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MECCANISMI NEUROADATTATIVI E REGOLAZIONE DELLA SFERA COGNITIVA

MECHANISMS OF NEUROADAPTATION AND REGULATION OF COGNITION

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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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There are many people I feel I should thank for their help and support during these 4 years of PhD, first of all my supervisors Ferdinando Nicoletti, Agata Copani and Zaf Bashir for their support and advice. In particular, I would like to thank Prof. Nicoletti for his amazing scientific contributions, and most of all for always believing in me. But in particular I'd like to offer my most heartfelt thanks to Prof. Bashir for his continual support and remarkable insight. I had a great time in the lab (yes, even while running experiments!), and I am glad I had the opportunity to spend some time in a place where scientific brightness was walking arm in arm with incredible human warmth.

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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

f EPSP, 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, phosphatidyl inositol 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

Table of Contents

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).

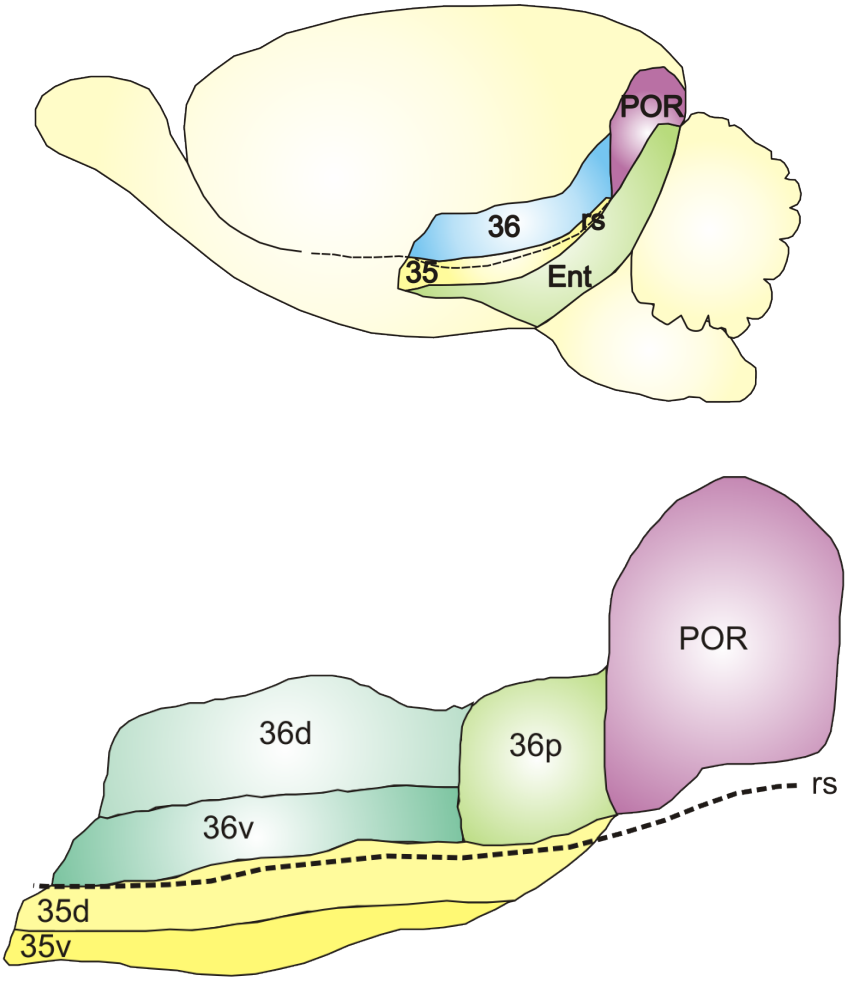

Figure 1.1.1 A schematic lateral view of the rat brain showing the perirhinal cortex and a schematic net of the perirhinal and postrhinal cortices of the rat, illustrating subdivisions within this portion of the temporal lobe. Areas 35 and 36 comprise the perirhinal cortex. Numbering refer to Brodmann's nomenclatures ('d', dorsal, 'v', ventral, 'p', postrhinal, 'rs', rhinal sulcus, 'POR', postrhinal cortex, 'Ent', entorhinal cortex). Modified from Burwell (2001).

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.

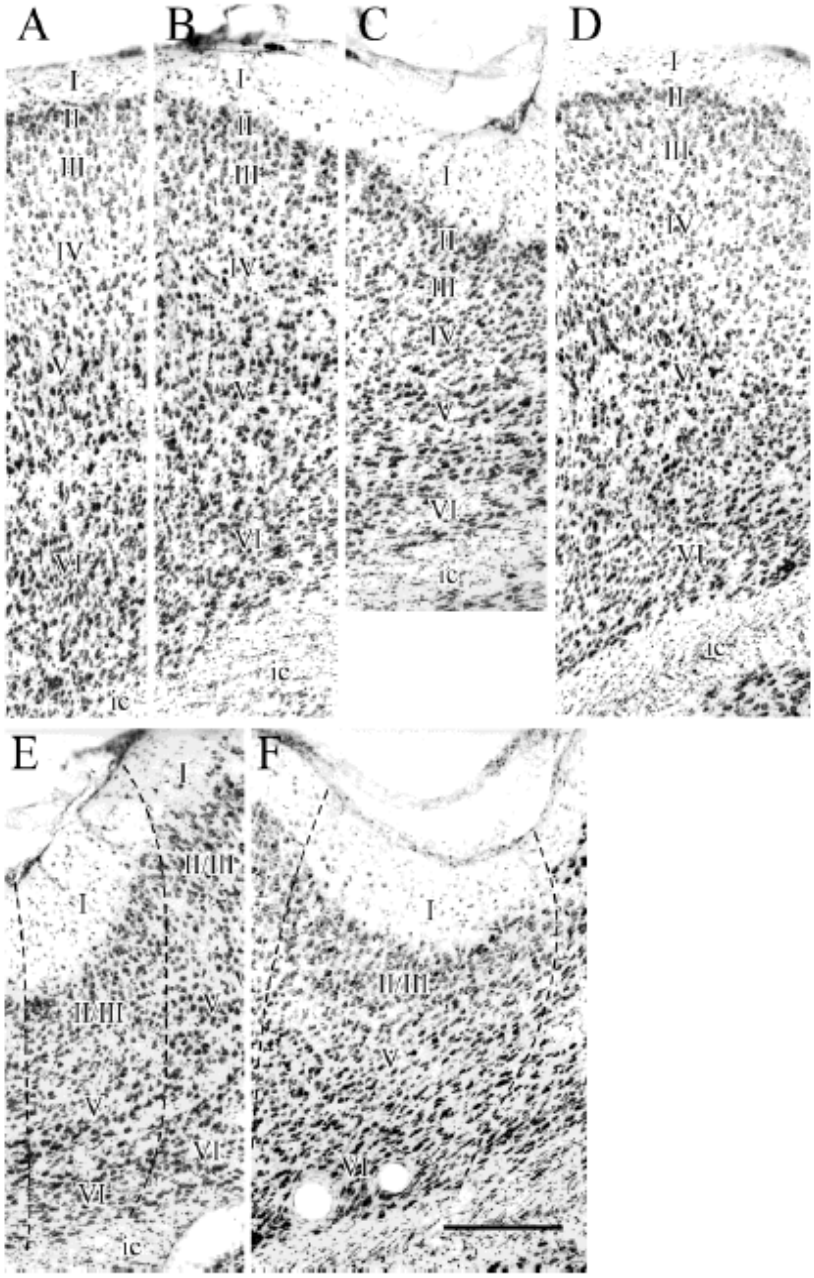

Figure 1.1.2 Photomicrographs of Nissl stained sections of area TE and perirhinal cortex. Sections from cortical layers of areas (A) TEv, (B) 36d, (C) 36v, (D) 36p, (E) 35d and (F) 35v are shown. The relative size of cortical layers are illustrated in the different sections, with a relatively smaller 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).

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

cortex. 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.

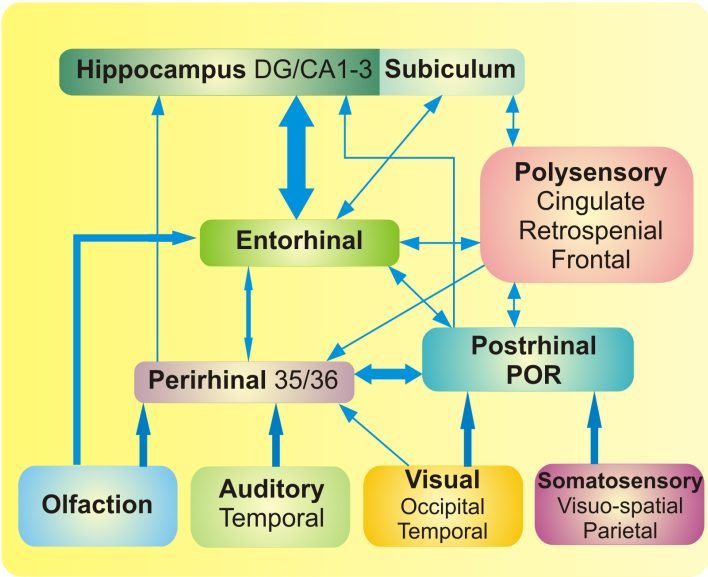

Figure 1.1.3 Schematic diagram illustrating the connectivity of the rat perirhinal cortex. The diagram shows parallel routes by which sensory information reaches the perirhinal cortex and from there the hippocampus. The thickness of the lines indicates the relative size of the projections. Modified from 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 Ca^{2+} 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).

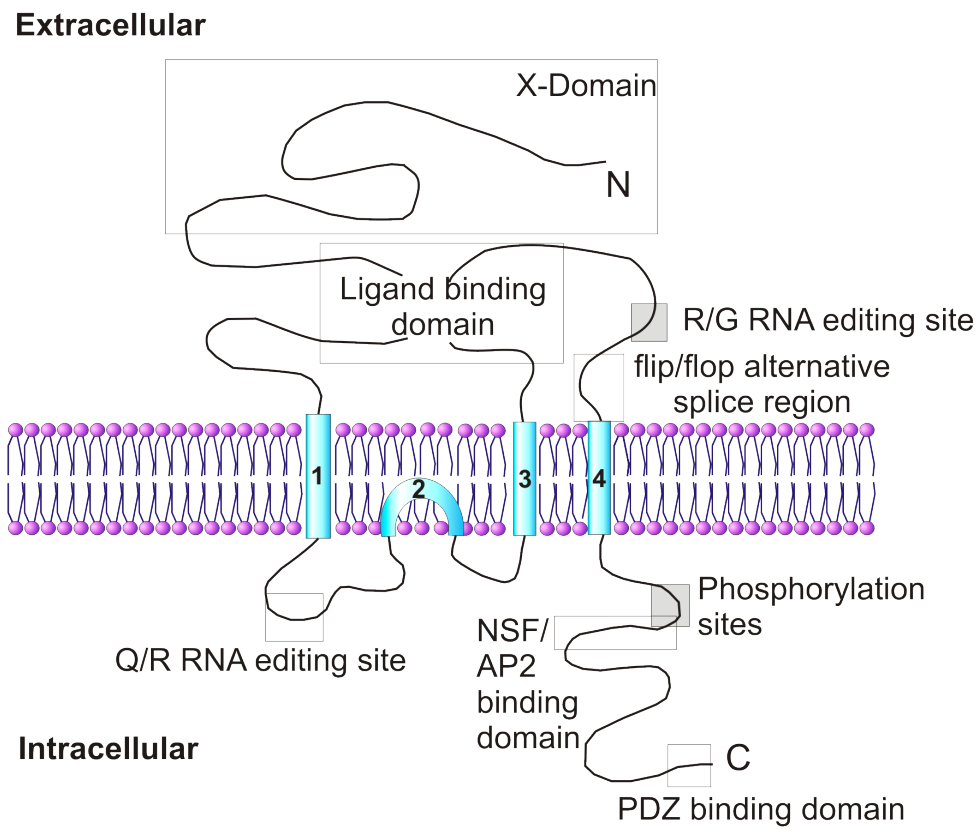

Figure 1.3.1. The topology of AMPAR subunit. Each subunit consists of an extracellular N-terminal domain, four hydrophobic regions (TM1–4), and an intracellular C-terminal domain. The ligand-binding site is a conserved amino acid pocket formed from a conformational association between the N terminus 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, amyotropic 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 membranem 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^{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

(Skeberdis, Chevaleyre et al. 2006). 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, 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).

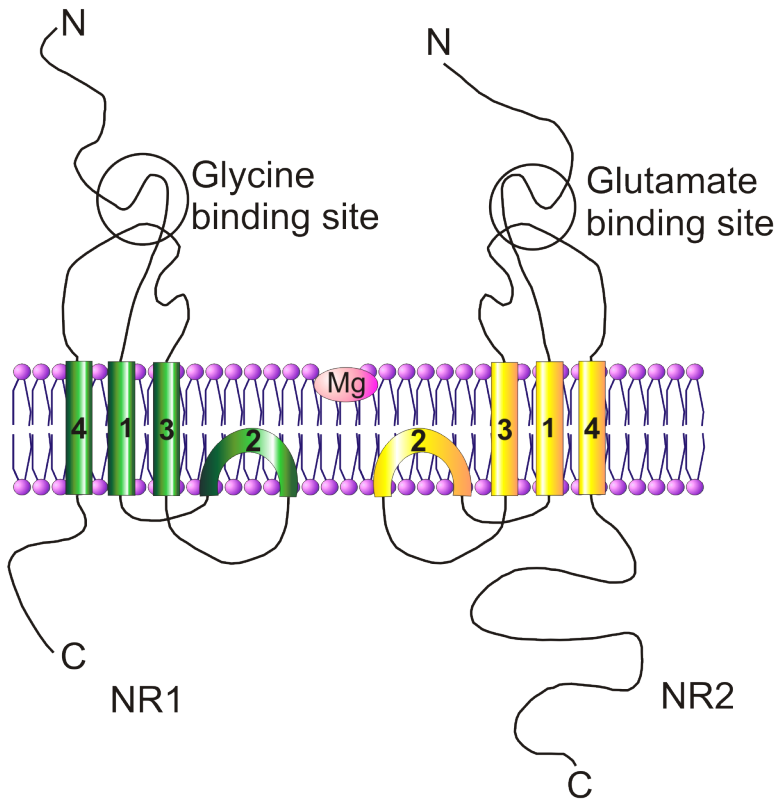

Figure 1.3.2 Schematic representation of the subunit transmembrane topography of NMDARs. A pair of NR1 and NR2 NMDAR subunits is shown to illustrate the transmembrane topography of these subunits. Their arrangement also shows the magnesium block of the pore-forming region made by the M2 regions in fully assembled NMDAR. Modified from Stephenson (2001).

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 excitotoxity, 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,

1997; Pin et al., 2003). 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).

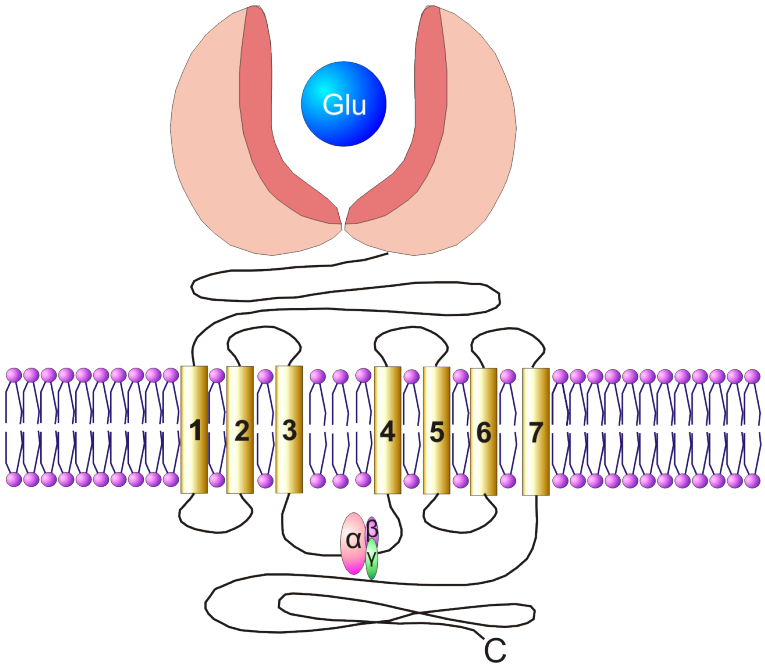

Figure 1.3.3 Schematic representation of the mGluR subunit structure. As is characteristic of metabotropic receptors, mGluRs have seven transmembrane domains. The intracellular loop between transmembrane regions III and IV is important for coupling to G-proteins. Modified from Conn and Pin (1997).

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 plasticites 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 (PKMξ) and phosphatidyl inositol 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 PKMξ 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 *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 *in vitro* and *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).

1.4.2.2 NMDAR-dependent LTD

1.4.2.2.1 Induction of LTD

NMDAR-dependent homosynaptic *de novo* LTD was first demonstrated in the CA1 region of the hippocampus, where *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^{2+} levels (Dudek and Bear, 1992; Mulkey and Malenka, 1992). As in the induction of LTP, Ca^{2+} enters through NMDARs when the neurone is depolarised and the Mg^{2+} block is relieved. The quantitative characteristics of the postsynaptic Ca^{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^{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 Ca^{2+} is crucial for the induction of LTD, and if the elevation is brief, at an equivalent Ca^{2+} concentration LTD will not be induced. In addition to Ca^{2+} entering through NMDARs, a role for Ca^{2+} 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 Ca^{2+} entry through L-type Ca^{2+} 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).

1.4.2.2.2 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^{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 ι\λ) (Nishizuka 1988; Nishizuka 1988). The conventional PKCs (α, βI, βII, and γ) are activated by both the lipid second messenger diacyl-glycerol (DAG) and Ca^{2+}. Novel PKCs (δ, θ, η, and ε) are activated by DAG, but not by Ca^{2+}. Atypical PKCs (ζ and ι\λ) 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**).

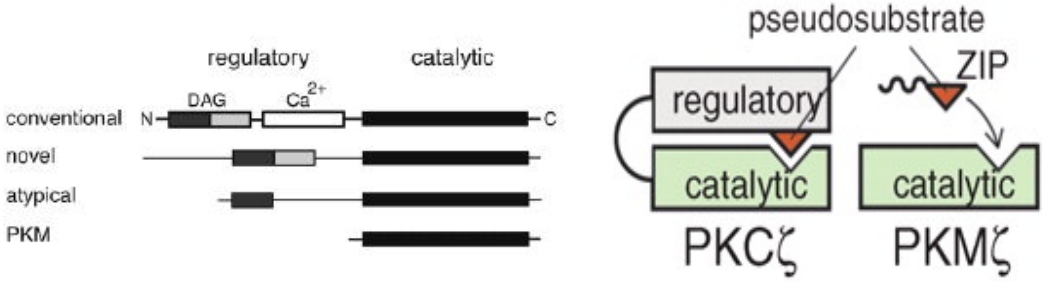

Figure 1.5.1: Left: a diagram representing the structure of PKC isoforms belonging to the different families. PKMζ possess only the independent catalytic domain of PKCζ, therefore being constitutively active (picture modified from Naik et. Al, 2000). Right: the diagram shows the site of action of the 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 an 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 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).

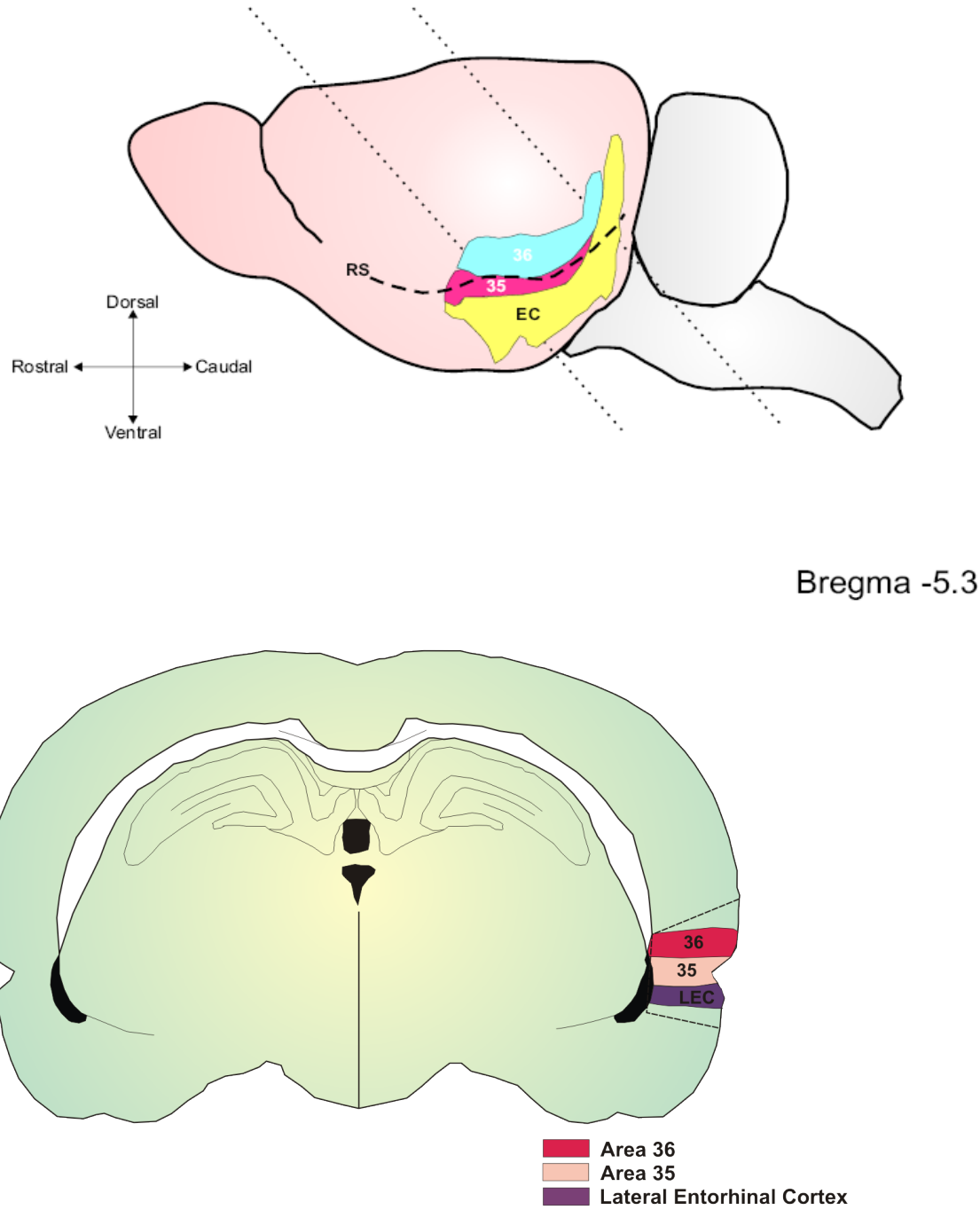

Figure 2.1 Location of the perirhinal cortex and adjacent cortices in the rat. A lateral view of the brain illustrates the perirhinal cortex, composed of Brodmann areas 35 and 36 along the rhinal sulcus (top). Dotted lines indicate the position at which scalpel cuts were made at 40° to the dorso-ventral 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%O_2/ 5%CO_2) 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

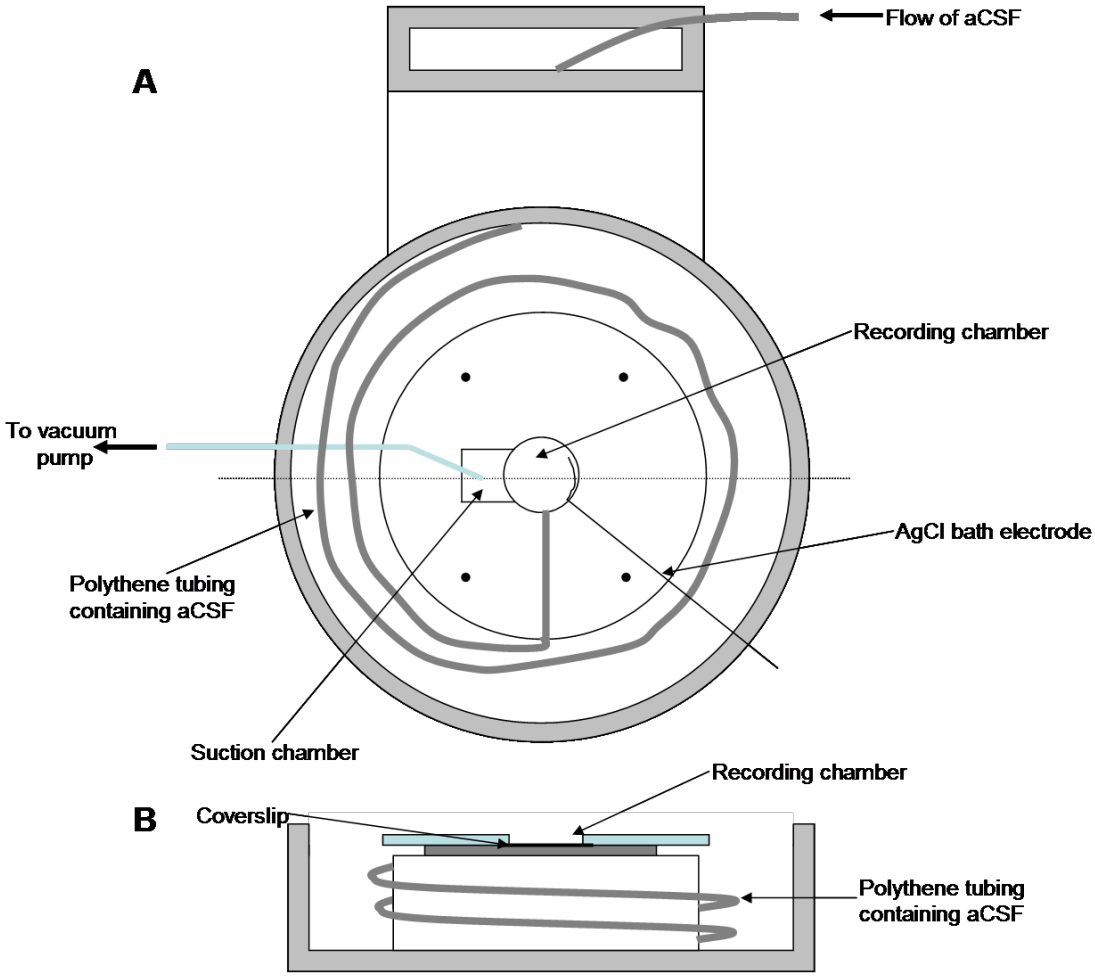

Figure 2.2 Diagram of the slice recording chamber. A) Plan view of the recording chamber, A polythene tube takes oxygenated aCSF to the recording chamber and once passed over the slice, it is removed by suction. B) Side view of the recording chamber, illustrating the coverslip sandwiched between the 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 $CaCl_2$ and 1mM $MgSO_4$.

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.

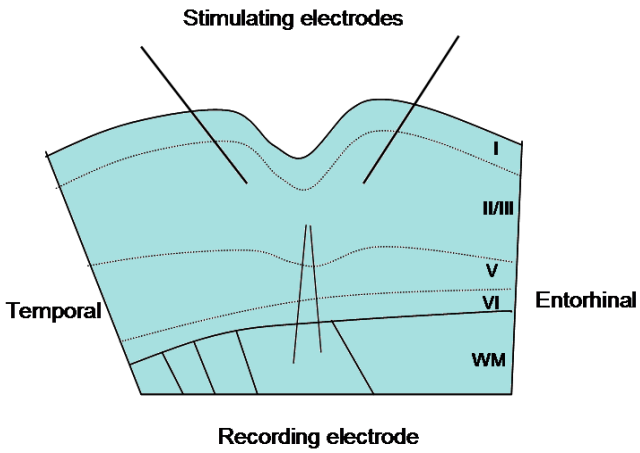

Figure 2.3 Illustration of the position of stimulating and recording electrodes within layers II/III of the perirhinal cortex. The recording electrode was positioned directly beneath the rhinal sulcus and stimulating electrodes were positioned either side of the recording electrode.

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 ddH_20, 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-morp holinyl)pyrido[2,3-d]pyrimidin-7-yl]-2-methoxybenzeneme thanol*, 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 PKMζ 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 nduce 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). Anysomicin 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 (NSAIDs or NAIDs), especially the specific inhibitors of ciclooxygenase-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 ± 8% of baseline, $p<0.01$ v baseline, n=6) is reversed following a long application of 3-HHA (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),

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.

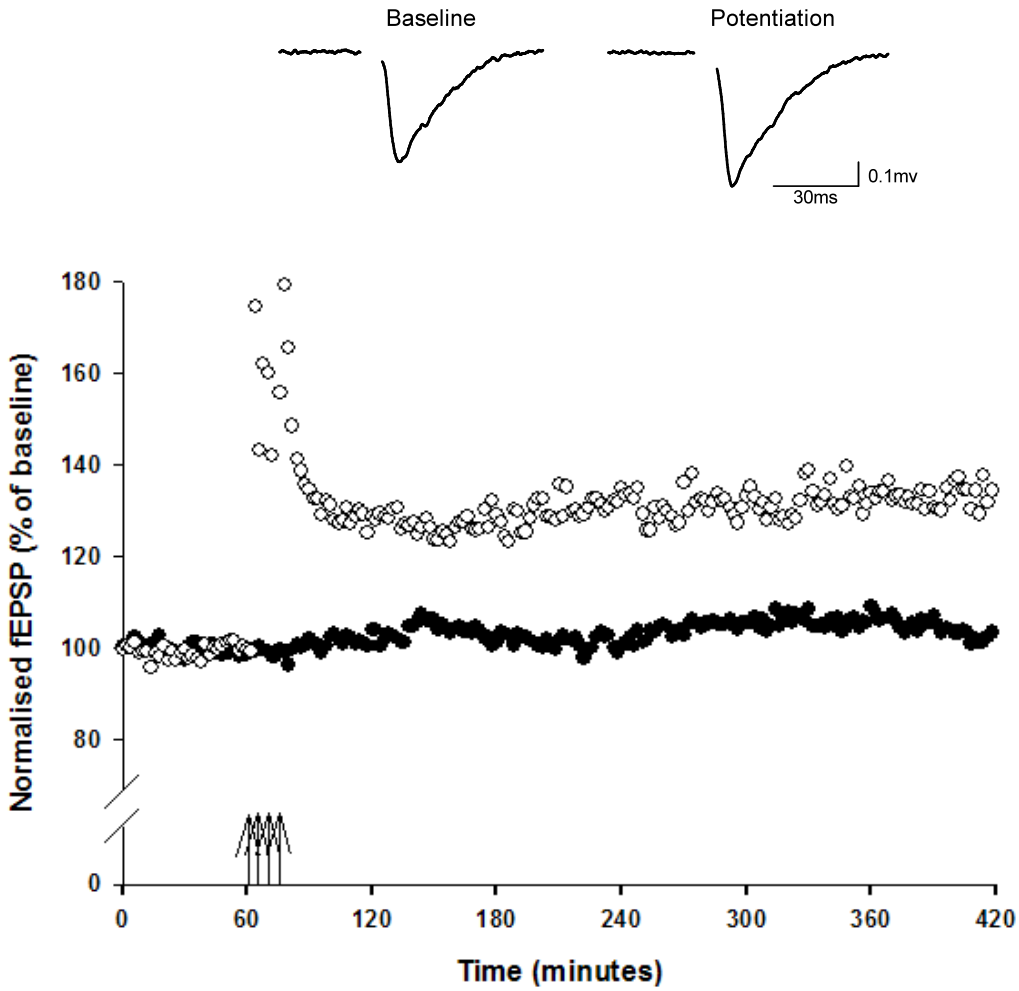

Figure 3.1 Single example of LTP in adult perirhinal cortex. P0 pathway (filled circles) was used as a control. A "spaced" tetra-burst stimulation (4x100Hz, 5 minutes interburst interval) produces a robust, stable LTP (open circles), which lasts for 6 hours after its induction. Upwards pointing arrows represent the moment when the stimulus was delivered. Traces are representative of the potentiation obtained.

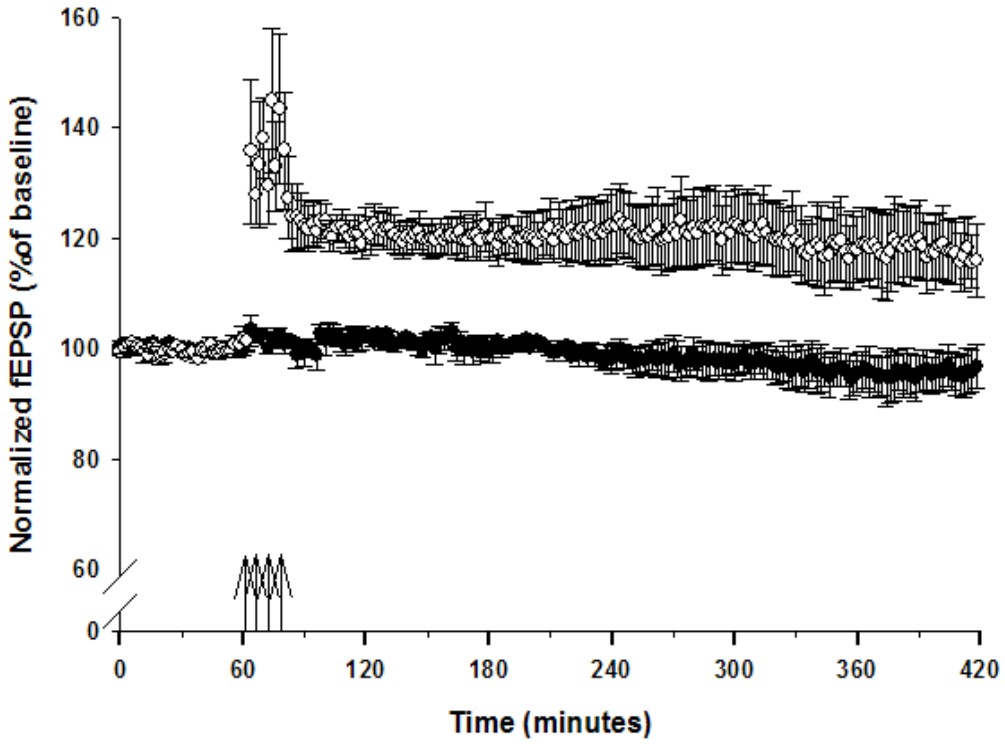

Figure 3.2 Pooled data for LTP in adult perirhinal cortex. The spaced tetra bust stimulation (4x100Hz) induces a stable and significant LTP (open circles, 126 ± 4% of baseline, $p<0.05$, n=4). The potentiation lasts for more than 5 hours.

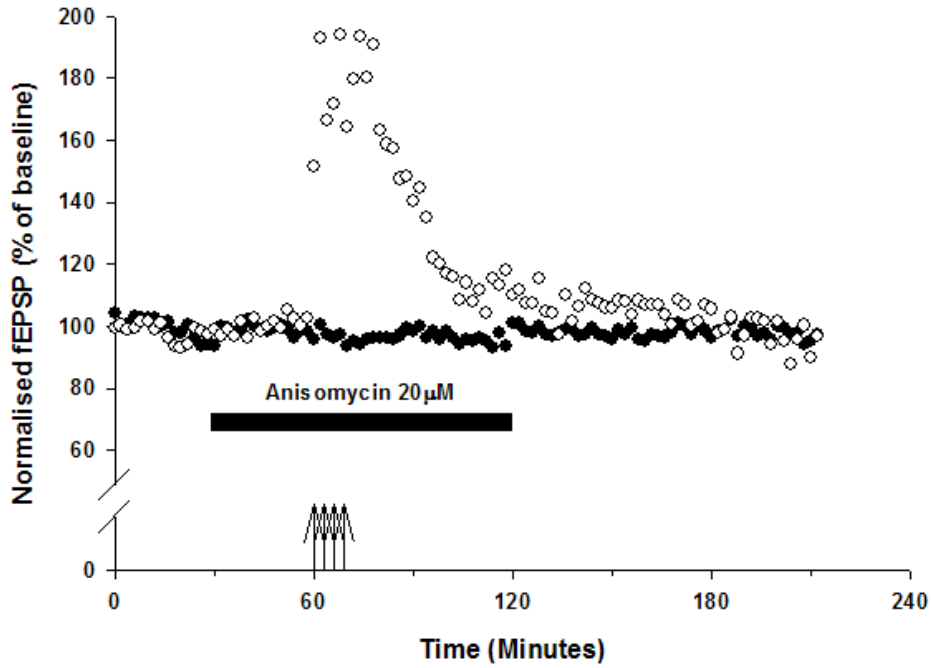

Figure 3.3 Single experiment for anisomycin on LTP in adult perirhinal cortex. The pre-incubation with Anisomycin 20μM is able to block the maintenance of LTP induced by a tetra-burst stimulation in P1 pathway (open circles). Upwards pointing arrows represent the moment when HFS was delivered. Anisomycin shows no effect on the control pathway P0 (filled circle).

