosphorylates and inactivates inhibitor 1, resulting in the activation of PP1 and/or PP2B, which can dephosphorylate AMPARs (Kemp and Bashir 2001). It has been reported that an increase in PP1 and protein phosphatase 2A (PP2A) activity occurs following the induction of LTD in vivo (Thiels, Norman et al. 1998) and that PP1 and PP2A inhibitors can block LTD (Mulkey, Herron et al. 1993). Moreover, phosphorylation of GluR2 has been shown to regulate the association of GluR2 with interacting proteins, such as GRIP/ABP and PICK1 (Seidenman, Steinberg et al. 2003), indicating that these interactions may be regulated during LTD, resulting in changes in the synaptic targeting of AMPARs.

A reduction in spine density is a postsynaptic structural change that is another proposed mechanism of LTD expression. The application of NMDA has been shown to cause a decrease in spine number in cultured hippocampal neurones (Halpain, Hipolito et al. 1998). The induction of LFS-LTD is accompanied by a marked shrinkage of dendritic spines in hippocampal slices and is dependent upon NMDARs and calcineurin activation (Zhou, Homma et al. 2004). Spine shrinkage is mediated by F-actin depolymerisation (Zhou, Homma et al. 2004). However, the blockade of PP1 inhibits LTD but has no effect upon spine shrinkage, indicating a divergent downstream pathways leading to LTD and spine shrinkage (Zhou, Homma et al. 2004).

1.4.2.3 mGluR-dependent LTD

A role for mGlu receptors in the induction of LTD was first demonstrated when it was shown that the group I/II antagonist α-methyl-4-carboxyphenylglycine (MCPG) blocked the induction of depotentiation in the CA1 region of the
hippocampus (Bashir, Jane et al. 1993; Bashir and Collingridge 1994). Many studies have described different roles for mGluRs for the induction of LTD that depend upon the brain region (Kemp and Bashir 2001; Malenka and Bear 2004). A role for group I and/or II mGluRs have been demonstrated in numerous areas of the brain, including the perirhinal cortex (Palmer, Irving et al. 1997; Fitzjohn, Kingston et al. 1999; Huber, Roder et al. 2001; Kemp and Bashir 2001; Moult, Schnabel et al. 2002; Huang, You et al. 2004; Moult, Gladding et al. 2006). DHPG-LTD does not occlude with NMDAR-LTD (Palmer et al., 1997; Fitzjohn et al., 1999) and depends upon activation of G-proteins and MAP kinase cascades (Fitzjohn, Palmer et al. 2001; Schnabel, Kilpatrick et al. 2001; Huang, You et al. 2004). There is a decrease in surface expressed AMPARs after DHPG treatment indicating that the removal of AMPARs from the synapse is an expression mechanism in this form of LTD (Moult et al., 2006).

1.4.3 Metaplasticity

Metaplasticity has been described as the modulation of synaptic plasticity by previously imposed activity (Abraham and Bear 1996; Jedlicka 2002; Woo and Nguyen 2002). The Bienenstock-Cooper-Munro (BCM) theory of metaplasticity describes a sliding scale that changes the ‘modification threshold,’ a parameter that determines if plasticity occurs, depending on the history of postsynaptic activity (Abraham and Bear 1996; Jedlicka 2002). An example of metaplasticity is depotentiation, which describes the depression of previously potentiated synapses. The process of applying LTP-inducing HFS protocols before LTD-inducing LFS protocols in this way is sometimes described as “priming.” It has also been shown that LTP protocols applied in the presence of D-AP5 do not induce LTP but are still able to prime synapses facilitating the subsequent induction of LTD (Kemp and Bashir, 2001).

1.4.4 Synaptic plasticity in the perirhinal cortex

Although numerous studies into synaptic plasticity in the brain have been made, only relatively recently has detailed investigation into synaptic plasticity in the perirhinal cortex begun. An initial study using evoked field recordings demonstrated that plasticity in the perirhinal cortex was both input- and layer-dependent (Ziakopoulos, Tillett et al. 1999). NMDAR-dependent LTP could only be induced in intermediate (layer II/III) pathways and not in superficial (layer I)
pathways. The magnitude of depotentiation (LFS following HFS) was found to be greatest in the temporal intermediate layer. These differences suggest that synaptic transmission in the perirhinal cortex could be differentially regulated. There are also differences in GABAergic transmission between temporal and entorhinal inputs (Garden, Kemp et al. 2002), which may be important in the control of neuronal activity in the perirhinal cortex. Indeed, inhibition of GABA_A receptors blocks LTP and also LTD, as well as inhibiting recognition memory and blocking the greater neuronal activation associated with viewing novel over familiar stimuli (Wan, Warburton et al. 2004).

The role of acetylcholine has been suggested to play a crucial role in learning and memory and has been investigated in the perirhinal cortex (Massey et al., 2001; Warburton et al., 2003). The application of the muscarinic receptor antagonist scopolamine blocked LTD in vitro and also recognition memory in vivo but LTP was unaffected (Warburton, Koder et al. 2003). This suggests that cholinergic mechanisms in the perirhinal cortex play an important role in synaptic plastic mechanisms, specifically LTD, which underlies recognition memory.

The role of mGluRs has been investigated as well (McCaffery et al., 1999; Cho et al., 2000; Cho et al., 2002; Harris et al., 2004). A form of LTD that is dependent upon the activation of both NMDA and mGlu receptors was found fairly recently (Cho et al., 2000). Further investigation of the cellular mechanisms of LTD induction at resting potentials revealed that the synergy between mGluR5 and group II mGluR receptors relies upon an increase in cAMP and that LTD was inhibited at resting potential when cyclosporin A was used to inhibit the phosphatase PP2B (Cho et al., 2002). Also, in perirhinal cultured neurons 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, Cho et al. 2004).

It has recently been reported that a developmental change in plasticity mechanisms occurs in the perirhinal cortex (Jo, Ball et al. 2006). LTD was shown to switch in a visual-experience manner from an mGluR5- to a mAChR-dependent form after eye opening. This age-related switch in mechanism
highlights the importance of knowing the age of the animals used in studies, as it could dictate which mechanisms are involved in plasticity, therefore needs to be known to be able to make accurate comparisons between studies.

A significant discovery that was reported in the perirhinal cortex regards the differential roles of NMDAR-subunits in bidirectional synaptic plasticity (Massey, Johnson et al. 2004). The induction of LTP requires NR2A-containing NMDARs (Massey, Johnson et al. 2004).

Additional to the above findings, Massey et al., (2004) offer an explanation to the difficulty in inducing LTD in adult tissue (described in Kemp and Bashir, 2001). Using evoked-field recordings, Massey et al., (2004) were able to induce LTD in adult tissue by blocking glutamate uptake, thus enabling greater activation of the extrasynaptically located NR2B-containing NMDARs. This suggests that a reason for the difficulty in inducing adult tissue may be attributable to a more efficient glutamate uptake mechanism than in younger tissue. In addition, there is a developmental transition from the NR2B-dominant form of NMDARs to the NR2A form that occurs at 14-21 days, which corresponds with a loss of LFS-induced LTD that is observed in adults, but not young animals (Okabe, Collin et al. 1998).

1.4.5 Plasticity in humans

Although there is strong evidence that correlates the underlying mechanisms of LTP with the mechanisms of learning and memory, there are very few studies in humans, although it appears that rodents and humans share much of the mechanisms of LTP (Chen et al., 1996; Cooke and Bliss, 2006). Hippocampal tissue taken from patients that have undergone excision as a treatment for temporal lobe epilepsy exhibit NMDAR-dependent LTP, the expression of which appears to involve CaMKII. Patients that have hippocampal foci for their epilepsy have impaired declarative memory, thereby providing a set of correlations with mechanisms previously identified in animals (Cooke and Bliss 2006). An interesting avenue for future research involves the use of non-invasive tetanic stimulation in conscious patients, which is now making it
possible to consider treatments based on the induction of long-lasting changes in cortical output using stimulation protocols similar to those that have been used to induce synaptic plasticity in animals. Repetitive transcranial magnetic stimulation (rTMS) has not yet been proven to act specifically by changing the efficacy of synaptic transmission, rather than increasing the excitability of the cell, its value as a therapeutic tool is the subject of investigation (Cooke and Bliss 2006). The application of rTMS (between 1-20Hz) has been shown to have anti-depressant effects and could potentially act as an alternative to electroconvulsive therapy as a treatment for depression (Nahas, Li et al. 2004). rTMS is also been investigated as a treatment for disrupted motor cortex output, such as akinesia and limb rigidity, which are symptoms associated with the loss of neurones from the substantia nigra that occurs in Parkinson’s disease (Goldberg, Boraud et al. 2002). The application of low frequency rTMS to the epileptic foci of severely epileptic patients has been shown to reduce the number of seizures (Tergau, Naumann et al. 1999). The LTD-like tetanus may either depotentiate over-potentiated synapses or compensate for other causes of neuronal hyperexcitability (Cooke and Bliss 2006).

1.5 PKMζ

The Protein Kinase Mζ is the constitutively active isoform of PKCζ. PKCζ is one of the Protein Kinase C (PKC). There are different isoforms of PKCs, which are divided into three groups: conventional (α, βI, βII, γ), novel (δ, θ, η, ε) and atypical (ζ and ιλ) (Nishizuka 1988; Nishizuka 1988). The conventional PKCs (α, βI, βII, and γ) are activated by both the lipid second messenger diacylglycerol (DAG) and Ca²⁺. Novel PKCs (δ, θ, η, and ε) are activated by DAG, but not by Ca²⁺. Atypical PKCs (ζ and ιλ) are activated by neither DAG nor Ca2+ directly, but by an alternate set of lipid second messengers, including arachidonic acid (Nakanishi and Exton 1992), phosphatidylinositol 3,4,5-trisphosphate (Nakanishi, Brewer et al. 1993), and ceramide (Muller, Ayoub et al. 1995). The M isoform of PKCζ is different because it consists only of the catalytic domain of the kinase (as shown in Figure 1.5.1). Therefore, lacking the regulatory domain, PKMζ is constitutively active. The inhibition of PKMζ activity,
in fact, can be obtained using a selective peptide inhibitor, ZIP, which blocks PKMζ by reconstituting the regulatory domain (Figure 1.5.1).

![Diagram](image)

**Figure 1.5.1**: Left: a diagram representing the structure of PKC isoforms belonging to the different families. PKMζ possesses only the independent catalytic domain of PKCζ, therefore being constitutively active (picture modified from Naik et al., 2000). Right: the diagram shows the site of action of the selective PKMζ inhibitory peptide ZIP. ZIP is a pseudosubstrate which acts by restoring the regulatory domain, missing in PKMζ (picture modified from Pastalkova et al., 2006).

In addition, PKMζ is the only isoenzyme of PKC family that shows a stable “M” isoform in the hippocampus (Sacktor, Osten et al. 1993; Naik, Benedikz et al. 2000). At first it was thought that the formation of PKMζ derived from an alternative splicing of the mRNA for PKCζ. However, following studies demonstrated that PKMζ is synthesized from a specific mRNA encoding only the independent catalytic domain (Hernandez, Blace et al. 2003), and it is widely expressed throughout the brain. The synthesis of PKMζ is induced by a high-frequency stimulation (Osten, Valsamis et al. 1996), and it has been demonstrated that PKMζ expression is increased during the maintenance of LTP (Sacktor, Osten et al. 1993); NMDA receptors seem to be required to obtain PKMζ synthesis (Sacktor, Osten et al. 1993; Osten, Valsamis et al. 1996).

The reason why this enzyme is raising a crescent interest amongst the scientific community is that PKMζ has been demonstrated to be both necessary and sufficient for the maintenance of LTP (Ling, Benardo et al. 2002; Serrano, Yao et al. 2005; Serrano, Friedman et al. 2008), but its activity is not required to induce the potentiation. The inhibition of PKMζ by a specific inhibitory
pseudosubstrate (ZIP) reverts an established potentiation, but shows no effect on the induction an on early phases of LTP (Serrano, Yao et al. 2005).

*In vivo* experiments demonstrated as well that the intracerebral infusion of the inhibitory peptide is able to revert the LTP in the hippocampus and specifically impairs spatial memory (Pastalkova, Serrano et al. 2006). In addition, the inhibition of PKMζ in the insular cortex irreversibly erases long-term memory associations (Shema, Sacktor et al. 2007). Also, recent studies confirmed the role of PKMζ in the maintenance of memory in the sensorimotor cortex (von Kraus, Sacktor et al. 2010) and amygdala (Cohen, Kozlovsky et al. 2010). Therefore, these studies demonstrate that PKMζ plays a key role in late-phases of LTP. In contrast, no role for PKMζ in the induction or maintenance of LTD has been shown; moreover, the synthesis and the activity of this protein appear to be down-regulated following LTD (Hrabetova and Sacktor 1996; Osten, Hrabetova et al. 1996; Hrabetova and Sacktor 2001).

Mechanisms underlying the PKMζ-dependent maintenance of synaptic potentiation are still under examination. One hypothesis is that PKMζ maintains LTP by regulating the trafficking of AMPA receptors (Ling, Benardo et al. 2006); moreover, other studies demonstrate that this trafficking depends on PKMζ interaction with NSF/GluR2 subunit (Yao, Kelly et al. 2008). A fascinating hypothesis is that PKMζ acts by inhibiting the endocytosis of NMDA already present on the synapse, rather than promoting their insertion in the cell membrane (Migues, Hardt et al. 2010).

The mechanisms underlying the regulation of PKMζ activity are still to be confirmed. So far, there are evidences pointing out that the synthesis of PKMζ is regulated by several protein kinases (PI3-Kinase, MAP kinase, PKA, CaM Kinase) (Osten, Valsamis et al. 1996; Kelly, Crary et al. 2007). PKMζ is known to be constitutively active, meaning that it doesn’t need any of the second messengers required by the full-length atypical PKCs to exert its function (Sacktor, Osten et al. 1993). However, as many other full-length PKCs, PKMζ does require phosphorylation of its activation loop (T410), probably by the phosphoinositide-dependent kinase 1 (PDK1) (Kelly, Crary et al. 2007).
PDK1 is a serin/threonin kinase that acts downstream to PI3K; through phosphorylation, PDK1 activates many kinases, including PKC. Therefore, PDK1 plays a crucial role in regulating important cellular processes like cell proliferation, differentiation and apoptosis (Bayascas 2008).

PDK1, though, regulates the activation of PKMζ in a rather peculiar way. PKC of the conventional and novel groups, in fact, are primed by phosphorylation by PDK1, and then fully activated by the intracellular second messengers signaling cascades. PKCs belonging to the atypical group, as PKCζ, instead, are immediately activated by a phosphorylation by PDK1 in an “on/off” fashion (Le Good, Ziegler et al. 1998; Dong, Zhang et al. 1999; Balendran, Biondi et al. 2000; Balendran, Hare et al. 2000).

Once phosphorylated by PDK1 and therefore fully active, PKMζ starts a positive feedback loop, auto-maintaining high levels of its own synthesis during late-phase LTP (Kelly, Crary et al. 2007).

Another way through which PKMζ might maintain sustained levels of its own synthesis involves protein interacting with NIMA 1 (Pin-1). The synthesis of PKMζ, in fact, is inhibited by Pin-1. Pin-1 represses protein translation in the dendrites, but signals mediated by the excitatory neurotransmitter glutamate, which induces LTP and promotes memory formation, inhibit Pin-1, allowing protein synthesis. Newly formed PKMζ, then, inhibits in turn Pin-1, therefore auto-sustaining its own persisting synthesis and activity (Sacktor 2010; Westmark, Westmark et al. 2010). In addition, it has been suggested (Westmark, Westmark et al. 2010) that Pin1 normally suppress protein translation via its interaction with 4E-BPs. 4E-BPs are phosphorylated by mTOR to remove the suppression of translation initiation (Hoeffer and Klann 2010). If PKMζ maintains elevated transmission through protein translation, then this is likely to involve the mTOR-dependent translation initiation pathway.