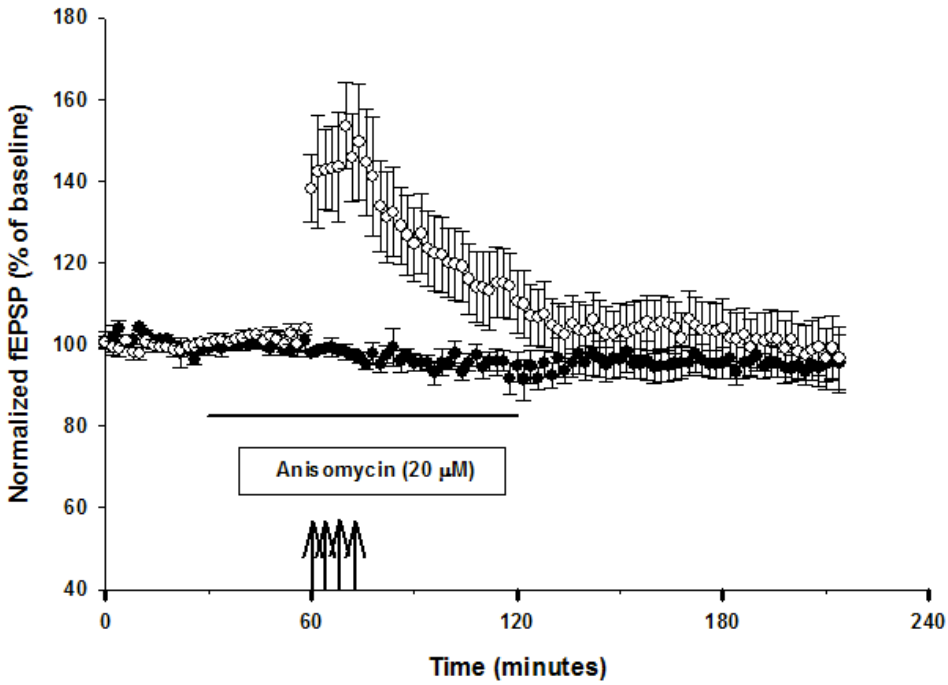

Figure 3.4 Pooled data for anisomycin on LTP in adult perirhinal cortex The pre-incubation with Anisomycin 20μM is able to block the maintenance of LTP (open circles 102 ± 5% of baseline, p>0.05 v baseline, n=5). Upwards pointing arrows represent the moment when HFS was delivered.

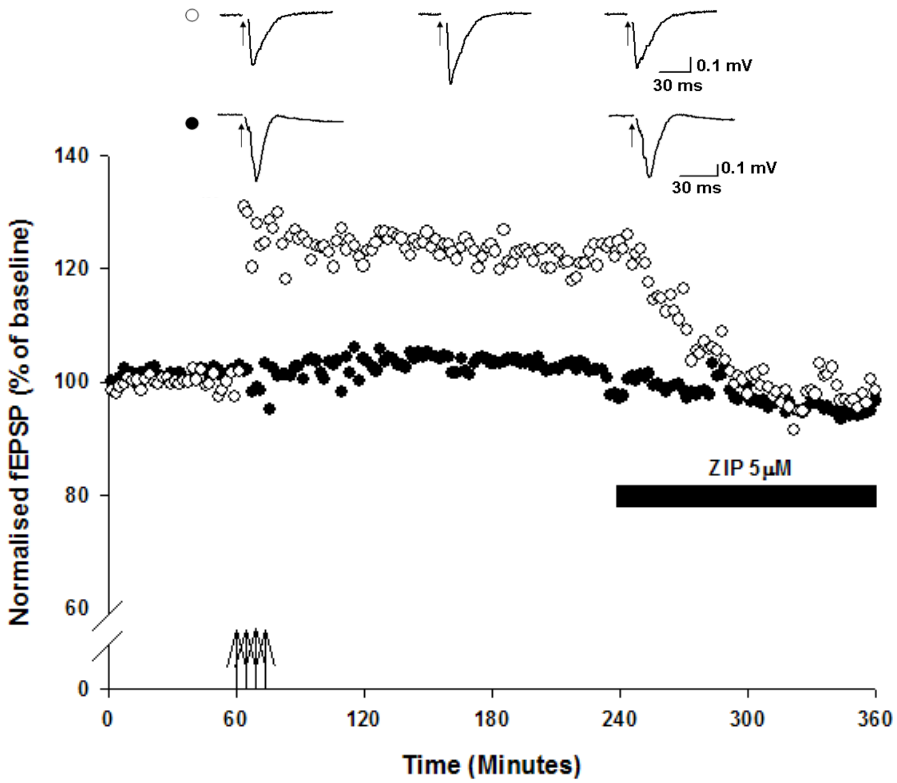

Figure 3.5 Single experiment for inhibition of PKMζ on LTP in adult perirhinal cortex The application of PKMζ inhibitor ZIP 5μM reverts the established LTP induced by a tetra-burst stimulation in P1 pathway (open circles). Upwards pointing arrows represent the moment when HFS was delivered. ZIP shows a small effect on the control pathway P0 (filled circles).

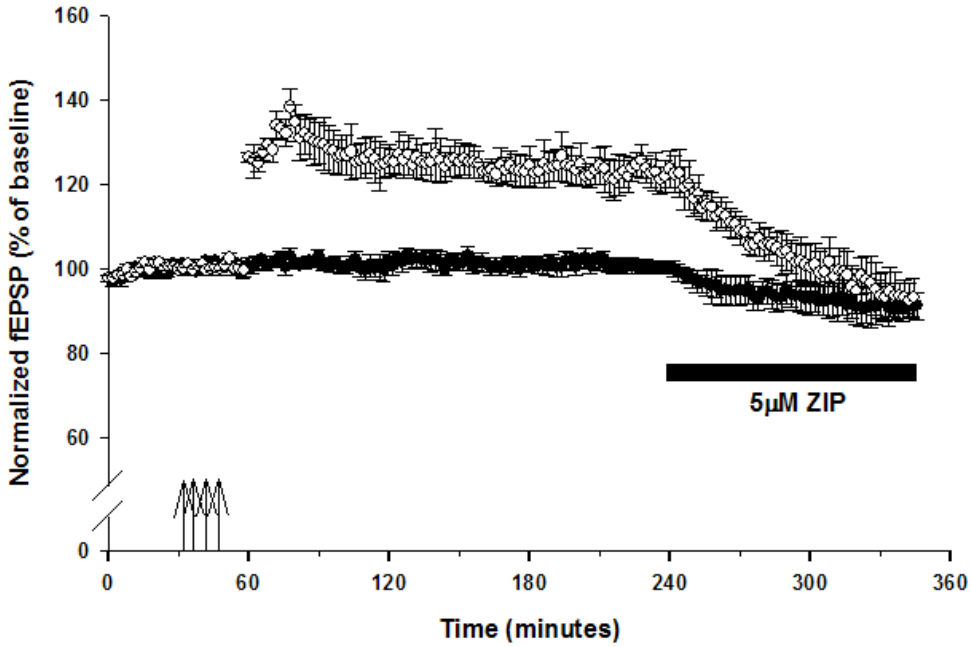

Figure 3.6 Pooled data for inhibition of PKMζ on LTP in adult perirhinal cortex ZIP 5μM completely reverts the established LTP (open circles, 98 ± 5 % of baseline, $p<0.01$, n=7) with no significant effect on the control input (filled circles, 93 ± 4 % of baseline, $p>0.05$).

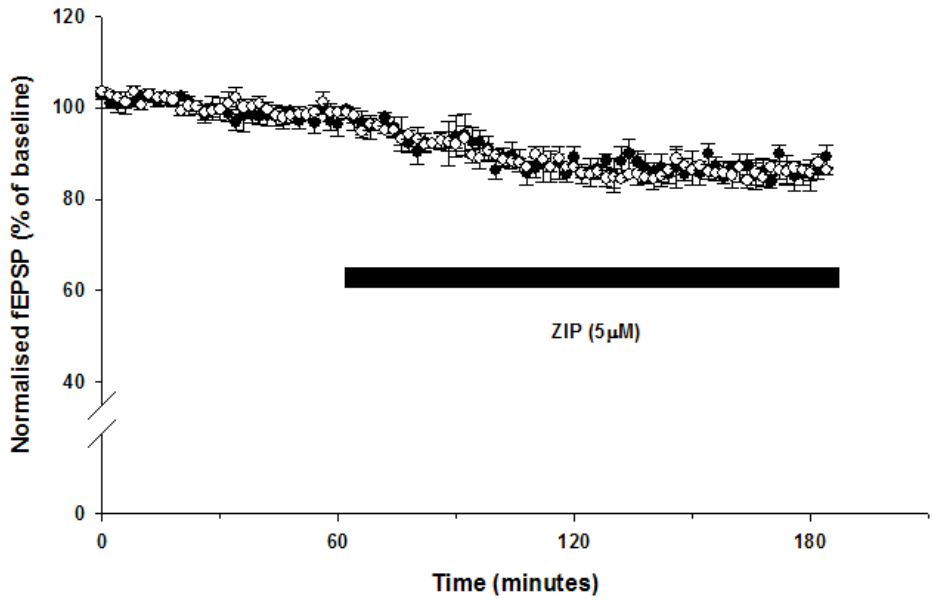

Figure 3.7 Pooled data for inhibition of PKMζ on baseline in adult perirhinal cortex ZIP 5μM produces a small, non-significant decrease on the baseline when bath-applied on adult perirhinal cortex. Temporal pathway (filled circles): 89 ± 2% of baseline, p>0.05. Entorhinal pathway (open circles): 88 ± 2% of baseline, p>0.05, n=4).

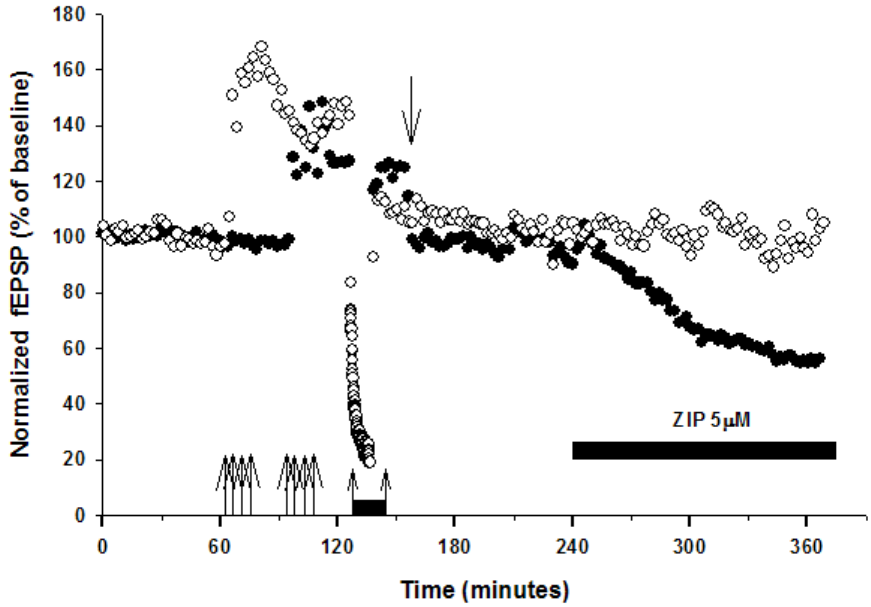

Figure 3.8 Single experiment of ZIP on depotentiation in adult perirhinal cortex ZIP 5μM shows no effect on a depotentiated pathway (P0, LTP followed by LFS protocol, filled circles). The inhibition of PKMζ produces a depression of the synaptic response in a potentiated pathway where the magnitude of 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.

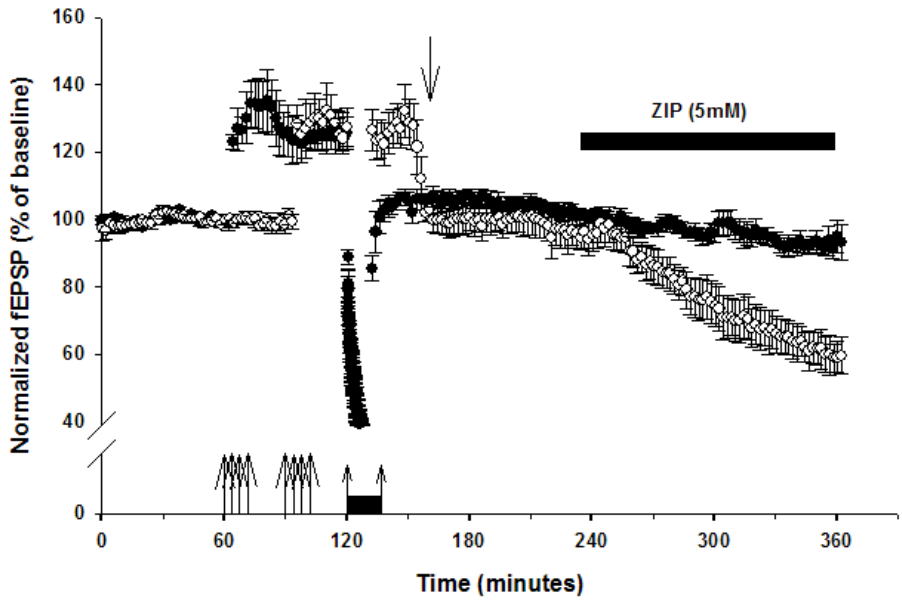

Figure 3.9 Pooled data for inhibition of PKMζ on baseline in adult perirhinal cortex ZIP 5μM shows no effect on a depotentiated pathway (filled circles:98 ± 4% of baseline, $p>0.05$, $n=5$). On the other input (open circles), after the induction of LTP, the amplitude of the response is decreased by a 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).

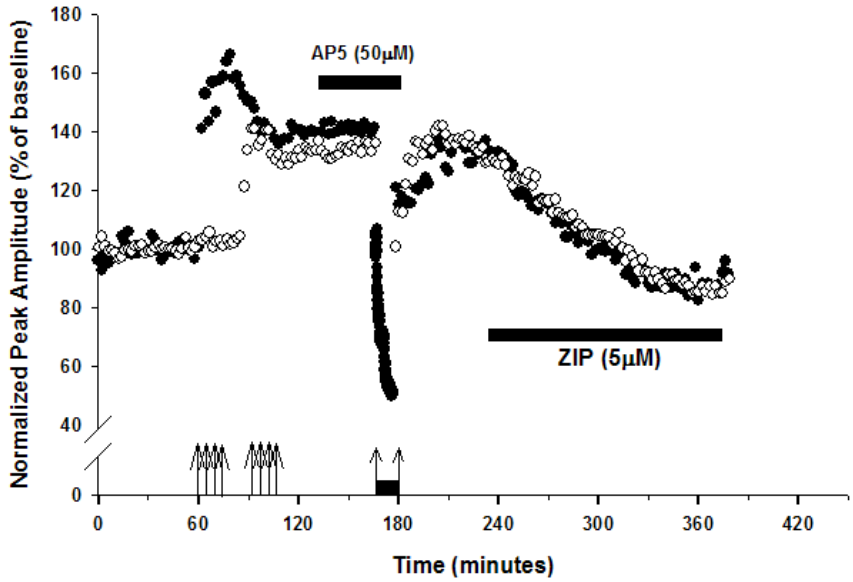

Figure 3.10 Single experiment of effect of inhibition of PKMζ on depotentiation in presence of AP5 in adult perirhinal cortex If the depotentiation is blocked by bath-application of AP5 (filled circles), ZIP produces a decrease in the synaptic response similar to the one that occurs in a potentiated pathway (open circles).

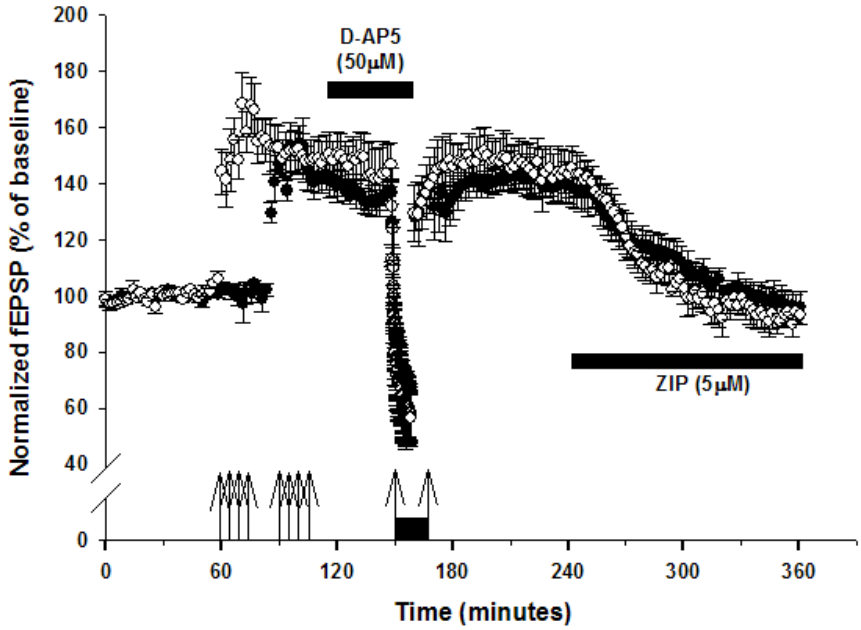

Figure 3.11 Pooled data for effect of inhibition of PKMζ on depotentiation in presence of AP5 in adult perirhinal cortex If the depotentiation is blocked by bath-application of AP5 (filled circles), ZIP is able to decrease the response, bringing it back to the baseline (102 ± 2% of baseline, $p<0.001$ 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).

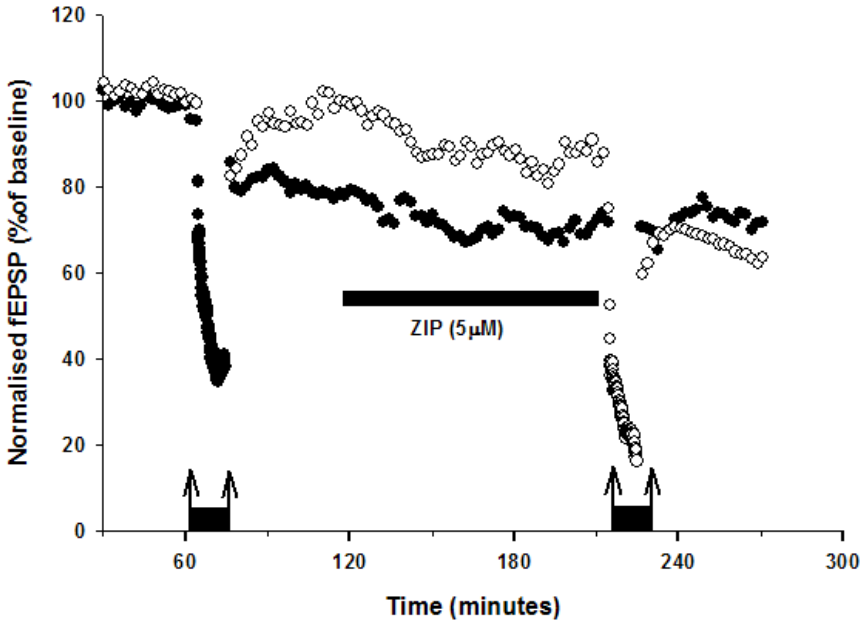

Figure 3.12 Single experiment of effect of inhibition of PKMζ on LTD induction in adult perirhinal cortex ZIP shows no effect on a depotentiated pathway (filled circle). Pre-incubation with ZIP does not affect the induction of LTD (open corcles).

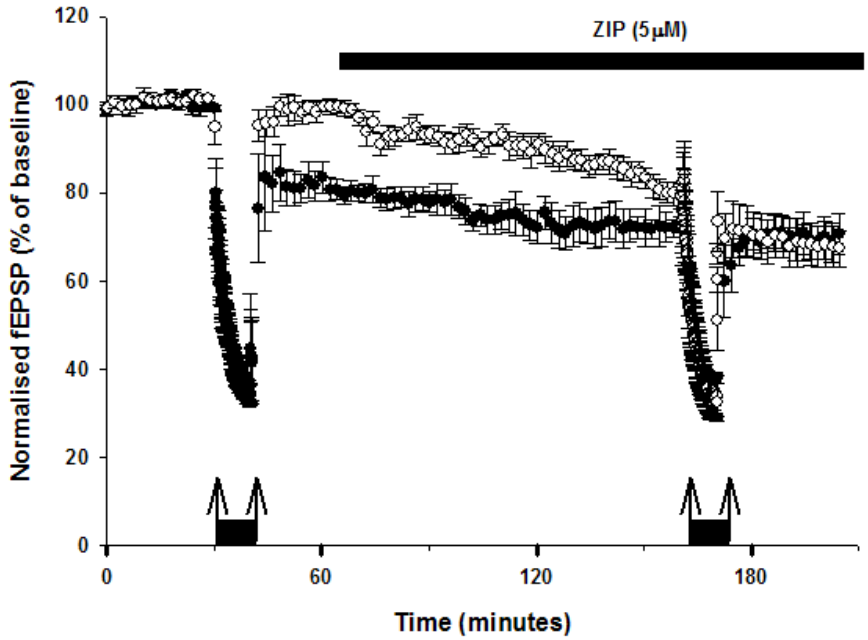

Figure 3.13 Pooled data for the effect of PKMζ inhibition on LTD induction in adult perirhinal cortex Inhibition of PKMζ has no effect on the induction of LTD in perirhinal cortex (Open circles 69 ± 4% of baseline, $p<0.01$ v baseline, n=5). There's no difference in the magnitude of the depression obtained 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).

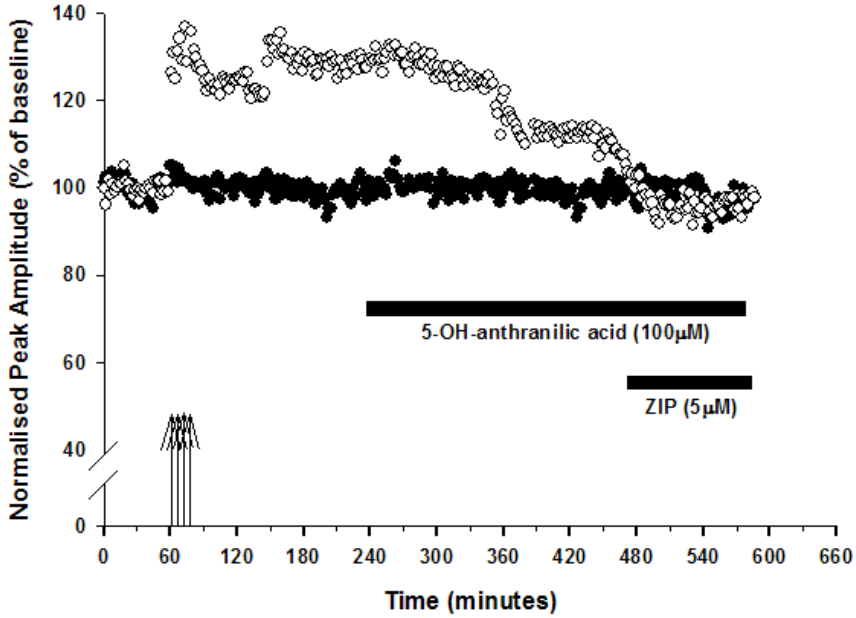

Figure 3.14 Single experiment of effect of PDK1 inhibition on LTP in adult perirhinal cortex The inhibition of PDK1 slowly but completely reverts an established LTP (open circles). Subsequent application of ZIP does not show any effect after the depotentiation is complete.

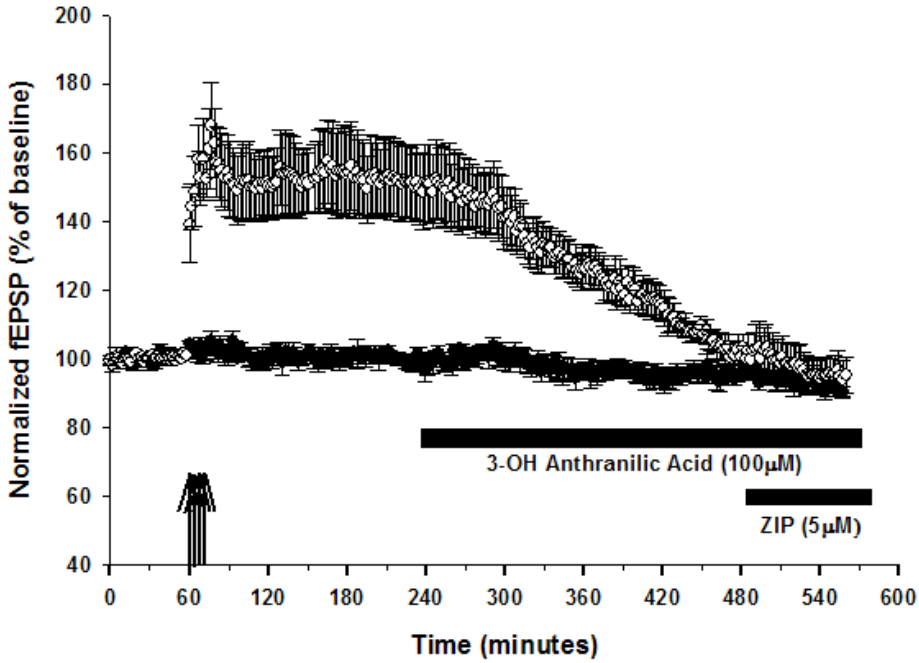

Figure 3.15 Pooled data for effect of PDK1 inhibition on LTP in adult perirhinal cortex The inhibition of PDK1 completely reverts the LTP (open circles, 104 ± 3% of baseline, $p<0.01$ v LTP, $p>0.05$ v baseline, n=6). The subsequent application of ZIP shows no more decrease of the synaptic transmission (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), CaM Kinase, 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, Tillett 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 synaptc 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 $\pm$ 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 $\pm$ 2% of baseline, $p < 0.001$, $n = 6$). However, ZIP had no effect on baseline transmission (97 $\pm$ 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.

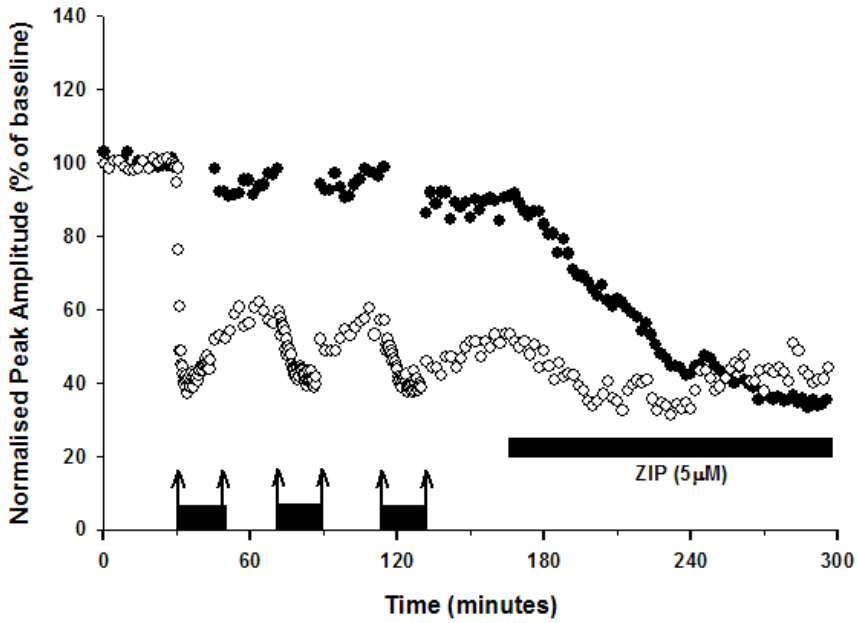

Figure 4.1 Single experiment of effect of PKMζ inhibition on P14 perirhinal cortex The application of ZIP produces a strong decrease in the basal synaptic transmission (filled circles). ZIP shows no effect on a pathway where a saturated LTD was previously obtained (open circles)

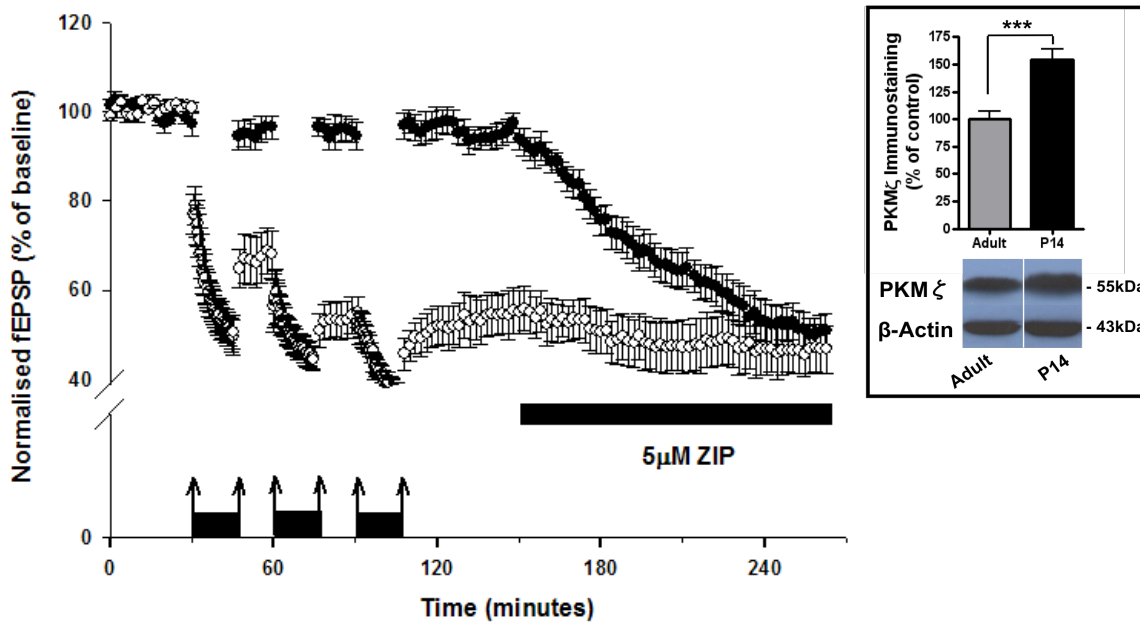

Figure 4.2 Pooled data for effect of PKMζ inhibition on P14 perirhinal cortex The inhibition of PKMζ produces a significant decrease in the synaptic response (filled circles 53 ± 4% of baseline, p<0.001 v baseline; n=7). When a saturated depression was obtained (open circles 54 ± 4 % of baseline, p<0.001) 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 PKMζ 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.

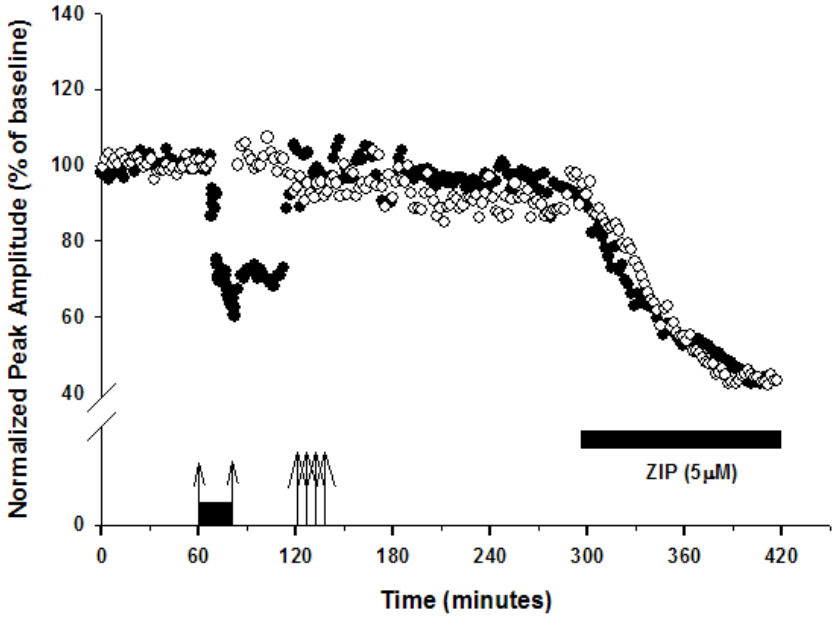

Figure 4.3 Single experiment of the effect of PKMζ inhibition on de-depression in P14 perirhinal cortex The administration of LFS followed by HFS protocol produces a de-depression in P14 perirhinal cortex, which brings the response back to the baseline (filled circles). The application of ZIP produces a decrease in the de-depressed pathway, similar to the one produced on the control pathway (open circles).

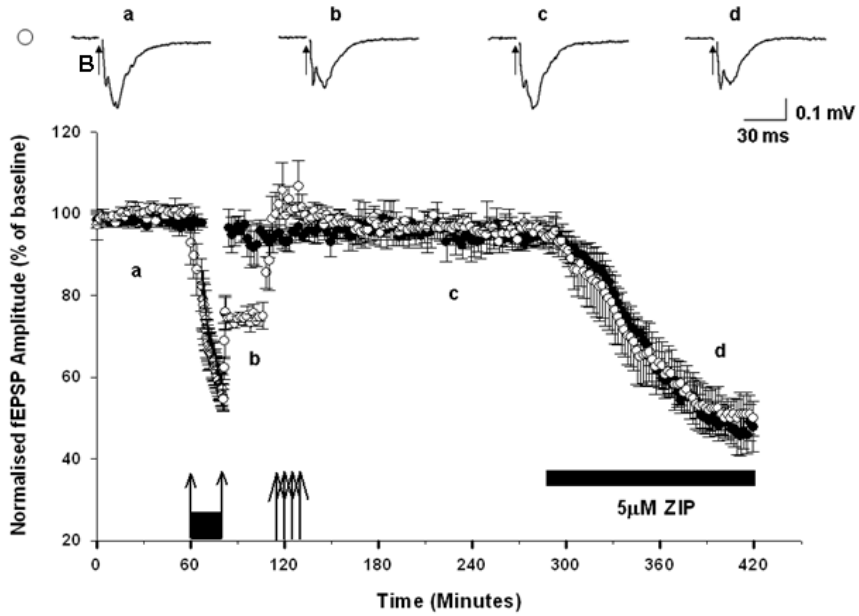

Figure 4.4 Pooled data for effect of PKMζ inhibition on de-depression inP14 perirhinal cortex LFS is able to induce a robust LTD in perirhinal cortex of P14 rats (open circles 74 ± 2 % of baseline, $p<0.001$, n=4). Thirty minutes following induction of LTD, delivery of HFS resulted in lasting potentiation (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, $p< 0.01$).

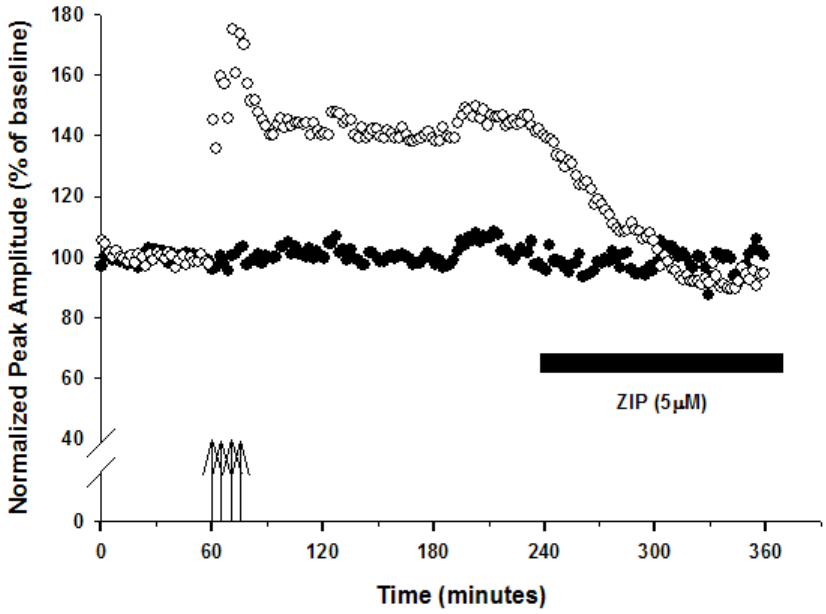

Figure 4.5 Single experiment of PKMζ inhibition LTP in P35 perirhinal cortex In P35 animals, HFS is able to promptly induce a robust and stable LTP (open circles), which is completely reversed by bath-application of ZIP**.**

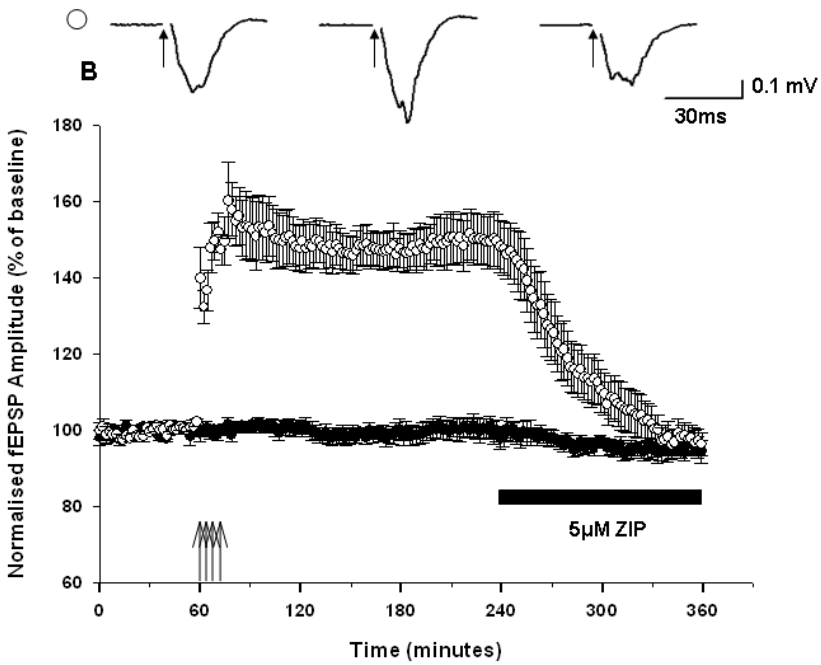

Figure 4.6 Pooled data for effect of PKMζ inhibition on LTP in P35 perirhinal cortex LTP was promptly induced in P35 perirhinal cortex (open circles 148 ± 5% of baseline, $p<0.001$, n=6) and the inhibition of PKMζ by bath application of 5μM ZIP completely reversed the established LTP (100 ± 2% of baseline, $p<0.001$). 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 PKMζ 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 PKMζ, 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 PKMζ.

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 PKMζ 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 PKMζ maintains LTP may involve AMPA receptor insertion into the synaptic membrane (Yao, Kelly et al. 2008). In addition, PKMζ 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.

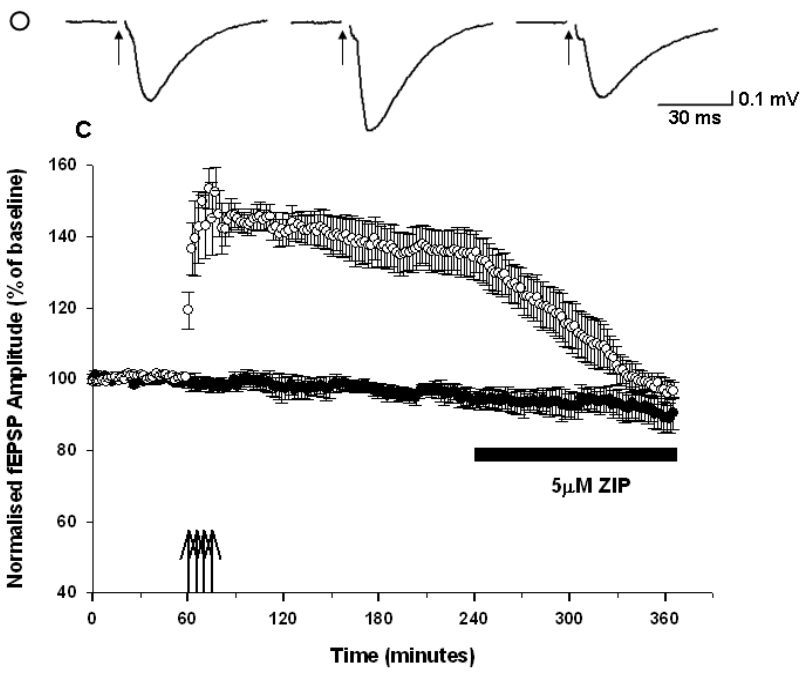

Figure 4.3.1 Pooled data for effect of PKMζ inhibition on LTP induced in P14 hippocampus (Peak Amplitude) LTP is easily induced in P14 hippocampus (open circles: 141 ± 5% of baseline; p<0.01; n = 4). The inhibition of PKMζ completely reverses the potentiation (100 ± 7% of baseline, p<0.01 v LTP) but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

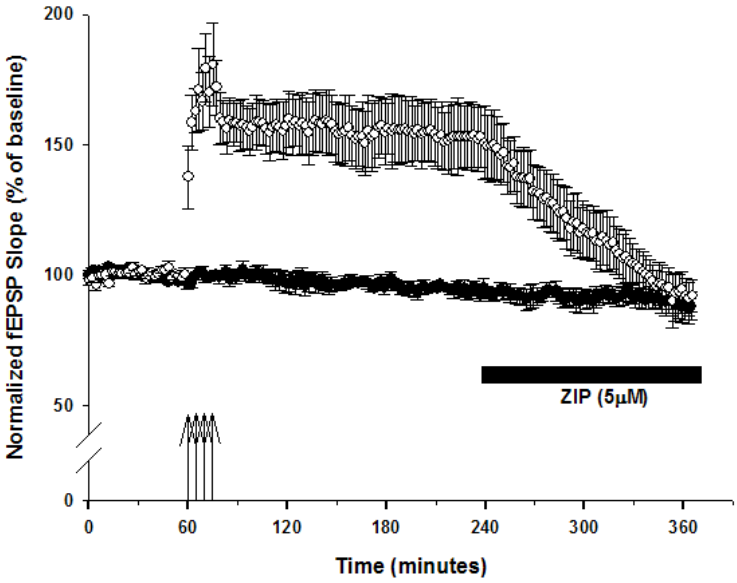

Figure 4.3.2 Pooled data for effect of PKMζ inhibition on LTP induced in P14 hippocampus (Slope) Measurements of variation of slope amplitude are consistent with variation in the peak amplitude. LTP is easily induced in P14 hippocampus (open circles: 157 ± 9% of baseline; p<0.01; n = 4). The inhibition 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 form 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 synthesys, 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.

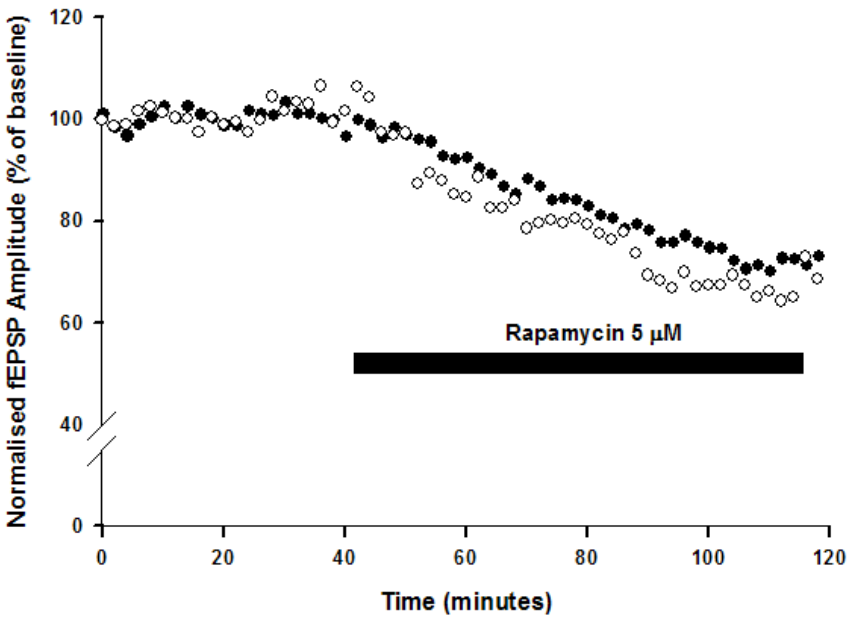

Figure 5.1.1 Single experiment on the effect of Rapamycin on basal synaptic transmission in P14 perirhinal cortex The application of the mTOR inhibitor rapamycin produces a substantial decrease in P14 baal synaptic transmission.

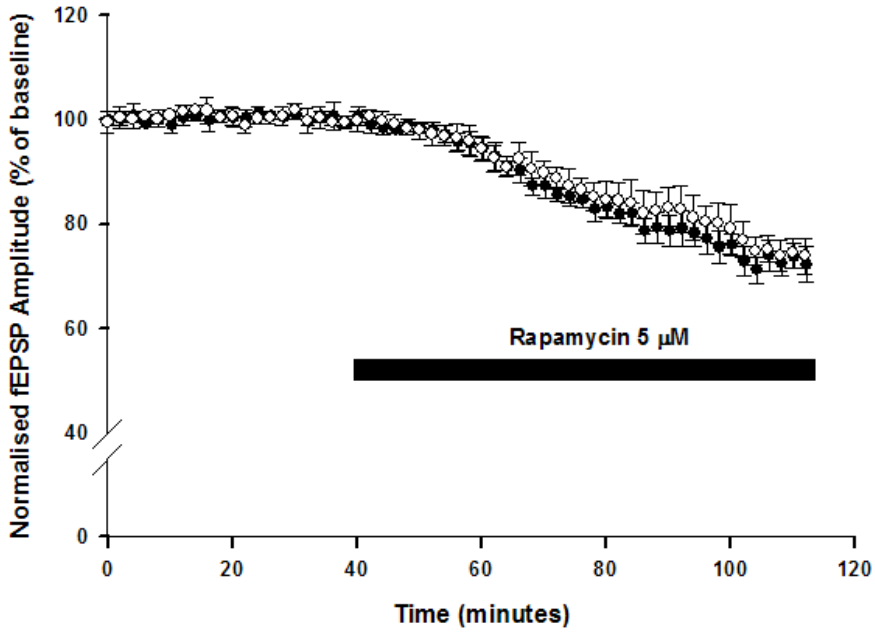

Figure 5.1.2 Pooled data for the effect of Rapamycin on basal synaptic transmission in P14 perirhinal cortex mTOR inhibition produced a substantial depression of basal transmission in P14 perirhinal cortex (temporal side, filled circles: 73 ± 3% of baseline, $p<0.001$; entorhinal side, open circles: 74 ± 3% of baseline, $p<0.001$, n=6).

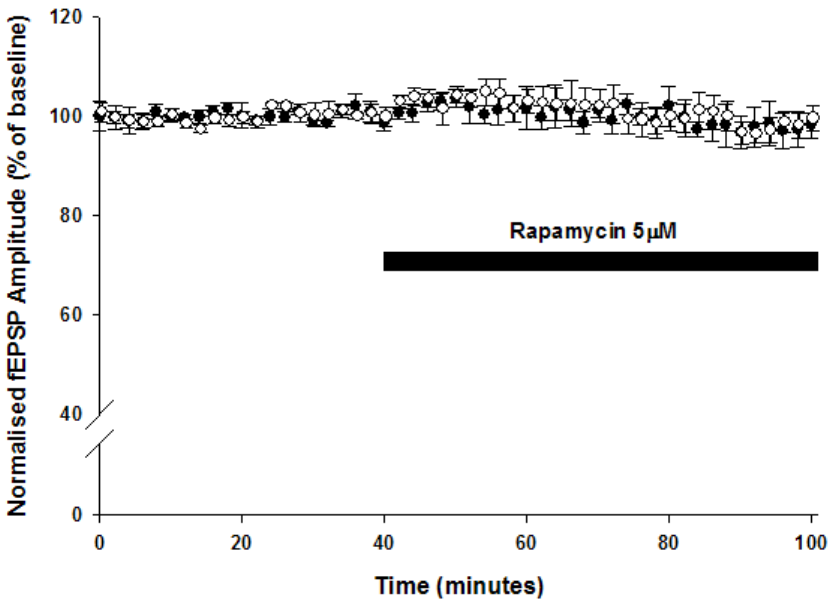

Figure 5.1.3 Pooled data for the effect of Rapamycin on basal synaptic transmission in adult perirhinal cortex No effect of mTOR inhibition by bath application of rapamycin was observed in perirhinal slices form adult animals (temporal side, filled circles: 98 ± 3% of baseline, p>0.05; entorhinal side, open circles: 100 ± 3% of baseline, p>0.05, n=4).

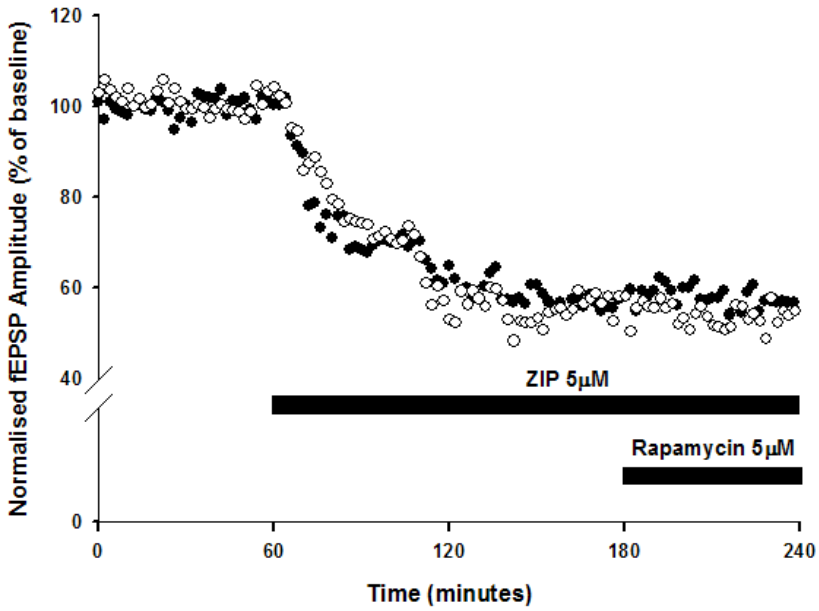

Figure 5.1.4 Single experiment on the effect of ZIP followed by Rapamycin on basal synaptic transmission in P14 perirhinal cortex The inhibition of PKMζ occludes any further depotentiation produced by Rapamycin.

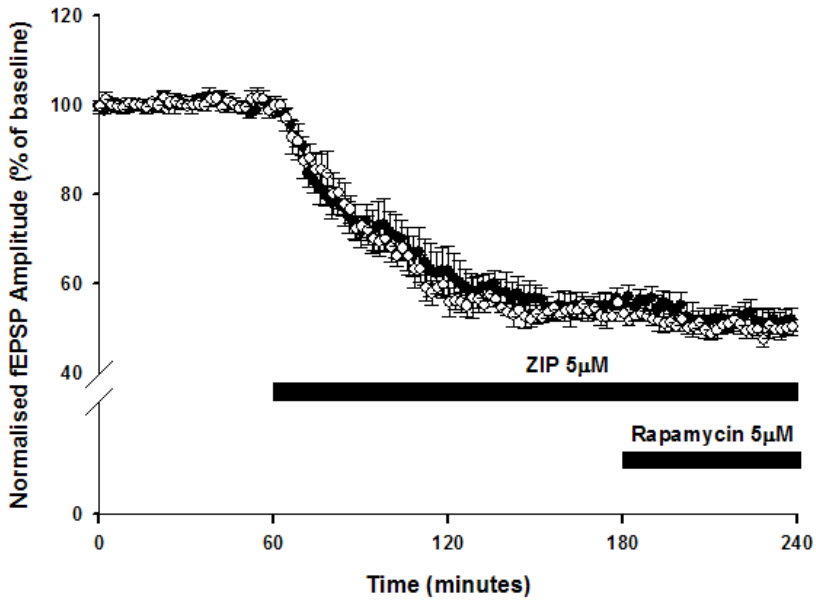

Figure 5.1.5 Pooled data for the effect of ZIP followed by Rapamycin on basal synaptic transmission in P14 perirhinal cortex Depression of transmission by inhibition of PKMζ in P14 perirhinal cortex occludes any subsequent depression of synaptic transmission by rapamycin (Temporal side, filled circles: 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)

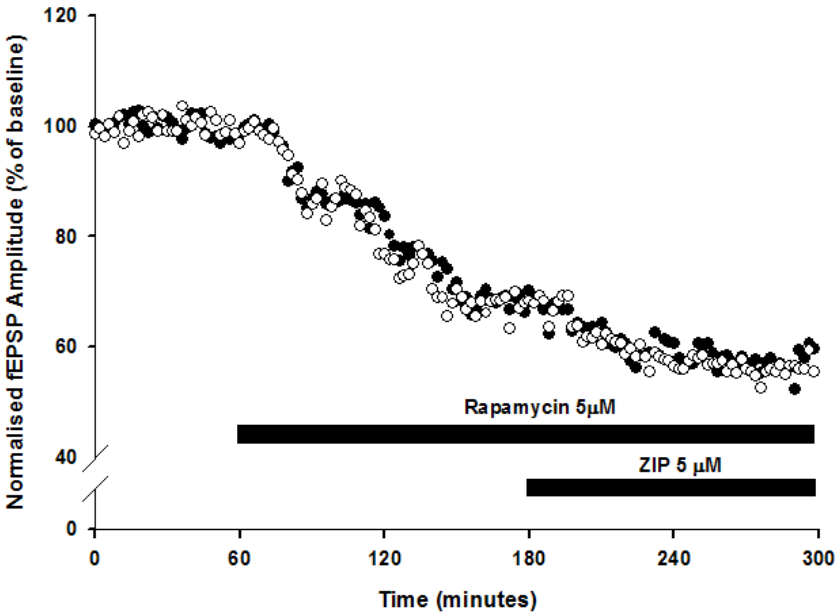

Figure 5.1.6 Single experiment of Rapamycin and ZIP on P14 perirhinal cortex Bath-application of the mTor inhibitor Rapamycin strongly decreases the baseline synaptic response in P14 perirhinal cortex. The subsequent inhibition of PLMζ produces a further small but significant decrease in the amplitude of the synaptic response.

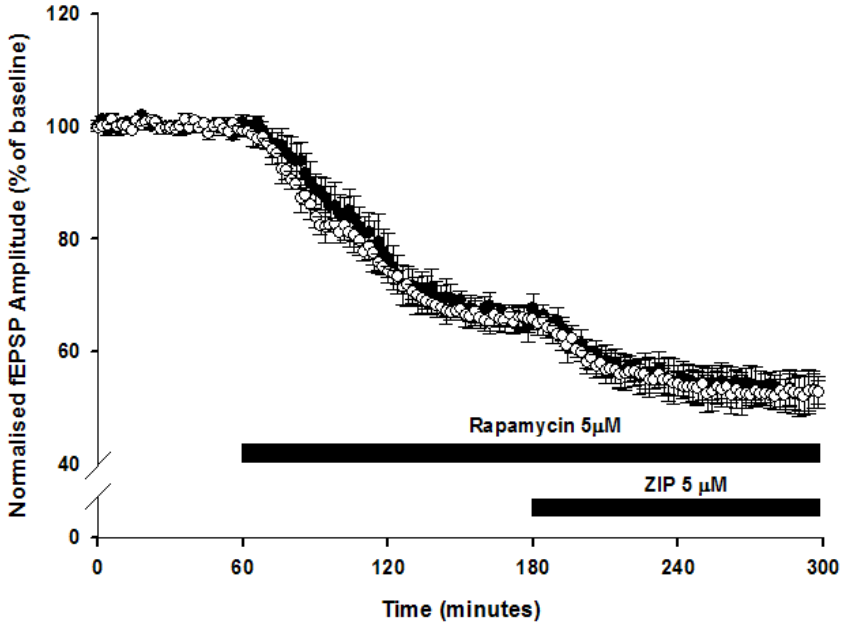

Figure 5.1.7 Pooled data for effect of Rapamycin and ZIP on P14 perirhinal cortex mTOR inhibition produced a substantial depression of basal synaptic transmission in P14 perirhinal cortex (Temporal side, filled circles: 67 ± 1% of baseline, $p<0.001$; Entorhinal side, open circles: 66 ± 2% of baseline, $p<0.001$, n=4) Subsequent inhibition of PKMζ 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).

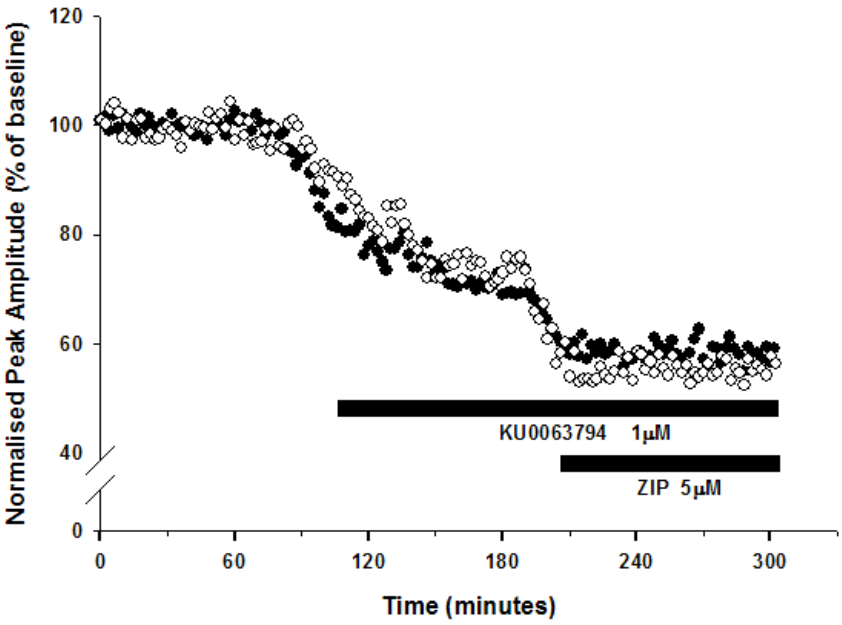

Figure 5.1.8 Single experiment on effect of KU0063794 and ZIP on P14 perirhinal cortex The inhibition of mTOR produces a wide depression in the synaptic transmission. ZIP shows no effect when bath applied afterwards.

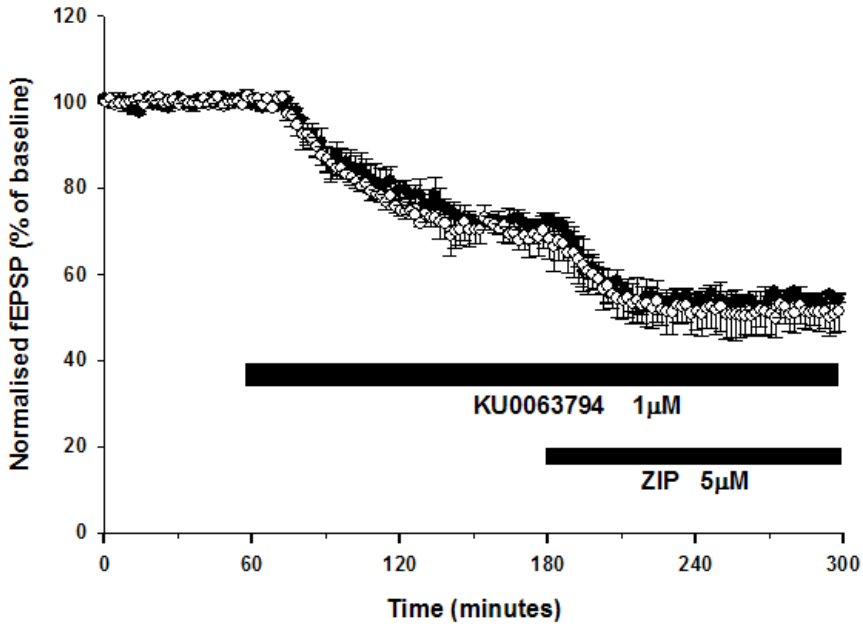

Figure 5.1.9 Pooled data for effect of KU0063794 and ZIP on P14 perirhinal cortex KU0063794 produces a significant decrease in the basal synaptic transmission, similar to the one produced by rapamycin (Temporal side, filled circles: 72 ± 2% of baseline, $p<0.01$; Entorhinal side, open circles 69 ± 2% 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).

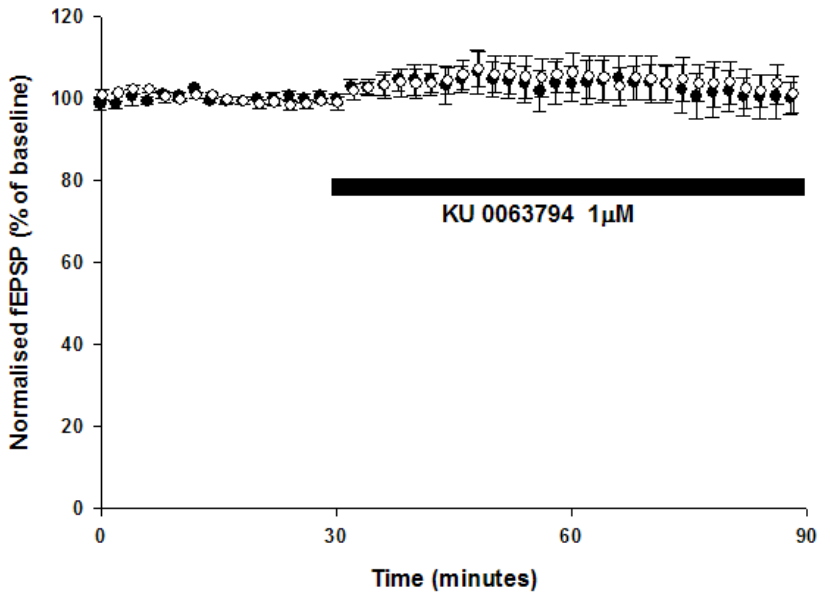

Figure 5.1.10 Pooled data for effect of KU0063794 adult perirhinal cortex No effect of mTOR inhibition by KU0063794 can be observed in adult perirhinal cortex (Temporal side, filled circles: 100 ± 4% of baseline, p>0.05; Entorhinal side, open circles 102 ± 4% of baseline, p>0.05, n=3).

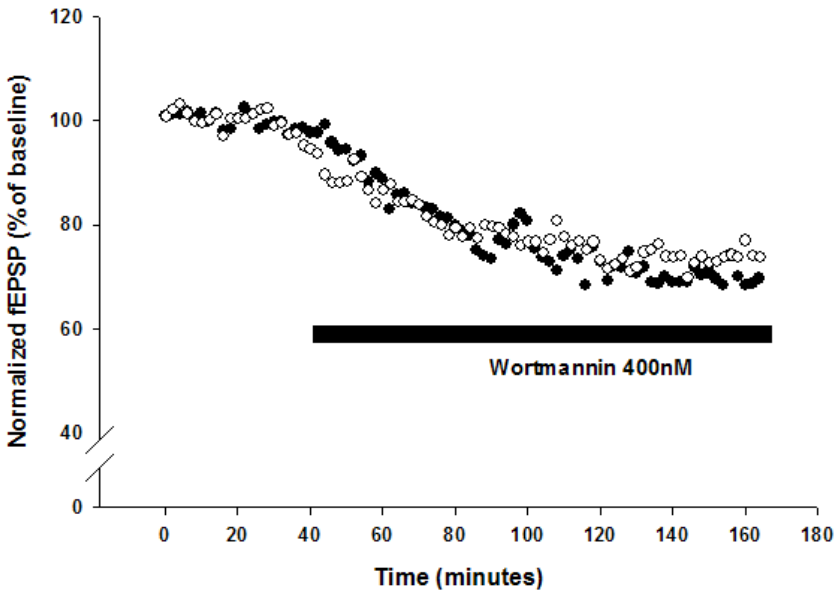

Figure 5.1.11 Single experiment on effect of Wortmannin on P14 perirhinal cortex Bath appliction of the PI3K inhibitor wortmannin produces a decrease in the basal synaptic response.

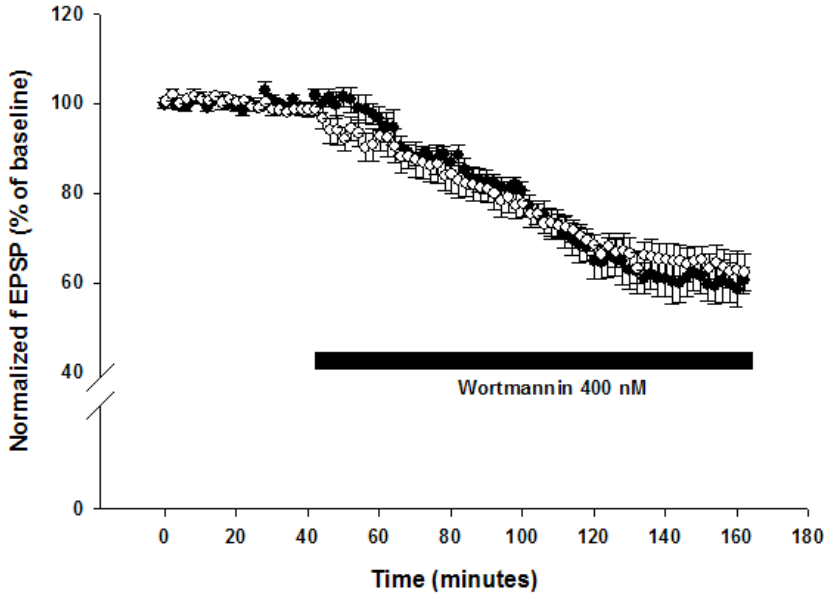

Figure 5.1.12 Pooled data for effect of Wortmannin on P14 perirhinal cortex Wortmannin is able to significantly decrease the synaptic transmission in P14 perirhinal cortex (Temporal side, filled circles : 61 $\pm$ 4% of baseline, $p<0.001$; Entorhinal side, open circles 64 $\pm$ 3% of baseline, $p<0.001$, $n=5$). No difference was found between the two pathways ($p>0.05$).

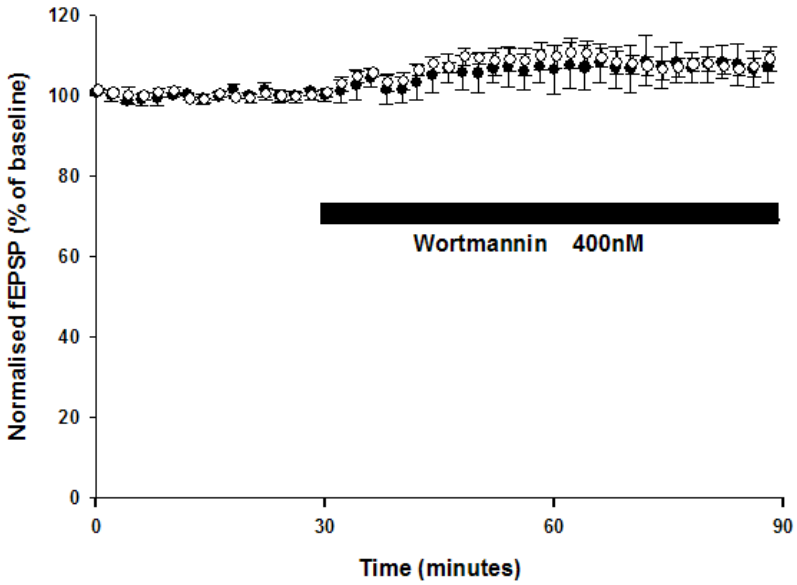

Figure 5.1.13 Pooled data for effect of Wortmannin on adult perirhinal cortex Inhibition of PI3K by bath application of Wortmannin does not show any effect on basal synaptic transmission in adult perirhinal cortex (Temporal side, filled circles: 107 ± 4% of baseline, p>0.05; Entorhinal side, open circles 107 ± 2% of baseline, p>0.05, n=3. No difference is found between the two pathways).

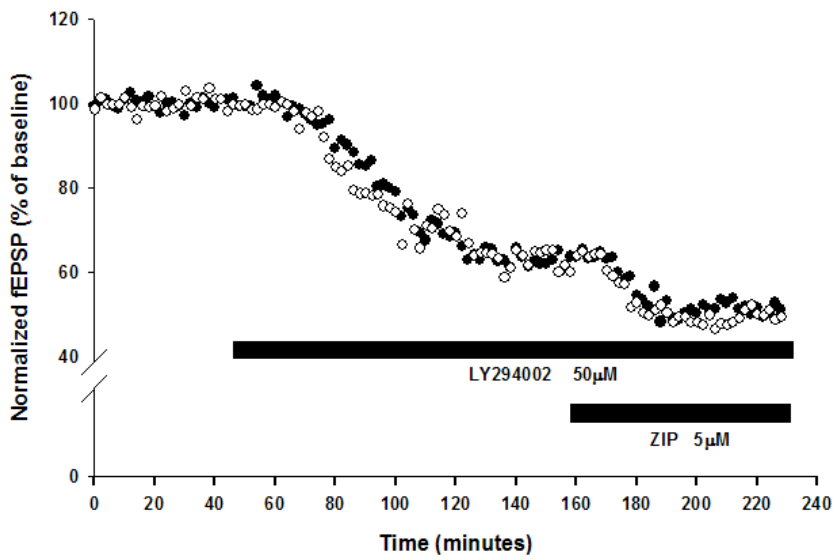

Figure 5.1.14 Single experiment of effect of LY294002 and ZIP on P14 perirhinal cortex Inhibition of PI3K by bath application of LY294002 is able to decrease the synaptic transmission. Subsequent inhibition of PKMζ produces a further depression of the evoked response.

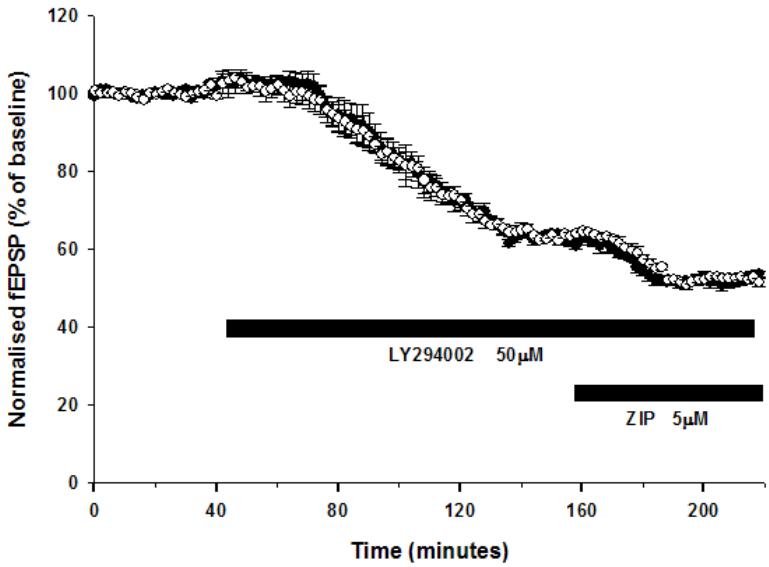

Figure 5.1.15 Pooled data for effect of LY294002 and ZIP on P14 perirhinal cortex LY294002 produces a significant decrease in the synaptic transmission (Temporal side, filled circles: 63 ± 0.5% of baseline, $p<0.001$; Entorhinal side, open circles 63 ± 0.6% of baseline, $p<0.001$, n=4). ZIP, bath applied 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 ($p>0.05$).

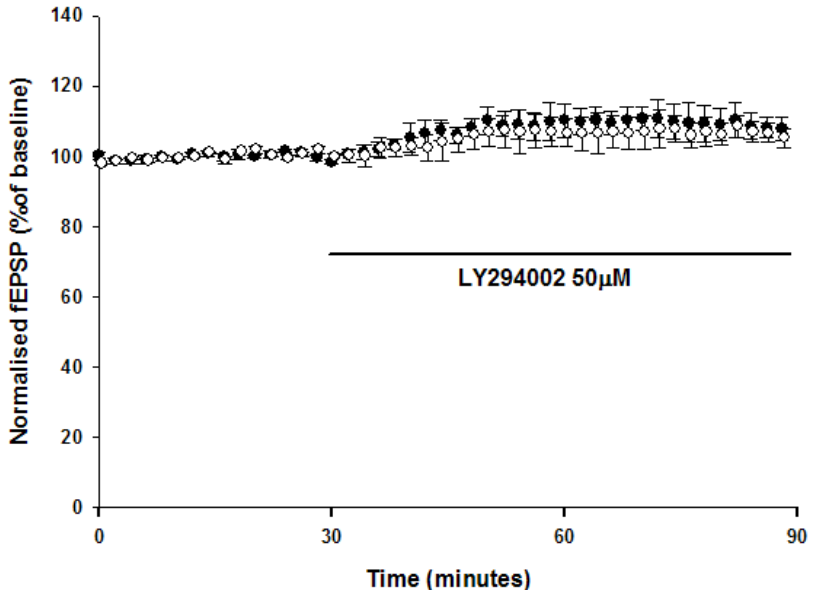

Figure 5.1.16 Pooled data for effect of LY294002 on adult perirhinal cortex PI3K inhibitor, LY294002, shows no effect on basal synaptic transmission in adult perirhinal cortex (Temporal side, filled circles: 108 ± 3% of baseline, p>0.05; Entorhinal side, open circles: 106 ± 3% of baseline, p>0.05, n=3). 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 singnificant 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 inosytol-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 asses 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 shows 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 ± 1% of baseline, $p<0.001$; Entorhinal side: 80 ± 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 ± 0.6% of baseline, $p<0.001$ v JNJ16259685; Entorhinal side: 59 ± 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 ± 0.7% of baseline, $p<0.01$ v MPEP; Entorhinal side: 51 ± 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.