1.6 Aim of the study

This study examinates the mechanisms of synaptic plasticity (namely potentiation and depression of the synaptic response) in perirhinal cortex, both in adult life and during the neurodevelopment, focusing on the role of PKMζ and its regulation.
2 METHODS AND MATERIALS
2.1 Electrophysiology

2.1.1 Animals

In some experiments performed in this study, perirhinal cortex slices were prepared from adult male Dark Agouti (DA) rats (Bantin & Kingman, Hull, UK, 7-15 weeks, 170-300g). In other experiments, perirhinal and hippocampal slices were prepared from P14 male DA rats. Animals were housed on a twelve-hour dark/light cycle with the dark-phase occurring during the daytime (0900-2100hrs). Samples of hippocampus and perirhinal cortex for molecular biology experiments were also collected from the same animals.

2.1.2 Preparation of perirhinal cortical and hippocampal 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 artificial cerebrospinal fluid (aCSF) for approximately one minute (aCSF composition in mM: NaCl, 124; KCl, 3; NaHCO3, 26; NaH2PO4, 1.25; CaCl2, 2; MgSO4, 1; D-glucose, 10; saturated with 95% oxygen and 5% carbon dioxide, pH 7.4). In order to obtain perirhinal cortex slices, the brain was placed on a piece of filter paper and hemisected by a mid-sagittal scalpel cut and one hemisphere was returned to the aCSF. The hemisphere was positioned with the midline face down and the majority of the frontal lobe was dissected with a cut at approximately 40° to the dorso-ventral axis. The cerebellum and a portion of the occipital lobe were removed by a single scalpel cut made at the same angle (Figure 2.1). The hemisphere was then glued (using cyanoacrylate adhesive) by its caudal end to a vibroslice stage and the tissue was supported medially by a block of Sylgard Ô (silicone elastomer, Dow Corning Ltd, Coventry, UK). 400 µm thick coronal slices were prepared using a vibroslice (Campden Instruments, Sileby UK). Slices included perirhinal areas 35 and 36 (Brodmann areas 35 & 36) and entorhinal and temporal cortices, which corresponded to rostro-caudal levels –3.80 to –5.80
mm relative to Bregma as described by Paxinos and Watson rat brain atlas and Burwell, 2001 (Paxinos and Watson 1998; Burwell 2001) (Figure 2.1).

**Figure 2.1 Location of the perirhinal cortex and adjacent cortices in the rat.** A lateral view of the brain illustrates the perirhinal cortex, composed of Brodmann areas 35 and 36 along the rhinal sulcus (top). Dotted lines indicate the position at which scalpel cuts were made at 40° to the dorso-ventral axis. A coronal schematic of the rat brain, which depicts the position of the perirhinal cortex (bottom). Dashed lines illustrate how 400 mm slices were trimmed during slicing. Adapted from thesis by Johnson BE (2003)
As for hippocampal slices, the cerebral hemisphere obtained as described above was glued by its lateral end to a vibroslice stage and cut in order to obtain 400 µm thick parasagittal slices including hippocampal areas CA1, CA2 and CA3. Once cut, slices were maintained in a holding chamber containing oxygenated aCSF at room temperature and were allowed to recover and equilibrate for at least one hour before recording commenced.

2.1.3 Field electrophysiological recording, equipment and techniques.

All the electrophysiological experiments in this thesis used the method of population field recording. The equipment and techniques used will be described in more detail. To achieve an optimal recording in the perirhinal cortex, various modifications were made, which will also be described below.

2.1.3.1 The rig

The rig was set up to enable the constant flow of oxygenated aCSF into the submerged recording chamber. The recording chamber consisted of a glass coverslip sandwiched between a Perspex disc and a metal disc, and sealed with silicone-based vacuum grease (see Figure 2.2). Slices were held in place by a nylon mesh stretched over a U-shaped piece of twisted temper annealed silver wire (0.55mm diameter, Advent Research Materials, Oxon, UK) affixed to a nylon mesh.

Oxygenated aCSF (95%O₂/5%CO₂) at a temperature of 28-29°C was pumped through polythene tubing (Fine bore polythene tubing, ID 1.4 mm, OD 1.9mm, Portex Ltd, UK) by a peristaltic pump (Watson-Marlow Ltd, UK) to a 2ml syringe. The syringe acted as a reservoir, where aCSF was again bubbled with oxygen and carbon dioxide to ensure saturation. The reservoir of aCSF in the syringe provided a constant supply to the recording chamber, eliminating the fluctuations in flow inherent in a peristaltic system. aCSF flowed through tubing into the recording chamber by force of gravity, with the flow rate set to 2-3ml/min by adjustment of the height of the syringe. Waste aCSF was removed by suction from the recording chamber (Charles Austen Pumps Ltd, UK) via a
needle. The recording chamber could contain a volume of 1.5-2ml depending on the positioning of the suction needle.

![Diagram of the slice recording chamber](image)

**Figure 2.2 Diagram of the slice recording chamber.** A) Plan view of the recording chamber, A polythene tube takes oxygenated aCSF to the recording chamber and once passed over the slice, it is removed by suction. B) Side view of the recording chamber, illustrating the coverslip sandwiched between the two parts of the recording chamber.

A Leica microscope (x40 magnification) was positioned directly above the recording chamber. Stimulation electrodes and recording electrode were mounted on magnetic stands (Narishige, Japan) onto an anti-vibration metal platform to minimise vibrations and prevent drift of equipment. The stimulation electrodes were positioned either side of the recording chamber and the recording electrode was mounted directly in front. All electrical equipment was earthed.
2.1.3.2 Glass recording pipettes

Recording electrodes 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 4-7MΩ. Micropipettes were filled with aCSF as internal solution (also called filling solution) using a fine Microfil™ needle (World Precision Instruments, USA) attached to a 1 ml syringe. A syringe filter (0.2 µm diameter pore size) was fitted between the syringe and Microfil™ needle to prevent any particles from obstructing the end of the glass micropipette that may have been present in the filling solution. The filled micropipette was then placed in an electrode holder (Axon Instruments, Union City, USA). This was fitted over a chlorided silver wire recording electrode (see below). The electrode holder was connected to a headstage (CV-4, Axon Instruments, USA) mounted on a micromanipulator (MWS-32, Narishige, Japan).

2.1.3.3 Bath and recording electrodes

Silver wire (0.25mm diameter, Advent Research Materials Ltd, UK) was used to make both recording and reference bath electrodes. The wire was chlorided in household 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. Silver chloride has a small redox potential, therefore minimises redox reactions occurring between the metal electrodes and salt-containing bathing solutions and reduces liquid-junction potential errors. The recording electrode was attached to the headstage and the reference bath electrode was secured around the perimeter of the recording chamber and grounded to the headstage.

2.1.3.4 Artificial cerebrospinal fluid composition

aCSF solutions (x1) were made up for dissection and experimentation fresh from 10x stock aCSF; this 1x solution contained 2mM CaCl₂ and 1mM MgSO₄.
2.1.3.5 Extracellular Field recording

A single slice was transferred to a submersion-type recording chamber containing recording aCSF. Before recording from the slice it was left for 15 minutes in the chamber to allow the tissue to acclimatise.

For recording, the glass micropipette was filled with standard aCSF as filling solution and lowered into the recording bath.

![Illustration of the position of stimulating and recording electrodes within layers II/III of the perirhinal cortex.](image)

**Figure 2.3 Illustration of the position of stimulating and recording electrodes within layers II/III of the perirhinal cortex.** The recording electrode was positioned directly beneath the rhinal sulcus and stimulating electrodes were positioned either side of the recording electrode.

With the aid of a microscope, the tip of the recording electrode was positioned midway between the two stimulating electrodes, immediately beneath the rhinal sulcus in cortical layers II/III (Figure 2.3). The tip of the pipette was slowly advanced into the slice using a hydraulic micromanipulator (MWS 32, Narishige, Japan). The amplifier settings were adjusted to recording voltage. Stimuli were delivered every 15 s alternately to each electrode (0.033Hz). Four input-specific consecutive fEPSPs (field excitatory postsynaptic potentials) were averaged for each stimulation input. A stimulus intensity of approximately 20V was initially used to evoke responses; this was adjusted to evoke responses with peak amplitudes in the range of −0.50 to −1.00 mV. The stimulus intensity was then adjusted to produce a peak amplitude ~70% of the maximal response. A period of stability lasting for 30 minutes or more was required to establish a baseline.
before adding drugs or delivering different protocols of stimulation, in order to induce potentiation or depression of the synaptic response in the slice. LTP was induced using an HFS protocol of 4 bursts of 100 stimuli at 100Hz, 1s duration (interburst time: 30s or 5 minutes). 5Hz LTD was induced using an LFS protocol of 3000 stimuli at 5Hz for duration of 10 minutes; 1Hz LTD was induced using an LFS protocol of 900 stimuli at 1Hz for a duration of 15 minutes. During LFS every 20 responses were averaged. 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. 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. In experiments run in hippocampus, the recording electrode was placed in the stratum radiatum; stimulating electrodes were placed on both sides (~0.5 mm) of the recording electrode. Both the amplitude of fEPSPs and the slope were measured. Analyses were carried as described for Perirhinal Cortex.

2.1.3.6 Data acquisition

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 WinLTP1.10 (Anderson and Collingridge 2001) with a sampling frequency of 10kHz. DC, Rm, Rs and synaptically-evoked currents were all recorded and saved to hard disk, and were reanalysed following completion of the experiment.

2.1.3.7 Data analysis

Data was reanalysed using the WinLTP1.10 reanalysis programme. Single sweeps were averaged offline every 4 sweeps, except during trains when sweeps were averaged every 20 sweeps. Reanalysed data was imported into Sigmaplot (Jandel Scientific, Germany) for analysis and for pooling purposes. Data for the whole experiment was normalised to the mean of the points that comprised the baseline. Data pooled across slices are expressed as means ±
SEM and effects of conditioning stimulation or drug applications were measured after LTD or LTP induction, or drug bath application, as described for each single experiment. Significance from baseline was tested using two tailed t-tests.

2.1.4 Pharmacological agents

Stock solutions were made by dissolving drugs into ddH₂O, HCl or DMSO, according to datasheets for each drug, and stored at -20°C. All stock solutions were made ≥ 100x the concentration required. Pharmacological compounds were bath applied as appropriate in different experiments.

Pharmacological agents were obtained from the following suppliers:

**Ascent Scientific, Bristol, UK**
MPEP 2-Methyl-6-(phenylethynyl)pyridine hydrochloride.

**Sigma-Aldrich, Poole, UK**
HAA, 3-Hydroxyanthranilic acid.

**Tocris, Bristol, UK**
Anysomicin, AP5 D-2-amino-5-phosphonopentanoate, KU 0063794 rel-5-[2-[(2R,6S)-2,6-dimethyl-4-morpholinyl]-4-(4-morpholinyl)pyrido[2,3-d]pyrimidin-7-yl]-2-methoxybenzenemethanol, LY294002 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride, LY456236 6-Methoxy-N-(4-methoxyphenyl)-4-quinazolinamine hydrochloride, Rapamycin, Wortmannin, ZIP Zeta Inhibitory Peptide.
2.2 Molecular Biology

2.2.1 Proteine Assay: Western Blot.
Perirhinal cortex was dissected from adult and P14 rats and stored frozen at -80°C. On the day of experiment, frozen tissue samples were lysed in 50mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA pH 8.0, 0.1% SDS, 1% Triton X-100 buffer, and supplemented with a protease inhibitor cocktail (Calbiochem, Gibbstown, NJ, USA). Protein concentration was determined via Bio-Rad Assay and 50µg of each protein sample was subjected to standard SDS-PAGE on 12% polyacrylamide gels, which were then electroblotted on mixed ester nitrocellulose membranes (Hybond-C Extra Amersham Bio). Filters were then blocked for 1h with 5% non-fat dry milk in TTBS buffer (100mM Tris-HCl, 0.9% NaCl, 0.1% Tween 20, pH 7.4). Blots were incubated overnight at 4°C with a polyclonal anti-PKCζ antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a monoclonal anti-β-actin antibody (1:5000, Sigma Aldrich, Gillingham, Dorset, UK). Blots were washed three times with TTBS buffer and then incubated for 1h with appropriate peroxidase-coupled anti-rabbit or anti-mouse IgG secondary antibodies, respectively; (1:10,000 Sigma Aldrich, Gillingham, Dorset, UK). All antibodies incubations were carried out in TTBS containing 5% non-fat dry milk. Blots were developed using BM Chemiluminescence Western Blotting Substrate (Roche, Burgess Hill, West Sussex, UK). Data are shown as levels of protein normalized to their actin (protein normally used as a control).
3 ROLE AND REGULATION OF PKM\(\zeta\) IN SYNAPTIC PLASTICITY IN ADULT PERIRHINAL CORTEX
3.1 Introduction

The primary function of perirhinal cortex is in processing recognition memory. Following the evidence that there is a decremental response in perirhinal neurons upon exposure to familiar objects (Brown and Xiang 1998), it has been proposed that mechanisms that rely upon a depression of synaptic transmission at perirhinal synapses could provide a model for understanding how the perirhinal cortex may process recognition memory (Brown and Aggleton, 2001; Brown and Bashir, 2002). Thus, most of the research has been focusing on LTD-like mechanisms in perirhinal cortex, therefore much less interest has been developed, instead, in LTP in this particular brain region. Although there are works confirming that it is possible to induce LTP in layers II/III of perirhinal cortex from adult rats (Ziakopoulos, Tillett et al. 1999; Massey, Johnson et al. 2004), the late phases of LTP maintenance were not investigated. So, the following experiments aim to determine whether it is possible, in fact, to induce a stable LTP in perirhinal cortex, lasting more than 3 hours, and which are the molecular mechanisms involved in the maintenance of this potentiation. It has been described before that in different brain areas (i.e. hippocampus) a strong HFS is able to induce a robust LTP, and that this potentiation depends in a continuous protein synthesis (Abraham and Williams 2003; Bozon, Davis et al. 2003; Bozon, Kelly et al. 2003; Lynch 2004; Miyamoto 2006). In early experiments (Frey, Krug et al. 1988; Abraham and Williams 2003; Bozon, Davis et al. 2003; Bozon, Kelly et al. 2003; Lynch 2004; Miyamoto 2006) it has been shown that a 3-h treatment with Anysomicin (a potent inhibitor of protein synthesis) immediately following multiple tetanization resulted in gradually developing loss of field excitatory postsynaptic potential in the hippocampus. In addition, (Scharf, Woo et al. 2002) Anysomicin blocks the induction of LTP when applied from 30 minutes to 1h after the delivery of HFS in vitro. The same group also demonstrated the a particular protocol, named “Spaced” tetra-bursts stimulation (4x100Hz bursts delivered at 5 minutes interburst interval) is able to induce a longer-lasting LTP, which also appear to be more dependent on the synthesis of new proteins (Scharf, Woo et al. 2002). Amongst these proteins, there’s an increasing number of works demonstrating that the constitutively active isoform of PKCζ, PKMζ, is necessary and sufficient for the maintenance
(but not the induction) of LTP, both in vitro and in vivo (Sacktor, Osten et al. 1993; Serrano, Yao et al. 2005; Pastalkova, Serrano et al. 2006; Shema, Sacktor et al. 2007; Serrano, Friedman et al. 2008). In contrast, no role for PKMζ in the induction or maintenance of LTD has been shown; moreover, the synthesis and the activity of this protein appear to be down-regulated following LTD (Hrabetova and Sacktor 1996; Osten, Hrabetova et al. 1996; Hrabetova and Sacktor 2001). Also, PKMζ is known to be constitutively active, meaning that it doesn’t need any of the second messengers required by the full-length atypical PKCs to exert its function (Sacktor, Osten et al. 1993). However, as many other full-length PKCs, PKMζ does require phosphorylation of its activation loop (T410), probably by the phosphoinositide-dependent kinase 1 (PDK1) (Kelly, Crary et al. 2007). Therefore, the questions we tried to address were (1) that it is in fact possible to induce a robust, stable LTP in adult perirhinal cortex, which would last for at least 5-6 hours; (2) whether this LTP is dependent or not on protein synthesis; (3) which is the role, if any, of PKMζ in the maintenance of the synaptic potentiation in perirhinal cortex; (4) whether PKMζ is in any way involved also in the induction and/or the maintenance of LTD in perirhinal cortex; (5) if it’s possible to modulate the activity of PKMζ, and consequently its effect on the maintenance of LTP through the inhibition of PDK1.