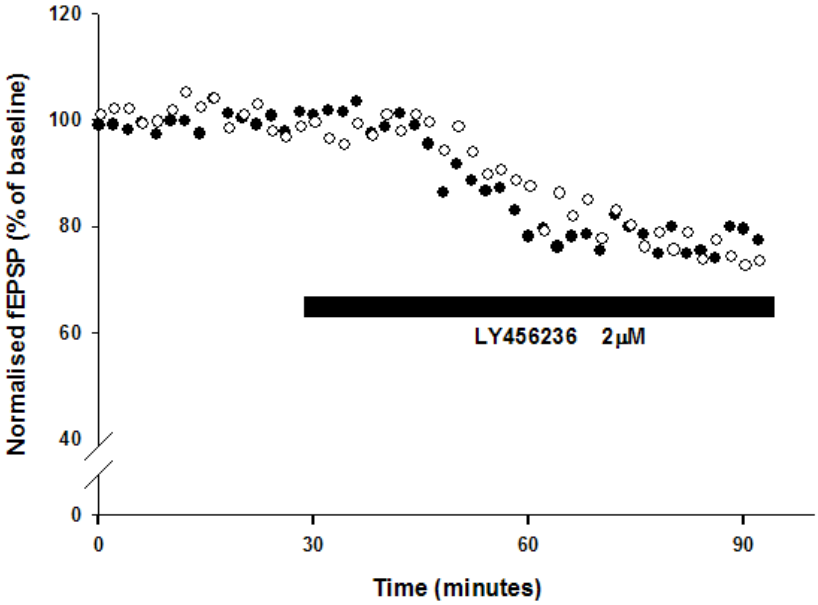

Figure 5.2.1 Single experiment on the effect of LY456236 on P14 perirhinal cortex The application of the selective mGlu1 antagonist, LY456236, produces a depression of the basal synaptic transmission in P14 perirhinal cortex.

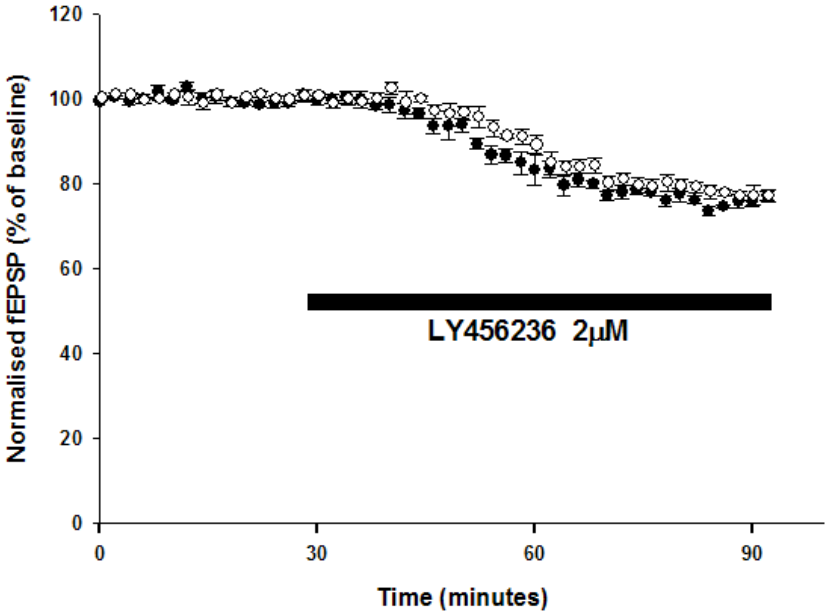

Figure 5.2.2 Pooled data for effect of LY456236 on P14 perirhinal cortex The selective mGlu1 receptor antagonist, LY456236, produces a decrease in the basal synaptic transmission in P14 perirhinal cortex (Temporal side, filled circles: 76 ± 0.7% of baseline, $p<0.001$; Entorhinal side, open circles 78 ± 01% of baseline, $p<0.001$, $n=4$).

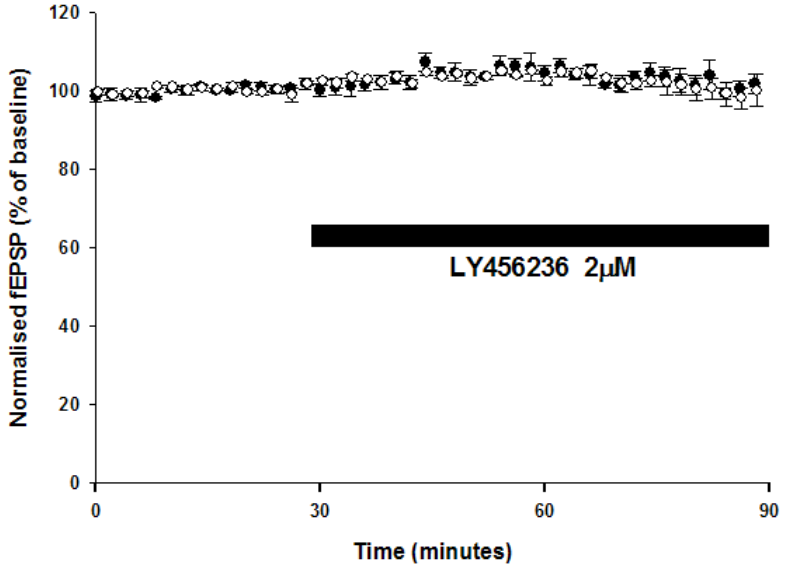

Figure 5.2.3 Pooled data for effect of LY456236 on adult perirhinal cortex No effect of the mGlu1 antagonist LY456236 was found on basal synaptic transmission in adult perirhinal cortex (Temporal side, filled circles: 101 ± 2% of baseline, p>0.05; Entorhinal side, open circles 99 ± 1.6% of baseline, p>0.05, n=3).

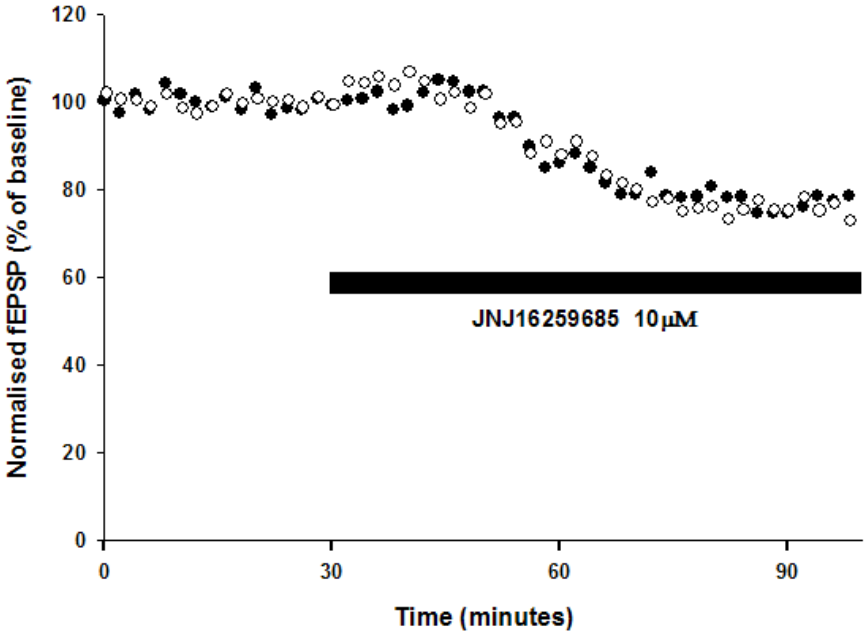

Figure 5.2.4 Single experiment on the effect of JNJ16259685 on P14 perirhinal cortex The application of the selective mGlu1 NAM, JNJ16259685, produces a depression of the basal synaptic transmission in P14 perirhinal cortex.

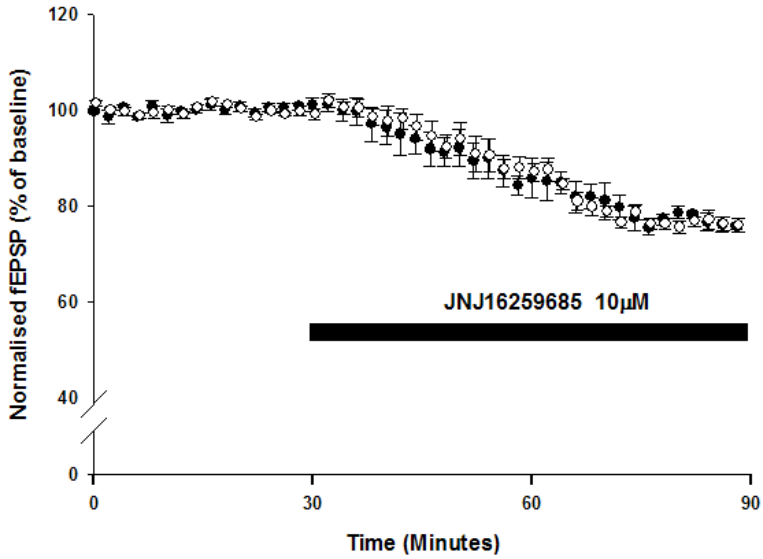

Figure 5.2.5 Pooled data for effect of JNJ16259685 on P14 perirhinal cortex Bath application of the selective mGlu1 NAM, JNJ16259685, produces a significant depression of the synaptic transmission from P14 animals (Temporal side, filled circles: 77 ± 1% of baseline, $p<0.001$; Entorhinal side, open circles: 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.

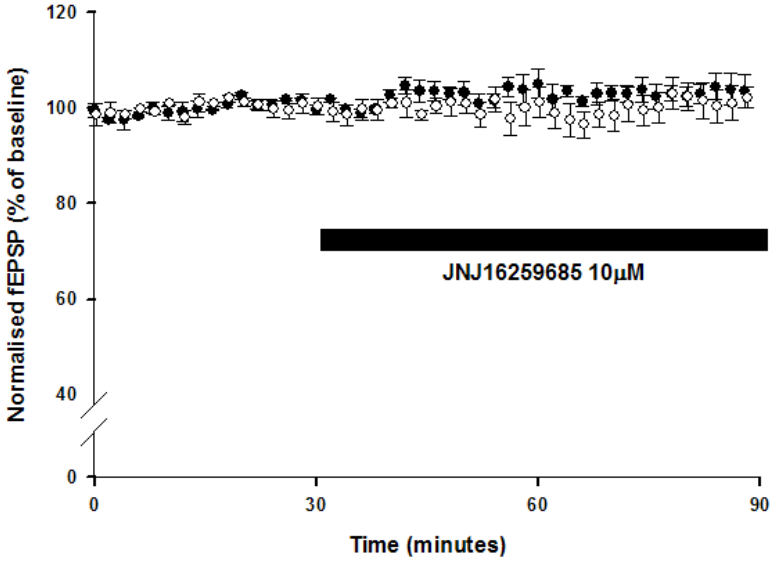

Figure 5.2.6 Pooled data for effect of JNJ16259685 on adult perirhinal cortex The application of JNJ16259685 shows no effect on adult perirhinal cortex (Temporal side, filled circles: 103 ± 2% of baseline, p>0.05; Entorhinal side, open circles: 101 ± 3% of baseline, p>0.05, n=3). No difference was found between the two pathways (p>0.05).

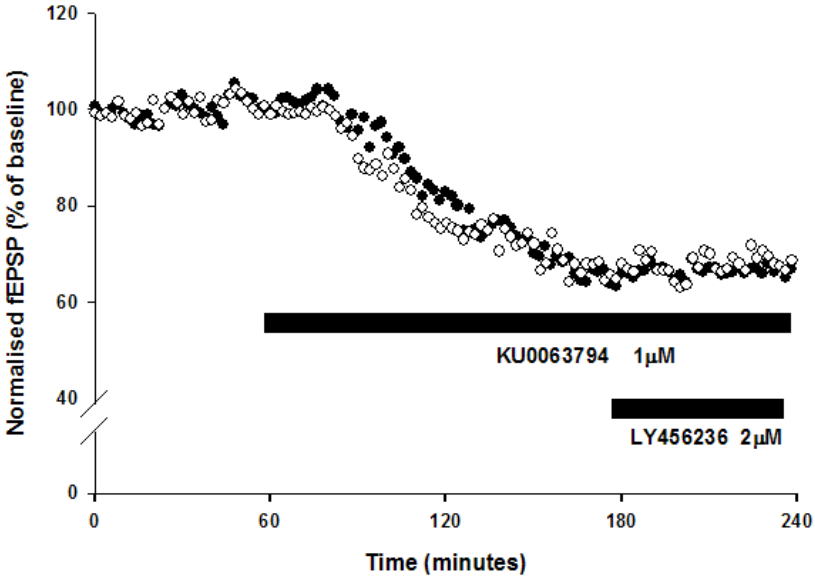

Figure 5.2.7 Single experiment on the effect of KU0063794 and LY456236 on P14 perirhinal cortex The application of the selective mGlu1 antagonist, LY456236, produces a depression of the basal synaptic transmission in P14 perirhinal cortex**.**

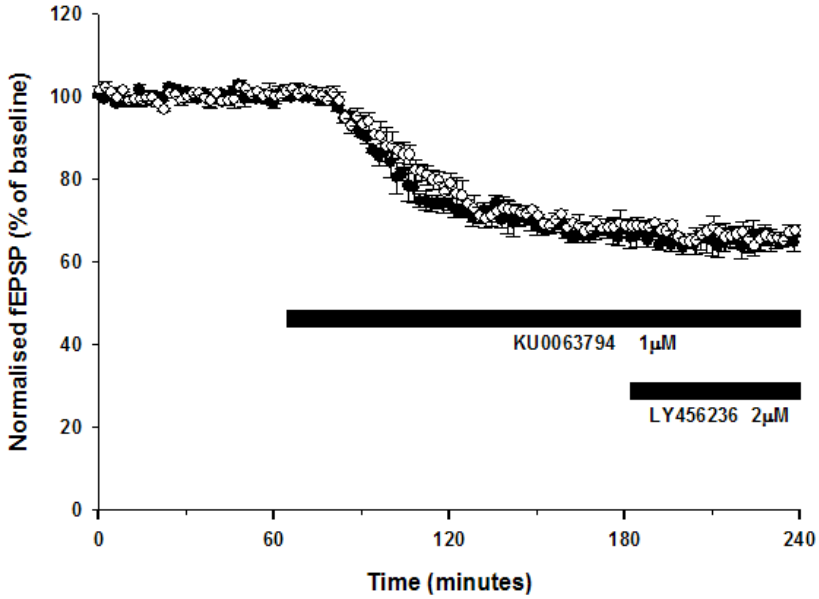

Figure 5.2.8 Pooled data for effect of KU0063794 and LY456236 on P14 perirhinal cortex The inhibition of mTOR by KU0063794 depresses the basal synaptic transmission (Temporal side, filled circles: 67 ± 1% of baseline, $p<0.01$; Entorhinal side, open circles 68 ± 1% of baseline, $p<0.01$, n=3). No further 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).

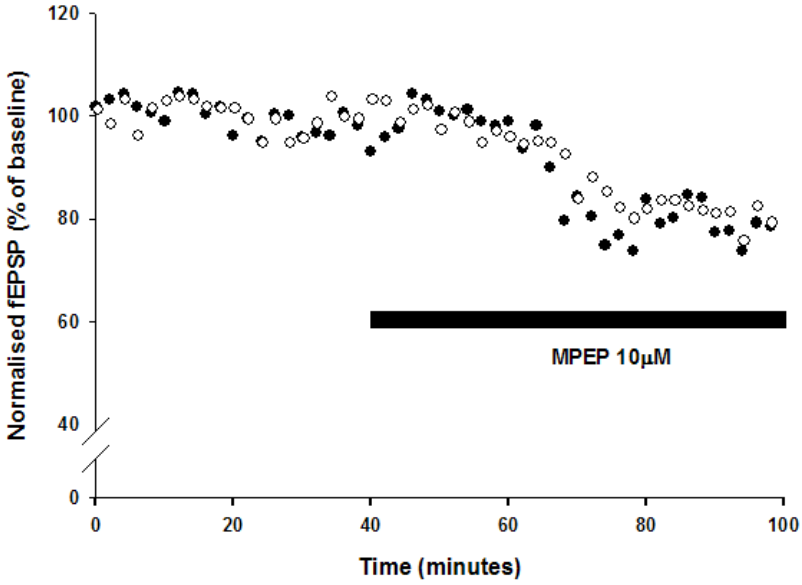

Figure 5.2.9 Single experiment on the effect of MPEP on P14 perirhinal cortex The application of the selective mGlu5 antagonist, MPEP, produces a depression of the basal synaptic transmission in P14 perirhinal cortex.

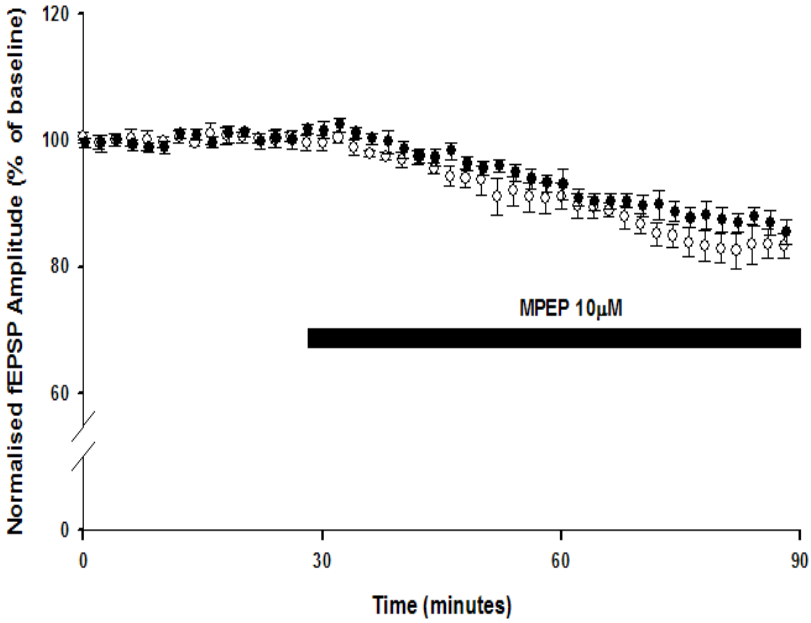

Figure 5.2.10 Pooled data for effect of MPEP on P14 perirhinal cortex The application of the selective mGlu5 antagonist MPEP produces a small but significant decrease in the synaptic transmission in P14 perirhinal cortex (Temporal side, filled circles: 87 ± 1% of baseline, $p<0.001$; Entorhinal side, open circles: 83 ± 2% of baseline, $p<0.001$, $n=7$).

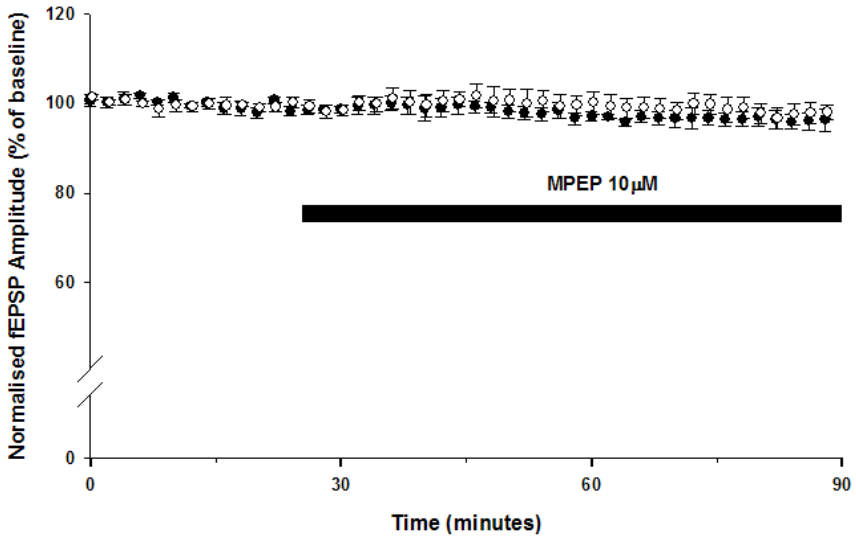

Figure 5.2.11 Pooled data for effect of MPEP on adult perirhinal cortex The application of MPEP shows no effect on adult perirhinal cortex (Temporal side, filled circles: 97 ± 2% of baseline, p>0.05; Entorhinal side, open circles: 99 ± 2% of baseline, p>0.05, n=5). No difference was found between the two pathways (p>0.05).

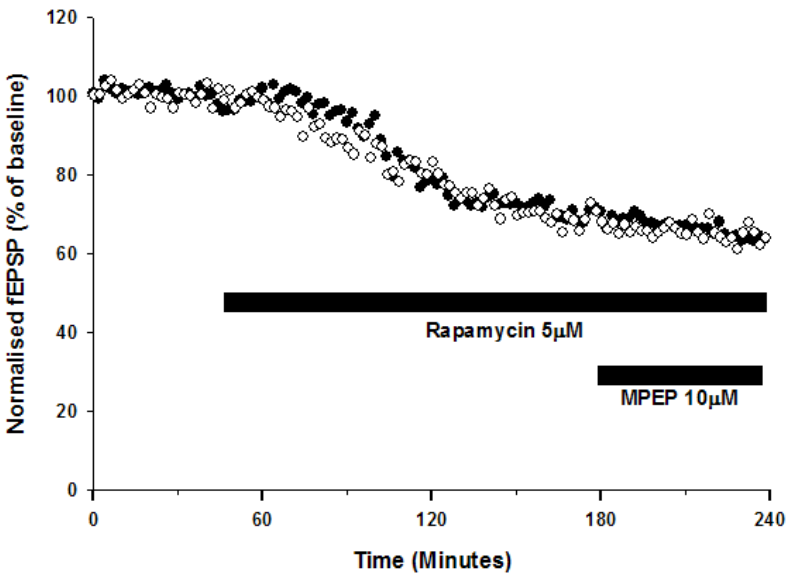

Figure 5.2.12 Single experiment on the effect of Rapamycin and MPEP on P14 perirhinal cortex The application of the mTOR inhibitor Rapamycin produces a decrease in the basal synaptic response. Subsequent application of the selective mGlu5 antagonist, MPEP, does not produce any further depression of the synaptic transmission.

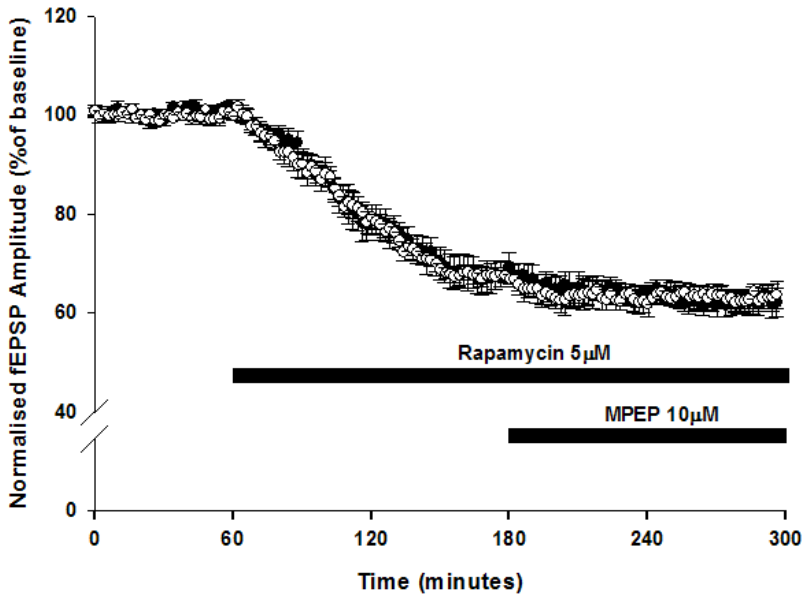

Figure 5.2.13 Pooled data for effect of Rapamycin and MPEP on P14 perirhinal cortex The inhibition of mTOR depressed the basal synaptic transmission, as observed before (Temporal side, filled circles: 67 ± 2% of baseline, $p<0.001$; Entorhinal side, open circles: 68 ± 1% of baseline, $p<0.001$, n=6). 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).

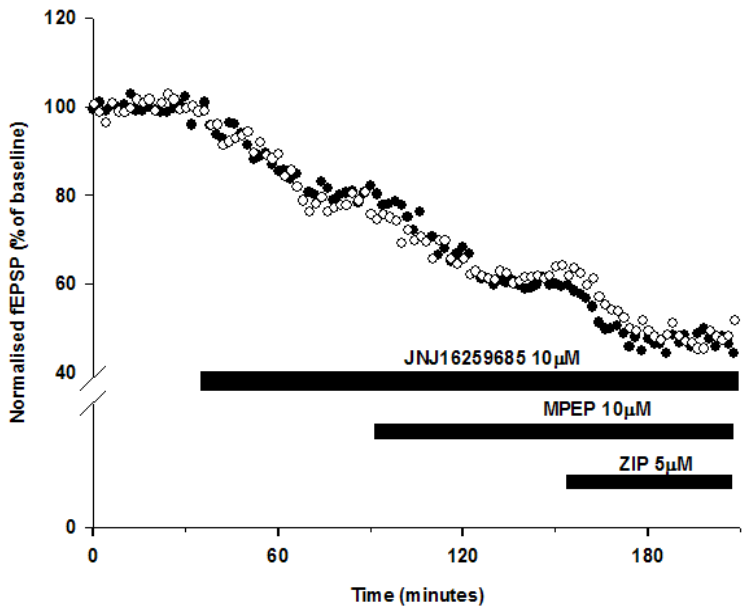

Figure 5.2.14 Single experiment on the effect of JNJ16259685, MPEP and ZIP on P14 perirhinal cortex The application of mGlu1 and mGlu5 show an additive effect in decreasing the basal synaptic response. The application of ZIP produces afurther, small decrease.

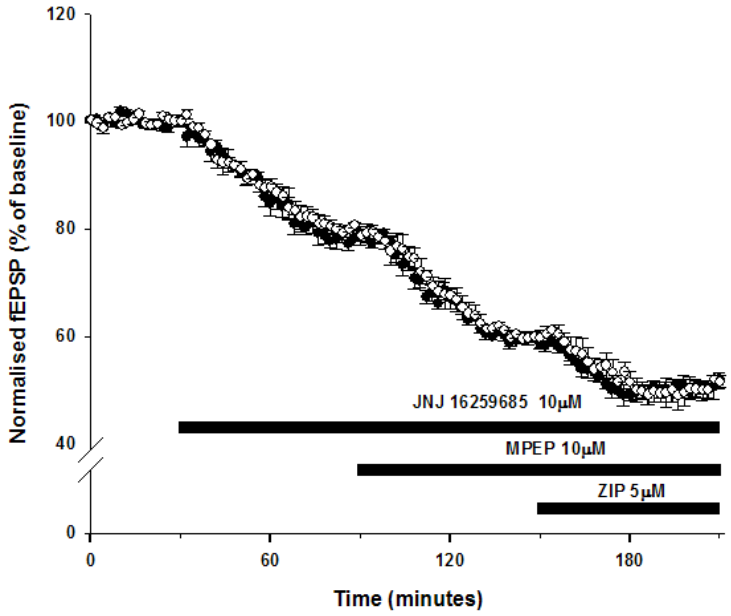

Figure 5.2.15 Pooled data for the effect of JNJ16259685, MPEP and ZIP on P14 perirhinal cortex Application of JNJ16259685 produces a significant decrease of the synaptic transmission in P14 perirhinal cortex (Temporal side, filled circles: 77 ± 1% of baseline, $p<0.001$; Entorhinal side, open circles: 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-depedent 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 loger 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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MECCANISMI NEUROADATTATIVI E REGOLAZIONE DELLA SFERA COGNITIVA

MECHANISMS OF NEUROADAPTATION AND REGULATION OF COGNITION

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Indice

1 INTRODUZIONE GENERALE

1.1 Memoria

Per memoria si intende quella funzione cerebrale superiore che permette l'immagazzinamento, la ritenzione ed il richiamo di informazioni acquisite durante l'esperienza.
La memoria viene suddivisa in diversi tipi, ciascuno dipendente da specifici circuiti neurali. Classicamente, si parla di memoria Dichiarativa e di memoria Non Dichiarativa (Mesulam, 1998). La memoria dichiarativa si suddivide a sua volta in Semantica ed Episodica. La memoria Semantica e' quella deputata ad immagazzinare fatti relativi al mondo circostante, mentre la memoria Episodica si riferisce al ricordo di esperienze personali (Tulving et al. 1988). Per memoria non dichiarativa, invece, si intende la memoria inconscia di procedure acquisite, nonche' la memoria emozionale (Mesulam, 1998).

E' stata dimostrata l'importanza di una particolare regione del cervello, il lobo temporale mediale (Medial Temporal Lobe, MTL) nei processi di memoria (Burwell and Amaral, 1998a,b). Questa regione cerebrale e' coinvolta anche nella memoria di riconoscimento, in un particolare tipo di memoria costituita dall'identificazione di un evento (o di un oggetto) e del suo riconoscimento come "familiare" (Brown and Aggleton, 2001).

Due strutture in particolare sono storicamente ritenute importanti per la memoria di riconoscimento, ossia ippocampo ed amigdala (Eichenbaum 1999). Tuttavia, di recente grande attenzione e' stata posta su altre porzioni cerbrali, in particolare le regioni che circondano l'ippocampo, ossia la corteccia peririnale e le cortecce paraippocampale ed entorinale (Brown and Aggleton, 2001).

1.2 Corteccia peririnale

1.2.1 Struttura e connessioni.

La corteccia peririnale nel ratto comprende due sottili porzioni di corteccia (aree 35 e 36 di Brodmann) localizzate al di sopra ed al di sotto del solco rinale. La corteccia peririnale e' sovrastata in senso dorso/rostrale dalla corteccia associativa temporale, mentre ventro/caudalmente ad essa si localizza la corteccia entorinale. La corteccia peririnale e' inoltre contornata rostralmente dalla corteccia insulare e caudalmente dalla corteccia postrinale, che mostra delle somiglianze con la corteccia paraippocampale nei primati (Burwell et al, 1995).

L'area 35 di Brodmann e' localizzata lungo il margine inferiore del fondo del solco rinale, nella sua porzione ventrale; l'area 36, invece, e' una zona piu'ampia, situata piu' dorsalmente, che include gran parte del margine dorsale del solco rinale.

Queste due aree sono ulteriormente suddivise in altre 5 aree in base alle loro differenze citoarchitettoniche: Area 35 dorsale e ventrale, Area 36 dorsale, ventrale e posteriore. La porzione ventrale dell'area 35 occupa approssimativamente il margine ventrale del solco rinale, mentre la porzione dorsale occupa il fondo del solco. L'area 35 e' costituita da corteccia agranulare (manca lo strato granulare IV), mentre l'area 36 presenta uno strato IV a cellule diradate. Sempre nella stessa area, lo strato II e' caratterizzato da aggregati di cellule rotondeggianti di medie dimensioni o poligonali, frapposte alle quali si trovano cellule piramidali, via via piu' numerose procedendo caudalmente (Burwell , 2001)

Per quanto riguarda le sue funzioni, la corteccia peririnale puo' essere definite come una corteccia associativa, ricevente informazioni da tutte le modalita' sensoriali, oltre che da altre areee associative come la corteccia prefrontale e quella entorinale.

Secondo gli studi di Deacon et al, 1983 e Burwell and Amaral, 1998a,b, l'area 36 riceve afferenze dalla corteccia postrinale, entorinale, temporale e dall'area 35. L'area 35, a sua volta, riceve afferenze dalla corteccia postrinale (principalmente dalle aree olfattorie), insulare ed entorinale, nonche' dall'area 36. Inoltre, in massima parte tramite connessioni con la corteccia postrinale, la corteccia peririnale, nel ratto, riceve numerose afferenze dalle regioni visive, olfattive, uditive e visuo-spaziali.
A sua volta, la corteccia peririnale invia proiezioni alla corteccia entorinale, alla quale risulta pertanto essere interconnessa. La corteccia entorinale fornisce la maggior parte degli impulsi corticali che raggiungono l'ippocampo (Burwell and Amaral, 1998a) e riceve da questo delle controproiezioni, chiudendo il circuito con la corteccia rinale.

1.2.2 Corteccia peririnale e memoria.

La corteccia peririnale e' una struttura di fondamentale importanza nei processi di memoria e in particolare nel riconoscimento degli oggetti, come dimostrato in numerosi studi

1.2.2.1 Test comportamentali

I test comportamentali piu' usati si basano essenzialmente sulla capacita' dell'animale da esperimento di riconoscere come "familiare" un oggetto esplorato in precedenza. Esistono due varianti: il "delayed matching" (DMS) ed il "delayed non-matching" (DNMS) (Mishkin and Delacour, 1975). Entrambi I test consistono essenzialmente di tre fasi: una prima fase di "presentazione" dell'oggetto, un intervallo di tempo di durata variabile ed infine la fase di test vera e propria. Durante la prima fase, all'animale viene presentato un oggetto che dovra' "esplorare" o spostare per ottenere una ricompensa. Al termine di questa fase, si lascia passare un intervallo di tempo variabile, seguito dall'ultima fase, in cui all'animale sono presentati una copia dell'oggetto gia' esplorato, considerato quindi "familiare", ed un nuovo oggetto. Nel test DMS,

l'animale dovra' riconoscere l'oggetto "familiare" per ottenere la ricompensa, mentre nel test DNMS, al contrario, otterra' la ricompensa se riconoscera' l'oggetto nuovo. Quest'ultimo test e' probabilmente il migliore per valutare la memoria di riconoscimento degli oggetti, in quanto nel DMS l'animale e' ricompensato se riconosce l'oggetto familiare, dunque coinvolge tanto la memoria di riconoscimento, quanto i processi di apprendimento associativo.
Nel test DNMS, infatti, l'animale e' ricompensato se riconosce l'oggetto che, in precedenza, non aveva esplorato e che quindi non aveva associato alla ricompensa; pertanto, risulta coinvolta esclusivamente la memoria di riconoscimento dell'oggetto.

Un altro test largamente usato e' il riconoscimento "spontaneo" degli oggetti (Ennaceur and Delacour, 1988), che si fonda sulla preferenza innata dei ratti ad esplorare oggetti nuovi piuttosto che oggetti familiari. Questo test non prevede nessuna ricompensa per l'animale, bensi' registra esclusivamente il tempo che il ratto spende nell'esplorare un nuovo oggetto, rapportandolo al tempo dedicato all'oggetto familiare. L'intervallo tra la fase di presentazione dell'oggetto e la fase di riconoscimento puo' essere allungato, aumentando di conseguenza il grado di difficolta' del test. Esistono inoltre diverse varianti; il ratto puo' dover semplicemente distinguere tra un oggetto familiare ed uno nuovo, oppure riconoscere cambiamenti spaziali (ossia se un oggetto e' stato spostato in uno spazio precedentemente vuoto) o cambiamenti d'oggetto (ossia se un oggetto e' stato cambiato di posto con un altro oggetto) (Dix and Aggleton, 1999).

1.2.2.2 Studi condotti tramite lesioni specifiche o incannulamento.

Numerosi studi sono stati condotti provocando lesioni corticali e valutando gli effetti di tali lesioni sulla memoria di riconoscimento degli oggetti tramite test DNMS (Ennaceur et al, 1996; Meunier et al, 1996; Nemanic et al, 2004; Buckley, 2005). Nei primati e' stato dimostrato che diverse regioni della corteccia rinale contribuiscono alla memoria di riconoscimento, e cioe' entorinale, peririnale e paraippocampale.

Lesioni selettive di queste regioni dimostrano che ognuna di esse ricopre un ruolo specifico nella memoria di riconoscimento. Lesioni della corteccia peririnale producono severi deficit nei test DNMS (Meunier et al., 1993;1996). Lesioni della corteccia entorinale producono solo deficit lievi e transitori (Meunier et al., 1993) mentre lesioni nella corteccia paraippocampale non hanno effetto nei test DNMS (Meunier et al., 1996). Analogamente, nel ratto lesioni della corteccia rinale in toto (Mumby and Pinel, 1994) o della sola peririnale (Wiig and Bilkey, 1994) producono severi deficit nei test DNMS e nel riconoscimento spontaneo degli oggetti (Ennaceur et al, 1996; Aggleton et al, 1997; Ennaceur and Aggleton, 1997; Bussey et al, 1999; Nemanic et al, 2004; Winters et al, 2004; Buckley 2005). Inoltre, e' stato dimostrato che lesioni della corteccia peririnale hanno effetti anche sulle memorie olfattiva, tattile e dell'appetito (Suzuki et al, 1993; Corodimas and Le Doux, 1995; Fortin et al, 2004).

L'importanza della corteccia peririnale in questo tipo di memoria appare molto maggiore di quella dell'ippocampo: lesioni dell'ippocampo, infatti, non modificano significativamente i test comportamentali di riconoscimento degli oggetti (Nemanic et al., 2004; Buckley, 2005). Tuttavia, danni ippocampali provocano deficit maggiori (rispetto a quelli indotti da lesioni della corteccia peririnale) nei test di memoria spaziale (Ennaceur et al, 1996; Murray et al., 1998; Aggleton et al, 2004; Winters et al, 2004; Murray et al., 2005). Cio' dimostra che l'interazione delle due strutture e' fondamentale in attivita' che richiedono sia la memoria spaziale sia la memoria di riconoscimento degli oggetti, in quanto lesioni dell'una o dell'altra struttura risultano in severi deficit ai test di riconoscimento dell'oggetto nello spazio (Gaffan and Parker, 1996; Bussey et al., 1999, 2000).