3.2 Results

3.2.1 Induction of LTP in adult perirhinal cortex

After a baseline recording of 60 minutes, LTP was induced on one pathway using a “spaced” tetra-bursts protocol (4x100Hz trains, 1s duration, delivered at 5 minutes interbursts interval) to induce a stronger LTP. No conditioning stimulation has been administered to the other pathway, which has been used as a control. Data were normalized to the 60 minutes baseline preceding the administration of the HFS protocol. Figure 3.1 shows a single example of LTP induced using the “Spaced” protocol. The pooled data (Figure 3.2) show that significant LTP (p<0.05) can be induced in one pathway, and the potentiation
remains stable for >5hs after the administration of the HFS protocol (126 ± 4% of baseline, n=4).

3.2.2 LTP in perirhinal cortex depends on newly synthesized proteins

After a baseline recording of 60 minutes, LTP was induced on one pathway using a “spaced” tetra-bursts protocol (4x100Hz trains, 1s duration, delivered at 5 minutes interbursts interval) to induce LTP (135 ± 6% of baseline, p<0.05, n=5). Anisomycin 20 µM was bath applied from 30 minutes before to 1h after the induction of LTP. No conditioning stimulation has been administered to the other pathway, which has been used as a control. Data were normalized to the 60 minutes baseline preceding the administration of the HFS. Figure 3.3 is a single experiment; the pooled data (Figure 3.4) show that Anisomycin 20µM significantly (p<0.05) prevents the lasting of LTP in perirhinal cortex (102 ± 5% of baseline, n=5), confirming that the maintenance of the potentiation requires the synthesis of new proteins.

3.2.3 Role of PKMζ in the maintenance of LTP in adult perirhinal cortex

Since, as shown before, it is actually possible to induce a stable, protein synthesis-dependent LTP in perirhinal cortex of adult (2-3 months) rats, and since there’s an increasing evidence that PKMζ plays a crucial role in the maintenance of LTP in the hippocampus, we decided to explore the role of this kinase in the maintenance of LTP also in perirhinal cortex. After a baseline recording of 60 minutes, LTP was induced on one pathway (LTP: 131 ± 5% of baseline, 60 min post HFS, P < 0.01, n=7). The selective Protein Kinase M ζ inhibitor peptide ZIP (5 µM) was bath applied 3 hours after the HFS. The concentration of 5µM has been chosen because it provides the maximal inhibition of PKMζ (Serrano, Yao et al. 2005) and it was bath applied 3 hours after the induction of LTP in order to be sure that the maintenance phase of LTP is examined. No conditioning stimuli have been administered to the other pathway, which has been used as a control. Data were normalized to the 60 minutes baseline preceding the administration of the HFS. Figure 3.5 is a single experiment; the pooled data (Figure 3.6) show that ZIP 5µM completely reverts
the established LTP (98 ± 5 % of baseline, P < 0.01, n = 7) with no significant effect on the control input (93 ± 4 % of baseline, P > 0.05, n = 7). Therefore in the mature cortical network PKMζ-dependent mechanisms are only active following induction of LTP but not during basal transmission. Experiments on the effect of ZIP on the baseline were carried to exclude the hypothesis that the small decrease in the synaptic transmission observed on the baseline following the application of ZIP could be due to a small cross-potentiation of the control pathway occurred during the administration of HFS. After 1 hour of baseline recording, ZIP 5µM has been bath applied. No conditioning stimulation was delivered to the slice. As shown in Figure 3.7, a small, non significant decrease of the basal synaptic transmission can be observed in both pathways (temporal: 89 ± 2% of baseline, p>0.05; entorhinal 88 ± 2% of baseline, p>0.05, n=4). Therefore, these data confirm that the inhibition of PKMζ has a minimum effect on the basal transmission. Since PKMζ maintains the LTP by regulating AMPA receptor trafficking in potentiated synapses (Yao, Kelly et al. 2008; Migues, Hardt et al. 2010), it is not completely unlikely that a small, non-specific activity of the Kinase on non-potentiated synapses could underlie this phenomenon.

3.2.4 Effect of the inhibition of PKMζ on a depotentiated pathway

According to different works (Hrabetova and Sacktor 1996; Osten, Hrabetova et al. 1996; Hrabetova and Sacktor 2001), the expression and the activity of PKMζ are increased after the induction of synaptic potentiation, and down-regulated by LTD-like mechanisms. Therefore, if this is the case, no effect of the inhibition of PKMζ should be observed in a de-depressed pathway, where the initial increase in the activity of this enzyme should be counter-balanced by its subsequent down-regulation determined by the induction of LTD. To stress this point, after 60 minutes baseline, the induction of LTP in one pathway (132 ± 5% of baseline, p<0.05, n=5) was followed by the administration of a 5Hz LFS protocol, in order to achieve a depotentiation of the pathway, which would bring the response back to the baseline (101 ± 2% of baseline, p>0.05, n=5). The subsequent bath application of ZIP shows no effect on a depotentiated pathway (98 ± 4% of baseline, p>0.05, n=5). On the other input, following the induction of LTP (128 ± 5% of baseline, p<0.01, n=5), the amplitude of the response is
decreased by a reduction of the stimulation intensity to match the depotentiation in the other input (99 ± 4% of baseline, p>0.05, n=5). This is aimed to exclude the bias that the effect of the inhibition of PKMζ could be in any way related to the sole actual amplitude of the synaptic response. In fact, even if the amplitude of the postsynaptic response, as measured, is the same of the other input, where the potentiation has been reversed by the administration of an LTD protocol, in this case the mechanisms leading to the expression of LTP are untouched, therefore, presumably, still fully working. The bath application of ZIP induces a significant reduction of the fEPSP amplitude in this pathway (65 ± 5% of baseline, p<0.01 v baseline, p<0.001 v the response measured after the reduction of the stimulation intensity, p<0.01 v the effect of ZIP in the depotentiated pathway, n=5), confirming that PKMζ is active after the induction of LTP, and that this activity is not related to the amplitude of the evoked response. Figure 3.8 shows a single experiment; the pooled data are shown in Figure 3.9.

3.2.5 Effect of the inhibition of PKMζ when the induction of LTD is blocked by AP5

In adult rat perirhinal cortex, LTD and synaptic depotentiation depend on the activity of NMDA receptors and can be blocked by the administration of the NMDA receptors antagonist D-AP5 (Massey, Johnson et al. 2004). In the experiments described above, we demonstrated that the administration of a depotentiation protocol (i.e. LFS) to a previously potentiated pathway, which brings the evoked response back to a baseline level, is also able to block the activity of PKMζ. It’s not clear, though, whether this effect is due to the sole administration of a LFS protocol, or if more complex intracellular changes, presumably LTD-related, are required. It is also known that NMDA activity is required to obtain PKMζ synthesis (Sacktor, Osten et al. 1993; Osten, Valsamis et al. 1996). Therefore we investigated the behaviour of the enzyme when the depotentiation is blocked by the NMDAR antagonist D-AP5. As shown in Figure 3.10 (single experiment) and 3.11 (pooled data), the induction of LTP in one pathway (148 ± 7% of baseline, p<0.01, n=5) was followed by the administration of a LFS protocol (5Hz) in presence of 50µM D-AP5 (from 30 minutes before
the induction of the depotentiation to the end of the protocol). If NMDARs are blocked, the LFS protocol fails to induce the depotentiation (148 ± 8% of baseline, p<0.01 v baseline, p>0.05 v LTP, n=5). The subsequent bath application of 5µM ZIP is able to decrease the response, bringing it back to the baseline (102 ± 2% of baseline, p<0.001 v LTP, p>0.05 v baseline, n=5). On the other input, no LFS was administered after the induction of a robust LTP (137 ± 4% of baseline, p<0.01, n=5); the application of ZIP 3 hours after the induction of LTP is able to revert the potentiation (100 ± 4% of baseline, p<0.001 v LTP, p>0.05 v baseline, p>0.05 v the “LTD” input after the administration of ZIP, n=5). These results suggest that when the depotentiation is blocked, PKMζ is still active and can be inhibited by ZIP. Thus, the activity of PKMζ requires the activity of NMDA receptors, and the down-regulation of this enzyme is determined by a proper synaptic depotentiation and consequent intracellular modifications.

3.2.6 Role of PKMζ in the induction of LTD

As discussed before, the synthesis of PKMζ is decreased during LTD, therefore its inhibition following the administration of an LFS protocol doesn’t seem to further decrease the amplitude of the synaptic response in slices of perirhinal cortex. Nevertheless, it’s not clear whether the inhibition of PKMζ before the administration of an LFS protocol has any effect on the induction of LTD. To examine this, after 30 minutes of baseline recording LTD was induced in one pathway, then the slice was incubated with 5µM ZIP. After 90 minutes of incubation, a 5Hz LFS was administered to the other pathway. ZIP was bath applied until the end of the experiment. Figure 3.12 shows a single experiment. Pooled data (Figure 3.13) show that the inhibition of PKMζ has no effect on the induction of LTD in perirhinal cortex (69 ± 4% of baseline, p<0.01 v baseline, n=5). Moreover, there’s no difference in the magnitude of the depression obtained after the LFS when the slice is pre-incubated with the inhibitor ZIP and the LTD in the control pathway (70 ± 4% of baseline, p>0.05 v experimental pathway, p<0.01 v baseline, n=5). Therefore, nor the induction of LTD neither the magnitude of the depression are influenced by the activity of PKMζ.
3.2.7 Role of PDK1 in the maintenance of LTP

Although the mechanisms underlying the regulation of the activity of PKMζ are still to be examined and confirmed, a pivotal role seems to be played by the phosphoinositide-dependent kinase 1 (PDK1). This kinase is activated by PI3-Kinase and phosphorylates PKMζ on its activation loop (T410); this phosphorylation alone is sufficient to fully activate PKMζ (Kelly, Crary et al. 2007). Following this theory, the inhibition of PDK1 should then lead to the block of PKMζ activity and, consequently, should negatively affect the maintenance of LTP. There are several specific PDK1 inhibitors, the most famous of all being the Non-steroidal anti-inflammatory drugs (NSAIgDs or NAIDs), especially the specific inhibitors of cicloxygenase-2 (Cox-2), called Coxibs. This group of drugs has been originally created as anti-inflammatory, analgesic drugs. Later it has been discovered that some coxibs are able to stop or slow down the cell proliferation of human cancer (Ryan, Rosita et al. 1999; Marnett and DuBois 2002; Asano and McLeod 2004; Asano and McLeod 2004; Koehne and Dubois 2004; Liao and Milas 2004; Brown and DuBois 2005; Kashfi and Rigas 2005; Breinig, Schirmacher et al. 2007), and it has been demonstrated that this effect occurs through the inhibition of PDK1 (Arico, Pattingre et al. 2002; Kulp, Yang et al. 2004). Another compound shows a very potent effect on the inhibition of PDK1: 3-Hydroxyanthranilic acid (3-HAA). It is a derivate of the metabolism of tryptophan, binds PDK1 in its ATP-binding site through H-bonds and blocks the auto-phosphorylation of this enzyme, crucial for its activity (Hayashi, Mo et al. 2007). To check whether the inhibition of PDK1 could have any effect on the maintenance of LTP in perirhinal cortex, and whether this effect could occur through the inhibition of the PDK1-dependent activation of PKMζ, we ran a set of experiments designed as shown in Figure 3.14. Following a 60 minutes baseline recording, LTP was induced on one pathway. No conditioning stimulation was delivered in the other pathway, which was used as a control. 3 hours after the induction of LTP, 3-HAA 100µM was bath applied, followed by ZIP 5µM. Pooled data (Figure 3.15) show that a robust LTP ($150 \pm 8\%$ of baseline, p<0.01 v baseline, n=6) is reversed following a long application of 3-HHA ($104 \pm 3\%$ of baseline, p<0.01 v LTP, p>0.05 v baseline, n=6). The subsequent application of ZIP shows no more decrease of the synaptic transmission ($99 \pm 5\%$ of baseline, p>0.05 v 3-HHA, n=6).
suggesting that the reversal of the synaptic potentiation observed following the inhibition of PDK1, could happen as the final result of the inhibition of the PDK1-dependent activation of PKMζ. Though, since PDK1 is active on a very wide range of targets, these experiments alone are not sufficient to prove that the observed reversal of synaptic potentiation occurs only through the inhibition of PKMζ. Other molecular mechanisms could as well be involved.
Figure 3.1 Single example of LTP in adult perirhinal cortex. P0 pathway (filled circles) was used as a control. A "spaced" tetra-burst stimulation (4x100Hz, 5 minutes interburst interval) produces a robust, stable LTP (open circles), which lasts for 6 hours after its induction. Upwards pointing arrows represent the moment when the stimulus was delivered. Traces are representative of the potentiation obtained.
Figure 3.2 Pooled data for LTP in adult perirhinal cortex. The spaced tetra burst stimulation (4x100Hz) induces a stable and significant LTP (open circles, 126 ± 4% of baseline, p<0.05, n=4). The potentiation lasts for more than 5 hours.
Figure 3.3 Single experiment for anisomycin on LTP in adult perirhinal cortex. The pre-incubation with Anisomycin 20 µM is able to block the maintenance of LTP induced by a tetra-burst stimulation in P1 pathway (open circles). Upwards pointing arrows represent the moment when HFS was delivered. Anisomycin shows no effect on the control pathway P0 (filled circle).

Figure 3.4 Pooled data for anisomycin on LTP in adult perirhinal cortex. The pre-incubation with Anisomycin 20 µM is able to block the maintenance of LTP (open circles 102 ± 5% of baseline, p>0.05 v baseline, n=5). Upwards pointing arrows represent the moment when HFS was delivered.
Figure 3.5 Single experiment for inhibition of PKM\(\zeta\) on LTP in adult perirhinal cortex. The application of PKM\(\zeta\) inhibitor ZIP 5\(\mu\)M reverts the established LTP induced by a tetra-burst stimulation in P1 pathway (open circles). Upwards pointing arrows represent the moment when HFS was delivered. ZIP shows a small effect on the control pathway P0 (filled circles).

Figure 3.6 Pooled data for inhibition of PKM\(\zeta\) on LTP in adult perirhinal cortex. ZIP 5\(\mu\)M completely reverts the established LTP (open circles, 98 ± 5 % of baseline, p<0.01, n=7) with no significant effect on the control input (filled circles, 93 ± 4 % of baseline, p>0.05).
Figure 3.7 Pooled data for inhibition of PKMζ on baseline in adult perirhinal cortex. ZIP 5µM produces a small, non-significant decrease on the baseline when bath-applied on adult perirhinal cortex. Temporal pathway (filled circles): 89 ± 2% of baseline, p>0.05. Entorhinal pathway (open circles): 88 ± 2% of baseline, p>0.05, n=4).
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Figure 3.8 Single experiment of ZIP on depotentiation in adult perirhinal cortex ZIP 5µM shows no effect on a depotentiated pathway (P0, LTP followed by LFS protocol, filled circles). The inhibition of PKMζ produces a depression of the synaptic response in a potentiated pathway where the magnitude of the response has been decreased by a reduction of the stimulation intensity (P1, open circles). The two upwards-pointing arrows joined by a rectangle represent the LFS protocol. The single downward-pointing arrow shows when the stimulation intensity was reduced on P1.

Figure 3.9 Pooled data for inhibition of PKMζ on baseline in adult perirhinal cortex ZIP 5µM shows no effect on a depotentiated pathway (filled circles: 98 ± 4% of baseline, p>0.05, n=5). On the other input (open circles), after the induction of LTP, the amplitude of the response is decreased by a reduction of the stimulation intensity to match the depotentiation in the other input. ZIP produces a significant decrease in the still potentiated pathway (65 ± 5% of baseline, p<0.001 v the response measured after the reduction of the stimulation intensity).
Figure 3.10 Single experiment of effect of inhibition of PKMζ on depotentiation in presence of AP5 in adult perirhinal cortex. If the depotentiation is blocked by bath-application of AP5 (filled circles), ZIP produces a decrease in the synaptic response similar to the one that occurs in a potentiated pathway (open circles).

Figure 3.11 Pooled data for effect of inhibition of PKMζ on depotentiation in presence of AP5 in adult perirhinal cortex. If the depotentiation is blocked by bath-application of AP5 (filled circles), ZIP is able to decrease the response, bringing it back to the baseline (102 ± 2% of baseline, p<0.001 v LTP, p>0.05 v baseline, n=5). On the other input (open circles), the application of ZIP 3h after the induction of LTP is able to revert the potentiation (100 ± 4% of baseline, p<0.001 v LTP, p>0.05 v baseline, p>0.05 v the “LTD” input after the administration of ZIP, n=5).
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Figure 3.12 Single experiment of effect of inhibition of PKMζ on LTD induction in adult perirhinal cortex ZIP shows no effect on a depotentiated pathway (filled circle). Pre-incubation with ZIP does not affect the induction of LTD (open circles).

Figure 3.13 Pooled data for the effect of PKMζ inhibition on LTD induction in adult perirhinal cortex Inhibition of PKMζ has no effect on the induction of LTD in perirhinal cortex (Open circles 69 ± 4% of baseline, p<0.01 v baseline, n=5). There’s no difference in the magnitude of the depression obtained after the LFS when the slice is pre-incubated with the inhibitor ZIP and the LTD in the control pathway (filled circles 70 ± 4% of baseline, p>0.05 v experimental pathway, p<0.01 v baseline).
**Figure 3.14** Single experiment of effect of PDK1 inhibition on LTP in adult perirhinal cortex The inhibition of PDK1 slowly but completely reverts an established LTP (open circles). Subsequent application of ZIP does not show any effect after the depotentiation is complete.

**Figure 3.15** Pooled data for effect of PDK1 inhibition on LTP in adult perirhinal cortex The inhibition of PDK1 completely reverts the LTP (open circles, 104 ± 3% of baseline, p<0.01 v LTP, p>0.05 v baseline, n=6). The subsequent application of ZIP shows no more decrease of the synaptic transmission (99 ± 5% of baseline, p>0.05 v 3-HHA, n=6). No effects of both drugs is shown on the control pathway (filled circles)
3.3 Discussion

These results show that a tetra-burst stimulation is able to induce a stable, long lasting LTP in adult perirhinal cortex. The HFS protocol used here was a so-called “spaced” tetra-burst stimulation, which is known to produce a longer-lasting LTP, which is more dependent on protein synthesis (Scharf, Woo et al. 2002). However, other protocols (i.e. 4X100 Hz bursts, 30’ interbursts interval) are able to induce LTP in adult perirhinal cortex, with no significant differences with the LTP induced by the “spaced” tetra-bursts stimulation (data not shown). This is of interest, since most of the studies so far have been focusing on LTD processes in perirhinal cortex, which underlie recognition memory. Therefore, not much is known on LTP in perirhinal cortex.

Our findings also show that the maintenance of LTP in adult perirhinal cortex seems to depend on newly synthesized proteins. The application of rapamycin, a potent inhibitor of protein synthesis, in fact, completely reverts the established potentiation. These results are perfectly consistent on what has been found in other brain areas, i.e. hippocampus, where the maintenance, but not the induction, of LTP is blocked by the application of anisomycin (Frey, Krug et al. 1988; Scharf, Woo et al. 2002). In other words, again, the maintenance of LTP relies on the synthesis of new proteins (Kelly, Mullany et al. 2000; Abraham and Williams 2003; Lynch 2004; Miyamoto 2006; Reymann and Frey 2007). Amongst the protein that may be involved in the maintenance of LTP, a crucial role seems played by kinases, like Protein Kinase C (PKC), CaMKINase, MAP Kinase, PKA (Reymann, Frey et al. 1988; Barria, Muller et al. 1997; Bozon, Kelly et al. 2003; Lisman 2003; Warburton, Glover et al. 2005; Miyamoto 2006).

There’s evidence that PKMζ plays a crucial role in the maintenance of LTP in the hippocampus (Sacktor, Osten et al. 1993; Serrano, Yao et al. 2005), and that is formed in a protein synthesis-dependent fashion following the induction of LTP (Osten, Valsamis et al. 1996). Our findings show that in adult perirhinal cortex as well, bath-application of the selective PKMζ inhibitor ZIP completely reverts an established LTP, when administered during the maintenance phase (i.e. 3 hours after the induction of LTP). Therefore, in perirhinal cortex, as well as in hippocampus, the maintenance of LTP relies on the continuous activity of
PKMζ. In contrast with previous works (Serrano, Yao et al. 2005), ZIP produces a small, non significant effect also on the basal synaptic transmission. This activity is not completely surprising, since is known that PKMζ maintains LTP by regulating the trafficking on the membrane of AMPA receptors (Ling, Benardo et al. 2006; Yao, Kelly et al. 2008; Migues, Hardt et al. 2010). Although the action of PKMζ is specific for potentiated synapses, it’s not completely unlikely that there is some small, less-specific effect also on the basal activity of some non-potentiated synapses. I.e., PKMζ could modify the activity-independent constitutive trafficking of AMPARs (McCormack, Stornetta et al. 2006). Alternatively, as suggested by prof. M.W. Brown, it could also be that what we consider a basal synaptic response of the neuronal pathway is already potentiated, in some amount. Since the animals used in this set of experiment are adults, it’s not unlikely that they have some memories encoded, which means that some synapses could be already potentiated in some amount, therefore showing an increased activity of PKMζ. When activated, PKMζ starts a positive feedback loop to sustain increases in its synthesis during LTP maintenance (Kelly, Crary et al. 2007), which lasts as long as the memory lasts. The application of the inhibitor ZIP, blocking the active PKMζ regardless on when the protein has been synthesized, might affect those pre-potentiated synapses.

Previous studies show that, whereas the synthesis of PKMζ is dependent on LTP there is a down-regulation of PKMζ following LTD (Hrabetova and Sacktor 1996; Osten, Hrabetova et al. 1996; Hrabetova and Sacktor 2001). Therefore, the inhibition of PKMζ in a depotentiated pathway should not show any effect on the synaptic transmission. A similar effect should be obtained when LTP (which induces PKMζ) is followed by LTD (which down-regulates PKMζ), a process that brings the synaptic response back to the baseline. This hypothesis is confirmed by the results showed in Figure 3.9, where no effect of ZIP is evident when the inhibitor is administered on a depotentiated pathway. Also, these results show that the effect of PKMζ inhibition does not depend on the measured amplitude of the synaptic response, but on its actual potentiation, underlying on various intracellular modifications. In fact, when the amplitude of the evoked potentiated synaptic response is actively decreased by a reduction
of the stimulation intensity, ZIP is still able to reduce the synaptic response. This happens because, although the size of the potentiated pathway matches the size of the depotentiated pathway, in the first case the mechanisms maintaining the LTP are still active. Therefore ZIP, inhibiting the active PKMζ, is able to produce an evident decrease of the synaptic transmission.

There are several evidences showing that PKMζ is down-regulated in LTD. LTD depends on NMDA receptors activity (Dudek and Bear 1992; Mulkey and Malenka 1992; Wexler and Stanton 1993; Stanton 1996), so, if these receptors are blocked by the antagonist D-AP5, a LFS protocol is not able to induce long term depression (Massey, Johnson et al. 2004). Therefore, the blockade of NMDA, not allowing the expression of LTD, should prevent the down-regulation of PKMζ induced by the LFS. To examine this, following the induction of LTP, slices were incubated with D-AP5. The administration of LFS on one pathway failed to induce a depotentiation; subsequent application of ZIP brings the response back to baseline levels. These findings, then, suggest that the administration of LFS alone is not sufficient to induce the expected down-regulation of PKMζ activity. The whole intracellular signaling, leading to the mechanisms underlying the actual depotentiation of the synaptic response (i.e. activation of Protein Phosphatases) are required in order to block the synthesis/activation of PKMζ. One possible explanation could be that protein phosphatase block the activity of some proteins responsible for the new synthesis/activation of PKMζ, i.e. PDK1, (Kelly, Crary et al. 2007). Therefore, if LFS, due to the AP5-dependent block of NMDA receptors, fails to activate these phosphatases, the intracellular signaling pathways leading to the activation of PKMζ are still fully working. This could explain the observed results.

In addition, as shown in Figure 3.9, since LTD down-regulates the activity of PKMζ, the application of ZIP after LFS has no effect on the synaptic response. Though, it wasn’t clear if PKMζ might have any effect in the induction of LTD. To examine this point, slices were pre-incubated with ZIP for 90 minutes, then LFS was administered. As expected, the inhibition of PKMζ has no effect in the induction of LTD. In fact, according to current knowledge, PKMζ does not seem to not be involved in LTD-like mechanisms. Also, PKMζ is not involved in the induction of LTP either (Serrano, Yao et al. 2005), but only in its
maintenance. Therefore, all evidences suggest once again that PKMζ activity is up- or down-regulated following conditioning stimulation, and it’s only involved in the late phases of the processes.

Finally, the last set of experiments started to examine the regulation of PKMζ activity. Although it seems to be universally agreed that PKMζ, once activated, self-maintains its own activity through a positive feedback loop, not much is known on the processes leading to the activation of PKMζ. The main protein involved in the activation of PKMζ was identified in PDK1. PKC of the conventional and novel groups, in fact, are primed by phosphorylation by PDK1, and then fully activated by the intracellular second messengers signaling cascades. PKCs belonging to the atypical group, as PKCζ, instead, are immediately activated activated by a phosphorylation by PDK1 in an “on/off” fashion (Le Good, Ziegler et al. 1998; Dong, Zhang et al. 1999; Balendran, Biondi et al. 2000; Balendran, Hare et al. 2000). Therefore, the inhibition of PDK1 activity should indirectly block the activity of PKMζ. Bath application of 3HAA slowly reverts the maintenance of LTP; when the response is back to the baseline, the application of ZIP does not produce any further decrease in the response. These results, then, confirm our hypothesis, that the inhibition of PDK1, preventing the phosphorylation and therefore the activation of PKMζ, reverts the established LTP. The lack of effect of ZIP confirms this hypothesis. 3HAA brings the response back to the baseline more slowly than ZIP. This could happen because the effect on PKMζ is indirect, requiring first the complete inhibition of PDK1 that, as a consequence, can’t phosphorylate PKMζ. Therefore, it takes more time for the whole process to be completely blocked. Anyway, although the results of the experiments are consistent with the hypothesis, PDK1 is a protein that affects many different intracellular pathways and processes, like cellular growth and proliferation through a PI3K/Akt/mTOR signaling cascade (Bayascas 2008; Kawauchi, Ogasawara et al. 2009). Therefore, it can’t be excluded that the observed effect occurs not only through a direct inhibition of PKMζ activity, but also via other mechanisms (i.e. via the inhibition of a more wide and unspecific mTOR-dependent protein synthesis).
4 ROLE OF PKMζ IN SYNAPTIC PLASTICITY IN PERIRHINAL CORTEX DURING NEURODEVELOPMENT
4.1 Introduction

The previous set of experiments demonstrated that it is, in fact, possible to induce LTP in adult perirhinal cortex, and this LTP can be completely reverted by the inhibition of PKMζ. Interestingly, some work carried on in this same lab shows that in young animals, i.e. at post natal day 14 (PND14 or P14), different protocols of HFS (100 Hz, 1s) that usually produce LTP in different brain areas (i.e. hippocampus) at different ages fail to induce LTP in perirhinal cortex (King and Bashir observation, unpublished). Even protocols that are known to induce LTP in adult perirhinal cortex, as 4xHFS (Ziakopoulos, Tillet et al. 1999; Massey, Johnson et al. 2004) or the “spaced” tetra-bursts stimulation (Scharf, Woo et al. 2002) didn’t produce LTP in neonatal perirhinal cortex. Neither two different theta burst stimulation protocols were effective in inducing LTP (observed by King and Bashir, unpublished). This phenomenon can find two possible explanations. One possibility is that the mechanisms for LTP simply do not exist at this stage of neurodevelopment, therefore all attempts to induce any kind of potentiation at this age result ineffective. An alternative explanation is, in contrast, that the mechanisms underlying the expression of LTP do exist but are already fully saturated; as a consequence, the induction of LTP is occluded. To examine these possibilities, LFS (1 Hz, 900 s) was first delivered to induce LTD; the subsequent delivery of HFS (100 Hz, 1s) resulted in lasting potentiation of synaptic transmission, which brought the synaptic response back to baseline levels (King and Bashir observations, unpublished). Therefore, these observations suggest that mechanisms leading to a long-term potentiation do exist in neonatal perirhinal cortex, but under basal conditions these mechanisms are most likely saturated and a long-lasting potentiation of the synapse is only observed if this saturation is reversed.

Since the activity of PKMζ is fundamental in the maintenance of LTP (Hrabetova and Sacktor 1996; Ling, Benardo et al. 2002), if LTP mechanisms are saturated under basal conditions then this may involve constitutive activation of PKMζ. If this is the case then inhibition of PKMζ should depress basal synaptic transmission. Also, if LTD reverses LTP maintenance by depression of PKMζ activity, the inhibition of PKMζ should have no effect on synaptic transmission following LTD. In addition, when a LFS-induced
depression of the synaptic transmission is reverted by the administration of HFS (de-depression), if this mechanism is dependent on the re-activation of PKMζ. HFS-induced, the application of ZIP should once again decrease the synaptic transmission. Also, we were interested in setting, if possible, a time-course for these phenomena during the different stages of neurodevelopment. In other words, when does the brain acquire the possibility to express LTP? We tested these hypotheses with the following set of experiments.
4.2 Results

4.2.1 Effect of the inhibition of PKMζ on basal synaptic transmission

Our hypothesis was that, in neonatal (P14) perirhinal cortex, the basal synaptic transmission is already set on very high levels, therefore occluding the experimental induction of any further potentiation. If PKMζ is involved in the maintenance of these high levels of basic synaptic transmission, its inhibition should lead to a decrease of the evoked synaptic response, whereas no effect should be observed in a fully-depotentiated pathway (see chapter 3.2.4). Figure 4.1 shows a representative single experiments, Pooled data in Figure 4.2. To test this idea, after a 30 minutes baseline recording, 3 sets of LFS were delivered to one input to obtain a saturated LTD (54 ± 4 % of baseline 30 min after last LFS, p < 0.001, n = 7; Figure 4.2). No conditioning stimulation was applied to the other input. Subsequent application of the PKMζ inhibitor ZIP depressed transmission in the control input (53 ± 4% of baseline 90 min after start of ZIP application, p < 0.001; n = 7; Figure 4.2) with no effect on the input in which LTD had been induced (94 ± 9 % compared to pre-ZIP level; p > 0.05, n = 7; Figure 4.2). The depression of basal synaptic transmission by ZIP suggests that basal transmission in P14 perirhinal cortex is maintained by PKMζ-mediated LTP-like mechanisms. The lack of effect of ZIP on the depotentiated pathway suggests that LTD is linked to a strong, activity-dependent, inhibition of PKMζ. Since these findings are very different from what we observed in adult, we therefore decided to assay PKMζ to determine whether differences in the levels of PKMζ could explain the differential effects of ZIP on neonatal basal synaptic transmission versus adult basal synaptic transmission. We found that the levels of PKMζ were significantly higher (p<0.01) in perirhinal cortex from P14 animals compared to adult animals (adults: 100 ± 7 %, n = 8; P14: 154 ± 9 %, n = 9; Insert in Figure 4.2). These findings provide more support to our hypothesis that the “potentiated” state of synaptic transmission in P14 perirhinal cortex might depend on higher expression and/or activity of PKMζ.
4.2.2 Effect of the inhibition of PKMζ in de-depression.