Inoltre, studi d'incannulazione della corteccia peririnale hanno migliorato la comprensione dei mecanismi alla base della memoria di riconoscimento. La somministrazione in loco, infatti, esclude il coinvolgimento di numerose altre strutture che potrebbe derivare invece dalla somministrazione sistemica delle sostanze in esame. Il coinvolgimento dei recettori dell'acido glutammico, sia ionotropici sia metabotropici, e' stato accertato da diversi studi (Winters and Bussey, 2005; Barker et al, 2006a,b), ed anche il ruolo della trasmissione

GABAergica e colinergica nella memoria di riconoscimento degli oggetti e' stata studiata, anche tramite test comportamentali (Warburton et al, 2003; Wan et al., 2004).

1.2.2.3 Immunoistochimica.

Utilizzando come modello la valutazione dell'espressione del gene c-fos, marker di attivazione neuronale, e' stato visto che nella corteccia peririnale l'attivazione di questo gene e' maggiore quando all'animale viene presentato un oggetto nuovo, rispetto a quando ne vede uno "familiare" (Zhu et al. 1995). Cio' non avviene allo stesso modo nell'ippocampo (Zhu et al, 1995).
Un'interessante variante di questo metodo consiste nel fare in modo che l'animale veda con un occhio un oggetto nuovo e con l'altro un oggetto familiare; in questo modo si puo' comparare l'attivita' di c-fos tra i due emisferi, minimizzando la variabilita' interindividuale. Studi condotti con questo metodo dimostrano che i neuroni della corteccia peririnale e della corteccia temporale mostrano un aumento nell'espressione di c-fos in risposta all'oggetto nuovo, cosa che non avviene in risposta all'oggetto familiare (Wan et al, 1999).

1.2.2.4 Trasduzione Virale

Infine, la corteccia peririnale e' stata studiata anche tramite tecniche di trasduzione virale. L'attivita' del fattore di trascrizione CREB e' stata impedita tramite l'induzione dell'espressione di una forma inattiva del CREB, il cui gene e' stato introdotto nel neurone grazie al virus. L'isoforma inattiva (dominante) del CREB dimerizza con il CREB nativo ma non e' in grado di legare il DNA. Cio' comporta: (i) deficit della memoria di ricognizione nei test comportamentali, (ii) blocco dell'LTP (iii) blocco dell'attivazione neuronale (Warburton et al, 2005).

1.3 PLASTICITA' SINAPTICA

Le sinapsi devono essere considerate elementi dinamici in grado di cambiare la propria struttura e le proprie funzioni in modo da adattarsi agli input che ricevono. Tali cambiamenti possono riguardare molteplici aree, ad esempio cambiamenti nell'espresione recettoriale, ma di base la plasticita' puo' essere considerata come un aumento (potenziamento) o una diminuzione (depressione) della trasmissione sinaptica. Generalmente, questi eventi si manifestano come cambiamenti nell'ampiezza dei potenziali postinaptici dipendenti dall'attivita' sinaptica stessa. Questo tipo di plasticita' puo' essere di durata variabile: a "breve termine" (da millisecondi a minuti) oppure a "lungo termine" (da ore a giorni).
In generale, si ritiene che la plasticita' prolungata sia alla base della capacita' del cervello di acquisire nuove informazioni (apprendimento) e conservarle nel tempo (memoria) (Bliss and Collingridge, 1993; Malenka and Bear, 2004).

La plasticita' a lungo termine, pertanto, viene comunemente suddivisa in LTP (Long-Term Potentiation) o LTD (Long-Term Depression). I miei studi si focalizzano in particolare sulla LTP.

La LTP e' caratterizzata da un incremento duraturo nella efficacia sinaptica, che puo' essere indotto tipicamente da una stimolazione ad alta frequenza (High Frequency Stimulation, HFS) di 100Hz ed e' ritenuta essere alla base dei cambiamenti che avvengono nel cervello durante l'apprendimento (Bliss and Collingridge, 1993).
La LTP e' stata studiata abbondantemente sulla regione CA1 dell'ippocampo, con attenzione sui vari meccanismi alla base di questo fenomeno nelle diverse sinapsi e su differenti circuiti cerebrali (Malenka and Bear, 2004).

La LTD (Long Term Depression), invece, e' in buona sostanza l'opposto dell'LTP, essendo caratterizzata da un decremento duraturo dell'efficacia sinaptica. Puo' essere indotta tramite stimolazioni a bassa frequenza (Low

Frequency Stimulation). Simile alla LTD e' il processo di depotenziamento indotto su sinapsi in precedenza potenziate e pertanto mostranti LTP. Questo processo in sostanza annulla la LTP tramite la stimolazione a bassa frequenza.
Similmente, una LTD indotta primitivamente puo' essere annullata tramite stimolazione ad alta frequenza, un processo che prende il nome di de-depressione (Kemp and Bashir, 2001; Collingridge et al, 2004).

Queste alterazioni "bi-direzionali" e reversibili della risposta sinaptica, dunque, rendono possible l'immagazzinamento dinamico delle informazioni neuronali (Collingridge et al., 2004).

L'induzione della LTP puo' essere bloccata antagonizzando i recettori NMDA, come dimostrato da studi condotti sulla regione CA1 dell'ippocampo (Collingridge et al, 1983); e' dunque intuibile che tali recettori ricoprono un ruolo primario in questo fenomeno.
Perche' si possa avere LTP e' necessario l'ingresso del Ca2+ all'interno della cellula, e cio' e' secondario all'attivazione degli NMDAR. Tali recettori sono inattivi quando I potenziali di membrana della cellula si trovano in una situazione di "riposo", in quanto il canale che essi formano e' bloccato dalla presenza di uno ione Mg2+. Tuttavia quando il neurone viene depolarizzato, tipicamente in seguito all'azione dei recettori NMDA per il glutammato, lo ione Mg2+ si stacca dal canale, liberandolo e permettendo l'ingresso nella cellula di Ma2+ e Ca2+. Cio' provoca la corrente di Ca2+ necessaria all'induzione della LTP (Malenka and Bear., 2004).
Tra le proteine intracellulari fondamentali per questo processo, un ruolo di rilievo spetta di sicuro alla Protein Kinasi Ca2+/calmodulina-dipendente di tipo II (CaMKII) (Lisman 2003). Durante l'attivita' sinaptica, questa proteina trasloca dal citoplasma e si lega ai recettori NMDA, dove rileva l'aumento delle correnti di Ca2+ e provoca la cascata di signalling intracellulare coinvolte nell'espressione della LTP.
Molto importante sembra essere anche la Protein Kinasi A (PKA); essa e' necessaria per l'induzione della LTP nell'immediato sviluppo postnatale

(Yasuda et al., 2003) ed inoltre blocca l'attivita' della Protein Fosfatasi 1 (PP1), inibitore di CaMKII, mantenendo l'attivazione della Kinasi (Brown et al., 2000).

I meccanismi post-sinaptici coinvolti nell'espressione della LTP NMDAR-dipendente sono principalmente due: l'aumento dei recettori AMPA nella sinapsi tramite meccanismi di "trafficking" e modificazioni degli stessi recettori AMPA tramite la fosforilazione della subunita' Glut1 (Malinow and Malenka, 2002; Malenka and Bear, 2004).
Tale fosforilazione si verifica in diversi siti della subunita' GluR1, ad opera della CaMK II e della PKC, e risulta in un aumento della conduttanza nei canali. CaMK II fosforila la Serina 831 durante la LTP (Barria et al, 1997). Tuttavia, nonostante topi knock-out per GluR1 non mostrino LTP, singole mutazioni di GluR1, come appunto la sostituzione della serina 831 con alanina, esitano solo in una lieve diminuzione della LTP (Lee et al., 2000).
Un altro sito di fosforilazione su GluR1 e' stato identificato nel residuo di Serina 818 (Boehm et al, 2006); la fosforilazione a questo livello aumenta la traslocazione in menmbrana di recettori AMPA contenenti GluR1.
Questo sito viene fosforilato in seguito alla stimolazione per l'induzione della LTP, e si suppone che la sua fosforilazione sia importante sia per lo stabilirsi della LTP, sia per facilitare l'interazione con proteine che mediano lo spostamento o l'ancoraggio dei recettori NMDA. (Boehm et al, 2006).
Per quanto riguarda il mantenimento dell'LTP, la maggior parte degi studi sembra essersi concentrata sulla fase iniziale, quella che dura 30-60 minuti dopo l'induzione, mentre meno si sa riguardo la fase tardiva della LTP e ai meccanismi che permettono a quest'ultima di durare per ore, giorni o addirittura settimane (Malenka and Bear, 2004). E' stato dimostrato che le fasi tardive della LTP richiedono la sintesi di nuove proteine e la trascrizione genica (Kelly et al., 2000; Bozon et al., 2003, Abraham and Williams, 2003; Lynch, 2004; Miyamoto, 2006; Reymann and Frey, 2007). In particolare, Scharf et al (2002) hanno dimostrato che la somministrazione di Anisomicina (25 μM), iniziata 20 minuti prima dell'induzione della LTP e continuata per i 15

minuti successivi, porta alla perdita del potenziamento, con ritorno ai valori di baseline, in circa un'ora.

Le molecole di signalling che sembrerebbero collegare l'induzione della LTP alla trascrizione di nuovi geni sono la Protein- Kinasi Calmodulino-dipendente di tipo IV (CaMK IV), la Protein Kinasi attivata da Mitogeni (MAPK) e la PKA, che induce infine la fosforilazione del fattore di trascrizione CREB (Lynch 2004; Warbuton et al, 2005; Miyamoto, 2006; Reymann and Frey, 2007). La fosforilazione di CREB puo' portare all'attivazione del gene c-fos (Ahn et al, 1998), il cui prodotto, Fos, rappresenta un marker accurato per i processi di memoria di riconoscimento (Warburton et al, 2003; Wan et al, 2004; Warburton et al., 2005). L'inibizione della fosforilazione di CREB nella corteccia peririnale, ottenuta mediante virus, blocca sia l'LTP sia la memoria di riconoscimento a lungo termine (Warburton et al., 2005). Altri studi hanno dimostrato che la fosforilazione di CREB e il mantenimento della LTP sono correlate alla memoria in altre regioni del cervello, come l'Ippocampo (Pittenger et al, 2002; Reymann and Frey, 2007). Il legame tra LTP ed altri tipi di memoria, ad esempio quella spaziale (ippocampo) e della paura (amigdala) e' dimostrato in diversi lavori (Morris, 2003; Sigurdsson et al, 2007). E' stato inoltre visto che bloccando il trafficking dei recettori contenenti GluR1 nel 10, 20 % circa dei neuroni responsabili della plasticita' e' sufficiente per impedire la formazione della memoria (Morris et al, 1986; Rumpel et al., 2005).

L'isoenzima della PKC, Protein Chinasi M Zeta (PKMζ)e la fosfatidil inositolo 3-fosfato kinasi (PI3K) sono implicate nello spostamento dei recettori NMDA contenenti GluR1 nelle sinapsi che hanno subito LTP, nonche' nel mantenimento della LTP (Ling et al, 2002, 2006; Sanna et al., 2002). L'iniezione di un inibitore della PKM nel giro dentato non blocca l'induzione della LTP; tuttavia l'inibizione della PKMζ annulla la LTP quando effettuata 5 ore dopo l'induzione della stessa (Serrano et al., 2005). Il peptide inibitore, inoltre, annulla la LTP nell'ippocampo anche in vivo e provoca una perdita della memoria spaziale recente, indicando che il mantenimento della LTP puo' essere alla base della memoria spaziale (Pastalkova et al, 2006). Questi studi, dunque, dimostrano che il suolo della PKM e' fondamentale nelle fasi tardive della LTP.

La LTP provoca anche cambiamenti a livello morfologico, quali crescita di nuove spine dendritiche, accrescimento di spine pre-esistenti e quindi della densita' post sinaptica (PSDs) (Abraham and Williams, 2003; Matsuzaki et al, 2004). Anche la polimerizzazione del citoscheletro di actina appare essere importante per la LTP, in quanto alcuni studi dimostrano che nelle spine dendritiche l'LTP provoca l'inibizione della depolimerizzazione dell'actina (Kim and Lisman, 2001; Fukazawa et al., 2003).

Esiste anche una LTP NMDAR-indipendente, localizzata principalmente nelle Mossy Fibers dell'ippocampo (Nicoll and Malenka, 1995; Bortolotto et al, 2003). Il meccanismo alla base di questa particolare forma di LTP, ancor controverso, sembra essere legato all'incremento del Ca2+ intracellulare nel terminale presinaptico, conseguente all'attivita' sinaptica stessa (Nicoll and Malenka, 1995; Mellor and Nicoll, 2001). Altri studi, invece, mostrano che la LTP nelle Mossy-fibres puo' essere scatenata dall'incremento di calcio postinaptico, dovuto per esempio al rilascio di Ca2+ dai compartimenti intracellulari, successivo all'attivazione dei recettori metabotropici del glutammato (mGluR) (Yeckel et al, 1999; Henze et al, 2000).

L'attivazione a livello presinaptico di recettori del Kainato, ed in particolare della subinita' GluR5, e' risultato importante per l'induzione della LTP nelle Mossy Fibres (Lauri et al, 2001; Bortolotto et al, 2003; Lauri et al, 2003). E' stato proposto che l'influsso di calcio attraverso i recettori per il Kainato provoca un rilascio del Ca2+ dai compartimenti intracellulari, conducendo ad un rapido incremento nel rilascio di glutammato da parte dei terminali presinaptici e quindi nell'innesco dei meccanismi che inducono la LTP (Lauri et al, 2003). Inoltre, sembra che elevate concentrazioni di Ca2+ nella soluzione extracellulare possano portare all'ingresso di un quantitativo sufficiente di ioni Ca2+ all'interno della cellula, tramite i canali per il calcio di tipo L, portando alla formazione di LTP presumibilmente tramite l'interazione del Calcio con la PKA. Pertanto, anche la concentrazione extracellulare di Ca2+ potrebbe influenzare l'induzione della LTP nelle Mossy Fibres (Lauri et al., 2003).

Il ruolo della PKA nell'induzione della LTP nelle Mossy Fibers e' stato oggetto di diversi studi (Malenka and Bear, 2004; Castillo et al, 1997; Sudhof, 2004), ma il meccanismo alla base rimane ancora poco chiaro.

1.4 Protein Kinasi Mζ (PKMζ)

Tra le numerose proteine implicate in questo fenomeno, una in particolare, la Protein Kinasi M Zeta (PKMζ) si è dimostrata cruciale per il mantenimento della LTP.

PKMζ è un particolare sottotipo dell'isoforma ζ della Protein Kinasi C (PKC). La famiglia delle PKC, infatti, comprende tre gruppi: le PKC "Convenzionali" (α, βI, βII, γ), le "Nuove" (δ, ε, η, θ) e le "Atipiche" (ζ, ι/λ) (Nishizuka 1988, 1995). Le PKC "Convenzionali" sono attivate da secondi messaggeri quali Diacilglicerolo (DAG) e Ca2+; le PKC "Nuove" sono attivate solo dal DAG, mentre le "Atipiche" né dall'uno né dall'altro, e sono invece regolate da un diverso set di secondi messaggeri, comprendente Acido Arachidonico (Nakanishi and Exton, 1992), Fosfatidilinositolo 3,4,5-trifosfato (Nakanishi et al., 1993) e Ceramide (Muller et al, 1995).

L'isoforma M della PKCζ è composta unicamente dal dominio catalitico dell'enzima **(Figura 1.4.1)**; pertanto, essendo priva del dominio regolatore, risulta essere costitutivamente attiva. È inoltre l'unico isoenzima della famiglia delle PKC a possedere una stabile isoforma di tipo M nell'Ippocampo (Sacktor et al, 1993; Naik et al, 2000). È inoltre espressa diffusamente nel tessuto cerebrale, ed è sintetizzata partendo da uno specifico mRNA che codifica esclusivamente per il dominio catalitico della proteina; contrariamente a quanto si riteneva in passato, dunque, essa non è semplicemente un prodotto di splicing (Hernandez et al., 2003). La sua sintesi è indotta da uno stimolo ad alta frequenza (Osten et al., 1996) ed è stato dimostrato che la sua espressione aumenta durante il mantenimento della LTP (Sacktor et al., 1993).

L'importanza di questo enzima risiede nel fatto che è stato dimostrato essere necessario e sufficiente per il mantenimento della LTP (Ling et al., 2002, Serrano et al., 2005), mentre non sembra rivestire alcun ruolo nell'induzione della stessa. Infatti, l'inibizione di PKMζ ad opera di uno pseudosubstrato inibitorio (ZIP) annulla un potenziamento già stabilito, ma non ha alcun effetto sull'induzione dello stesso, né sulle fasi precoci della LTP (Serrano et al., 2005). ZIP agisce ricostituendo il dominio catalitico, di cui PKMζ è priva **(Figura 1.4.1).**

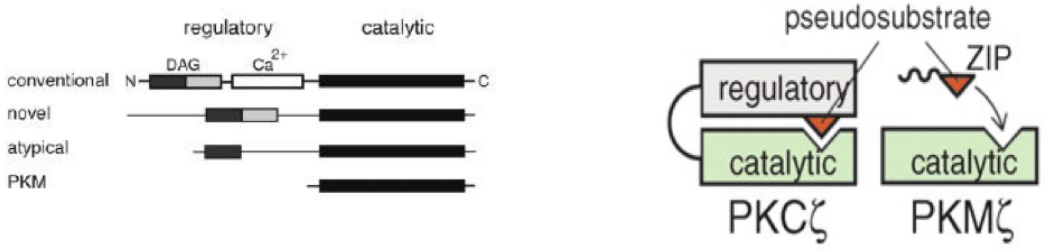

Figura 1.4.1: Sinistra: l'immagine mostra la struttura delle varie isoforme di PKC appartenenti a diverse famiglie. PKMζ è costituita unicamente dal dominio catalitico di PKCζ, pertanto e' costitutivamente attiva (Naik et. Al, 2000). Destra: la figura mostra il sito di azione del peptide inibitore selettivo di PKMζ, ZIP. ZIP è uno pseudo substrato che agisce ripristinando il dominio regolatore non presente in PKMζ(Pastalkova et al., 2006).

In esperimenti condotti *in vivo*, è stato dimostrato che il peptide inibitore è in grado di annullare la LTP nell'ippocampo e provoca una perdita specifica della memoria spaziale recente (Pastalkova et al, 2006). Inoltre, il blocco di PKMζ nella corteccia insulare provoca la cancellazione della memoria a lungo termine, in maniera apparentemente irreversibile (Shema et al., 2007).
Questi studi, dunque, dimostrano che il ruolo della PKMζ è fondamentale nelle fasi tardive della LTP.

I meccanismi tramite i quali PKMζ mantiene il potenziamento sinaptico sono ancora oggetto di studi. L'ipotesi al momento più accreditata è che l'enzima sia coinvolto nel trafficking dei recettori AMPA (Ling et al,. 2006); recenti studi inoltre dimostrano che tale trafficking è dipendente dall'interazione N-Ethylmalemide-sensitive Factor/GluR2 (Yao et al., 2008). Inoltre, recenti studi

suggeriscono che PKMζ regoli il trafficking degli AMPA non favorendone l'inserzione in membrana, ma bloccandone la rimozione (Migues, Hardt et al. 2010).

Anche la regolazione dell'attività di PKMζ resta ancora in attesa di conferma. La teoria al momento più accreditata è che la sintesi di PKMζ sia regolata da diverse protein kinasi (PI-3-Kinasi, MAP Kinasi, Protein Kinasi A, CAM Kinasi); la proteina viene poi attivata tramite fosforilazione sul suo "activation loop" (pT410) da parte della Protein Kinasi-1 Fosfoinositide-dipendente (PDK1). Una volta fosforilata, e dunque attiva, PKMζ dà origine ad un loop di feedback positivo che auto mantiene l'incremento della sua sintesi durante il mantenimento del potenziamento sinaptico (Kelly et al., 2007). In effetti, il meccanismo con cui PDK1 promuove l'attivazione di PKMζ è leggermente diverso dal suo effetto sugli altri enzimi su cui agisce. PDK-1 è una serin/treonin kinasi, la cui attività è secondaria all'attività della PI-3-Kinasi. Essa attiva, fosforilandole, diverse kinasi, tra cui PKC; ha, dunque, un ruolo fondamentale nella regolazione di processi quali proliferazione cellulare, differenziamento ed apoptosi. La differenza nell'azione di PDK1 sulle PKC dotate di dominio regolatore, rispetto all'azione esercitata sulle "atipiche"; tra cui PKCζ, consiste nel fatto che la fosforilazione dell'activation loop sulle PKC convenzionali le "predispone" ad una piena attivazione conseguente alla cascata di secondi messaggeri, che eliminano l'inibizione dovuta al dominio regolatore (il quale le mantiene in una conformazione ancora inattiva, nonostante la fosforilazione sull'"activation loop"). PKCζ, invece, una volta fosforilata è immediatamente attiva (meccanismo "on/off") (Le Good et al., 1998; Dong et al., 1999; Balendran et al., 2000a/b).
Recenti studi hanno dimostrato che l'attività di PDK1 può essere bloccata dal Celecoxib, un membro della famiglia dei Farmaci Antiinfiammatori Non Steroidei (FANS), più precisamente appartenente al gruppo dei Coxib, inibitori specifici dell'attività della Cicloossigenasi 2 (Cox2) (Arico et al., 2002; Kulp et al., 2004). Un altro composto, tuttavia, si è dimostrato capace di inibire l'attivazione di PDK-1: l'acido 3-Idrossiantranilico (3HAA), un prodotto di ossidazione del triptofano. Esso lega PDK-1 nel suo binding site per l'ATP

tramite legami idrogeno e blocca l'autofosforilazione in questo sito, essenziale per l'attività dell'enzima (Hayashi et al., 2007).

Un'altra via attraverso cui PKMζ potrebbe mantenere alti i ivelli della sua propria sintesi comprendono proteine note come "protein interacting with NIMA 1" (Pin-1). La sintesi di PLMζ, infatti, e' inibita da Pin-1. Pin-1 blocca la sintesi protica nei dendriti, ma attivita' di signalling mediate dal glutammato, che inducono LTP e stimolano la fomrazione di memoria, inibiscono Pin-1, permettendo la sintesi proteica. La neo-sintetizzata PKMζ, quindi, inibisce a sua volta Pin-a, auto-mantenendo la propria sintesi ed attivita' (Sacktor 2010; Westmark, Westmark et al. 2010). Inoltre, è stato proposto (Westmark, Westmark et al. 2010) che Pin1 normalmente blocchi la sintesi proteica tramite l'interazione con 4E-BPs. 4E-BPs sono fosforilate da mTOR in modo da rimuovere l'inibizione della sintesi proteica (Hoeffer and Klann 2010). Se PKMζ mantiene elevati livelli di trasmissione sinaptica tramite la sintesi proteica, allora questo meccanismo probabilmente coinvolge processi mTOR-dipendenti.

1.5 Scopo dello studio

Date queste premesse, e dopo aver dimostrato che anche nella corteccia peririnale il mantenimento della LTP dipende dalla sintesi proteica, il mio lavoro si è dunque indirizzato allo studio, sempre tramite tecniche di elettrofisiologia (registrazione in field) del ruolo di PKMζ nel mantenimento della LTP nella corteccia peririnale nell'adulto, nonché dei vari meccanismi alla base della regolazione dell'attività dell'enzima. Parallelamente, abbiamo studiato l'attività di PKMζ durante il neurosviluppo, vagliandone gli effetti su animali più giovani, più precisamente a 14 (P14) e 35 giorni di vita (P35).

2 MATERIALI E METODI

2.1 Elettrofisiologia

2.1.1 Animali

Gli esperimenti di elettrofisiologia sono stati condotti su fettine di corteccia peririnale ottenute da ratti maschi Dark Agouti (DA) adulti (7-15 settimane, 170-300 gr.), oppure a 14 o 35 giorni di vita post natale (P14 e P35, rispettivamente). Gli animali sono stabulati con un ciclo luce/buio di 12 ore, con la fase di buio fissata durante il giorno (dalle ore 9.00 alle ore 21.00).

2.1.2 Preparazione di fettine di corteccia peririnale e ippocampo:

Gli animali sono anestetizzati con una miscela gassosa contentente ossigeno ed isoflurano finche' il riflesso di ammiccamento non fosse piu' evocabile. I ratti sono stati in seguito decapitati utilizzando una ghigliottina. Il cervello viene rapidamente rimosso ed immerso in liquido cerebrospinale artificiale (aCSF) freddo (composizione dell' aCSF, mM: NaCl, 125; KCl, 2; NaHCo3, 26; NaH2PO4, 1.25; CaCl2, 1; MgSO4, 6; D-Glucosio, 10; pH 7.4). L' aCSF, sempre fresco, viene preparato prima dell'esperimento da uno stock 10X.
Il cervello e' poi posizionato su carta da filtro e sottoposto ad un taglio lungo il solco sagittale mediale, utilizzando un bisturi, in modo da essere suddiviso nei suoi due emisferi. Un emisfero e' riposizionato nell'aCSF freddo, mentre l'altro viene adagiato sulla faccia mediale per la dissezione. La maggior parte del lobo frontale e' asportato tramite un taglio, angolato di circa 40°, lungo l'asse dorso-ventrale. Il cervelletto ed una porzione del lobo occipitale sono asportati tramite un secondo taglio, condotto con la stessa angolatura. L'emisfero e' poi adagiato con la porzione caudale e fissato tramite un collante (cianoacrilato) su un supporto da microtomo; il tessuto e' inoltre supportato medialmente da un blocco di Sylgard TM (elastomero di silicone). Il cervello viene in seguito tagliato coronalmente, utilizzando un microtomo, in fettine di 400μm di spessore. Le fettine includono le aree 35 e 36 di Brodmann (corteccia Peririnale), piu' le corteccie entorinale e temporale, che corrispondono ai livelli rostro-caudali da -3.80 a -5.80 mm dal Bregma, in accordo a quanto riportato nell'Atlante di

Paxinos e Watson (1998). Una volta tagliate, le fettine sono posizionate in un contenitore contenente aCSF ossigenato, a temperatura ambiente, e lasciate riposare per almeno un'ora prima di essere sottoposte alle procedure di registrazione.
Per quanto riguarda le fettine di ippocampo, gli emisferi cerebrali venivano incollati dal lato laterale ad un supporto da vibratomo e tagliati in modo da ottenere fettine parasagittali dello spessore di 400μm. le fettine includono le aree ippocampali CA1, CA2 e CA3. Una volta tagliate, le fette venivano poste in un contenitore con aCSF ossigenato e mantenute a temperatura ambiente a riposare e riequilibrarsi per almeno un'ora prima dell'inizio delle registrazioni.

2.1.3 Extracellular Field Recording

2.1.3.1 Apparato e tecnica.

L'apparato (Rig) e' strutturato in modo da permettere un flusso costante di aCSF ossigenato all'interno della camera di registrazione. La camera di registrazione consiste in un vetrino coprioggetto posizionato tra un disco di metallo ed un disco di Perspex, sigillati tra loro tramite silicone. Le fettine di corteccia sono mantenute sempre nella medesima posizione da fili di Nylon fissati ad un supporto a forma di U, ottenuto intrecciando due pezzi di filo d'argento (0.55 mm di diametro).
Il liquido cerebrospinale, ossigenato (95% O_2/ 5% CO_2), mantenuto alla temperatura di 28º C ±1, viene incanalato in tubi di poliene (diametro interno, 1.4mm) tramite una pompa peristaltica, fino a gocciolare in una siringa da 2 ml che funge da reservoir. Nella siringa, l'aCSf viene nuovamente ossigenato per assicurare una saturazione costante. Il reservoir di aCSF fornisce un rifornimento costante alla camera di registrazione e d elimina inoltre le fluttuazioni di flusso dovute alla pompa peristaltica. Il volume di flusso e' fissato a 2-3 ml/minuto; l'aCSF in eccesso viene eliminato dalla camera di registrazione tramite una pompa di suzione, attraverso un ago. La camera di registrazione contiene circa 1.5-2 ml di liquido.
Direttamente al di sopra della camera di registrazione e' posizionato un microscopio (ingrandimento, 40X); gli elettrodi di stimolazione e l'elettrodo di

registrazione sono montati su supporti magnetici su una piattaforma di metallo, per evitare spostamenti degli stessi. Gli elettrodi di stimolazione sono posizionati ai due lati della camera di registrazione, mentre l'elettrodo di registrazione si trova davanti ad essa.
Tutti gli apparecchi elettrificati sono collegati ad una terra per eliminare interferenze.

Gli elettrodi di registrazione sono ottenuti da capillari di borosilicato (diametro esterno, 1.5mm; diamentro interno, 0.86 mm). I capillari sono modellati in micropipette utilizzando un apposito macchinario. Le micropipette vengono poi riempite con aCSF utilizzando un ago sottile ed una siringa da 1 ml; tra l'ago e la siringa e' posizionato un filtro per evitare che qualsiasi particella estranea possa trovarsi all'interno della pipetta, disturbando il processo di registrazione. La pipetta e' montata ad un supporto, contenente al suo interno un filo d'argento clorurato, che funge da elemento di registrazione vero e proprio. Il tutto e' poi fissato ad un supporto, montato su un micromanipolatore.

L'elettrodo di registrazione e l'elettrodo di riferimento sono ottenuti tramite un filo d'argento (0.25 mm di diametro). Il filo viene clorurato tramite immersione overnight in comune ipoclorito di sodio. Questo processo serve a ridurre il rumore di fondo ed eventuali interferenze durante la registrazione. Il cloruro d'argento che si viene a creare, infatti, ha bassi potenziali redox, minimizzando in questo modo eventuali reazioni di ossidoriduzione che potrebbero verificarsi tra gli elettrodi metallici e il bagno di aCSF.
L'elettrodo di registrazione e' collegato ad un box di registrazione, mentre l'elettrodo di riferimento e' fissato lungo il perimetro della camera di registrazione e collegato a terra sempre per mezzo del box sopracitato.

Gli elettrodi di stimolazione sono ottenuti intrecciando due fili di nickel-cromo (diametro 0.05mm). Ogni elettrodo viene fatto passare in una pipetta di vetro, che funge da protezione all'elettrodo stesso, alla quale vengono fissati tramite Blue Tack TM. Gli elettrodi sono montati su supporti magnetici e collegati a unita' di stimolazione (una per ogni elettrodo) situate all'interno del rig.

2.1.3.2 Extracellular Field Recording: procedura, acquisizione e rielaborazione dei dati.

Viene posizionata una singola fettina nella camera di registrazione, curando che rimanga completamente sommersa dall'aCSF.

La pipetta di vetro contenente l'elettrodo di registrazione viene a sua volta riempita con aCSF e posizionata immediatamente al di sotto del solco rinale, tra gli strati corticali II/III, facendo in modo che la punta tocchi appena la superficie della fettina.

Gli elettrodi di stimolazione, invece, vengono posizionati (sempre all'interno della corteccia peririnale), uno in senso dorso-rostrale sul versante temporale (area 35/36) e l'altro in senso ventro-caudale sul versante entorinale (area 35/corteccia entorinale) del solco rinale. Gli elettrodi devono essere quanto piu' possibile equidistanti (circa 1-2 mm) dall'elettrodo di registrazione, che si trova nel mezzo.

Gli elettrodi vengono stimolati alternativamente ogni 15s (0.033Hz); viene calcolata la media di quattro fEPSPs (field excitatory postsynaptic potentials) consecutivi per ognuno dei due input. Per ottenere la risposta, si utilizza inizialmente un input di circa 20V, modulato per ottenere risposte con picchi di ampiezza di valore compreso tra -0.50 e -1.00 mV. L'intensita' di stimolazione viene poi diminuita fino ad ottenere un'ampiezza pari a circa il 65-70% della risposta massimale. Si determina un periodo di registrazione costante (baseline) di circa 60 minuti prima di procedere all'induzione dell'LTP. Un periodo prolungato di baseline si e' reso necessario per assicurarsi che la risposta fosse realmente stabile e costante.

Per quanto riguarda gli esperimenti condotti in ippocampo, l'elettrodo di registrazione viene posto in area CA1, e i due elettrodi di stimolazione sono posizionati in modo da stimolare uno le Shaffer Collateral e l'altro la Perforant Path.

L'LTP e' stata indotta negli esperimenti-pilota utilizzando due protocolli distinti: quattro impulsi da 100 Hz, della durata di un secondo ciascuno, somministrati o ogni 20 secondi oppure ogni 5 minuti, come descritto da Scharf et al, 2002. In seguito il primo protocollo e' stato abbandonato e la LTP e' sempre stata indotta con quattro stimolazioni somministrate ogni 5 minuti, in quanto quest'ultima procedura risulta essere la piu' adatta per studiare la fase tardiva della LTP. Il

periodo di registrazione e' stato prolungato per circa 6 ore dopo l'induzione della LTP (controlli). Il depotenziamento è stato indotto tramite protocolli di Low Frequency Stimulation (LFS) a 5 HZ (3000 stimoli in 10 minuti) negli esperimenti condotti su tessuti adulti, o 1Hz (900 stimoli, 15 minuti) quando gli esperimenti venivano condotti su P14.
Maggiori dettagli sui metodi sperimentali di registrazione verranno presentati insieme ai singoli esperimenti.

Al termine dell'esperimento, si procede ad un wash-out con aCSF non contenente Ca2+. Cio' elimina la componente sinaptica della risposta e dunque permette l'esclusione della componente non-sinaptica dall'analisi dei picchi di ampiezza (fEPSP).
I dati ottenuti vengono registrati su un PC utilizzando il software Ltpm100 (Anderson and Collingridge, 2001), tramite il quale vengono anche, in seguito, rianalizzati e normalizzati rispetto ai valori della baseline. I dati vengono presentati graficamente come medie normalizzate ± Errore standard (SEM). I valori sono espressi come relativi alla baseline e le analisi statistiche sono state condotte utilizzando il paired student's t- test.

2.1.4 Agenti farmacologici

Soluzioni di stock sono state preparate dissolvendo le sostanze in dd$H_2$0, HCl o DMSO, in base a quanto ripostato sui datasheets di ciascun composto, aliquotate e conservate a -20°C. Tutte le soluzioni di stock sono state preparate ≥ 100x la concentrazione finale necessaria.

I composti sono stati ottenuti dai seguenti fornitori:

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-morp holinyl)pyrido[2,3-d]pyrimidin-7-yl]-2-methoxybenzeneme thanol*, 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 Biologia Molecolare

2.2.1 Western Blot.

La corteccia peririnale e' stata preparata da ratti adulti e aPND14 e conservata congelata a -80°C. Il giorno dell'esperimento, campioni di tessuto congelato sono stati lisati in 50mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA pH 8.0, 0.1% SDS, 1% Triton X-100 buffer, ed e' stato aggiunto in cocktail di inibitori di proteasi (Calbiochem, Gibbstown, NJ, USA). La concentrazione proteica di ogni campione e' stata determinate via Bio-Rad Assay e aliquote da 50µg di ciascun campione sono state sottoposte a standard SDS-PAGE e corse sugel di poliacrilamide al 12%, che sono stati in seguito trasferiti su membrane di nitrocellulosa (Hybond-C Extra Amersham Bio). Le membrane sono state poi bloccate per 1h con TTBS buffer contenente latte in polvere al 5% (100mM Tris-HCl, 0.9% NaCl, 0.1% Tween 20, pH 7.4). Le membrane sono state infine incubate overnight a 4°C con un anticorpo policlonale anti-PKCζ (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) o con un anticorpo monoclonale anti- β-actina (1:5000, Sigma Aldrich, Gillingham, Dorset, UK). Le membrane sono state lavate tre volte con TTBS e poi incubate per 1h con anticorpi secondare accoppiati a perossidasi, anti-rabbit or anti-mouse IgG rispettivamente (1:10,000 Sigma Aldrich, Gillingham, Dorset, UK). Tutte le incubazioni con anticorpi sono state condotte in un buffer di TTBS e latte al 5%. Le membrane sono state infine sviluppate utilizzando BM Chemiluminescence

Western Blotting Substrate (Roche, Burgess Hill, West Sussex, UK). I dati ottenuti sono stati graficati come livelli di proteina (misurati alla densitometria) normalizzati per I rispettivi livelli di actina (proteina strutturale normalmente utilizzata come controllo).