As explained earlier, it has been observed that the only form of synaptic potentiation in perirhinal cortex of P14 rats occurs only following a previous depotentiation. The potentiation brings the response back to baseline levels, but it does not produce further increase. The pathway should nevertheless have started the molecular machinery responsible for the maintenance of the potentiation, as it would happen when inducing LTP. If the high levels of synaptic transmission at baseline are maintained by the activity of PKMζ, which is up- or down-regulated following HFS and LFS (Hrabetova and Sacktor 1996), its inhibition by ZIP should be able to decrease the amplitude of the response. Figure 4.3 is a representative example of the experiment: after 30 minutes of baseline recording, LFS was delivered in one pathway to induce LTD, followed by HFS to obtain de-depression of the pathway. The other input was used as a control. Pooled data (Figure 4.4) show that LFS is able to induce a robust LTD in perirhinal cortex of P14 rats (74 ± 2 % of baseline, p < 0.001, n = 4). Thirty minutes following induction of LTD, delivery of HFS resulted in lasting potentiation (to 99 ± 8 % of original pre-LTD baseline, 60 min post HFS; p < 0.001 v LTD, p > 0.05 v baseline, n=4; Figure 4.4). Bath application of ZIP depressed synaptic transmission in both the potentiated (50 ± 5% of baseline; p< 0.01) and the control pathways (48 ± 3% of baseline, p< 0.01). These results suggest, then, that the maintenance of LTP, induced following LTD, depends on activation of PKMζ. This confirms that in immature perirhinal cortex basal synaptic transmission seems to be in a fully potentiated state and that this relies on PKMζ activity. Furthermore, the data suggest that PKMζ activity can be both up and down regulated in P14 perirhinal cortex in an activity-dependent manner.

4.2.3 Role of PKMζ in P35 perirhinal cortex

The experiments run so far have given us evidences that perirhinal cortex shows different properties in adult and neonatal animals. The maintenance of LTP is promptly reversed by the inhibition of PKMζ in adult animals (Figure 3.6). In P14 animals, instead, no induction of LTP is possible, and the bath application of ZIP produces a significant decrease in the synaptic transmission
(Figure 4.2). Considering these findings, it was interesting to examine at which point during the development the peririnhal cortex acquires the ability to produce a “classic” LTP, or rather, to switch from a “saturated potentiation” already occurring as a baseline, to a situation where the synapse are ready to be potentiated once again. To examine this matter, we decided to run a set of experiments on young animals, at 35 days post-natal (PND35 or P35). At this age, in fact, rats are already active and show a vivacious explorative behaviour.

As shown in Figure 4.5, showing a representative single experiment, after 60 minutes of baseline recording, a “Spaced” tetra-burst stimulation protocol was administered in one input to induce a stable, long lasting potentiation. No conditioning stimulation was delivered to the other input, used as a control. Three hours after the induction of LTP, ZIP was bath applied. Pooled data (Figure 4.6) show that LTP was promptly induced in P35 perirhinal cortex (148 ± 5% of baseline, p<0.001, n = 6) and the inhibition of PKMζ by bath application of 5 µM ZIP completely reversed the established LTP (100 ± 2% of baseline, p < 0.001, n = 6). However, ZIP had no effect on baseline transmission (97 ± 2% of baseline, p>0.05, n = 6). These results, then, provide some good evidence that the decline in contribution of LTP/PKMζ mechanisms to basal transmission in perirhinal cortex most likely occurs between P14 and P35.
Role of PKM\(\zeta\) in synaptic plasticity in perirhinal cortex during neurodevelopment

**Figure 4.1** Single experiment of effect of PKM\(\zeta\) inhibition on P14 perirhinal cortex. The application of ZIP produces a strong decrease in the basal synaptic transmission (filled circles). ZIP shows no effect on a pathway where a saturated LTD was previously obtained (open circles).

**Figure 4.2** Pooled data for effect of PKM\(\zeta\) inhibition on P14 perirhinal cortex. The inhibition of PKM\(\zeta\) produces a significant decrease in the synaptic response (filled circles 53 ± 4% of baseline, p<0.001 v baseline; n=7). When a saturated depression was obtained (open circles 54 ± 4% of baseline, p<0.001) subsequent application of ZIP shows no effect (94 ± 9% compared to pre-ZIP level; p>0.05). The insert shows the differences in the expression of PKM\(\zeta\) in perirhinal cortex from P14 and adult rats. Pooled data illustrated in the histogram (above) show that PKM levels are significantly (***p<0.001) higher in P14 than in adult (adults: 100 ± 7 %, n=8; P14: 154 ± 9 %, n=9). Below, single example of western blot.
Figure 4.3 Single experiment of the effect of PKMζ inhibition on de-depression in P14 perirhinal cortex The administration of LFS followed by HFS protocol produces a de-depression in P14 perirhinal cortex, which brings the response back to the baseline (filled circles). The application of ZIP produces a decrease in the de-depressed pathway, similar to the one produced on the control pathway (open circles).

Figure 4.4 Pooled data for effect of PKMζ inhibition on de-depression in P14 perirhinal cortex LFS is able to induce a robust LTD in perirhinal cortex of P14 rats (open circles 74 ± 2 % of baseline, p<0.001, n=4). Thirty minutes following induction of LTD, delivery of HFS resulted in lasting potentiation (to 99 ± 8 % of original pre-LTD baseline, 60 min post HFS; p<0.001 v LTD, p > 0.05 v baseline). Bath application of ZIP depressed synaptic transmission in both the potentiated (50 ± 5% of baseline; p<0.01) and the control pathways (filled circles, 48 ± 3% of baseline, p< 0.01).
Figure 4.5 Single experiment of PKMζ inhibition LTP in P35 perirhinal cortex In P35 animals, HFS is able to promptly induce a robust and stable LTP (open circles), which is completely reversed by bath-application of ZIP.

Figure 4.6 Pooled data for effect of PKMζ inhibition on LTP in P35 perirhinal cortex LTP was promptly induced in P35 perirhinal cortex (open circles 148 ± 5% of baseline, p<0.001, n=6) and the inhibition of PKMζ by bath application of 5µM ZIP completely reversed the established LTP (100 ± 2% of baseline, p<0.001). ZIP shows no effect on baseline transmission in a control pathway (filled circles 97 ± 2% of baseline, p>0.05). Above, representative traces of the evoked response.
4.3 Discussion

In contrast with the observations made in adult perirhinal cortex, in young animals (P14) none of the HFS protocols that are known to normally be able to induce LTP is effective. These findings are quite surprising, considering that in younger animals a higher level of plasticity is expected, and that in other brain areas, i.e. visual cortex and hippocampus, it's relatively easy to induce a robust and stable LTP using common HFS protocols (Malenka and Bear 2004).

One possible explanation to the finding that it's not possible to induce LTP in perirhinal cortex of P14 animals is that the intracellular mechanisms leading to the potentiation of the synaptic response simply do not exist at this stage of neurodevelopment. An alternative hypothesis is that these mechanisms are present, but are already fully saturated, hence a potentiation is only possible following a depression of the synaptic transmission, a process known as de-depression (as observed by R.King and Z.I. Bashir, but also shown in Figure 4.3 and 4.4). Therefore, these results suggest that the lack of LTP in neonatal perirhinal cortex can be explained by constitutive LTP-like maintenance mechanisms are responsible of keeping basal transmission in an already potentiated state during early development. The reasons why synaptic transmission is maintained in an enhanced state is not known but these mechanisms may promote or stabilise synaptic connections in the immature cerebral cortex (Hua and Smith 2004; Cline and Haas 2008; Hanse, Taira et al. 2009). Whether similar mechanisms operate in other brain regions and at what time during development will require further investigation.

Since PKMζ is sufficient to maintain LTP, and basal synaptic transmission in P14 perirhinal cortex seems to be in an already LTP-like potentiated state, PKMζ appeared as an interesting target to examine. Results show, in fact, that the inhibition of its activity produces a strong depression in the synaptic transmission. Interestingly, the size of this depression matches the one achieved after the induction of a fully-saturated LTD by three trains of LFS. As expected, the application of ZIP shows no effect on the depotentiated pathway, probably because PKMζ has been down-regulated by the repeated LFS, as described before (Hrabetova and Sacktor 1996; Hrabetova and Sacktor 2001).
Therefore, these findings suggest that PKM\(\zeta\) is, in fact, the main responsible for the maintenance of the basal synaptic transmission in a potentiated state.

The hypothesis that the high levels of basal synaptic transmission in P14 perirhinal cortex are maintained by LTP-like mechanisms, which depend on the activity of PKM\(\zeta\), is strengthened also by the finding that ZIP is able to decrease the synaptic response in a de-depressed pathway. In other words, if we consider the basal synaptic transmission as “LTP”, and the depressed synaptic transmission as the basal transmission, the administration of HFS produces a “new LTP”, maintained by PKM\(\zeta\).

This phenomenon, to our knowledge, only happens in perirhinal cortex at this stage (P14 of neurodevelopment). In P14 hippocampus, in fact, it is possible to induce LTP quite easily, and the potentiation is reverted by application of ZIP (Serrano, Yao et al. 2005). Experiments in P14 hippocampus have been repeated as an internal control; HFS is able to induce a robust LTP, which is reversed by the application of ZIP (showed later in Figure 4.3.1 and 4.3.2). Also, the occlusion of LTP in perirhinal cortex occurs during a limited period of time, since it is possible to induce a robust LTP in perirhinal slices of animals at 35 day post-natal (P35). Therefore, this occlusion of the induction of LTP appears to be specific for one particular brain region (perirhinal cortex) at a particular time in neurodevelopment (around PND14).

The mechanisms that reduce the role of LTP and PKM\(\zeta\) during development of perirhinal cortex are not known but might derive from LTD-like processes that underlie visual recognition memory (Brown and Bashir 2002; Griffiths, Scott et al. 2008).

The insertion of AMPA receptors may be important in the stabilisation of synaptic connections in the immature CNS (Hua and Smith 2004; Cline and Haas 2008; Hanse, Taira et al. 2009), and it has been demonstrated that the mechanisms through which PKM\(\zeta\) maintains LTP may involve AMPA receptor insertion into the synaptic membrane (Yao, Kelly et al. 2008). In addition, PKM\(\zeta\) has very recently been shown to be important for synapse stabilisation in the retino-tectal pathway in developing xenopus (Liu, Tari et al. 2009). These
results show that LTP-like mechanisms relying on PKMζ play a critical role in maintenance of synaptic stability during development of higher centres of the mammalian CNS.

Summarizing, the hypothesis is that synapses are maintained in a high level of potentiation by PKMζ in early stages of neurodevelopment. This phenomenon stabilizes the young synapses, “priming” them to develop different plasticity mechanisms later during the development. I.e., LTD-like mechanisms depending on experience, could bring the synaptic transmission back to a state where both up- and down-regulation of the synapses are possible, therefore permitting the storage of new information and other forms of plasticity. It would be interesting to test whether the eye-opening plays any sort of role in this process; P14 animals, in fact, have still their eyes closed, and it makes sense that perirhinal cortex, being involved in recognition memory, shows phenomena of synaptic plasticity dependent on input received by visual cortex.
Role of PKMζ in synaptic plasticity in perirhinal cortex during neurodevelopment

Figure 4.3.1 Pooled data for effect of PKMζ inhibition on LTP induced in P14 hippocampus (Peak Amplitude) LTP is easily induced in P14 hippocampus (open circles: 141 ± 5% of baseline; p<0.01; n = 4). The inhibition of PKMζ completely reverses the potentiation (100 ± 7% of baseline, p<0.01 v LTP) but had no significant effect on basal transmission in the non-tetanised input (filled circles, 92 ± 4% of baseline, p>0.05, n = 4)

Figure 4.3.2 Pooled data for effect of PKMζ inhibition on LTP induced in P14 hippocampus (Slope) Measurements of variation of slope amplitude are consistent with variation in the peak amplitude. LTP is easily induced in P14 hippocampus (open circles: 157 ± 9% of baseline; p<0.01; n = 4). The inhibition of PKMζ completely reverses the potentiation (99 ± 5% of baseline, p<0.01) but had no significant effect on basal transmission in the non-tetanised input (open circles, 101 ± 0.6% of baseline, p>0.05, n = 4)
5 REGULATION OF PKMζ IN PERIRHINAL CORTEX DURING NEURODEVELOPMENT
5.1 Role of PI3K and mTOR in the regulation of PKMζ activity

5.1.1 Introduction

The experiments run so far provided some good evidences that PKMζ maintains enhanced synaptic transmission in LTP. Nevertheless, the underlying mechanisms underlying this phenomenon occurs are still not known.

One possible mechanism involves PKMζ regulation of protein synthesis through local dendritic translation (Westmark, Westmark et al. 2010). Translation initiation relies at least in part on the activation of mammalian target of rapamycin (mTOR), the inhibition of which prevents LTP (Hoeffer and Klann 2010). The mammalian target of rapamycin is involved in many ways in neuronal plasticity; along with its crucial role in the maintenance of LTP and memory (Kelleher, Govindarajan et al. 2004; Helmstetter, Parsons et al. 2008; Klann and Sweatt 2008; Swiech, Perycz et al. 2008; Costa-Mattioli, Sossin et al. 2009; Richter and Klann 2009; Hoeffer and Klann 2010), a dysregulation of mTOR activity is linked to different cognitive diseases, like Alzheimer’s disease (Pei and Hugon 2008; Swiech, Perycz et al. 2008; Ma, Hoeffer et al. 2010), fragile X syndrome (Sharma, Hoeffer et al. 2010), tuberous sclerosis (Ehninger, de Vries et al. 2009; Sampson 2009) and many others (Swiech, Perycz et al. 2008; Hoeffer and Klann 2010). It is also known (Kelly, Crary et al. 2007) that the inhibition of mTOR not only blocks the maintenance of LTP, but also decreases the synthesis and activity of PKMζ. Therefore, to examine if basal synaptic transmission in P14 perirhinal cortex is maintained by protein translation we investigated effects of inhibiting mTOR. It has been suggested (Westmark, Westmark et al. 2010) that PKMζ may maintain LTP by protein translation through a signalling cascade involving regulation of Pin1 – a protein that interacts with 4E-BPs that normally suppress protein translation. 4E-BPs are phosphorylated by mTOR to remove the suppression of translation initiation (Hoeffer and Klann 2010). If PKMζ maintains elevated transmission through protein translation, then this is likely to involve the mTOR-dependent translation initiation pathway.
In addition to this, we wanted to examine another crucial step in the intracellular signaling that may lead to the mTOR-mediated regulation of PKMζ activity. It is known that one of the main enzymes that play a crucial role in regulating the activity of mTOR is the phosphoinositide-3-kinase (PI3K) (Sabatini 2006; Tsokas, Ma et al. 2007; Gobert, Topolnik et al. 2008; Costa-Mattioli, Sossin et al. 2009; Kawauchi, Ogasawara et al. 2009). PI3K is also involved in the expression and maintenance of LTP (Horwood, Dufour et al. 2006; Karpova, Sanna et al. 2006; Tsokas, Ma et al. 2007; Gobert, Topolnik et al. 2008; Sui, Wang et al. 2008; Bruel-Jungerman, Veyrac et al. 2009), and is upstream in the signaling cascade that leads to the activation of PDK1 (Duronio 2008; Carnero 2009; Costa-Mattioli, Sossin et al. 2009; Kawauchi, Ogasawara et al. 2009; Carnero 2010; Hoeffer and Klann 2010), which is thought to phosphorylate PKMζ in its activation loop, activating it (Kelly, Crary et al. 2007). In particular, PDK1 phosphorylates the activation loop sites of PKCζ in a PI3-kinase-dependent manner (Le Good, Ziegler et al. 1998). Moreover, the inhibition of PI3K blocks the maintenance of LTP and down-regulates the synthesis of PKMζ (Kelly, Crary et al. 2007). Interestingly, it has been demonstrated that the activation of PI3K is also required for AMPA receptors insertion during LTP in cultured hippocampal neurons (Man, Wang et al. 2003), which is also the mechanism through which PKMζ is thought to maintain LTP (Ling, Benardo et al. 2006; Yao, Kelly et al. 2008; Migues, Hardt et al. 2010; Sacktor 2010).