3 RUOLO E REGOLAZIONE DI PKMζ NELLA PLASTICITÀ SINAPTICA IN CORTECCIA PERIRINALE DI ADULTO

La funzione primaria della corteccia peririnale è nella memoria di riconoscimento degli oggetti. In seguito alla dimostrazione che vi è una risposta decrementale nei neuroni peririnale in seguito all'esposizione oggetti familiari (Brown e Xiang 1998), è stato proposto che i meccanismi che si basano su una depressione della trasmissione sinaptica nelle sinapsi di corteccia peririnale possano fornire un modello per comprendere come la corteccia peririnale sia coinvolta nel matenimento della memoria di riconoscimento (Brown e Aggleton, 2001; Brown e Bashir, 2002). Pertanto, la maggior parte della ricerca si è focalizzata sui meccanismi LTD-simili nella corteccia peririnale, quindi molto meno interesse è stato generato, al contrario, nello studio della LTP in questa particolare regione del cervello. Nonostante in letteratura siano presenti lavori che confermano la possibilità di indurre LTP negli strati II/III della corteccia peririnale di ratti adulti (Ziakopoulos, Tillett et al 1999; Massey, Johnson et al 2004), i meccanismi alla base del mantenimento delle fasi tardive LTP non sono mai stati ampiamente indagati . Così, gli esperimenti che seguono mirano a stabilire se è possibile indurre una LTP stabile nella corteccia peririnale, della durata di più di 3 ore, e quali sono i meccanismi molecolari coinvolti nella manutenzione di questo potenziamento. È stato dimostrato in passato in diverse aree cerebrali (ippocampo) che una forte HFS è in grado di indurre una robusta LTP, e che questo potenziamento dipende da una continua sintesi proteica (Abraham e Williams 2003;. Bozon, Davis et al 2003; Bozon , Kelly et al 2003;. Lynch 2004; Miyamoto 2006). Nei primi esperimenti (Frey, Krug et al 1988;. Abraham e Williams, 2003; Bozon, Davis et al 2003;. Bozon, Kelly et al 2003;. Lynch 2004; Miyamoto 2006) è stato dimostrato che un trattamento di 3 ore con Anisomicina (un potente inibitore della sintesi proteica) subito dopo la somministrazione di HFS provoca gradualmente la diminuzione del potenziale eccitatorio postsinaptico nell'ippocampo. Inoltre, (Scharf, Woo et al. 2002) Anisomicina blocca l'induzione di LTP se applicata da 30 minuti a 1 ora dopo la somministrazione di HFS in vitro. Lo stesso gruppo di ricerca ha dimostrato anche un protocollo particolare, chiamato

"Spaced" tetra-burst stimulation (4x100Hz trains somministrati a intervalli di 5 minuti), è in grado di indurre una LTP di maggiore durata, che sembra anche essere maggiormante dipendente dalla sintesi di nuovi proteine (Scharf, Woo et al. 2002). Un crescente numero di lavori dimostra che l'isoforma costitutivamente attiva di PKCζ, PKMζ, è necessaria e sufficiente per il mantenimento (ma non l'induzione), della LTP, sia in vitro che 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). Al contrario, nessun ruolo per PKMζ nell'induzione o il mantenimento di LTD è stato dimostrato; inoltre, la sintesi e l'attività di questa proteina sembrano essere down-regolate in seguito a LTD (Hrabetova e Sacktor 1996;. Osten, Hrabetova et al 1996; Hrabetova e Sacktor 2001). Inoltre, PKMζ è nota per essere costitutivamente attiva, nel senso che non ha bisogno di nessuno dei secondi messaggeri richiesti dalle full-length PKCs per esercitare la loro funzione (Sacktor, Osten et al. 1993). Tuttavia, come molte altre PKCs full-length, PKMζ necessita di essere fosforilata sul suo activation loop (T410), probabilmente dalla kinasi fosfoinositide-dipendente di tipo 1 (PDK1) (Kelly, Crary et al. 2007). Pertanto, le tematiche affrontate in questo lavoro sono (1) determinare che sia di fatto possibile indurre una robusta, stabile in LTP nella corteccia peririnale di ratto adulto, che si mantenga per almeno 5-6 ore (2) determinare se questa LTP è dipendente o no dalla sintesi proteica, (3) determinare il ruolo di PKMζ nel mantenimento del potenziamento sinaptico nella corteccia peririnale; (4) definire se PKMζ è in alcun modo coinvolta anche nella induzione e / o il mantenimento di LTD nella corteccia peririnale, (5) se è possibile modulare l'attività di PKMζ, e di conseguenza il suo effetto sul mantenimento della LTP attraverso l'inibizione della PDK1.

3.1 Risultati

3.1.1 Induzione di LTP in corteccia peririnale di ratto adulto

Dopo una registrazione di base di 60 minuti, LTP è stata indotta in una pathway utilizzando una tetra "Spaced" tetra-burts stimulation (4x100Hz trains, durata 1s, all intervallo di 5 minuti tra le stimolazioni). Nessuna stimolazione di condizionamento è stata somministrata all'altra pathway, che è stata usata come controllo. I dati sono stati normalizzati ai valori di baseline dei 60 minuti che precedono la somministrazione del protocollo HFS. La **figura 3.1** e **3.2** mostrano che é possible indurre una significativa LTP ($p < 0,05$), e il potenziamento rimane stabile per > 5h dopo la somministrazione del protocollo di HFS (126 ± 4% della baseline, n = 4).

3.1.2 La LTP in corteccia peririnale dipende dalla sintesi di nuove proteine

Dopo una registrazione di baseline di 60 minuti, LTP è stata indotta in una pathway utilizzando uno "Spaced" tetra-burts stimulation protocol (LTP: 135 ± 6% della baseline, $p < 0.05$, n = 5). Anisomicina 20μM è stata da 30 minuti prima ad 1 ora dopo l'induzione della LTP. Nessuna stimolazione è stata somministrata all'altra pathway, che è stato usata come controllo. I dati sono stati normalizzati ai 60 minuti di baseline che precedono la somministrazione del HFS. Le **figure 3.3** e **3.4** mostrano che l'anisomicina impedisce il mantenimento della LTP in corteccia peririnale (102 ± 5% di base, n = 5), a conferma che il mantenimento della potenziamento richiede la sintesi di nuove proteine.

3.1.3 **Ruolo di PKMζ nel mantenimento della LTP nella corteccia peririnale dell'adulto.**

Dopo un periodo di registrazione di baseline di circa 60 minuti, LTP viene indotta utilizzando il protocollo descritto in precedenza (LTP: 131 ± 5% della baseline, 60 min post HFS, $p < 0.01$, n=7). Il potenziamento così ottenuto viene registrato per le successive tre ore, dopodiché viene somministrato l'inibitore ZIP (5μM). Questa concentrazione di ZIP e' stata scelta in quanto e' quella che produce il massimo effetto inibitorio su PKM (Serrano et al., 2005). L'inibitore viene somministrato dopo 180 minuti dall'induzione della LTP in quanto ci permette di essere sicuri di trovarci sicuramente in fase di mantenimento del potenziamento. In seguito alla somministrazione di ZIP, si osserva un graduale ma rapido (120 minuti) ritorno ai valori di baseline nel pathway potenziato (**Figura 3.5** e **3.6**, 98 ± 5%; n=7, $p<0.01$. L'induzione della LTP è visualizzata come frecce con la punta rivolta verso l'alto). L'altro pathway e' utilizzato come controllo; ZIP dimostra di avere un piccolo, non significativo effetto anche sulla trasmissione sinaptica della pathway di controllo (93 ± 4 % of baseline, $P > 0.05$, n = 7).

Questi risultati ci permettono di affermare che, come nel'ippocampo, il mantenimento della LTP in corteccia peririnale è dipendente dall'attività di PKMζ, poiché l'inibizione specifica di questa proteina è sufficiente per annullare il potenziamento sinaptico.

Un minimo effetto del composto si evidenzia però anche nel pathway di controllo, non potenziato, con una lieve diminuzione (non statisticamente significativa) della risposta (circa 15-20%). Per fugare il dubbio che tale effetto fosse dovuto ad un casuale, lieve potenziamento delle pathway di controllo in seguito all'induzione di LTP sull'altra pathway (contaminazione), ulteriori esperimenti si sono resi necessari.

3.1.4 Attività di PKMζ su pathways non potenziate.

Per testare l'attività di PKMζ in pathways non potenziate, ZIP 5μM è stato somministrato dopo 60 minuti di registrazione, utilizzati come baseline di riferimento. Anche in questo caso è stato possibile notare una lieve depressione della risposta sinaptica, ancora nell'ordine del 15-20% (**Figura 3.7**, pathway temporale: 89 ± 2% della baseline, $p>0.05$; entorinale 88 ± 2% of baseline, $p>0.05$, $n=4$). Questi dati confermano che l'inibizione di PKMζ possiede un minimo effetto anche sulla trasmissione sinaptica di base. Una ipotesi che potrebbe spiegare questo effetto si fonda sul principio che PKMζ è in grado di incrementare la trasmissione sinaptica aumentando il numero di recettori AMPA postsinaptici (Ling et al., 2006). Si può pertanto ipotizzare che PKMζ possa essere coinvolta anche nella trasmissione sinaptica basale, modulando il trafficking costitutivo dei recettori AMPA (McCormack, Stornetta et al. 2006).

3.1.5 Ruolo di PKMζ nel depotenziamento sinaptico.

Dopo aver dimostrato il ruolo cruciale di PKMζ nel mantenimento della LTP, e posto che l'attività di questo enzima aumenta dopo induzione del potenziamento sinaptico, ci siamo domandati quale effetto un depotenziamento potrebbe avere sull'attività della kinasi.
Come mostrato in **figura 3.8** e **3.9**, in questi esperimenti l'induzione di LTP (132 ± 5% della baseline, $p<0.05$, $n=5$) in una delle pathways dopo una baseline di 60 minuti è stata seguita dalla somministrazione di un protocollo di depotenziamento (LFS, 5Hz) che riportava la trasmissione sinaptica a valori di baseline (101 ± 2% della baseline, $p>0.05$, $n=5$). 180 minuti dopo l'induzione della LTD, è stato somministrato l'inibitore ZIP (5μM). Tuttavia, il composto non ha mostrato di avere alcun effetto sulla risposta sinaptica, suggerendo

una down-regulation dell'attività di PKMζ in seguito al depotenziamento (98±4%, p>0.05, n=5).

Per quanto riguarda la seconda pathway, invece, dopo l'induzione della LTP (128 ± 5% della baseline, p<0.01, n=5), è stata diminuita l'intensità di stimolazione (freccia rivolta verso il basso) in modo da ottenere delle risposte sinaptiche di ampiezza simile a quelle del pattern depotenziato (99 ± 4% della baseline, p>0.05, n=5), mantenendo però il potenziamento indotto dalla somministrazione del protocollo di LTP. In questo caso, ZIP si è dimostrato capace di ridurre l'ampiezza della risposta sinaptica (65 ± 5% della baseline, p<0.01 v baseline, p<0.001 v la risposta misurata dopo al riduzione del'intensitá di misurazione, p<0.01 v l'effetto di ZIP nel pathway depotenziato, n=5), dimostrando dunque che PKMζ è ancora attiva.

3.1.6 Ruolo di PKMζ nel depotenziamento sinaptico in presenza dell'antagonista dei recettori NMDA per il glutammato, D-AP5.

Nella corteccia peririnale, la LTD (e dunque il depotenziamento sinaptico) è dipendente dall'attivazione dei recettori NMDA e può essere bloccata dall'antagonista dei recettori NMDA, D-AP5 (Massey et al., 2004). Poiché abbiamo dimostrato che l'attività di PKMζ è bloccata in seguito a depotenziamento, ci siamo chiesti quale potesse essere il comportamento dell'enzima quando il depotenziamento fosse inibito. Pertanto, come mostrato in **figura 3.10** e **3.11**, dopo l'induzione di LTP su una pathway (●,148 ± 7% della baseline, p<0.01, n=5), il protocollo di depotenziamento è stato somministrato in presenza dell'antagonista NMDA, D-AP5 (50μM, applicato 30 minuti prima della somministrazione della LFS fino alla fine del protocollo stesso). In questo caso, il depotenziamento è bloccato da D-AP5 (148 ± 8% della baseline, p<0.01 v baseline, p>0.05 v LTP, n=5) e ZIP, somministrato sempre 180 minuti dopo l'induzione di LTP, è ancora capace di ridurre la risposta sinaptica (102 ± 2% della baseline, p<0.001 v LTP, p>0.05 v baseline, n=5).

La pathway depotenziata, dunque, si comporta esattamente come la pathway di controllo (○), dove solo la LTP è stata indotta (137 ± 4% della baseline, $p<0.01$, n=5). Pertanto, se il depotenziamento è bloccato, l'attività di PKMζ non è inibita e l'enzima è ancora attivo, potendo essere in seguito inibito da ZIP (100 ± 4% della baseline, $p<0.001$ v LTP, $p>0.05$ v baseline, $p>0.05$ v ZIP nell'altra pathway, n=5). Quindi la down regulation dell'attività della kinasi è determinata da un depotenziamento sinaptico effettivo e dalle modificazioni intracellulari che ne conseguono (ad esempio attivazione di determinate fosfatasi che potrebbero, direttamente o indirettamente, avere un effetto sullo stato di PKMζ).

3.1.7 Ruolo di PKMζ nell'induzione di LTD nella corteccia peririnale di ratti adulti.

Una volta stabilito il ruolo di PKMζ nel mantenimento dell'LTP, e avendo dimostrato che la sua attivita' e' inibita dalla somminstrazione di un protocollo di depotenziamento, abbiamo voluto investigare sull'eventuale ruolo della kinasi nell'induzione di Long Term Depression (LTD), meccanismo ritenuto fondamentale nei processi di apprendimento in corteccia Peririnale (Brown and Aggleton, 2001). Come mostrato nella **figura 3.12** e **3.13**, Un protocollo di Low Frequency Stimulation (LFS) e' stato somministrato dopo una baseline di 30 minuti nella pathway di controllo (●) ottenendo LTD (69 ± 4% della baseline, $p<0.01$ v baseline, n=5)). 30 minuti dopo, ZIP 5μM e' stato perfuso nella camera di registrazione per 90 minuti, dopodiche' lo stesso protocollo di LFS e' stato indotto nell'altra pathway (○). Nonostante si noti una lieve, non significativa riduzione dell'ampiezza della risposta sinaptica (in entrambe le pathways) in seguito all'incubazione con ZIP (come osservato in precedenza, vedi punto 4), anche qui il protocollo di LFS si dimostra comunque in grado di indurre una riduzione dell'ampiezza della risposta sinaptica che si mantiene stabile per i successivi 30 minuti di registrazione (LTD) (70 ± 4% della baseline, $p>0.05$ v la pathway di controllo, $p<0.01$ v baseline, n=5). Tali

evidenze permettono di concludere che la preventiva inibizione dell'attivita' di PKMζ non influisce sulla possibilita' di indurre LTD in corteccia peririnale di ratto adulto, ne' sull'ampiezza della depressione indotta (non c'e' differenza tra l'LTD indotta in assenza di ZIP e quella indotta in sua presenza).

3.1.8 Effetto dell'inibizione sequenziale di PDK-1 e PKMζ sul mantenimento della LTP

Poiché PDK-1 è stato individuato come il fattore fondamentale in grado di permettere la piena attivazione di PKMζ, ulteriori studi sono stati condotti per verificarne il ruolo nel mantenimento della LTP, in combinazione con l'inibitore selettivo di PKMζ, ZIP. Per questo, l'acido 3-idrossi antranilico (3HAA) 100μM è stato somministrato 180 minuti dopo l'induzione della LTP in una pathway (**Figura 3.14** e **3.15**, 150 ± 8% della baseline, $p<0.01$ v baseline, n=6), lasciando l'altra come controllo. Anche in questo caso, in presenza di 3HAA la risposta sinaptica torna gradualmente ai valori di baseline (104 ± 3% della baseline, $p<0.01$ v LTP, $p>0.05$ v baseline, n=6); una volta raggiunti i valori di baseline, la successiva applicazione di ZIP 5μM non provoca ulteriore significativa diminuzione dell'ampiezza della risposta sinaptica (99 ± 5% della baseline, $p>0.05$ v 3-HHA, n=6). Tali risultati ci permettono di confermare il ruolo di PDK1 nel mantenimento della LTP e forniscono un'ulteriore indizio a supporto che tale effetto possa essere correlato all'attivazione di PKMζ dipendente da PDK-1. Tuttavia, dal momento che PDK1 è attivo su una gamma molto ampia di target cellulari, questi esperimenti da soli non sono sufficienti a dimostrare che l'annullamento del potenziamento sinaptico avviene solo attraverso l'inibizione della PKMζ. Altri meccanismi molecolari potrebbero essere coinvolti.

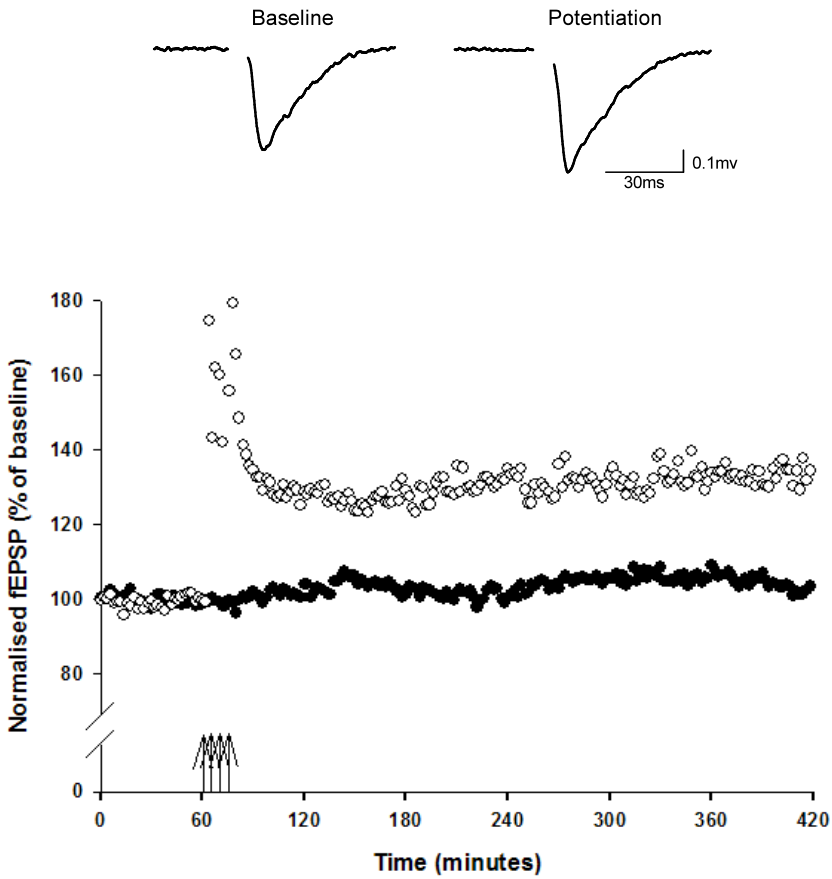

Figura 3.1 Singolo esperimento di induzione di LTP in corteccia peririnale di adulto. La pathway P0 (cerchi neri) e' usata come controllo. Un protocollo di "spaced" tetra-burst stimulation (4x100Hz, 5 minutes interburst interval) produce una consistente e stabile LTP (cerchi bianchi), che si mantiene per almeno 6 ore dalla sua induzione. Le frecce rivolte verso l'alto rappresentano il momento in cui lo stimolo e' stato somministrato. Le tracce mostrate in alto rappresentano un esempio di risposta sinaptica evocata, a livelli di baseline e potenziata.

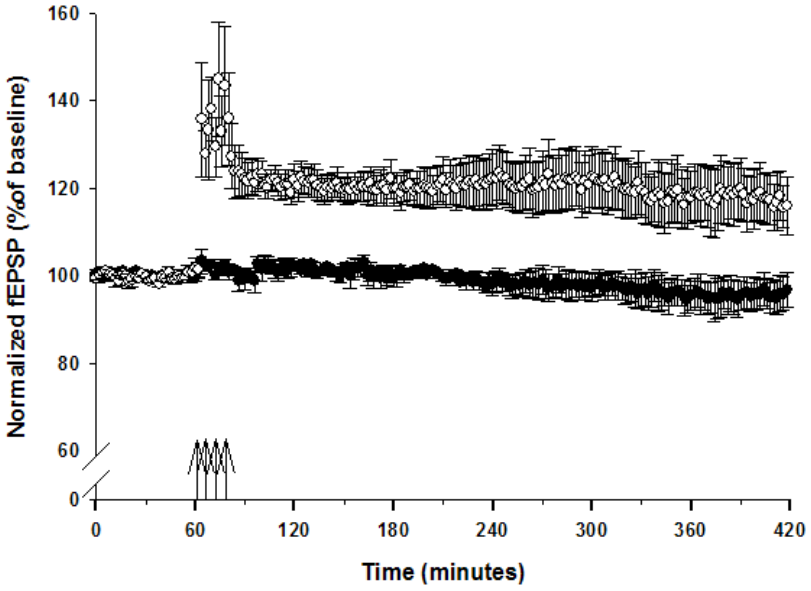

Figura 3.2 Dati totali per l'induzione di LTP nella corteccia peririnale di adulto il protocollo di spaced tetra bust stimulation (4x100Hz) induce una consistente e stabile LTP (cerchi bianchi, 126 ± 4% della baseline, $p<0.05$, n=4). Il potenziamento dura per oltre 5 ore.

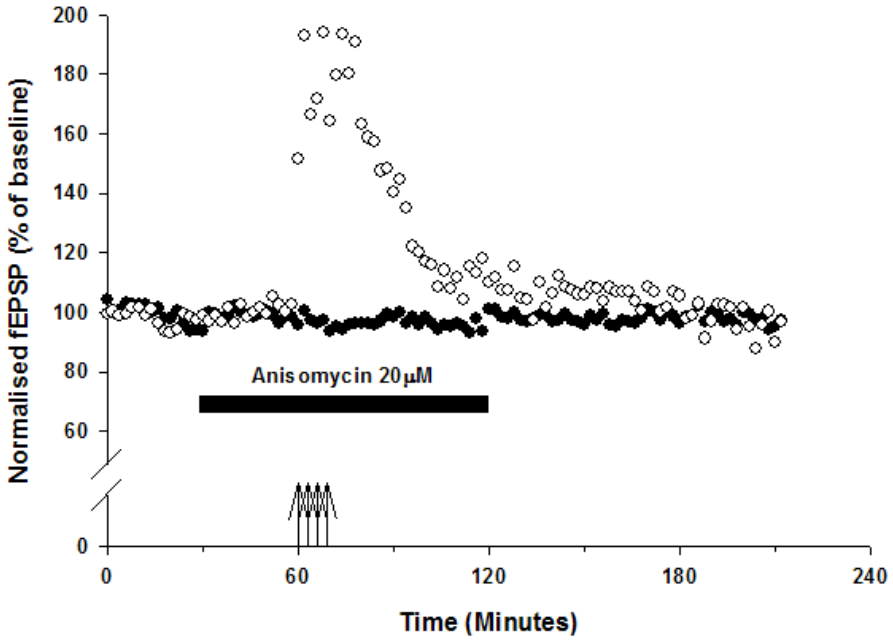

Figura 3.3 Singolo esempio dell'effetto di anisomicina su LTP in corteccia peririnale di adulto. L'incubazione con anisomicina 20μM blocca il mantenimento della LTP nella pathway P1 (cerchi bianchi). L'anisomicina non sembra avere effetto sulla pathway di controllo (cerchi neri).

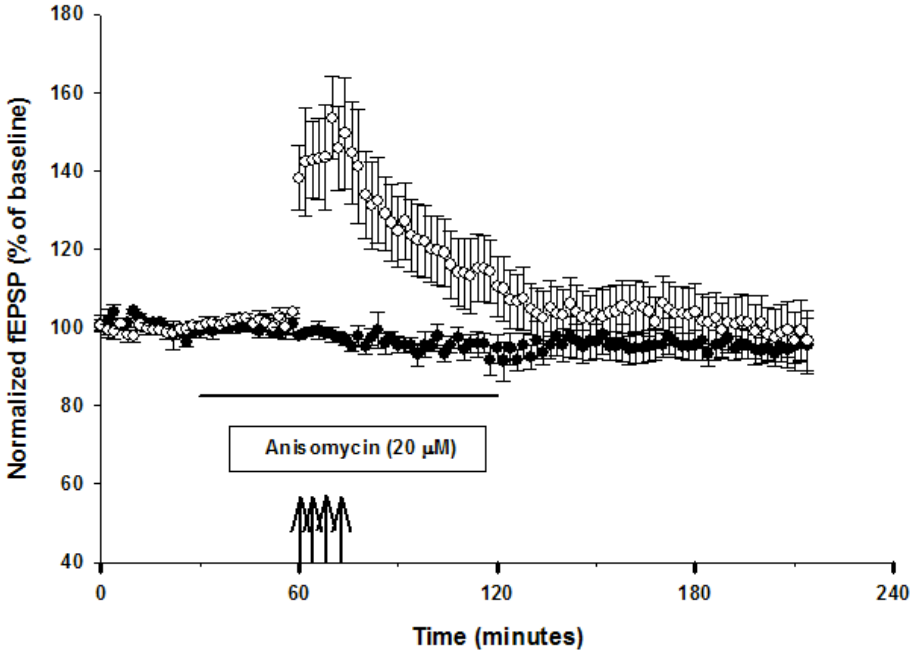

Figura 3.4 Dati totali sull'effetto di anisomicina su LTP nella corteccia peririnale dell'adulto L'incubazione con anisomicina 20μM bocca il mantenimento di LTP (cerchi bianchi, 102 ± 5% della baseline, p>0.05 v baseline, n=5).

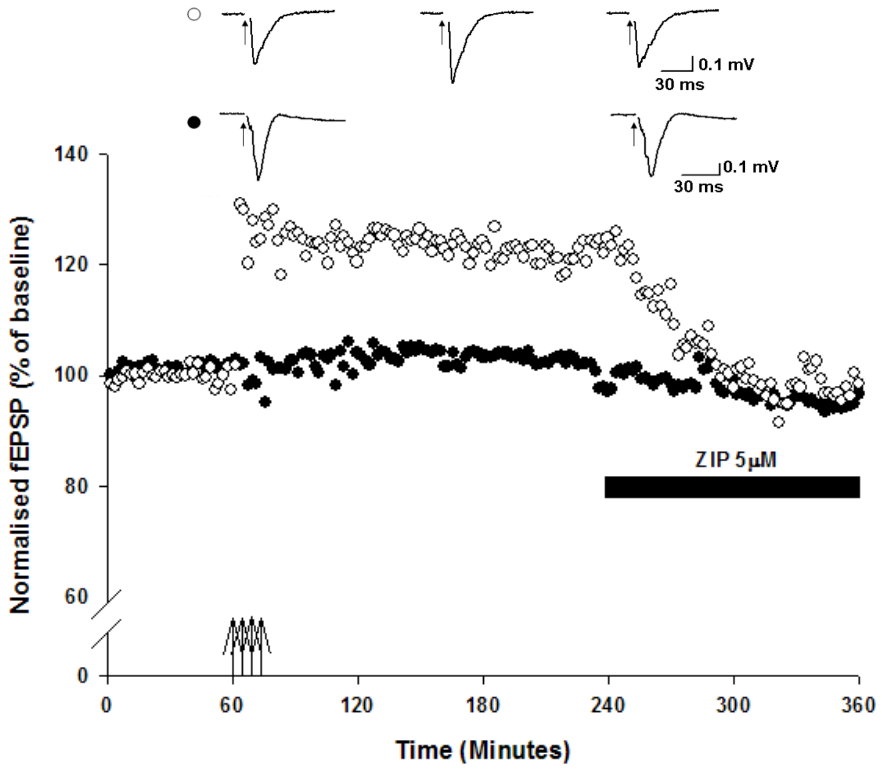

Figura 3.5 Singolo esperimento sull'effetto dell' inibizione di PKMζ su LTP in corteccia peririnale di adulto L'applicazione dell;inibitore di PKMζ, ZIP 5μM, annulla il mantenimento dell'LTP indotto da tetra-burst stimulation in P1 (cerchi bianchi). Le frecce all'insu' indicano il momento di somministrazione della HFS. ZIP mostra un piccolo effetto anche sulla pathway di controllo (P0, cerchi neri).

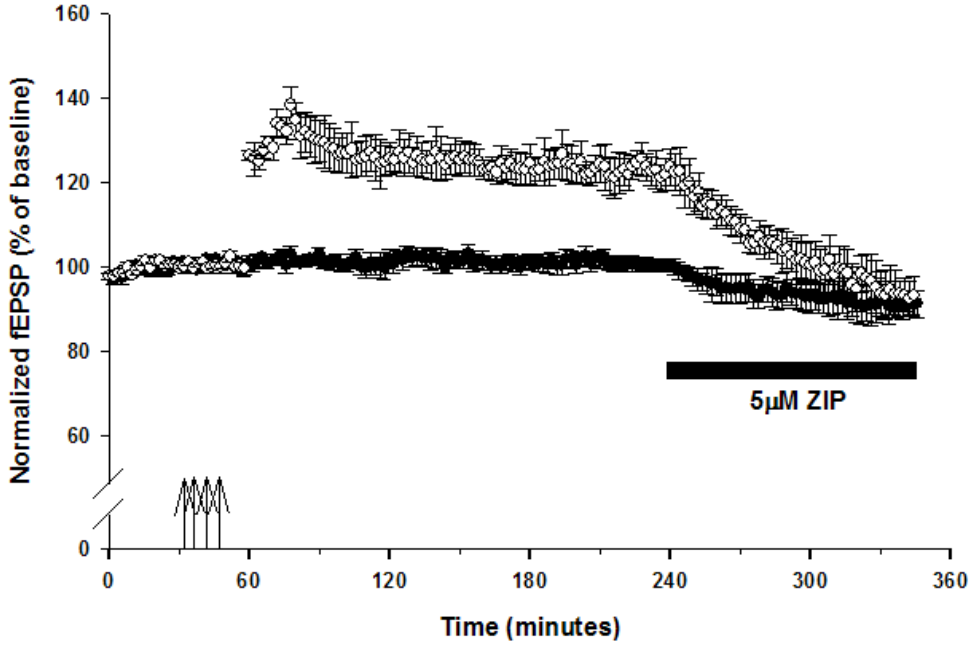

Figura 3.6 Dati totali sull'effetto dell' inibizione di PKMζ su LTP in corteccia peririnale di adulto ZIP 5μM annulla completamente il mantenimento di LTP (cerchi bianchi, 98 ± 5 % della baseline, $p<0.01$, $n=7$) senza mostrare un effetto significativo sull'input di controllo (cerchi neri, 93 ± 4 % della baseline, $p>0.05$).

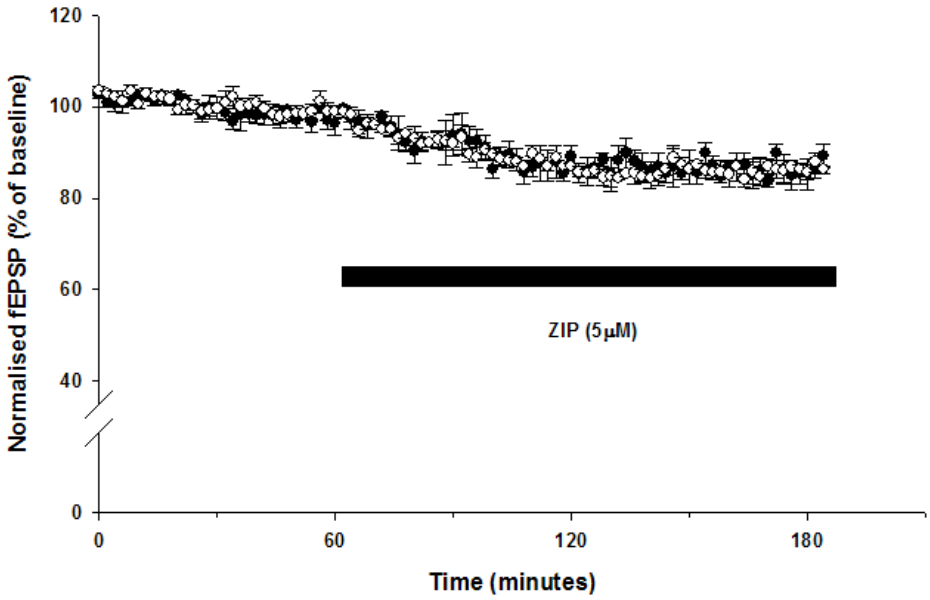

Figura 3.7 Dati totali sull'effetto dell'inibizione di PKMζ sulla trasmissione sinaptica basale in corteccia peririnale di adulto ZIP 5μM produce una piccola, non significativa diminuzione della risposta sinaptica di base. Pathway temporale pathway (cerchi neri: 89 ± 2% della baseline, $p>0.05$. Pathway entorinale (cerchi bianchi): 88 ± 2% della baseline, $p>0.05$, $n=4$).

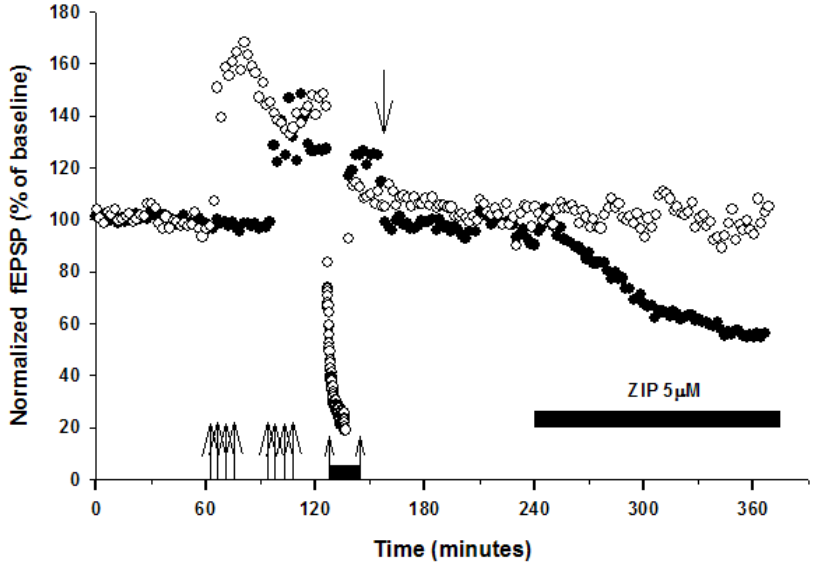

Figura 3.8 Singolo esperimento sull'effetto di ZIP nel depotenziamento in corteccia peririnale di adulto ZIP 5μM non ha effetto in una pathway depotenziata (P0, cerchi neri, HFS seguito da LFS) l'inibizione di PKMζ deprime la risposta sinaptica in una pathway potenziata dove l'ampiezza della risposta sia stata diminuita da una riduzione dell'intensita' di stimolazione (P0, cerchi bianchi). Le due frecce all'insu' unite dal rettangolo nero rappresentano la LFS. La freccia rivolta all'ingiu' rappresenta la riduzione dell'intensita' di stimolazione in P1.

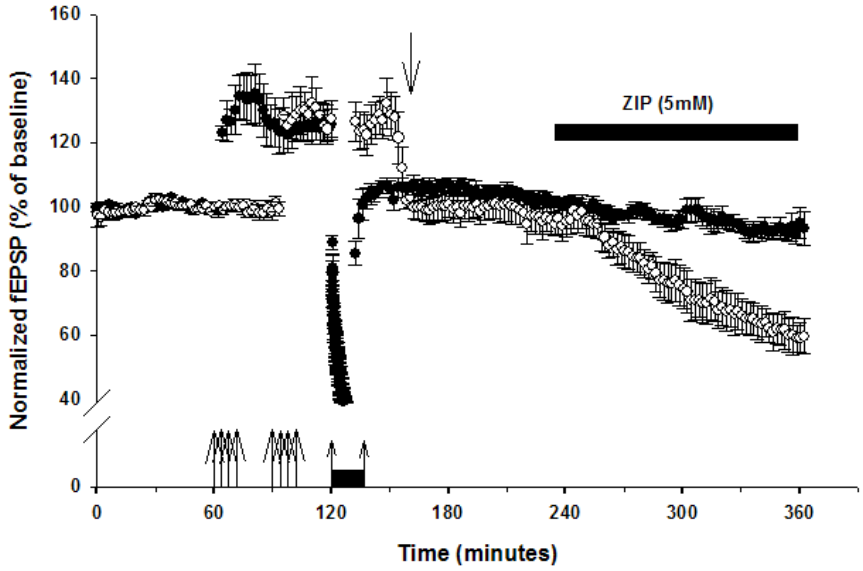

Figura 3.9 Dati totali sull'effetto di ZIP nel depotenziamento in corteccia peririnale di adulto ZIP 5μM non ha effetto su una pathway depotenziata (cerchi neri:98 ± 4% della baseline, $p>0.05$, $n=5$). Dopo l'induzione di LTP nell'altro input (cerchi bianchi), l'ampiezza della risposta sinaptica e' diminuita da una riduzione dell'intensita' di stimolazione in modo da ottenere gli stessi valori dell'altro input. ZIP deprime significativamente questa pathway, ancora potenziata (65 ± 5% della baseline, $p<0.001$ v la risposta misurata dopo la riduzione dell'intensita' di stimolazione).