Therefore, the following sets of experiments are aimed to investigate the role of mTOR and PI3K in synaptic plasticity PKMζ-linked in perirhinal cortex during neurodevelopment.
5.1.2 Results

5.1.2.1 Role of mTOR in basal synaptic transmission

As described earlier in this chapter, mTOR plays a crucial role in regulating protein synthesis. Protein synthesis has also been demonstrated to be required for the maintenance of LTP. Our previous observations led us to think that in neonatal (P14) perirhinal cortex, the basal transmission is fully potentiated as if some LTP-like mechanism underlies the basal transmission at this stage. If this is true, the inhibition of protein synthesis through the inhibition of mTOR should decrease the basal synaptic transmission in P14 perirhinal cortex. To examine this, after a 40 minutes baseline recording, rapamycin (5µM) was bath applied (Figure 5.1.1). Pooled data (Figure 5.1.2) show that mTOR inhibition produced a substantial depression of basal transmission in P14 perirhinal cortex (temporal side: 73 ± 3% of baseline, p<0.001; entorhinal side: 74 ± 3% of baseline, p<0.001, n=6). However, no effect of mTOR inhibition by bath application of rapamycin was observed in perirhinal slices from adult animals (Pooled data in Figure 5.1.3: temporal side: 98 ± 3% of baseline, p>0.05; entorhinal side 100 ± 3% of baseline, p>0.05, n=4). This result suggests that ongoing protein translation controls basal levels of synaptic transmission in P14 but not in adult perirhinal cortex.

5.1.2.2 Effect of mTOR inhibition on PKMζ activity in perirhinal cortex

We found that mTOR-mediated ongoing protein synthesis controls basal synaptic transmission in P14 perirhinal cortex. Previous findings suggested that mTOR is important for the regulation of PKMζ synthesis (Kelly, Crary et al. 2007) and that PKMζ may maintain LTP through a signaling pathway involving its regulation of Pin-1 (Westmark, Westmark et al. 2010). The interaction of Pin-1 with 4E-BPs suppresses protein translation; the phosphorylation of 4E-BPs by mTOR removes the suppression of translation initiation. Therefore, the inhibition of mTOR activity should lead to an indirect inhibition of PKMζ functions, whereas the previous inhibition of PKMζ should occlude any subsequent depression of the synaptic transmission generated by the inhibition of mTOR.
This latest scenario is illustrated in Figure 5.1.4; after 1 hour of baseline recording, ZIP was bath applied in perirhinal cortex slices from P14 rats, followed by rapamycin 5µM. Pooled data (Figure 5.1.5) show that the depression of transmission by inhibition of PKMζ in P14 perirhinal cortex occludes any subsequent depression of synaptic transmission by rapamycin (Temporal side: ZIP 55 ± 3% of baseline, p<0.001; Rapamycin 52± 3% of baseline, p> 0.05 v ZIP; Entorhinal side: ZIP 54 ± 1% of baseline; Rapamycin 51± 1% of baseline, p>0.05 v ZIP; n=4). This occlusion result suggests that PKMζ maintains basal synaptic transmission in P14 perirhinal cortex through, at least in part, mTOR-dependent protein translation.

On the other hand, the application of ZIP following depression of synaptic transmission by rapamycin (Figure 5.1.6) resulted in a small but significant depression of the synaptic transmission (pooled data shown in Figure 5.1.7): Temporal side: Rapamycin 67 ± 1% of baseline, p<0.001; ZIP 52± 3% of baseline, p<0.01 v Rapamycin; Entorhinal side: Rapamycin 66 ± 2% of baseline, p<0.001; ZIP 52± 3% of baseline, p<0.01 v Rapamycin; n=4). The depression by ZIP after rapamycin was smaller than the depression of basal transmission produced by ZIP alone. This suggests that although PKMζ maintains basal transmission through mTOR-dependent protein translation some additional mechanism may also be involved.

To further strengthen these findings, this last set of experiments has been repeated using another mTOR inhibitor, KU0063794. This compound is a very potent and selective inhibitor of mTOR (Garcia-Martinez, Moran et al. 2009). Figure 5.1.8 shows a single experiment where, after 1 hour of baseline recording from P14 perirhinal cortex, KU0063794 1µM was bath applied, followed by ZIP. Pooled data (Figure 5.1.9) show that KU0063794 produces a significant decrease in the basal synaptic transmission, similar to the one produced by rapamycin (Temporal side: 72 ± 2% of baseline, p<0.01; Entorhinal side 69 ± 2% of baseline, p<0.01). The following application of ZIP (5µM) produces a further small but significant depression of the synaptic transmission (Temporal side: ZIP 54 ± 0.6% of baseline, p<0.01 v KU0063794; Entorhinal side: ZIP 51 ± 3% of baseline, p<0.01 v KU0063794; n=4). It’s also interesting to note that the combination of these drugs determines a total depression of the synaptic transmission of the same amount of the depression.
produced by the application of ZIP. No effect of mTOR inhibition by KU0063794 can be observed in adult perirhinal cortex (Pooled data in Figure 5.1.10: Temporal side: 100 ± 4% of baseline, p>0.05; Entorhinal side 102 ± 4% of baseline, p>0.05, n=3).

5.1.2.3 Role of PI3K in basal synaptic transmission

The phosphoinositide-3-kinase (PI3K) is known to play an important role in the regulation of mTOR (Sabatini 2006; Tsokas, Ma et al. 2007; Gobert, Topolnik et al. 2008; Costa-Mattioli, Sossin et al. 2009). Furthermore, PI3K is involved in the expression and maintenance of LTP (Horwood, Dufour et al. 2006; Karpova, Sanna et al. 2006; Tsokas, Ma et al. 2007; Gobert, Topolnik et al. 2008; Sui, Wang et al. 2008). For these reasons, we thought it was an important target to investigate in order to further clarify the mechanisms underlying the potentiated synaptic transmission that we observed in P14 perirhinal cortex. To stress this point, after a 40 minutes baseline the “classic” PI3K inhibitor wortmannin (400nM) was bath applied to the slice (Figure 5.1.11). Pooled data (Figure 5.1.12) show that wortmannin is able to significantly decrease the synaptic transmission in P14 perirhinal cortex (Temporal side: 61 ± 4% of baseline, p<0.001; Entorhinal side 64 ± 3% of baseline, p<0.001, n=5). No difference was found between the two pathways (p>0.05). Wortmannin does not decrease basal synaptic transmission in adult perirhinal cortex (pooled data shown in Figure 5.1.13: Temporal side: 107 ± 4% of baseline, p>0.05; Entorhinal side 107 ± 2% of baseline, p>0.05, n=3. No difference between the two pathways). These results confirm that PI3K is in some way involved in the maintenance of basal synaptic transmission in P14, but not adult, perirhinal cortex, presumably through the inhibition of the mTOR-mediated protein synthesis.

5.1.2.4 Effect of PI3K inhibition on PKMζ activity in perirhinal cortex

Since PI3K is important in maintaining high levels of synaptic transmission in P14 perirhinal cortex, possibly through mTOR-mediated protein synthesis, and mTOR seems to be involved in the regulation of PKMζ activity, it was interesting
to investigate whether the inhibition of PI3K could itself affect in any way the activity of PKMζ in P14 perirhinal cortex. In order to do so, after 40 minutes of baseline recording (as shown in Figure 5.1.14), a potent and selective inhibitor of PI3K, LY294002 50µM was bath-applied, followed by ZIP. Pooled data (Figure 5.1.15) show that LY294002 produces a significant decrease in the synaptic transmission in P14 perirhinal cortex (Temporal side: 63 ± 0.5% of baseline, p<0.001; Entorhinal side 63 ± 0.6% of baseline, p<0.001, n=4). ZIP, bath applied after the synaptic response was again stabilized, shows a further small, but significant, decrease in the fEPSP amplitude (Temporal side: 53 ± 1% of baseline, p<0.01 v LY294002; Entorhinal side 52 ± 0.6% of baseline, p<0.01 v LY294002). No difference was found between the two pathways (p>0.05). It’s important to note that the selective PI3K inhibitor, LY294002, produces a decrease in the synaptic response of the same magnitude as the one produced by wortmannin. LY294002 shows no effect on basal synaptic transmission in adult perirhinal cortex (Figure 5.1.16, pooled data: Temporal side: 108 ± 3% of baseline, p>0.05; Entorhinal side 106 ± 3% of baseline, p>0.05). These results suggest that PI3K is involved in the maintenance of the potentiated baseline response in P14 (but not adult) perirhinal cortex by someway regulating the activity of PKMζ, possibly through the regulation of mTOR. An alternative explanation is that PI3K modulates the activity of PKMζ by acting on the activation of PDK1.
Figure 5.1.1 Single experiment on the effect of Rapamycin on basal synaptic transmission in P14 perirhinal cortex The application of the mTOR inhibitor rapamycin produces a substantial decrease in P14 basal synaptic transmission.

Figure 5.1.2 Pooled data for the effect of Rapamycin on basal synaptic transmission in P14 perirhinal cortex mTOR inhibition produced a substantial depression of basal transmission in P14 perirhinal cortex (temporal side, filled circles: 73 ± 3% of baseline, p<0.001; entorhinal side, open circles: 74 ± 3% of baseline, p<0.001, n=6).
Figure 5.1.3 Pooled data for the effect of Rapamycin on basal synaptic transmission in adult perirhinal cortex. No effect of mTOR inhibition by bath application of rapamycin was observed in perirhinal slices from adult animals (temporal side, filled circles: 98 ± 3% of baseline, p>0.05; entorhinal side, open circles: 100 ± 3% of baseline, p>0.05, n=4).
Figure 5.1.4 Single experiment on the effect of ZIP followed by Rapamycin on basal synaptic transmission in P14 perirhinal cortex. The inhibition of PKMζ occludes any further depotentiation produced by Rapamycin.

Figure 5.1.5 Pooled data for the effect of ZIP followed by Rapamycin on basal synaptic transmission in P14 perirhinal cortex. Depression of transmission by inhibition of PKMζ in P14 perirhinal cortex occludes any subsequent depression of synaptic transmission by rapamycin (Temporal side, filled circles: ZIP 55 ± 3% of baseline, p<0.001; Rapamycin 52± 3% of baseline, p> 0.05 v ZIP; Entorhinal side, open circles: ZIP 54 ± 1% of baseline; Rapamycin 51± 1% of baseline, p>0.05 v ZIP; n=4)
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Figure 5.1.6 Single experiment of Rapamycin and ZIP on P14 perirhinal cortex. Bath-application of the mTor inhibitor Rapamycin strongly decreases the baseline synaptic response in P14 perirhinal cortex. The subsequent inhibition of PLM\(\zeta\) produces a further small but significant decrease in the amplitude of the synaptic response.

Figure 5.1.7 Pooled data for effect of Rapamycin and ZIP on P14 perirhinal cortex. mTOR inhibition produced a substantial depression of basal synaptic transmission in P14 perirhinal cortex (Temporal side, filled circles: 67 ± 1% of baseline, p<0.001; Entorhinal side, open circles: 66 ± 2% of baseline, p<0.001, n=4) Subsequent inhibition of PKM\(\zeta\) produces a small but significant decrease in the synaptic transmission (temporal side, ZIP 52± 3% of baseline, p<0.01 v Rapamycin; entorhinal side ZIP 52± 3% of baseline, p<0.01 v Rapamycin; n=4).
Figure 5.1.8 Single experiment on effect of KU0063794 and ZIP on P14 perirhinal cortex  The inhibition of mTOR produces a wide depression in the synaptic transmission. ZIP shows no effect when bath applied afterwards.

Figure 5.1.9 Pooled data for effect of KU0063794 and ZIP on P14 perirhinal cortex  KU0063794 produces a significant decrease in the basal synaptic transmission, similar to the one produced by rapamycin (Temporal side, filled circles: 72 ± 2% of baseline, p<0.01; Entorhinal side, open circles 69 ± 2% of baseline, p<0.01). The following application of ZIP (5µM) produces a further small but significant depression of the synaptic transmission (Temporal side: ZIP 54 ± 0.6% of baseline, p<0.01 v KU0063794; Entorhinal side: ZIP 51 ± 3% of baseline, p<0.01 v KU0063794; n=4).

Figure 5.1.10 Pooled data for effect of KU0063794 adult perirhinal cortex
No effect of mTOR inhibition by KU0063794 can be observed in adult perirhinal cortex (Temporal side, filled circles: 100 ± 4% of baseline, p>0.05; Entorhinal side, open circles 102 ± 4% of baseline, p>0.05, n=3).
Figure 5.1.11 Single experiment on effect of Wortmannin on P14 perirhinal cortex  Bath application of the PI3K inhibitor wortmannin produces a decrease in the basal synaptic response.

Figure 5.1.12 Pooled data for effect of Wortmannin on P14 perirhinal cortex  Wortmannin is able to significantly decrease the synaptic transmission in P14 perirhinal cortex (Temporal side, filled circles : 61 ± 4% of baseline, p<0.001; Entorhinal side, open circles 64 ± 3% of baseline, p<0.001, n=5). No difference was found between the two pathways (p>0.05).
Figure 5.1.13 Pooled data for effect of Wortmannin on adult perirhinal cortex. Inhibition of PI3K by bath application of Wortmannin does not show any effect on basal synaptic transmission in adult perirhinal cortex (Temporal side, filled circles: 107 ± 4% of baseline, p>0.05; Entorhinal side, open circles 107 ± 2% of baseline, p>0.05, n=3. No difference is found between the two pathways).
Figure 5.1.14 Single experiment of effect of LY294002 and ZIP on P14 perirhinal cortex. Inhibition of PI3K by bath application of LY294002 is able to decrease the synaptic transmission. Subsequent inhibition of PKMζ produces a further depression of the evoked response.

Figure 5.1.15 Pooled data for effect of LY294002 and ZIP on P14 perirhinal cortex. LY294002 produces a significant decrease in the synaptic transmission (Temporal side, filled circles: 63 ± 0.5% of baseline, p<0.001; Entorhinal side, open circles 63 ± 0.6% of baseline, p<0.001, n=4). ZIP, bath applied after the synaptic response was again stabilized, shows a further small, but significant, decrease in the fEPSP amplitude (Temporal side: 53 ± 1% of baseline, p<0.01 v LY294002; Entorhinal side 52 ± 0.6% of baseline, p<0.01 v LY294002). No difference was found between the two pathways (p>0.05).
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Figure 5.1.16 Pooled data for effect of LY294002 on adult perirhinal cortex
PI3K inhibitor, LY294002, shows no effect on basal synaptic transmission in adult
perirhinal cortex (Temporal side, filled circles: 108 ± 3% of baseline, p>0.05; Entorhinal
side, open circles: 106 ± 3% of baseline, p>0.05, n=3). No difference was found
between the two pathways (p>0.05)
5.1.3 Discussion

The results obtained so far show that PKMζ maintains high levels of basal transmission in P14 but not in adult perirhinal cortex. The underlying mechanisms by which this occurs are still not known, but a plausible hypothesis could be that PKMζ maintains these high levels of synaptic transmission in P14 perirhinal cortex via regulation of protein synthesis through local dendritic translation (Muslimov, Nimmrich et al. 2004; Westmark, Westmark et al. 2010). It has been suggested (Westmark et al 2010) that PKMζ may maintain LTP by protein translation through a signalling cascade involving regulation of Pin1 – a protein that interacts with 4E-BPs that normally suppress protein translation. 4E-BPs are phosphorylated by mTOR to remove the suppression of translation initiation (Hoeffer and Klann 2010): If PKMζ maintains elevated transmission through protein translation then this is likely to involve the mTOR-dependent translation initiation pathway. Therefore, if this is true, the inhibition of mTOR should produce a depression in the basal synaptic transmission in P14 perirhinal cortex. The results previously shown confirm that mammalian target of rapamycin (mTOR) could be involved in this process. It is known, in fact, that translation initiation relies at least in part on the activation of mTOR, the inhibition of which prevents LTP (Hoeffer and Klann 2010). Different inhibitors of mTOR (rapamycin and KU0063794) show the same effect on basal synaptic transmission in P14 perirhinal cortex, that is a wide decrease in the response, without affecting the basal synaptic transmission in adult perirhinal cortex. Two different mTOR inhibitors have been used in order to strengthen the preliminary findings and exclude that rapamycin could have any different action on other elements that might influence the results. In fact, even if rapamycin is considered a potent and selective mTOR inhibitor, there are some evidences that this compound might have also some effect on high-voltage activated Ca(2+) channels (Regimbald-Dumas, Fregeau et al. 2010; Suh, Leal et al. 2010). Some of these channels seems to be involved in LTP in the amygdala (Pinard, Mascagni et al. 2005), so testing also another compound which shouldn’t have effect on these channels would provide a control and strengthen the findings. Both the compounds, though, showed the same effect in depressing the synaptic response, suggesting that ongoing protein translation...
controls basal levels of synaptic transmission in P14 but not in adult perirhinal cortex.

The depression of the synaptic response obtained by inhibition of mTOR, though, is smaller than the one produced by the inhibition of the sole PKMζ. When ZIP is bath applied in P14 perirhinal cortex following the maximal depression of the synaptic transmission produced by mTOR inhibition, a further depression of the response is observed. Interestingly, the total depression of the synaptic transmission produced by the consecutive inhibition of mTOR first, and then PKMζ, is of the same amount of the one produced by the inhibition of PKMζ alone. These results suggest that, although PKMζ maintains basal transmission through mTOR-dependent protein translation, some additional mechanism may also be involved. One hypothesis is that mTOR only regulates the new synthesis of PKMζ, but has no effect on that part of the enzyme that is already active; thus, the complete inhibition of PKMζ could be obtained only through the selective inhibitor ZIP. This hypothesis is consistent with the finding that the depression of transmission by inhibition of PKMζ in P14 perirhinal cortex occludes any subsequent depression of synaptic transmission that might be produced by rapamycin.

Therefore, protein translation, critical for the maintenance of LTP and memory (Hoeffer and Klann 2010), may be a possible route for PKMζ-dependent maintenance of LTP (Westmark et al 2010). According to these results, mTOR-dependent protein translation is also important for the regulation of basal transmission in young, but not adult, perirhinal cortex.

Another mechanism that could play a role in the PKMζ-dependent maintenance of the potentiated synaptic response in P14 perirhinal cortex involves the phosphoinositide-3-kinase (PI3K). As better described before, PI3K is known to play an important role in the regulation of mTOR (Sabatini 2006; Tsokas, Ma et al. 2007; Gobert, Topolnik et al. 2008; Costa-Mattioli, Sossin et al. 2009). Furthermore, PI3K is involved in the expression and maintenance of LTP (Horwood, Dufour et al. 2006; Karpova, Sanna et al. 2006; Tsokas, Ma et al. 2007; Gobert, Topolnik et al. 2008; Sui, Wang et al. 2008). Finally, PI3K is known to regulate PDK1 activity, crucial for the activation of PKMζ (Kelly, Crary et al. 2007)
Therefore, in a potentiated pathway, as the ones in P14 perirhinal cortex appear to be, the inhibition of PI3K should lead to a depression of the synaptic transmission. The results showed before confirm this hypothesis: bath application of the PI3K inhibitors Wortmannin or LY294002 produces a significant depression of the synaptic transmission. In this case as well, two different PI3K inhibitors were used to increase the preciseness of the experiments. Both the compounds appear to have the same effect. Once again, though, the depression of the synaptic transmission produced by the inhibition of PI3K is smaller than the depression produced by the direct inhibition of PKMζ. The subsequent application of ZIP further decreases the response, and the final depression of the synaptic transmission is eventually of the same amount of the one produced by ZIP alone. These findings are similar to the results of the experiments carried using mTOR inhibitors. Once again, the hypothesis is that PKMζ is downstream to this signaling cascade, but only its new synthesis is under the control of PI3K/mTOR. The inhibition of PI3K-dependent effectors, though, has no effect on already active PKMζ. Also, PI3K controls PDK1, crucial for the activation of PKMζ. Therefore, it can’t be excluded that the effect observed by the inhibition of PI3K occurs via an indirect inhibition of PKMζ activity through the inhibition of PDK1.
5.2 Role of Group I Metabotropic Glutamate Receptors in the regulation of PKMζ during neurodevelopment

5.2.1 Introduction

As described in the previous chapters, PKMζ seems to maintain an elevated level of synaptic transmission in P14 perirhinal cortex by acting downstream of some intracellular signaling mechanism involving PI3K and mTOR. It is well known that activation of a variety of glutamate receptors, including metabotropic glutamate receptors (mGluRs), can trigger signalling cascades that underpin LTP and regulate mTOR (Hoeffer and Klann 2010). Also, it is known PI3K, a kinase involved in learning and memory, is activated by the complex calcium/calmodulin (Joyal, Burks et al. 1997; Wang, Fibuch et al. 2007) and by the G-protein subunit βγ (Lopez-Ilasaca 1998). It is also known that Group I mGluRs (mGluR1 and mGluR5) are intracellularly coupled to the trimeric protein Gq: the α subunit of Gq induces the hydrolysis of phosphoinositides, with formation of diacylglycerol (DAG) and inositol-3-phosphate, and increase in the intracellular concentration of Ca++. The βγ subunit activates PI3K (Pin and Duvoisin 1995).

There are several evidences of the interaction between mGlu receptors and PI3K; Group I mGluRs activate PI3K, leading to a pathway which shows important neuroprotective effects. Through the activation of PI3K, Group I mGluRs prevent neuronal apoptosis (Rong, Ahn et al. 2003), promote the PI3K-dependent activation of both Akt and mTOR (Hou and Klann 2004), regulate microglial activation (Chong, Kang et al. 2005), is neuroprotective to Aβ peptide in animal models of Alzheimer’s disease (Liu, Gong et al. 2005) and show neuroprotective effects in animal models of cerebral ischemia (Scartabelli, Gerace et al. 2008).

Finally, there are some evidences showing that mGluRs are involved in the regulation of protein synthesis (Weiler and Greenough 1993; Angenstein, Greenough et al. 1998), so it’s not unlikely that they might affect also the synthesis of PKMζ.
Starting from the knowledge that Group I mGluRs, i.e. mGlu5, are highly expressed early in the neurodevelopment (Casabona, Knopfel et al. 1997; Copani, Casabona et al. 1998), these receptors appeared to be good candidates to maintain the high levels of basal synaptic transmission we observed in perirhinal cortex, possibly through PI3K/mTOR-mediated mechanisms.

So, in order to examine the role of Group I mGluRs in maintaining basal synaptic transmission in P14, and their possible role in regulating PKMζ activity, we run the following sets of experiments.
5.2.2 Results

5.2.2.1 Role of mGluR1 on basal synaptic transmission

In order to determine if a continuous activation of mGlu1 can contribute to the maintenance of the basal synaptic transmission in P14 perirhinal cortex, after a 30 minutes of baseline recording, a selective mGlu1 antagonist, LY456236 2μM was bath applied for 1 hour (Figure 5.2.1). Pooled Data (Figure 5.2.2) show that LY456236 produces a decrease in the basal synaptic transmission in P14 perirhinal cortex (Temporal side: 76 ± 0.7% of baseline, p<0.001; Entorhinal side 78 ± 01% of baseline, p<0.001, n=4). No effect of LY456236 is observed in perirhinal cortex when the compound is bath applied on slices from adult (2-3 weeks) animals, as shown in Figure 5.2.3 (Temporal side: 101 ± 2% of baseline, p>0.05; Entorhinal side 99 ± 1.6% of baseline, p>0.05, n=3).

These experiments have been repeated using another mGluR1 inhibitor, the Negative Allosteric Modulator (NAM) JNJ16259685. After 30 minutes of baseline recording, bath application of 10μM JNJ16259685 produces a significant decrease in the basal synaptic transmission in slices of perirhinal cortex from P14 animals (single example in Figure 5.2.4; pooled data in Figure 5.2.5. Temporal side 77 ± 1% of baseline, p<0.001; Entorhinal side 76 ± 1% of baseline, p<0.001, n=4). The effect on basal synaptic transmission of JNJ16259685 is of the same magnitude of the decrease produced by the other mGlu1 antagonist LY456236, therefore confirming the previous results. The application of JNJ16259685 shows no effect on adult perirhinal cortex (pooled data in Figure 5.2.6, Temporal side 103 ± 2% of baseline, p>0.05; Entorhinal side 101 ± 3% of baseline, p>0.05, n=3).

The depression by both mGlu1 antagonist is smaller than that produced by either inhibition of PKMζ or inhibition of mTOR, indicating that although the continuous activation of mGlu1 receptors most likely contributes to sustained basal transmission in P14 cortex, other mechanisms might be involved in this phenomenon. In order to confirm that the observed decrease on basal synaptic transmission in P14 perirhinal cortex following the blockade of mGlu1 occurs through an mTOR-dependent mechanism, after 60 minutes baseline recording, the m-TOR inhibitor KU0063794 1μM was bath-applied, followed by the mGlu1 antagonist LY456236 (Figure 5.2.7). Pooled data (Figure 5.2.8) show that the
inhibition of mTOR depressed the basal synaptic transmission, as observed before (chapter 5.1.2.2) (Temporal side: 67 ± 1% of baseline, p<0.01; Entorhinal side 68 ± 1% of baseline, p<0.01, n=3). No further decrease of the synaptic transmission is observed following the subsequent application of LY456236 (Temporal side 65 ± 2% of baseline, p>0.05; Entorhinal side 66 ± 1% of baseline, p>0.05, n=3). Therefore, these results suggest that mGlu1 maintains the basal synaptic transmission in p14 perirhinal cortex through an mTOR-dependent mechanism.

5.2.2.2 Role of mGluR5 on basal synaptic transmission

To assess whether mGlu5, as well as mGlu1, could contribute to the maintenance of high levels of basal synaptic transmission in P14 perirhinal cortex, the mGlu5 antagonist MPEP 10µM was bath applied after 30 minutes of baseline recording (Figure 5.2.9). Pooled data (Figure 5.2.10) show that the block of mGlu5 produces a small but significant decrease in the synaptic transmission in P14 perirhinal cortex (Temporal side 87 ± 1% of baseline, p<0.001; Entorhinal side 83 ± 2% of baseline, p<0.001, n=7). These results confirm the role of mGlu5 in the maintenance of basal synaptic transmission. No effect of the inhibition of mGlu5 is observed on adult slices of perirhinal cortex (pooled data in Figure 5.2.11, Temporal side 97 ± 2% of baseline, p>0.05; Entorhinal side 99 ± 2% of baseline, p>0.05, n=5).

In order to confirm that the observed decrease on basal synaptic transmission in P14 perirhinal cortex occurs through an mTOR-dependent mechanism, after 60 minutes baseline recording, the m-TOR inhibitor Rapamycin 5µM was bath applied, followed by the mGlu5 antagonist MPEP (Figure 5.2.12). Pooled data (Figure 5.2.13) show that the inhibition of mTOR depressed the basal synaptic transmission, as observed before (chapter 5.1.2.2) (Temporal side: 67 ± 2% of baseline, p<0.001; Entorhinal side 68 ± 1% of baseline, p<0.001, n=6). No further decrease of the synaptic transmission is observed following the subsequent application of MPEP (Temporal side 63 ± 1% of baseline, p>0.05; Entorhinal side 64 ± 1% of baseline, p>0.05, n=3).

So, the depression of basal transmission induced by MPEP in P14 perirhinal cortex was not observed following rapamycin-induced depression. This
suggests that also an mGlu5/mTOR dependent signalling cascade maintains basal synaptic transmission in neonatal cortex.

5.2.2.3 Effect of the combined antagonism of mGlu1 and mGlu5 on the maintenance of basal synaptic transmission on P14 perirhinal cortex

Since our previous findings show that the selective antagonism of either mGlur1 or mGlu5 in P14 perirhinal cortex produces a depression in the synaptic transmission of more or less 20% each, it was interesting to find out whether this effect was additive, and if it occurred through the inhibition of the activity of PKMζ. To test this hypothesis, after a 30 minutes baseline, the selective mGlu1 NAM JNJ16259685 was bath applied on perirhinal cortex slice from P14 rat, followed by the selective mGlu5 antagonist MPEP (Figure 5.2.14). The selective PKMζ inhibitor, ZIP, was bath applied after MPEP. Pooled data (Figure 5.2.15) confirm that the blockade of mGlu1 produces a significant decrease of the synaptic transmission in P14 perirhinal cortex (Temporal side: $77 \pm 1\%$ of baseline, $p<0.001$; Entorhinal side: $80 \pm 0.8\%$ of baseline, $p < 0.001$; n=4). The subsequent bath application of MPEP in P14 cortex further decreases the basal synaptic transmission in an additive fashion (Temporal side: $59 \pm 0.6\%$ of baseline, $p<0.001$ v JNJ16259685; Entorhinal side: $59 \pm 0.6\%$ of baseline, $p<0.001$ v JNJ16259685; n=4). Subsequent bath application of ZIP, though, produces a further small, but significant, depression of the synaptic transmission (Temporal side: $51 \pm 0.7\%$ of baseline, $p<0.01$ v MPEP; Entorhinal side: $51 \pm 1\%$ of baseline, $p>0.01$ v MPEP; n=4). Therefore, the ZIP-induced depotentiation of the baseline is not fully occluded by the blockade of both mGluR1 and mGluR5, this suggesting once again that the activity of PKMζ is likely to be regulated by Group I mGluRs, although not completely.
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Figure 5.2.1 Single experiment on the effect of LY456236 on P14 perirhinal cortex The application of the selective mGlu1 antagonist, LY456236, produces a depression of the basal synaptic transmission in P14 perirhinal cortex.

Figure 5.2.2 Pooled data for effect of LY456236 on P14 perirhinal cortex The selective mGlu1 receptor antagonist, LY456236, produces a decrease in the basal synaptic transmission in P14 perirhinal cortex (Temporal side, filled circles: 76 ± 0.7% of baseline, p<0.001; Entorhinal side, open circles 78 ± 01% of baseline, p<0.001, n=4).
Figure 5.2.3 Pooled data for effect of LY456236 on adult perirhinal cortex No effect of the mGlu1 antagonist LY456236 was found on basal synaptic transmission in adult perirhinal cortex (Temporal side, filled circles: 101 ± 2% of baseline, p>0.05; Entorhinal side, open circles 99 ± 1.6% of baseline, p>0.05, n=3).
Figure 5.2.4 Single experiment on the effect of JNJ16259685 on P14 perirhinal cortex
The application of the selective mGlu1 NAM, JNJ16259685, produces a depression of the basal synaptic transmission in P14 perirhinal cortex.