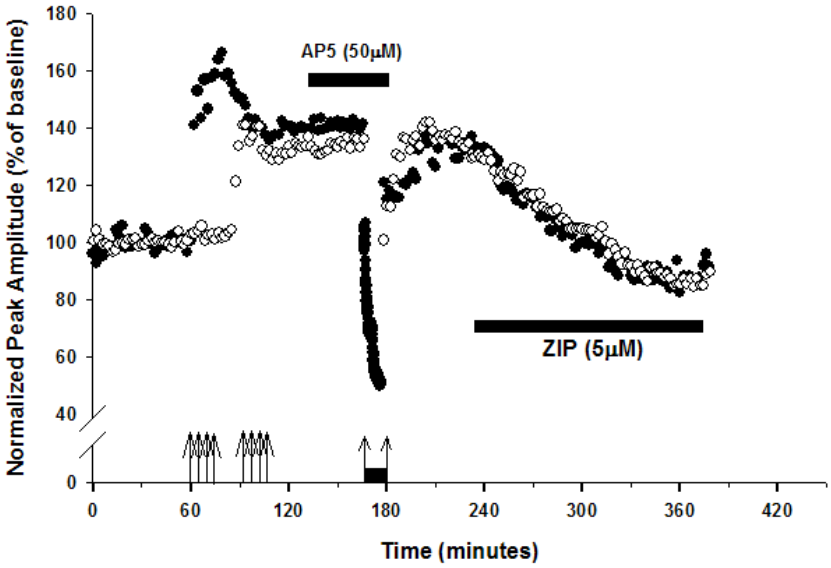

Figura 3.10 Singolo esperimento sull'effetto dell'inibizione di PKMζ sul depotenziamento in presenza di AP5 in corteccia peririnale di adulto Quando il depotenziamento e' boccato da AP5 (cerchi neri), ZIP determina una riduzione della risposta sinaptica simile a quella prodotta in una pathway potenziata (cerchi bianchi).

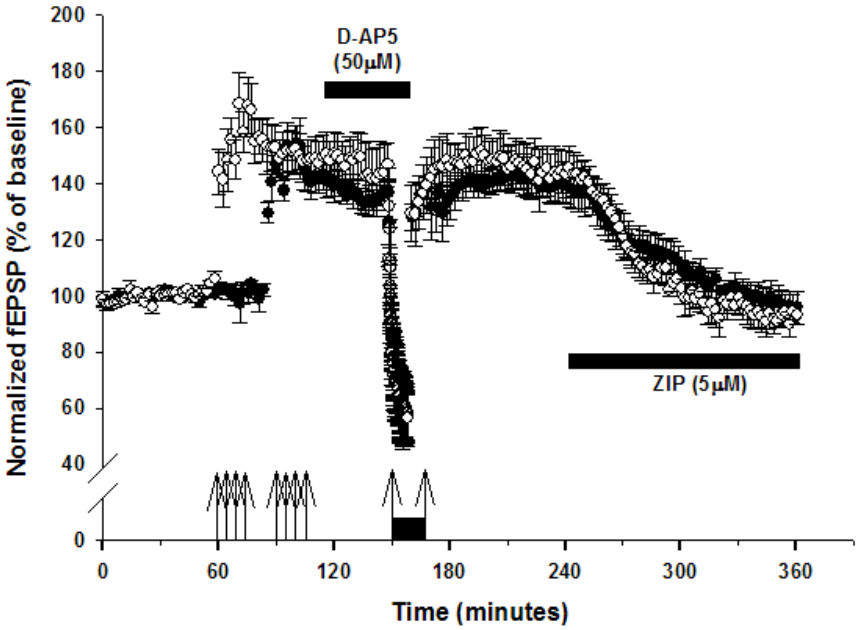

Figura 3.11 Dati totali dell'effetto dell'inibizione di PKMζ sul depotenziamento in presenza di AP5 in corteccia peririnale di adulto Quando il depotenziamento e' boccato da AP5 (cerchi neri), ZIP diminuisce la risposta sinaptica, riportandola a valori di baseline (102 ± 2% della baseline, $p<0.001$ v LTP, $p>0.05$ v baseline, n=5). Sull'altro input (cerchi bianchi), l'applicazione di ZIP 3 ore dopo l'induzione di LTP annulla il potenziamento (100 ± 4% of baseline, $p<0.001$ v LTP, $p>0.05$ v baseline, $p>0.05$ v "LTD" input dopo l'applicazione di ZIP, n=5).

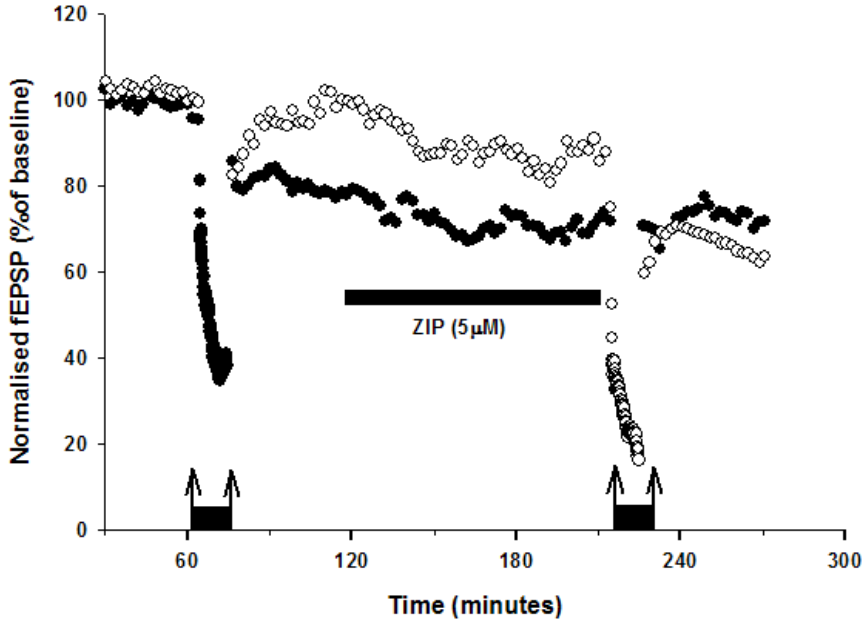

Figura 3.12 Singolo esperimento sull'effetto dell'inibizione di pKMζ nell'induzione di LTD nella corteccia peririnale di adulto ZIP non ha effetto su una pathway depotenziata (cerchi neri). Una Pre-incubazione con ZIP non influisce sull'induzione di LTD (cerchi bianchi).

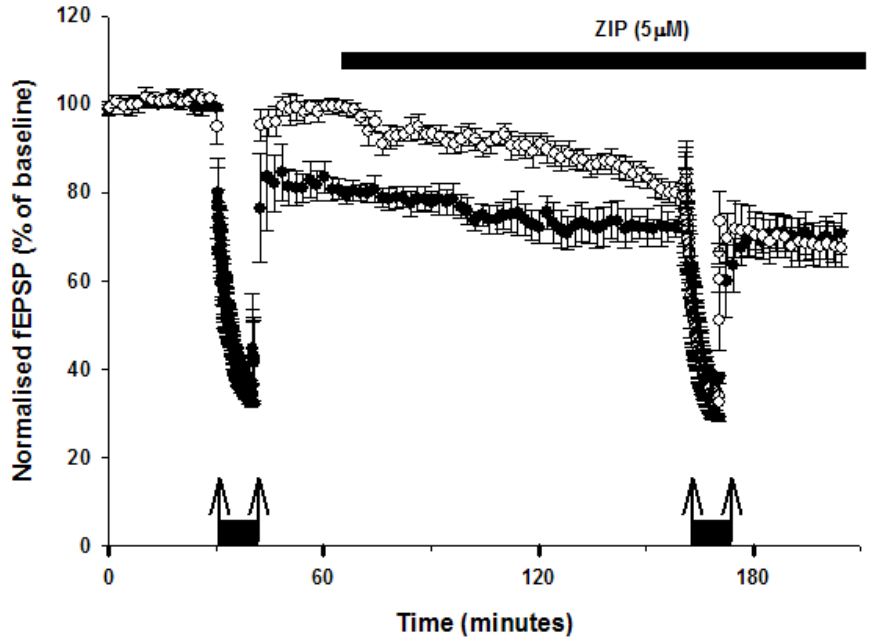

Figura 3.13 Dati totali sull'effetto dell'inibizione di pKMζ nell'induzione di LTD nella corteccia peririnale di adulto L'inibizione di PKMζ non ha effetto sull'induzione di LTD in corteccia peririnale (cerchi bianchi 69 ± 4% della baseline, $p<0.01$ v baseline, n=5). Non c'e' differenza d'ampiezza tra la depressione indotta da LFS in seguito a pre-incubazinoe con ZIP e la LTD ottenuta nella pathway di controllo (cerchi neri, 70 ± 4% of baseline, $p>0.05$ v pathway sperimentale, $p<0.01$ v baseline).

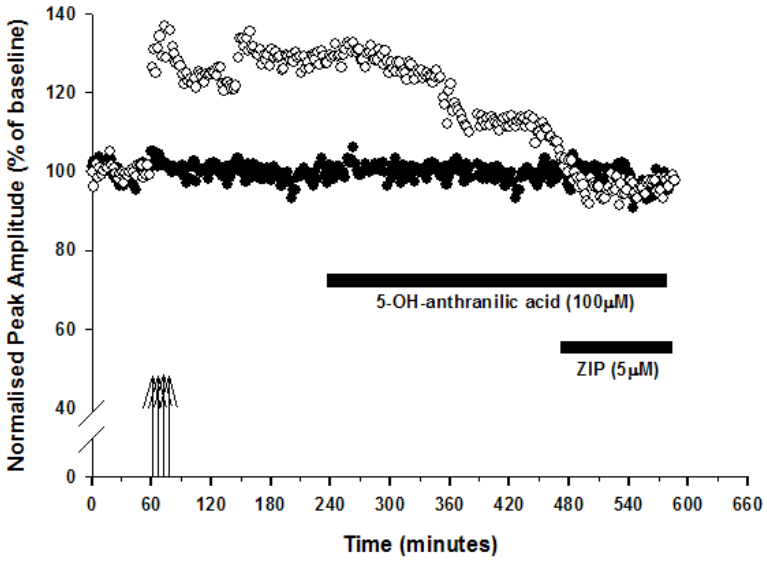

Figura 3.14 Singolo esperimento sull'effetto dell'inibizione di PDK1 su LTP in corteccia peririnale di adulto L'inibizione di PDK1 annulla la LTP lentamente ma competamente (cerchi bianchi). La successiva applicazione di ZIP non mostra alcun effetto sulla trasmissione sinaptica.

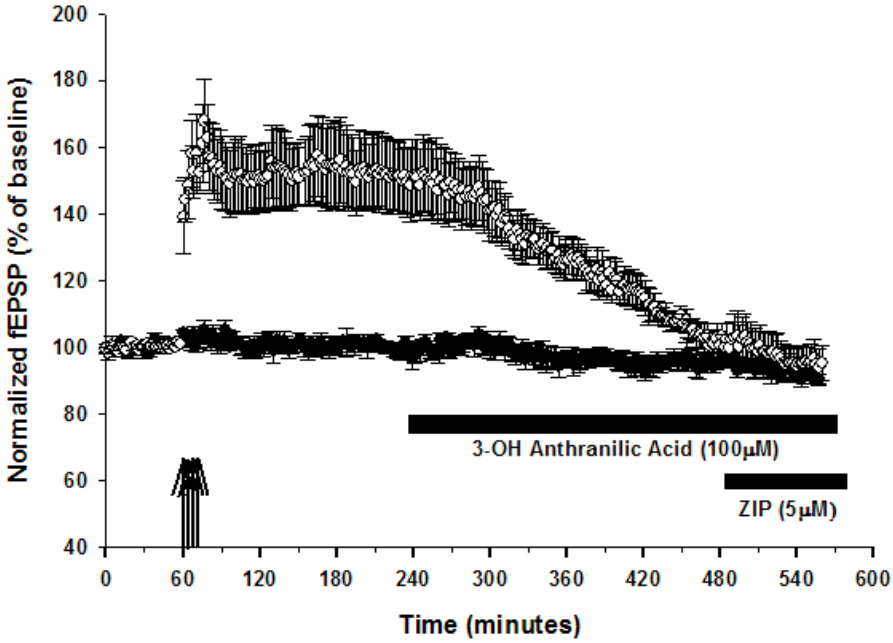

Figura 3.15 Dati totali per l'effetto dell'inibizione di PDK1 sulla LTP in corteccia peririnale di adulto L'inibizione di PDK1 annulla completamente la LTP (cerchi bianchi, 104 ± 3% della baseline, $p<0.01$ v LTP, $p>0.05$ v baseline, n=6). La successiva applicazione di ZIP non provoca ulteriori diminuzioni dell'ampiezza della risposta sinaptica (99 ± 5% of baseline, $p>0.05$ v 3-HHA, n=6). Nessuno dei due composti modifica sostanzialmente la trasmissione sinaptica nella pathway di controllo (cerchi neri)

3.2 Discussione

Questi risultati dimostrano che una tetra-burst stimulation è in grado di indurre una stabile e duratura LTP corteccia peririnale di ratti adulti. Il protocollo HFS utilizzato qui era una cosiddetta stimolazione "Spaced", che sembra produrre una LTP di più lunga durata e più dipendente dalla sintesi proteica (Scharf, Woo et al. 2002). Tuttavia, altri protocolli (ad esempio 4 stimoli a100Hz, somministrati a intervalli di 30 secondo) sono in grado di indurre LTP in corteccia peririnale di adulto, senza differenze significative con l'LTP indotta dalla stimolazione "Spaced" (dati non riportati). Questi risultati sono interessanti, poiché la maggior parte degli studi finora si sono concentrati sui processi LTD nella corteccia peririnale, che sono alla base della memoria di riconoscimento. Pertanto, non si sa molto sui meccanismi di LTP nella corteccia peririnale.

Questi risultati mostrano anche che il mantenimento di LTP in corteccia peririnale di adulto sembra dipendere dalla sintesi di nuove proteine. L'applicazione di anisomicina, un potente inibitore della sintesi proteica, annulla completamente il potenziamento stabilito. Questi risultati sono perfettamente in linea con quanto è stato trovato in altre aree cerebrali, ad esempio l'ippocampo, in cui è possibile bloccare il mantenimento, ma non l'induzione di LTP mediante l'applicazione di anisomicina (Frey, Krug et al 1988;. Scharf, Woo et al . 2002). In altre parole, ancora una volta, il mantenimento della LTP si basa sulla sintesi di nuove proteine (Kelly, Mullany et al 2000;. Abramo e Williams, 2003; Lynch 2004; Miyamoto 2006; Reymann e Frey 2007). Tra le proteine che possono essere coinvolte nel mantenimento di LTP, un ruolo cruciale sembra svolto dalle kinasi, come la proteina kinasi C (PKC), CaM kinasi, MAP kinasi, 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)

Ci sono prove che PKMζ svolga un ruolo cruciale nel mantenimento della LTP nell'ippocampo (Sacktor, Osten et al 1993;. Serrano, Yao et al 2005.), e che

la formazione di nuove molecole di PKMζ dipenda dalla sintesi di nuove proteine indotta da LTP (Osten, Valsamis et al. 1996). Questi risultati dimostrano che nella corteccia peririnale adulta, l'applicazione dell' inibitore selettivo di PKMζ, ZIP, annulli completamente l' LTP, quando somministrato durante la fase di mantenimento (cioè 3 ore dopo l'induzione della LTP). Pertanto, in corteccia peririnale, così come in ippocampo, il mantenimento della LTP si basa sulla continua attività di PKMζ. In contrasto con i lavori precedenti (Serrano, Yao et al. 2005), si osserva che ZIP produce un piccolo effetto non significativo anche sulla trasmissione sinaptica basale. Questa azione non è del tutto sorprendente, poiché è noto che PKMζ mantiene LTP attraverso la regolazione del traffico di membrana dei recettori AMPA (Ling, Benardo et al 2006;. Yao, Kelly et al 2008;. Migues, Hardt et al 2010).. Sebbene l'azione di PKMζ è specifica per le sinapsi potenziate, non è del tutto improbabile che ci sia qualche piccolo effetto meno specifico anche sull' attività basale di alcune sinapsi non-potenziate. Vale a dire, PKMζ potrebbe modificare il trafficking costitutivo dei recettori AMPA (McCormack, Stornetta et al. 2006). In alternativa, come suggerito dal prof. MW Brown, potrebbe anche essere che quella che noi consideriamo una risposta basale sinaptica della via neuronale, sia in realta' già potenziato, ad un certo livello. Dal momento che gli animali utilizzati in questo set di esperimenti sono adulti, non è improbabile che posseggano qualche ricordo codificato nella corteccia peririnale, il che significa che alcune sinapsi potrebbero essere già potenziate in una certa quantità, e quindi mostrare un aumento di attività del PKMζ. Quando viene attivata, PKMζ avvia un ciclo di feedback positivo di auto mantenimento della propria sintesi durante il mantenimento di LTP (Kelly, Crary et al. 2007), che dura finché dura la memoria. L'applicazione del'inibitore ZIP, bloccando la PKMζ attiva, a prescindere da quando la proteina è stata sintetizzata, puo' andare ad incidere sull'attivita' di dette sinapsi pre-potenziate.

Studi precedenti mostrano che, mentre la sintesi di PKMζ dipende da LTP, PKMζ e' down-regolata in seguito a LTD (Hrabetova e Sacktor 1996; Osten,

Hrabetova et al 1996;. Hrabetova e Sacktor 2001). Pertanto, l'inibizione di PKMζ in una pathway depotenziata non dovrebbe mostrare alcun effetto sulla trasmissione sinaptica. Un effetto simile poterbbe essere ottenuto anche quando LTP (che induce PKMζ) è seguita da LTD (che down-regola PKMζ), un processo che riporta la risposta sinaptica a livelli di baseline. Questa ipotesi è confermata dai risultati riportati in figura 3.9, dove nessun effetto di ZIP è evidenziabile quando l'inibitore viene somministrato in una pathway depotenziata. Inoltre, questi risultati mostrano che l'effetto di inibizione PKMζ non dipende dall'ampiezza della risposta sinaptica, misurata ma dal suo reale stato di potenziamento, e da diverse sottostanti modificazioni intracellulari. Infatti, quando l'ampiezza della risposta sinaptica potenziata è attivamente diminuita da una riduzione della intensità di stimolazione, ZIP è ancora in grado di ridurre la risposta sinaptica. Ciò accade perché, anche se le dimensioni della risposta sinaptica nella pathway potenziata sono della stessa ampiezza di quelle della pathway depotenziata, nel primo caso i meccanismi di mantenimento della LTP sono ancora attivi. Pertanto ZIP, inibendo la PKMζ attiva, è in grado di produrre una diminuzione evidente della trasmissione sinaptica.

Ci sono numerosi lavori che dimostrano che PKMζ è down-regolata nella LTD. LTD dipende dall'attività recettori NMDA (Dudek e Bear 1992; Mulkey e Malenka 1992; Wexler e Stanton 1993; Stanton 1996), quindi, se questi recettori sono bloccati dall'antagonista D-AP5, un protocollo di LFS non è in grado di produrre LTD (Massey, Johnson et al. 2004). Pertanto, il blocco di NMDA, non consentendo l'espressione di LTD, dovrebbe impedire la down-regulation di PKMζ indotta dalla LFS. Per esaminare questa ipotesi, dopo l'induzione della LTP, le fette di corteccia peririnale di adulto sono state incubate con il D-AP5. La somministrazione di LFS su un percorso non è riuscita a indurre un depotenziamento; la successiva applicazione di ZIP riporta la risposta al livelli di base. Questi risultati, quindi, suggeriscono che la somministrazione di LFS da sola non è sufficiente ad indurre l'attesa down-regolation dell'attività PKMζ. I fenomeni di signalling intracellulare alla base del depotenziamento effettivo della risposta sinaptica (cioè l'attivazione della

proteina fosfatasi) sono obbligatori al fine di bloccare la sintesi / attivazione di PKMζ. Una possibile spiegazione potrebbe essere che alcune protein fosfatasi possano bloccare l'attività di alcune proteine responsabili della sintesi / attivazione di PKMζ, come ad esempio PDK1, (Kelly, Crary et al. 2007). Pertanto, se LFS, a causa del blocco AP5-dipendente dei recettori NMDA, non riesce ad attivare queste fosfatasi, le vie di segnalazione intracellulare che portano all'attivazione di PKMζ sono ancora completamente funzionanti. Questo potrebbe spiegare i risultati osservati.

Inoltre, come mostrato nella Figura 3.9, poiché LTD down-regola l'attività di PKMζ, l'applicazione della ZIP dopo LFS non ha alcun effetto sulla risposta sinaptica. Nonostante cio' non era chiaro se PKMζ potesse avere qualche effetto l'induzione di LTD. Per esaminare questo punto, le fette sono state pre-incubate con ZIP per 90 minuti, poi LFS è stato somministrato. Come previsto, l'inibizione della PKMζ non ha alcun effetto nella induzione di LTD. In effetti, secondo le conoscenze attuali, PKMζ non sembra essere coinvolta in meccanismi LTD-simili. Inoltre, PKMζ non è coinvolta nella induzione di LTP (Serrano, Yao et al. 2005), ma solo nel suo mantenimento. Pertanto, tutte le evidenze suggeriscono ancora una volta che l'up- o down-regulation dell'attività di PKMζ sono secondarie all'induzion di LTP o LTD, e sono coinvolte solo nel mantenimento delle fasi tardive di tali processi.

Infine, l'ultima serie di esperimenti ha iniziato ad esaminare la regolazione dell'attività di PKMζ. Anche se sembra essere universalmente accettato che PKMζ, una volta attivato, auto-mantiene la propria attività attraverso un ciclo di feedback positivo, non si sa molto sui processi che portano all'attivazione di PKMζ. La principale proteina coinvolta nella attivazione di PKMζ è stata identificata in PDK1. PKC "convenzionali" e "nuove", infatti, sono innescate dalla fosforilazione ad opera di PDK1, e successivamente vengono pienamente attivate dalle cascate di signalling dei secondi messaggeri intracellulari. PKCS appartenenti al gruppo atipico, come PKCζ, invece, sono immediatamente attivate dalla fosforilazione da parte di PDK1, in un modo

"on / off" (LeGood, Ziegler et al 1998;. Dong, Zhang et al 1999;. Balendran, Biondi et al 2000;. Balendran, Hare et al 2000). Pertanto, l'inibizione dell'attività PDK1 dovrebbe indirettamente bloccare l'attività di PKMζ. L'applicazione di 3HAA annulla lentamente il mantenimento della LTP, e quando la risposta è di nuovo i valori di baseline, l'applicazione di ZIP non produce alcuna ulteriore calo nella risposta. Questi risultati, quindi, confermano la nostra ipotesi, ossia che l'inibizione di PDK1, impedendo la fosforilazione e quindi l'attivazione di PKMζ, cancella la LTP. La mancanza di effetto di ZIP conferma questa ipotesi. 3HAA porta la risposta al basale più lentamente di ZIP. Questo può accadere perché l'effetto sul PKMζ è indiretto, e richiede prima l'inibizione completa di PDK1 che, di conseguenza, non può fosforilare PKMζ. Quindi, ci vuole più tempo per poter completamente bloccare l'intero processo. In ogni caso, anche se i risultati degli esperimenti sono coerenti con l'ipotesi, PDK1 è una proteina che agisce su molti processi intracellulari, come la crescita e proliferazione cellulare attraverso una cascata di signalling che coinvolge PI3K/Akt/mTOR (Bayascas 2008; Kawauchi, Ogasawara et al . 2009). Pertanto, non si può escludere che l'effetto osservato non si verifichi solo attraverso una inibizione diretta dell'attività PKMζ, ma anche attraverso altri meccanismi (ad esempio attraverso l'inibizione di una sintesi proteica più ampia e meno specifica mTOR-dipendente).

4 RUOLO DI PKMζ NELLA PLASTICITÁ SINAPTICA NELLA CORTECCIA PERIRINALE DURANTE IL NEUROSVILUPPO

4.1 Introduzione

La precedente serie di esperimenti ha dimostrato che possibile indurre LTP in corteccia peririnale di adulti, e che questa LTP può essere completamente annullata dall'inibizione di PKMζ. È interessante notare che alcuni lavori realizzati in questo stesso laboratorio mostrano che negli animali giovani, cioè al quattordicesimo giorno post-natale (PND14 o P14), diversi protocolli di HFS (100 Hz, 1s) che di solito producono LTP in differenti aree del cervello (ippocampo) in età diverse, non riescono a indurre LTP in corteccia peririnale (osservazione King e Bashir, inedito). Anche protocolli che sono noti per indurre LTP in corteccia peririnale di adulti, come 4xHFS (Ziakopoulos, Tillett et al 1999;. Massey, Johnson et al 2004.) o la "Spaced" tetra-burst stimolation (Scharf, Woo et al 2002). non hanno prodotto LTP in corteccia peririnale neonatale. Nepure due diversi protocolli di stimolazione theta burst sono stati efficaci nell'indurre la LTP (osservata da King e Bashir, inedito). Questo fenomeno può trovare due spiegazioni possibili. Una possibilità è che i meccanismi di LTP semplicemente non esistono in questa fase del neurosviluppo, quindi tutti i tentativi di indurre qualsiasi tipo di potenziamento a questa etá risultano inefficaci. Una spiegazione alternativa è, al contrario, che i meccanismi alla base della espressione della LTP esistono, ma sono già completamente saturi, di conseguenza, l'induzione di LTP è occlusa. Per esaminare queste possibilità, LFS (1 Hz, 900 s) è stato somministrato per indurre LTD; la successiva somministrazione di HFS (100 Hz, 1s) ha determinato un potenziamento della trasmissione sinaptica, che ha riportato la risposta sinaptica ai livelli basali (osservato da King e Bashir, non pubblicato). Pertanto, queste osservazioni suggeriscono che meccanismi che portano ad un potenziamento a lungo termine esistono in corteccia peririnale neonatale, ma in condizioni basali questi meccanismi sono molto probabilmente saturi e un potenziamento di lunga durata della sinapsi è possibile solo se la saturazione è preventivamente revertita.

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Dal momento che l'attività di PKMζ è fondamentale per il mantenimento della LTP (Hrabetova e Sacktor 1996;. Ling, Benardo et al 2002), se i meccanismi di LTP sono saturi in condizioni basali allora questo fenomeno potrebbe derivare da un'attivazione costitutiva della PKMζ. Se questo è il caso, allora l' inibizione di PKMζ dovrebbe deprimere la trasmissione sinaptica basale. Inoltre, se LTD annulla il mantenimento di LTP determinando una diminuzione dell'attività di PKMζ, l'inibizione della PKMζ non dovrebbe avere alcun effetto sulla trasmissione sinaptica successiva a LTD. Inoltre, quando una depressione LFS-indotta della trasmissione sinaptica è revertita dalla somministrazione di HFS (de-depressione), se questo meccanismo dipende dalla riattivazione di PKMζ HFS-indotta, l'applicazione di ZIP dovrebbe diminuire di nuovo la trasmissione sinaptica. Inoltre, eravamo interessati a determinare, se possibile, un time-course per questi fenomeni durante le varie fasi di sviluppo neurologico. In altre parole, quando il cervello acquisisce la possibilità di esprimere LTP? Abbiamo testato queste ipotesi con la seguente serie di esperimenti.

4.2 Risultati

4.2.1 Attivita' di PKMζ in pathway depotenziate in corteccia peririnale di P14.

La nostra ipotesi era che, in corteccia peririnale neonatale (P14), la trasmissione sinaptica basale è già impostata su livelli molto elevati, occludendo l'induzione sperimentale di qualsiasi ulteriore potenziamento. Se PKMζ è coinvolta nel mantenimento di questi alti livelli di trasmissione di base, la sua inibizione dovrebbe portare ad una diminuzione della risposta evocata sinaptica, mentre nessun effetto deve essere osservato in una pathway completamente depotenziata.

Dopo una registrazione di baseline della durata di 30 minuti, tre LFS (1Hz) sono state somministrate nella stessa pathway a distanza di circa 15 minuti l'una dall'altra, in modo da ottenere una LTD saturata (nessuna differenza statisticamente significativa tra i valori registrati dopo il secondo train di LFS e il terzo) di notevole ampiezza (54 ± 4 % della baseline 30 min dopo l'ultima LFS, $p < 0.001$, $n = 7$; **Figura 4.1**). L'altra pathway e' stata lasciata come controllo. La perfusione di ZIP 5uM e' stata iniziata 40 minuti dopo l'ultimo train di LFS e l'esperimento e' stato condotto a termine in presenza del composto.

I risultati dimostrano che ZIP non ha alcun effetto sulla pathway depotenziata (94 ± 9 % della risposta sinaptica alla fine dell'ultimo train di LFS; $p > 0.05$, $n = 7$) ma riduce siginificativamente l'ampiezza della risposta sinaptica nella pathway non depotenziata (53 ± 4% della baseline baseline 90 min dopo l'applicazione di ZIP, $p < 0.001$).

Un'ulteriore interessante constatazione e' che la diminizione della risposta sinaptica nella pathway non depotenziata successiva all'applicazione di ZIP porta tali valori a coincidere con quelli ottenuti nella pathway completamente depotenziata.

La mancanza di effetto di ZIP sulla pathway depotenziata suggerisce che LTD è legata ad una forte inibizione della PKMζ, attività-dipendente. Dal momento che questi risultati sono molto diversi da ciò che si osserva nell'adulto, abbiamo quindi deciso di misurare l'espressione di PKMζ nella corteccia peririnale di neonati e adulti per determinare se esistono differenze nell'espressione di PKMζ che potrebbero spiegare gli effetti diversi di ZIP sulla trasmissione sinaptica basale nei P14 e nell'adulto. I risultati ottenuti dimostrano che i livelli di PKMζ sono significativamente più alti ($p < 0,01$) nella corteccia peririnale di P14 rispetto agli animali adulti (adulti: 100 ± 7%, n = 8; P14: 154 ± 9%, n = 9; **Inserto in Figura 4.1**). Questi risultati forniscono un maggiore sostegno alla nostra ipotesi che lo stato "potenziato" della trasmissione sinaptica nella corteccia peririnale P14 potrebbe dipendere dall' aumentata espressione e / o attività di PKMζ.
Questi risultati permettono pertanto di confermare il coinvolgimento di PKMζ nel mantenimento degli elevati livelli di attivita' sinaptica riscontrabili nella corteccia peririnale di P14.

4.2.2 Attivita' di PKMζ nel de-depotenziamento in corteccia peririnale di P14.

Come dimostrato in precedenza (osservazioni di R. King, dati non pubblicati) nella corteccia peririnale di P14 non e' possibile indurre LTP ma e' possibile ottenere il de-depotenziamento di una pathway in precedenza depotenziata, somministrando un normale protocollo per l'induzione di LTP. Pertanto abbiamo voluto studiare l'effetto dell'inibizione di PKMζ in una pathway de-depotenziata in corteccia peririnale di P14.
Se i livelli elevati della trasmissione sinaptica basale sono mantenuti dall'attività dei PKMζ, che è up- o down-regolata in seguito a HFS e LFS, rispettivamente (Hrabetova e Sacktor 1996), la sua inibizione per opera di ZIP dovrebbe essere in grado di diminuire l'ampiezza della risposta sinaptica.

Dopo una baseline di 60 minuti, una LFS e' stata somministrata in una pathway in modo da indurre un depotenziamento (**Figura 4.2** 74 ± 2 % of baseline, $p < 0.001$, $n = 4$) ed e' stata seguita, 30 minuti dopo, da HSF in modo da ottenere un de-depotenziamento (99 ± 8 % dell'originaria, pre-LTD baseline, 60 min post HFS; $p < 0.001$ v LTD, $p > 0.05$ v baseline, n=4); l'altra pathway e' stata lasciata come controllo.
180 minuti dopo il de-depotenziamento, la fettina e' stata perfusa con ZIP μM e l'esperimento e' stato condotto a termine in presenza del composto; in seguito all'inibizione di PKMζ si evidenzia una simile diminuzione dell'ampiezza della risposta sinaptica in entrambe le pathways (controllo: 48 ± 3% of baseline, $p< 0.01$; Pathway de-depotenziata: 50±5%; n=4; $p<0.01$)
Pertanto, l'attivita' di PKMζ si dimostra la stessa tanto in una pathway de-depotenziata quanto in una pathway di controllo, che noi ipotizziamo essere "naturalmente potenziata"; l'inibizione dell'attivita' della kinasi, infatti, provoca una riduzione dell'ampiezza della risposta sinaptica in ambo le pathways, e tale riduzione appare essere di simile entita' in entrambe.

4.2.3 Ruolo di PKMζ nella trasmissione sinaptica in corteccia peririnale di P35.

Considerando questi risultati, è stato interessante esaminare a che punto durante lo sviluppo, la corteccia peririnale acquisisce la capacità di produrre una "classica" LTP, o meglio, quando acquisisce l'abilitá di passare da un "potenziamento saturo" già presenti alla baseline, ad una situazione in cui le sinapsi sono pronte per essere potenziate ancora una volta.
A questo punto il passo successivo è stato analizzare i meccanismi di plasticità sinaptica nella corteccia peririnale di ratti di età intermedia tra gli adulti ed i P14. Per esaminare la questione, abbiamo deciso di eseguire una serie di esperimenti su animali giovani, a 35 giorni post-natale (PND35 o P35). A questa età, infatti, i ratti sono già attivi e mostrano un vivace comportamento esplorativo.

In primo luogo, sono stati condotti degli esperimenti per essere sicuri che fosse possibile ottenere una robusta e stabile LTP in questi animali. Una volta dimostrato ciò, abbiamo testato l'attività di PKMζ esattamente come era stato fatto per l'adulto.

Come mostrato in **figura 4.3**, dopo un periodo di registrazione di baseline di circa 60 minuti, una robusta LTP è stata indotta su una pathway utilizzando 4 stimoli a 100Hz (1 s) distanziati tra loro di 5 minuti (148 ± 5% della baseline, $p<0.001$, $n = 6$). La LTP è stata mantenuta per 180 minuti, dopodiché è stato somministrato ZIP (5μM). Anche in questo caso, come nell'adulto, ciò che si osserva è una rapida diminuzione della risposta sinaptica, con perdita completa del potenziamento e ritorno ai valori di baseline in circa 120 minuti (100 ± 2%; $n=6$; $p<0.001$). ZIP non ha effetto sulla trasmisione sinaptica basale nella pathway di controllo transmission (97 ± 2% della baseline, $p>0.05$, $n = 6$).

Queste osservazioni permettono di affermare che, almeno per quanto riguarda il potenziamento, già a 35 giorni il comportamento delle sinapsi in corteccia peririnale è simile a quello di sinapsi adulte. Ciò potrebbe essere messo in relazione con modificazioni sinaptiche post natali probabilmente dovute ai meccanismi di plasticità neuronale conseguenti allo sviluppo e all' "esperienza" (ad esempio, apertura degli occhi).

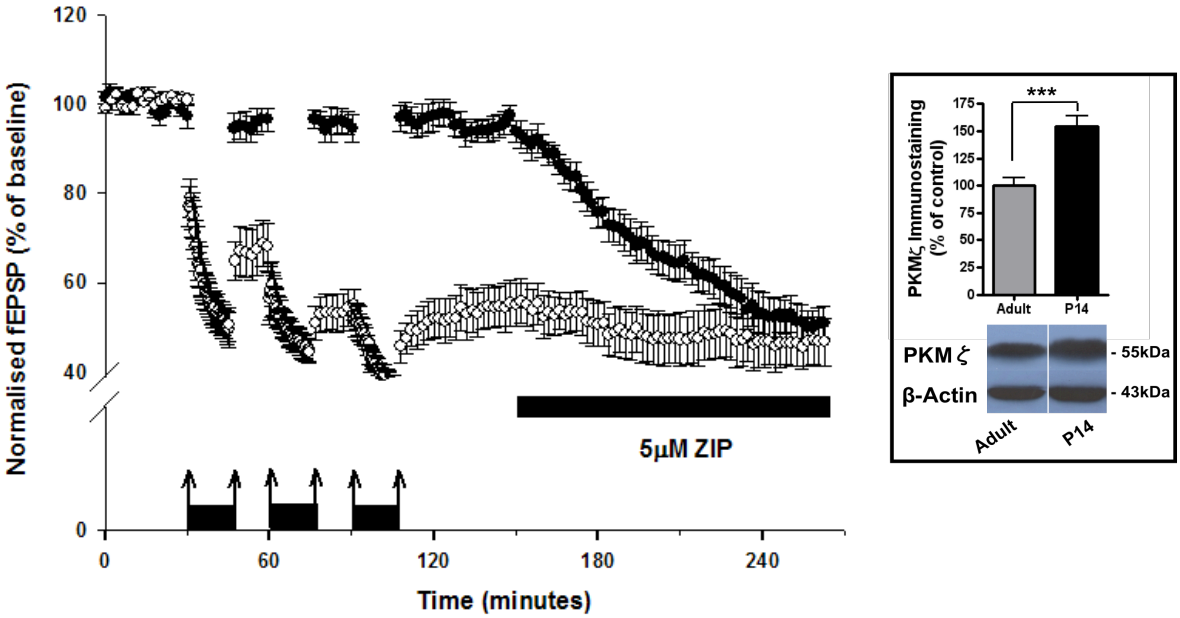

Figura 4.1 Effetto dell'inibizione di PKMζ nella corteccia peririnale di P14 L'inibizione di PKMζ produce un a significativa diminuzione nella risposta sinaptica (cerchi neri, 53 ± 4% della baseline, p<0.001 v baseline; n=7). Una volta ottenuta un depotenziamento saturo (cerchi bianchi 54 ± 4 % della baseline, p<0.001) la successive applicazinone di ZIP non mostra alcun effetto (94 ± 9% compared to pre-ZIP level; p>0.05). L'inserto mostra le differenze nell'esperssione di PKMζ nella corteccia peririnale di ratti P14 e adulti. L'istogramma (in alto) mostra che I livelli di PKMζ a=sono significativamente (***p<0.001) piu' alti in corteccia peririnale di P14 che negli adulti (adults: 100 ± 7 %, n=8; P14: 154 ± 9 %, n=9). In basso, un esempio delle bande del western blot.