Figure 5.2.5 Pooled data for effect of JNJ16259685 on P14 perirhinal cortex
Bath application of the selective mGlu1 NAM, JNJ16259685, produces a significant depression of the synaptic transmission from P14 animals (Temporal side, filled circles: 77 ± 1% of baseline, p<0.001; Entorhinal side, open circles: 76 ± 1% of baseline, p<0.001, n=4). The effect on basal synaptic transmission of JNJ16259685 is of the same magnitude of the decrease produced by the other mGlu1 antagonist LY456236.
Figure 5.2.6 Pooled data for effect of JNJ16259685 on adult perirhinal cortex The application of JNJ16259685 shows no effect on adult perirhinal cortex (Temporal side, filled circles: 103 ± 2% of baseline, p>0.05; Entorhinal side, open circles: 101 ± 3% of baseline, p>0.05, n=3). No difference was found between the two pathways (p>0.05).
Figure 5.2.7 Single experiment on the effect of KU0063794 and LY456236 on P14 perirhinal cortex The application of the selective mGlu1 antagonist, LY456236, produces a depression of the basal synaptic transmission in P14 perirhinal cortex.

Figure 5.2.8 Pooled data for effect of KU0063794 and LY456236 on P14 perirhinal cortex The inhibition of mTOR by KU0063794 depresses the basal synaptic transmission (Temporal side, filled circles: 67 ± 1% of baseline, p<0.01; Entorhinal side, open circles 68 ± 1% of baseline, p<0.01, n=3). No further decrease of the synaptic transmission is observed following the subsequent application of LY456236 (Temporal side 65 ± 2% of baseline, p>0.05; Entorhinal side 66 ± 1% of baseline, p>0.05, n=3).
Figure 5.2.9 Single experiment on the effect of MPEP on P14 perirhinal cortex
The application of the selective mGlu5 antagonist, MPEP, produces a depression of the basal synaptic transmission in P14 perirhinal cortex.

Figure 5.2.10 Pooled data for effect of MPEP on P14 perirhinal cortex
The application of the selective mGlu5 antagonist MPEP produces a small but significant decrease in the synaptic transmission in P14 perirhinal cortex (Temporal side, filled circles: 87 ± 1% of baseline, p<0.001; Entorhinal side, open circles: 83 ± 2% of baseline, p<0.001, n=7).
Figure 5.2.11 Pooled data for effect of MPEP on adult perirhinal cortex. The application of MPEP shows no effect on adult perirhinal cortex (Temporal side, filled circles: 97 ± 2% of baseline, p>0.05; Entorhinal side, open circles: 99 ± 2% of baseline, p>0.05, n=5). No difference was found between the two pathways (p>0.05).
Figure 5.2.12 Single experiment on the effect of Rapamycin and MPEP on P14 perirhinal cortex The application of the mTOR inhibitor Rapamycin produces a decrease in the basal synaptic response. Subsequent application of the selective mGlu5 antagonist, MPEP, does not produce any further depression of the synaptic transmission.

Figure 5.2.13 Pooled data for effect of Rapamycin and MPEP on P14 perirhinal cortex The inhibition of mTOR depressed the basal synaptic transmission, as observed before (Temporal side, filled circles: 67 ± 2% of baseline, p<0.001; Entorhinal side, open circles: 68 ± 1% of baseline, p<0.001, n=6). No further decrease of the synaptic transmission is observed following the subsequent application of MPEP (Temporal side 63 ± 1% of baseline, p>0.05; Entorhinal side 64 ± 1% of baseline, p>0.05, n=3).
Figure 5.2.14 Single experiment on the effect of JNJ16259685, MPEP and ZIP on P14 perirhinal cortex The application of mGlu1 and mGlu5 show an additive effect in decreasing the basal synaptic response. The application of ZIP produces a further, small decrease.

Figure 5.2.15 Pooled data for the effect of JNJ16259685, MPEP and ZIP on P14 perirhinal cortex Application of JNJ16259685 produces a significant decrease of the synaptic transmission in P14 perirhinal cortex (Temporal side, filled circles: 77 ± 1% of baseline, p<0.001; Entorhinal side, open circles: 80 ± 0.8% of baseline, p<0.001; n=4). The subsequent bath application of MPEP further decreases the synaptic transmission in an additive fashion (Temporal side: 59 ± 0.6% of baseline, p<0.001 v JNJ16259685; Entorhinal side: 59 ± 0.6% of baseline, p<0.001 v JNJ16259685). Subsequent bath application of ZIP produces a further small, but significant, depression of the synaptic transmission (Temporal side: 51 ± 0.7% of baseline, p<0.01 v MPEP; Entorhinal side: 51 ± 1% of baseline, p>0.01 v MPEP).
5.2.3 Discussion

Previous results show that it is possible that a high level of synaptic transmission in P14 perirhinal cortex is maintained by PKMζ, which is in turn controlled by PI3K- and mTOR-dependent processes. But what maintains such a high level of activity of PKMζ? One possible candidate was identified in metabotropic glutamate receptors (mGluRs), in particular Group I mGluRs.

As described before, it is widely known that metabotropic glutamate receptors play many different roles in neuronal plasticity, especially in learning and memory processes. Group I mGluRs, intracellularly coupled with Gq, have as ultimate effect the hydrolysis of Phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to diacyl glycerol (DAG) and inositol triphosphate (IP$_3$) signal transduction pathway, which leads to an increase of the intracellular concentration of Ca$^{2+}$. Group I mGluRs are also known to regulate the activity of both PI3K and mTOR, so they appeared to be an interesting subject to investigate, in order to further clarify the mechanisms leading to the sustained activity of PKMζ.

When mGlu1 or mGlu5 activity is blocked by the application of selective antagonists, a small but significant depression of the synaptic transmission is observed. The blockade of these receptors shows no effect on the synaptic transmission in adult perirhinal cortex. Therefore, these receptors seem to be involved in the maintenance of the high levels of synaptic transmission in P14 perirhinal cortex, although the mechanisms responsible to this effect are not known. Following the hypothesis that basal synaptic transmission is maintained by PKMζ possibly via a PI3K/mTOR-dependent protein translation, the idea was that Group I mGluRs, regulating the activity of both PI3K and mTOR, could be upstream to the whole signaling pathway. In fact, when either mGlu1 or mGlu5 antagonists are applied on the slice following the depression of the synaptic transmission produced by the inhibition of mTOR, no further decrease of the response is observable. These findings then support the hypothesis that the mTOR regulation of synaptic transmission may rely at least in part on activation of mGlu1 and mGlu5 receptors. It’s important to remember that mTOR-dependent translation has previously been shown to be involved in synaptic plasticity and it is known that group I mGluRs can regulate mTOR-dependent...
protein translation (Hoeffer and Klann 2010). Moreover, the inhibition of both mGlu1 and mGlu5 appear to depress the synaptic transmission in an additive fashion. Once again, the depression produced by both mGlu1 and mGlu5 antagonist is smaller than the one produced by the direct inhibition of PKMζ, and the subsequent application of ZIP produces a further small decrease in the synaptic transmission. The total depression of the synaptic transmission obtained by the application of the three compounds is of the same magnitude of the one produced by ZIP alone. Once again, then, these results suggest that, even if ongoing activation of Group I mGlu receptors widely contributes to maintained basal transmission in P14 cortex, other mechanisms are also likely to play a role.

Taken together, these results suggest that an mGlu1/5-PKMζ-mTOR dependent signalling cascade maintains basal synaptic transmission in neonatal cortex.
6 GENERAL DISCUSSION
6.1 General Discussion

There is a growing interest in the scientific community about PKMζ. It has been well demonstrated in the last few years that PKMζ is crucial in the maintenance of LTP in different brain areas (especially hippocampus and insular cortex). The inhibition of PKMζ reverts an established LTP both in vitro and in vivo, and completely erases the encoded memories in behavioural experiments of different kind (Sacktor, Osten et al. 1993; Serrano, Yao et al. 2005; Pastalkova, Serrano et al. 2006; Shema, Sacktor et al. 2007; Serrano, Friedman et al. 2008). The experiments run in adult perirhinal cortex confirm the role of PKMζ in maintaining the potentiation also in this brain area, crucial for recognition memory. Also, the activity of PKMζ is down-regulated by the administration of LFS protocols, when they are able to lead to a proper, stable LTD (or de-depression). According to this, PKMζ shows no effect on the induction of LTD, as it does on the induction of LTP, in which it’s not involved. In general, the results confirm what is known on PKMζ, as described in previous studies, although this is the first time that such studies are carried in perirhinal cortex. Also, experiments on the inhibition of PDK1 seem to confirm the crucial role of this protein in the regulation of PKMζ activity, although, as explained before, it’s impossible to state for sure that the observed effect on the maintenance of LTP occurs only via a PDK1-dependet inhibition of PKMζ activity. PDK1, in fact, regulates many different cellular processes, most of them involved in cell proliferation. Nevertheless, the results are encouraging and might provide a base for further work aimed to better clarify the intracellular signaling involved in the regulation of PKMζ. The regulation of PKMζ activity is, in fact, a very fascinating matter, because this protein alone seems to be sufficient for the maintenance of LTP (Ling, Benardo et al. 2002), but the mechanisms leading to its activation are still not clear. It’s kind of amazing, in the first place, that only one protein seems to be responsible for the maintenance of such an important and complicated process, as memory storage. Also, the up-regulation of PKMζ following LTP and its down-regulation following LTD (or other way round: LTP following up-regulation of PKMζ and LTD following down-regulation of PKMζ) appear to be phenomena of a surprising straightforwardness. As for intracellular mechanisms leading to the
activation of PKMζ, it has been hypothesized that PKMζ is activated by different kinases, such as PI3K, CaMKII and associated kinases, which are up-regulated within 10 minutes post- HFS (Osten, Valsamis et al. 1996; Sacktor 2008). Levels of PKMζ, instead, only increase after 10 minutes post-HFS (Sacktor 2008). Therefore, PKMζ is likely to represent a final common pathway for the induction kinases, capable of maintaining the synaptic potentiation through persistent kinase activity. The mechanisms by which PKMζ maintains this persistent kinase activity need to be clarified as well. In fact, the half-life of PKMζ is a few hours at most, though its effects can last for a much longer time (possibly more than one month) (Sacktor 2008). It has been suggested that, once activated, PKMζ self-maintains its synthesis through a positive-feedback loop (Kelly, Crary et al. 2007), but the actual transcription factors responsible for this long-lasting transcriptional upregulation need still to be identified. CREB is thought to be involved (Muslimov, Nimmrich et al. 2004), but more studies will be needed to obtain conclusive evidences.

Experiments carried on P14 animals provide a different scenario. As opposed to what was observed in adults, not only it’s not possible to induce LTP in P14 perirhinal cortex, but the inhibition of PKMζ significantly decreases the synaptic response both in the baseline and in a de-depressed pathway. Further experiments provided evidence that PKMζ maintains these LTP-like levels of synaptic response via a PI3K/mTOR-dependent signaling cascade. Also, these processes appear to be under the regulation of Group I metabotropic glutamate receptors. PI3K, mTOR and Group I mGluRs are crucial in synaptic plasticity and their roles have been widely discussed in Chapters 5.1.1, 5.1.3, 5.2.1 and 5.2.3. Now, it’s interesting to examine the meaning of these observations taken together. Without doubt, such a peculiar behaviour of perirhinal cortex raises many questions, since so far there’s no evidence of a similar behaviour in any other brain region. PKMζ is now labeled as “the protein that maintains LTP” (Hrabetova and Sacktor 1996; Drier, Tello et al. 2002; Ling, Benardo et al. 2002; Pastalkova, Serrano et al. 2006), therefore such a big effect of its inhibition on a baseline could be quite surprising, at first. Nevertheless, the results shown in Chapter 4.2 provide a strong support to the hypothesis that in perirhinal cortex the basal synaptic transmission in already
potentiated on its own, as if it was in a “permanent state of LTP”. In this picture, the effect of the inhibition of PKMζ makes more sense. Apparently, at this stage of neurodevelopment in perirhinal cortex, the main role of PKMζ in maintaining high levels of synaptic transmission should be read in a different way, that is stabilisation of the immature synapses. A possible involvement of PKMζ in stabilising immature synapses during neurodevelopment has been recently demonstrated in xenopus (Liu, Tari et al. 2009). Also, although the reasons why synaptic transmission is maintained in an enhanced state are not known, it’s possible that these mechanisms promote or stabilise synaptic connections in the immature cerebral cortex (Hua and Smith 2004; Cline and Haas 2008; Hanse, Taira et al. 2009). This stabilisation of immature synapses may occur through a mechanism that relies on glutamatergic transmission and AMPA receptor insertion into the neuronal membrane (Rajan, Witte et al. 1999; Haas, Li et al. 2006; Hanse, Taira et al. 2009). Since there’s increasing agreement on the evidence that PKMζ maintains LTP through regulation of AMPA receptor trafficking (Ling, Benardo et al. 2006; Yao, Kelly et al. 2008; Migues, Hardt et al. 2010), it’s not hasty to say that these mechanisms of synapse stabilisation are essentially the same as those that operate in the expression/maintenance of long-term potentiation (LTP). Therefore stabilisation of and transmission at immature synapses is potentially under the control of LTP-like induction, expression and maintenance mechanisms.

Later on during the development, this “basal” potentiation is gradually lost, until it’s possible to start inducing LTP in perirhinal cortex. This is already evident at PND35, and persists during adult life.

The mechanisms that reduce the role of LTP and PKMζ during development of perirhinal cortex are not known but might arise from LTD-like processes that underlie visual recognition memory (Brown and Bashir 2002; Griffiths, Scott et al. 2008). The eye opening could be an important element involved in this phenomenon. Perirhinal cortex is strongly connected to visual cortex, so one very interesting hypothesis is that LTD-like processes might happen in perirhinal cortex as a consequences of new inputs coming from visual cortex. It’s worth to highlight once again that at PND14, rats have their eyes still closed. The hypothesis that the first visual experiences may produce some form of plasticity
in the visual cortex, which in turn modifies the synaptic connections in perirhinal
cortex, is undoubtedly fascinating, but it would require further investigations. A
way to do this could be trying to induce LTP in visually deprived adult rats. If the
mechanisms leading to the depression of basal synaptic transmission, that
allows the synapse to be potentiated following adequate stimulation, depend on
visual inputs, dark-reared adult animals should not show any LTP, exactly as it
happens in P14.

Another interesting possibility is that the neuronal stabilization provided by
PKMζ occurs in order to prevent developmental disorders. Mis-regulated protein
synthesis is important in developmental disorders, like autism and mental
retardation (Hoeffer and Klann 2010). It is possible that PKMζ, maintaining
some sort of stability in the synapses, prevents also the occurring of some kind
of aberrant dendritic protein translation that could produce abnormal synaptic
transmission and connectivity during early development. Therefore
PKMζ/mTOR-dependent mechanisms could be also critical for normal
development of the central nervous system.

So far, there’s no work carried on humans, but obviously this protein is of
great interest especially in diseases involving memory. Some preliminary works
demonstrate that PKMζ aggregates with limbic neurofibrillary tangles and
AMPA receptors in Alzheimer disease (Crary, Shao et al. 2006). Also, there are
evidences that dysregulation of mTOR signalling are involved in many
pathologies, like Alzheimer disease, X-fragile syndrome, tuberous sclerosis and
so on (Pei and Hugon 2008; Swiech, Perycz et al. 2008; Ehninger, de Vries et
al. 2009; Hoeffer and Klann 2010; Ma, Hoeffer et al. 2010; Malter, Ray et al.
2010; Sharma, Hoeffer et al. 2010), and the experiments described in Chapter
5.1 show a link between mTOR and PKMζ activity. Finally, PKMζ might play a
role also in psychiatric diseases, such as Post-Traumatic Stress Disorder.
Preliminary works have shown, in fact, (Cohen, Kozlovsky et al. 2010) that
inactivation of PKMζ in different brain areas reduces PSTD-like behaviour.

Therefore, PKMζ is a very interesting topic to develop, and further insight in
its activity might produce incredibly useful tools to better understand the
mechanisms underlying synaptic stabilization in young neurons and the
maintenance of memory in adult life.
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