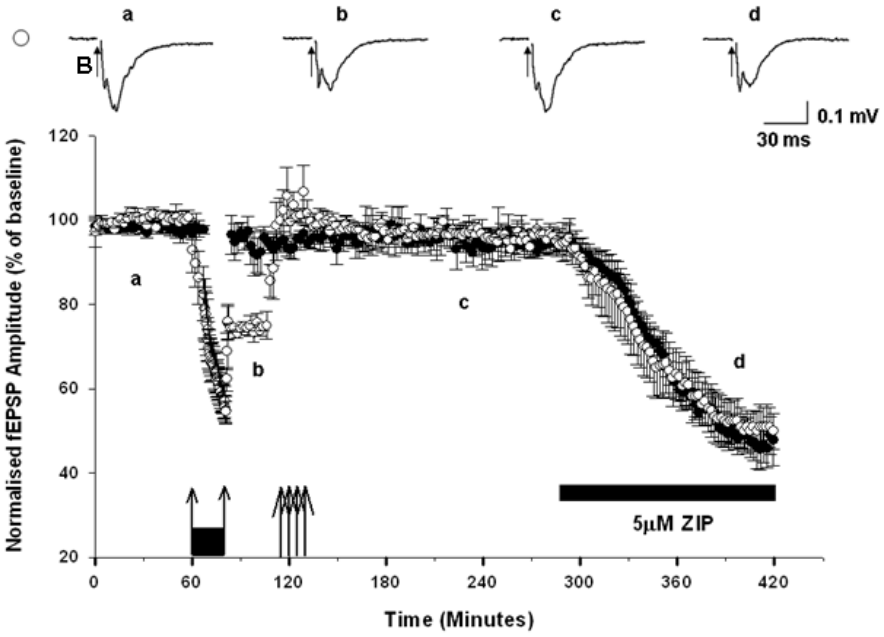

Figura 4.2 Effetto dell'inibizione di PKMζ nel de-depotenziamento nella corteccia peririnale di P14 LFS induce una robusta LTD (cerchi bianchi 74 ± 2 % della baseline, p<0.001, n=4). Trenta minuti dopo l'induzione di LTD, la somministrazione di HFS induce uno stabile potenziamento (99 ± 8 % della baseline pre-LTD, 60 min post HFS; p<0.001 v LTD, p > 0.05 v baseline). ZIP deprime la trasmissione sia nell pathway potenziata (50 ± 5% of baseline; p< 0.01) sia in quella di controllo (cerchi neri, 48 ± 3% della baseline, p< 0.01).

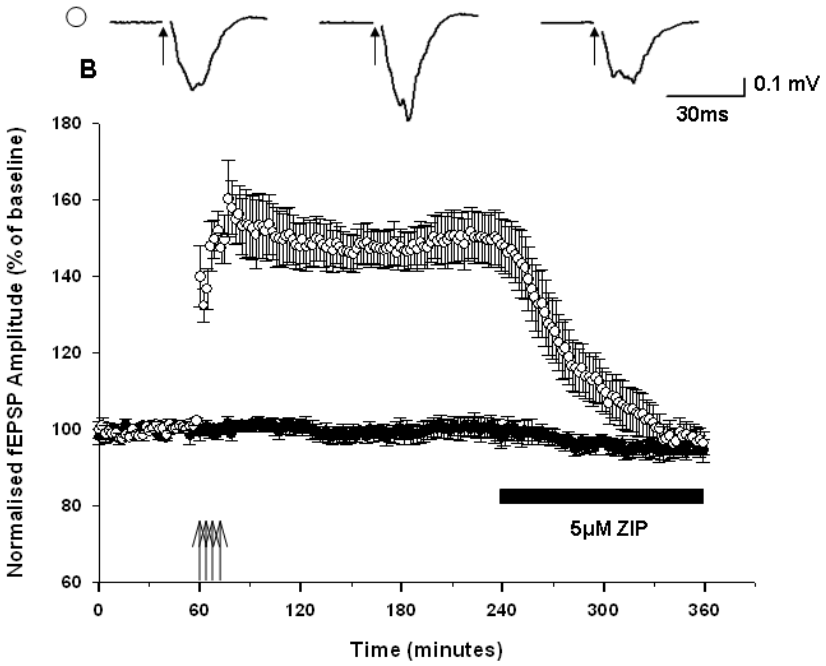

Figura 4.3 Effetto dell'inibizione di PKMζ sulla LTP nella corteccia peririnale di P35 LTP e' facilmente indotta nella corteccia peririnale di P14 (cerchi bianchi 148 ± 5% della baseline, p<0.001, n=6) e l'inibizione di PKMζ ad opera di ZIP 5μM annulla completamente l'LTP (100 ± 2% of baseline, p<0.001). ZIP non sembra avere effetto sulla baseline nella pathway di controllo (cerchi neri, 97 ± 2% della baseline, p>0.05). In alto, tracce rappresentative delle risposte evocate.

4.3 Discussione

In contrasto con le osservazioni formulate nella corteccia peririnale adulta, negli animali giovani (P14), nessuno dei protocolli HFS noti per essere normalmente in grado di indurre LTP è efficace. Questi risultati sono abbastanza sorprendenti, visto che negli animali più giovani un più alto livello di plasticità è previsto, e che in altre aree cerebrali, cioè corteccia visiva e l'ippocampo, è relativamente facile indurre una robusta e stabile LTP utilizzando i comuni protocolli di HFS (Malenka and Bear 2004).

Una possibile spiegazione per spiegare il fatto che non è possibile ottenere LTP nella corteccia peririnale di P14 è che i meccanismi intracellulari che conducono al potenziamento della risposta sinaptica semplicemente non esistono, in questa fase di sviluppo neurologico. Un'ipotesi alternativa è che questi meccanismi sono presenti, ma sono già completamente saturi, quindi un potenziamento è possibile solo a seguito di una depressione della trasmissione sinaptica, un processo noto come de-depressione o de-depotenziamento. Pertanto, questi risultati suggeriscono che la mancanza di LTP in neonatale corteccia peririnale può essere spiegata dalla presenza di meccanismi costitutivi LTP-simili responsabili di mantenere trasmissione basale in uno stato già potenziato nelle fasi precoci dello sviluppo. Le ragioni per cui la trasmissione sinaptica è mantenuta in uno stato potenziato non è noto ma questi meccanismi possono promuovere o stabilizzare le connessioni sinaptiche nella corteccia cerebrale immatura (Hua e Smith 2004; Cline e Haas 2008; Hanse, Taira et al 2009). Se meccanismi simili operano in altre regioni del cervello e in quali momenti durante lo sviluppo non e' noto.

Poiché PKMζ è sufficiente a mantenere LTP, e la trasmissione sinaptica basale in corteccia peririnale di P14 sembra essere in uno stato già potenziato, PKMζ è apparso come un obiettivo interessante da esaminare. I risultati mostrano, infatti, che l'inibizione della sua attività produce una forte

depressione nella trasmissione sinaptica. È interessante notare che le dimensioni di questa depressione corrispondono a quelle ottenute dopo l'induzione di una LTD completamente a. Come previsto, l'applicazione di ZIP non mostra alcun effetto sul sulla pathway depotenziata, probabilmente perché PKMζ è stata down-regolata dai ripetuti train di LFS, come descritto in precedenza (Hrabetova e Sacktor 1996; Hrabetova e Sacktor 2001). Pertanto, questi risultati suggeriscono che PKMζ è il principale responsabile per il mantenimento della trasmissione sinaptica basale in uno stato potenziato.

L'ipotesi che gli elevati livelli basali di trasmissione sinaptica nella corteccia peririnale P14 sono mantenuti da meccanismi LTP-like, che dipendono dalle attività di PKMζ, è rafforzata anche dalla constatazione che ZIP è in grado di diminuire la risposta sinaptica in una pathway de-depresso. In altre parole, se si considera la trasmissione sinaptica basale come "LTP", e la trasmissione sinaptica depressa come la trasmissione basale, la somministrazione di HFS produce una "nuova LTP", mantenuta tale da PKMζ.

Questo fenomeno, a nostra conoscenza, si verifica solo nella corteccia peririnale in questa fase (P14 di sviluppo neurologico). In ippocampo di P14, infatti, è possibile indurre LTP abbastanza facilmente, e il potenziamento viene ripristinato mediante l'applicazione di ZIP (Serrano, Yao et al. 2005). Esperimenti in ippocampo P14 sono stati ripetuti come un controllo interno; HFS è in grado di indurre una robusta LTP, che si annulla con l'applicazione di ZIP (mostrato in **figura 4.3.1 e 4.3.2**). Inoltre, l'occlusione della LTP in corteccia peririnale avviene durante un periodo di tempo limitato, dal momento che è possibile indurre una robusta LTP in fettine di peririnale di animali a 35 giorni post-natale (P35). Pertanto, questa occlusione della induzione di LTP sembra essere specifica per una particolare regione del cervello (corteccia peririnale) in un momento particolare nel neurosviluppo (circa PND14).

I meccanismi che riducono il ruolo della LTP e PKMζ durante lo sviluppo della corteccia peririnale non sono noti, ma potrebbero derivare da processi LTD-simili che sono alla base della memoria di riconoscimento visivo (Brown e Bashir 2002; Griffiths, Scott et al 2008).

L'inserimento dei recettori AMPA puo' essere importante per la stabilizzazione delle connessioni sinaptiche nel sistema nervoso centrale immaturo (Hua e Smith 2004; Cline e Haas 2008;. Hanse, Taira et al 2009), ed è stato dimostrato che i meccanismi attraverso i quali PKMζ sostiene l'LTP includono l'inserimento dei recettori AMPA nella membrana sinaptica (Yao, Kelly et al. 2008). Inoltre, PKMζ ha recentemente dimostrato di essere importante per la stabilizzazione delle sinapsi della via retino-tettale nello Xenopus in via di sviluppo (Liu, Tari et al. 2009). Questi risultati mostrano che i meccanismi LTP-simili dipendenti da PKMζ giocano un ruolo critico nel mantenimento della stabilità sinaptica durante lo sviluppo di centri superiori del sistema nervoso centrale dei mammiferi.

Riassumendo, l'ipotesi è che le sinapsi sono mantenute in un elevato livello di potenziamento da parte PKMζ nei primi stadi di sviluppo neurologico. Questo fenomeno stabilizza le sinapsi giovani, "preparandole" a sviluppare meccanismi di plasticità differenti in seguito durante lo sviluppo. Vale a dire, i meccanismi LTD-like derivanti dall'esperienza, potrebbero portare la trasmissione sinaptica a uno stato in cui up- e down-regulation delle sinapsi sono possibili, permettendo quindi l'archiviazione delle informazioni nuove e altre forme di plasticità. Sarebbe interessante verificare se l'apertura degli occhi possiede alcun tipo di ruolo in questo processo; i P14, infatti, hanno ancora gli occhi chiusi, e ha senso che la corteccia peririnale, essendo coinvolta in memoria di riconoscimento, mostri fenomeni di plasticità sinaptica dipendenti da input ricevuti da corteccia visiva.

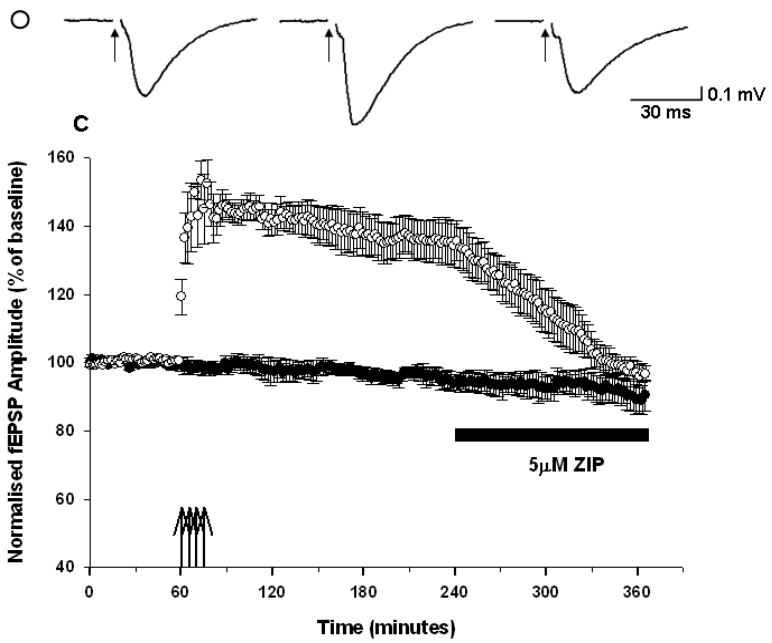

Figura 4.3.1 Effetto dell'inibizione di PKMζ su LTP in P14 ippocampo (Peak Amplitude) LTP e' facilmente indotta nell'ippocampo di P14 (cerchi bianchi: 141±5% della baseline; p<0.01; n = 4). L'inibizione di PKMζ reverte completamente il potenziamento (100 ± 7% of baseline, p<0.01 v LTP) ma non ha effetto sulla trasmissione basale del controllo (cerchi neri 92 ± 4% della baseline, p>0.05, n = 4)

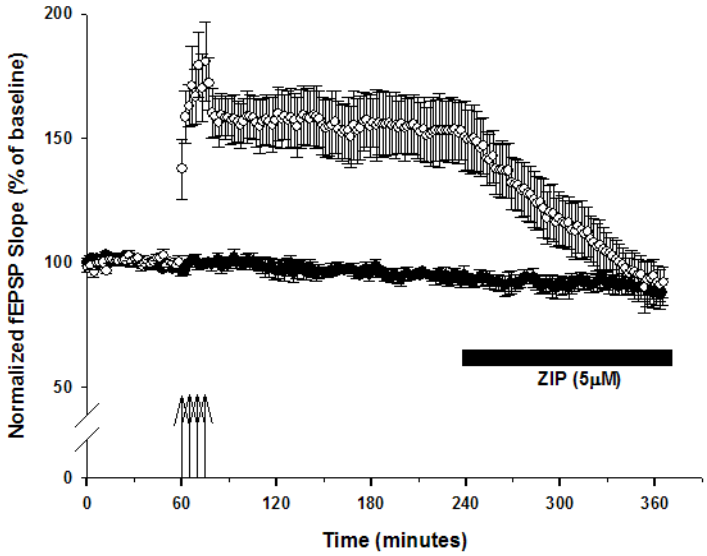

Figura 4.3.2 Effetto dell'inibizione di PKMζ su LTP in P14 ippocampo (Slope) Le misurazioni delle variazioni della slope sono consistenti con le variazioni del picco di ampiezza della risposta. LTP é facilente inducibile nell'ippocampo di P14 (cerchi bianchi: 157 ± 9% della baseline; p<0.01; n = 4). L'inibizione di PKMζ reverte completamente il potenziamento (99 ± 5% della baseline, p<0.01) ma non mostra effetti significativi sulla trasmissione basale nell'input di controllo (cerchi neri, 101 ± 0.6% della baseline, p>0.05, n = 4)

5 REGOLAZIONE DI PKMζ NELLA CORTECCIA PERIRINALE DURANTE IL NEUROSVILUPPO

5.1 Ruolo di PI3K e mTOR nella regolazione dell'attivitá di PKMζ

5.1.1 Introduzione

Gli esperimenti finora condotti forniscono alcune buone evidenze che PKMζ mantenga l'aumentata trasmissione sinaptica nella LTP. Tuttavia, i meccanismi sottostanti tramite i quali questo fenomeno si verifica non sono ancora noti.

Un possibile meccanismo comporta la regolazione tramite PKMζ della sintesi proteica locale attraverso meccanismi di traslazione a livello dendritico (Westmark, Westmark et al. 2010). L'inizio della traslazione si basa, almeno in parte, sull'attivazione del mammalian target of rapamycin (mTOR), l'inibizione del quale impedisce la LTP (Hoeffer e Klann 2010). mTor è coinvolto in molti modi nella plasticità neuronale, inoltre possiede un ruolo cruciale nel mantenimento di LTP e memoria (Kelleher, Govindarajan et al 2004;. Helmstetter, Parsons et al 2008;. Klann e Sweatt 2008; Swiech, Perycz et al 2008;. Costa-Mattioli, Sossin et al 2009;. Richter e Klann 2009; Hoeffer e Klann 2010). Inoltre, la disregolazione di attività di mTOR è legata a diverse malattie cognitive, come la malattia di Alzheimer (Pei e Hugon 2008; Swiech, Perycz et al 2008; Ma, Hoeffer et al 2010), la sindrome da X fragile (Sharma, Hoeffer et al 2010), la sclerosi tuberosa (Ehninger, de Vries et al 2009;. Sampson 2009) e molte altre (Swiech, Perycz et al 2008;. Hoeffer e Klann 2010). E anche noto (Kelly, Crary et al. 2007) che l'inibizione di mTOR blocchi non solo il mantenimento della LTP, ma diminuisca anche la sintesi e l'attività di PKMζ. Pertanto, per verificare se la trasmissione sinaptica basale nella corteccia peririnale di P14 è dipendente da meccanismi di traslazione proteica abbiamo studiato gli effetti sulla peririnale di inibitori di mTOR. E' stato suggerito (Westmark, Westmark et al 2010) che PKMζ potrebbe mantenere la LTP tramite meccanismi di traslazione proteica, attraverso una cascate di signalling che coinvolgono la regolazione di Pin1 - una proteina che

interagisce con 4E-BP, e che di norma sopprime la traduzione proteica. 4E-BP sono fosforilati dagli mTOR per rimuovere l'inibizione all'inizio della traduzione (Hoeffer e Klann 2010). Se PKMζ mantiene elevata la trasmissione sinaptica attraverso la traduzione delle proteine, è quindi probabile che coinvolga i processi di iniziazione della sintesi proteica mTOR-dipendenti.

Oltre a questo, abbiamo voluto esaminare un altro step fondamentale nella segnalazione intracellulare che puo' portare alla regolazione dell'attivita' di PKMζ mediata da mTOR. E' noto che uno dei principali enzimi che svolgono un ruolo cruciale nel regolare l'attività di mTOR è la fosfoinositide-3-chinasi (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 è anche coinvolta nella espressione e mantenimento di 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), ed è a monte nella cascata di segnali che porta all'attivazione di PDK1 (Duronio 2008; Carnero 2009;. Costa-Mattioli, Sossin et al 2009; Kawauchi, Ogasawara et al. 2009; Carnero 2010; Hoeffer e Klann 2010), che si pensa sia fondamentale per fosforilare PKMζ nel suo loop di attivazione, attivandola completamente (Kelly, Crary et al 2007). In particolare, PDK1 fosforila i loop di attivazione di PKCζ in modo PI3-kinasi-dipendente (Le Good, Ziegler et al. 1998). Inoltre, l'inibizione della PI3K blocca il mantenimento di LTP e down-regola la sintesi di PKMζ (Kelly, Crary et al. 2007). È interessante notare che è stato dimostrato che l'attivazione della PI3K è necessaria anche per l'inserimento in membrana dei recettori AMPA durante LTP in colture di neuroni dell'ippocampo (Man, Wang et al. 2003), che è anche il meccanismo attraverso il quale si pensa che PKMζ mantenga la LTP (Ling, Benardo et al 2006;. Yao, Kelly et al 2008;. Migues, Hardt et al 2010;. Sacktor 2010).

Pertanto, i seguenti gruppi di esperimenti sono volti a studiare il ruolo di mTOR e PI3K in plasticità sinaptica PKMζ-dipendente nella corteccia peririnale nel corso dello sviluppo neurologico.

5.1.2 Risultati

5.1.2.1 Ruolo di mTOR nella trasmissione sinaptica basale

Come descritto in precedenza in questo capitolo, mTOR ha un ruolo cruciale nella regolazione della sintesi proteica. E' stato anche dimostrato che la sintesi proteica é necessaria per il mantenimento della LTP. Le nostre osservazioni precedenti ci hanno portato a pensare che nella corteccia peririnale neonatale (P14), la trasmissione sinaptica basale è completamente potenziato come se qualche meccanismo LTP-simile fosse alla base della trasmissione basale in questa fase. Se questo è vero, l'inibizione della sintesi proteica attraverso l'inibizione di mTOR dovrebbe diminuire la trasmissione sinaptica basale nella corteccia peririnale di P14. Per esaminare cio', dopo una registrazione di baseline di 40 minuti, rapamicina (5 micron) è stato applicata (**Figura 5.1.1**). L'inibizione di mTOR produce una depressione sostanziale della trasmissione basale nella corteccia peririnale di P14 (lato temporale: 73 ± 3% del valore basale, p <0,001; lato entorinale: 74 ± 3% del valore basale, p < 0,001, n = 6). Tuttavia, nessun effetto dell'inibizione di mTOR mediante l'applicazione della rapamicina è stato osservato su fettine di peririnale da animali adulti (**Figura 5.1.2**: temporale: 98 ± 3% del valore basale, p> 0.05; entorinale 100 v 3% basale, p> 0.05, n = 4). Questo risultato suggerisce che la continui meccanismi di sintesi proteica mantengonoi livelli basali di trasmissione sinaptica in P14, ma non nella corteccia peririnale adulta.

5.1.2.2 Effetto della inibizione mTOR sull'attività di PKMζ in corteccia peririnale

La sintesi di nuove proteine mediata da mTOR mantiene alti livelli di trsamissione sinaptica e ci sono evidenze che anche la sintesi di PKMζ a livello dendritico sia regolata da mTOR. Se questo e' vero, l'inibizione

dell'attività di mTOR dovrebbe portare ad una inibizione indiretta di PKMζ, mentre l'inibizione di PKMζ dovrebbe occludere ogni successiva depressione della trasmissione sinaptica generato dalla successiva inibizione di mTOR.

Questo ultimo scenario è illustrato in **Figura 5.1.3**: dopo 1 ora di registrazione di baseline, ZIP è stato applicato in fette corteccia peririnale di ratti P14, seguita da 5μM rapamicina. La depressione di trasmissione determinata dall'inibizione di PKMζ in corteccia peririnale di P14 occlude ogni successiva depressione della trasmissione sinaptica indotta da rapamicina (lato temporale: ZIP 55 ± 3% del valore basale, p <0,001; Rapamicina 52 ± 3% del valore basale, p> 0,05 v di ZIP; lato entorinale: ZIP 54 ± 1% del valore iniziale; Rapamycin 51 ± 1% del valore basale, p> 0,05 v di ZIP; n = 4). Questo risultato suggerisce che l'occlusione PKMζ mantiene la trasmissione sinaptica basale in corteccia peririnale di P14 attraverso, almeno in parte, la sintesi proteica mTOR-dipendente.

D'altra parte, l'applicazione di ZIP a seguito della depressione della trasmissione sinaptica da rapamicina (**Figura 5.1.4**) ha comportato una piccola ma significativa depressione della trasmissione sinaptica (lato temporale: Rapamicina 67 ± 1 % del valore basale, p <0,001; ZIP 52 v 3% del valore basale, p <0,01 V Rapamicina; lato entorinale: Rapamicina 66 ± 2% del valore basale, p <0,001; ZIP 52 ± 3% del valore basale, p <0,01 V Rapamicina n = 4). La depressione prodotta da ZIP quando applicato dopo dopo la rapamicina è stata inferiore alla depressione della trasmissione basale prodotta da ZIP da solo. Questo suggerisce che, sebbene PKMζ mantenga la trasmissione basale attraverso la sintesi proteica mTOR-dipendente qualche ulteriore meccanismo può anche essere coinvolto.

Per rafforzare ulteriormente questi risultati, questa ultima serie di esperimenti è stata ripetuta utilizzando un altro inibitore di mTOR, KU0063794. Questo composto è un inibitore molto potente e selettivo di mTOR (Garcia-Martinez, Moran et al. 2009). Dopo 1 ora di registrazione di base da P14 corteccia

peririnale, 1μM KU0063794 è stato applicato bagno, seguito da ZIP. I dati totali (**figura 5.1.5**) mostrano che KU0063794 produce una significativa riduzione della trasmissione sinaptica basale, simile a quella prodotta da rapamicina (lato temporale: 72 ± 2% del valore basale, p <0.01; entorinale laterale 69 ± 2% basale, p <0,01). La seguente applicazione di ZIP (5 μM) produce una ulteriore piccola ma significativa depressione della trasmissione sinaptica (lato temporale: ZIP 54 ± 0,6% del valore basale, p <0,01 V KU0063794; lato entorinale: ZIP 51 ± 3% del valore basale, p < 0,01 V KU0063794; n = 4). È anche interessante notare che la combinazione di questi farmaci determina una depressione totale della trasmissione sinaptica dello stesso ampiezza della depressione prodotta mediante l'applicazione di ZIP da solo. Nessun effetto derivante dall'inibizione di mTOR da parte di KU0063794 puo' essere osservato nella corteccia peririnale di ratti adulti (**Figura 5.1.6**: lato temporale: 100 ± 4% del valore basale, p> 0.05; lato entorinale 102 ± 4% del valore basale, p> 0,05, n = 3).

5.1.2.3 **Ruolo di PI3K in basale trasmissione sinaptica**

La fosfoinositide 3-chinasi (PI3K) svolge un ruolo importante nella regolazione di mTOR (Sabatini 2006; Tsokas, Ma et al 2007;.. Gobert, Topolnik et al 2008; Costa-Mattioli, Sossin et al 2009.). Inoltre, PI3K è coinvolta nella espressione e mantenimento di 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). Per queste ragioni, abbiamo pensato che fosse un importante obiettivo da indagare al fine di chiarire ulteriormente i meccanismi alla base della trasmissione sinaptica potenziata che abbiamo osservato in corteccia peririnale di P14. Per indagare questo punto, dopo una baseline di 40 minuti l'inibitore "classico" di PI3K (wortmannin 400nm) è stato perfuso nella fetta. I dati mostrati in **Figura 5.1.7** mostrano che wortmannin è in grado di diminuire significativamente la trasmissione sinaptica nella corteccia peririnale di P14 (temporale: 61 ± 4% di base, p<0,001; entorinale 64 ± 3% del valore basale, p <0,001 , n = 5). Nessuna differenza è stata osservata tra le due pathway (p>

0,05). Wortmannin non diminuisce la trasmissione sinaptica basale in corteccia peririnale di ratti adulti (**Figura 5.1.8**: lato temporale: 107± 4% del valore basale, p> 0.05; lato entorinale 107 ± 2% del valore basale, p> 0.05, n = 3. Nessuna differenza tra le due pathway). Questi risultati confermano che PI3K è in qualche modo coinvolta nel mantenimento della trasmissione sinaptica basale in corteccia peririnale di P14, ma non nell'adulto, presumibilmente attraverso l'inibizione della sintesi delle proteine mTOR-mediata.

5.1.2.4 Effetto della inibizione PI3K sull'attività di PKMζ in corteccia peririnale

Poiché PI3K è importante per mantenere alti livelli di trasmissione sinaptica nella corteccia peririnale di P14, possibilmente attraverso la sintesi proteica mTOR-mediata, e mTOR sembra essere coinvolto nella regolazione dell'attività di PKMζ, è stato interessante indagare se l'inibizione della PI3K poteva incidere in alcun modo sull'attività di PKMζ in corteccia peririnale di P14. Per fare ciò, dopo 40 minuti di registrazione di baseline, un potente inibitore selettivo di PI3K, LY294002 50μM, è stato infuso nella fettina, seguito da ZIP. I dati in **Figura 5.1.9** mostrano che LY294002 produce una significativa riduzione della trasmissione sinaptica nella corteccia peririnale di P14 (lato temporale: 63 ± 0,5% del valore basale, p <0,001; lato entorinale 63 ± 0,6% del valore basale, p <0,001 , n = 4). ZIP, applicato dopo che la risposta sinaptica si sianuovamente stabilizzata, produce una ulteriore piccola, ma significativa diminuzione di ampiezza della fEPSP (lato temporale: 53 ± 1% del valore basale, p <0.01 v LY294002; lato entorinale 52 ± 0,6% del valore basale , p <0,01 v LY294002). Nessuna differenza è stata trovata tra le due pathway (p>0,05). È importante notare che l'inibitore selettivo di PI3K, LY294002, produce una diminuzione nella risposta sinaptica della stessa entità di quella prodotta da wortmannin. LY294002 non mostra alcun effetto sulla trasmissione sinaptica basale in corteccia peririnale di ratti adulti (**Figura 5.1.10**: temporale: 108 ± 3% del valore basale, p> 0.05;

entorinale 106 ± 3% del valore basale, p> 0,05). Questi risultati suggeriscono che PI3K è coinvolta nel mantenimento della risposta basale potenziata in corteccia peririnale di P14 (ma non di adulto), controllando in qualche modo l'attività di PKMζ, possibilmente attraverso la regolazione di mTOR. Una spiegazione alternativa è che PI3K modula l'attività dei PKMζ agendo sulla attivazione di PDK1.

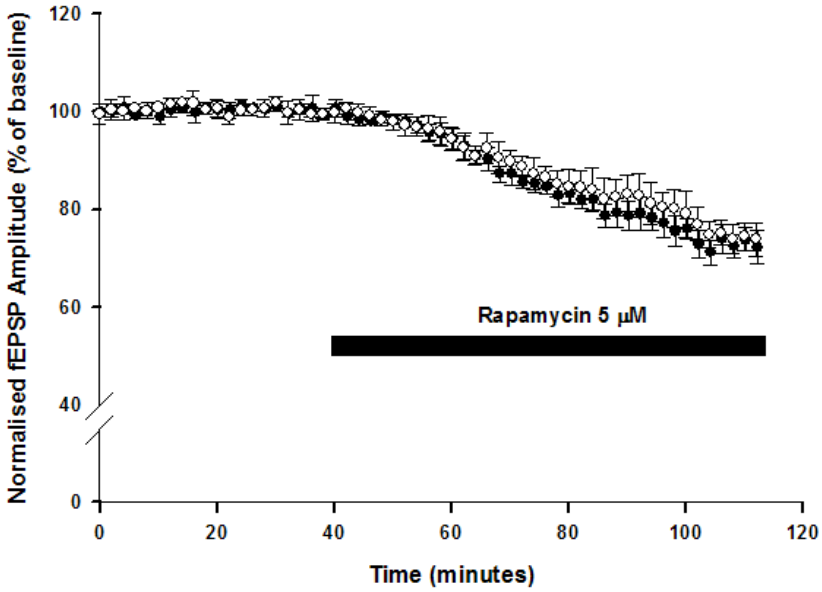

Figura 5.1.1 Effetto di Rapamicina sulla trasmissione sinaptica basale in corteccia peririnale di P14 L'inibizione di mTOR produce una sostanziale depressione della trasmissione basale (temporale, cerchi neri: 73±3% della baseline, p<0.001; entorinale, cerchi bianchi: 74±3% della baseline, p<0.001, n=6).

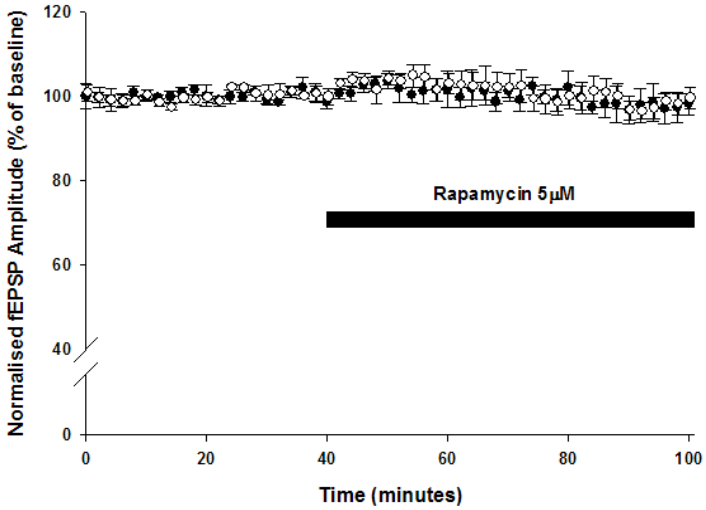

Figure 5.1.2 Effetto di rapamicina sulla trasmissione sinaptica basale in corteccia peririnale di adulto L'inibizione di mTOR tramite l'applicazione di rapamicin non dimostra di aver effetto sulla trasmissione sinaptica in fettine di peririnale di animali adulti (lato temporale, cerchi neri: 98 ± 3% della baseline, p>0.05; lato entorinale, cerchi bianchi: 100 ± 3% della baseline, p>0.05, n=4).

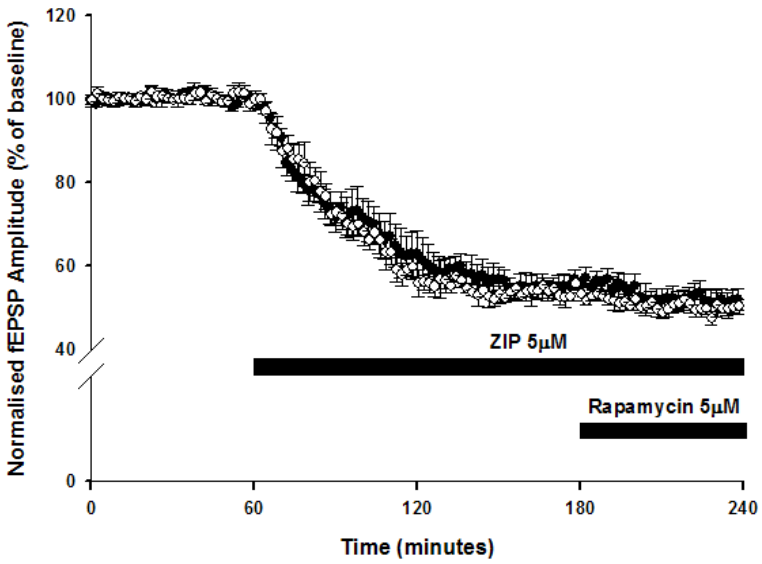

Figura 5.1.3 Effetto di ZIP seguito da Rapamycin sulla trasmissione sinaptica basale in P14 La depressione della trasmissione in corteccia peririnale di P14mediante inibizione della PKMζ occlude ogni successiva depressione della trasmissione sinaptica da rapamicina (lato temporale, cerchi neri: ZIP 55 ± 3% del valore basale, p <0,001; Rapamycin 52 ± 3% del valore basale, p> 0,05 v ZIP; lato entorinale, cerchi bianchi: ZIP 54 ± 1% del valore iniziale; Rapamycin 51 ± 1% del valore basale, p> 0,05 v ZIP; n = 4)

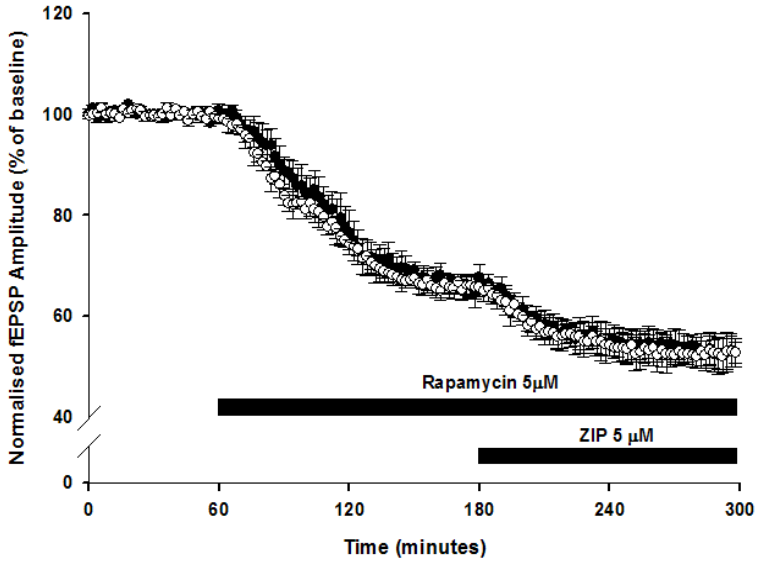

Figura 5.1.4 Effetto di rapamicina e zip sulla corteccia peririnale di P14 L'inibizione di mTOR produce una depressione sostanziale della trasmissione sinaptica basale in P14 (lato temporale, cerchi neri: 67 ± 1% del valore basale, p <0,001; lato entorinale, cerchi bianchi: 66 ± 2% della baseline, p<0,001, n = 4) La successive inibizione di PKMζ produce una piccola ma significativa riduzione della trasmissione sinaptica (lato temporale, ZIP 52 ± 3% del valore basale, p <0,01 v Rapamicina; lato entorinale ZIP 52 ± 3% del valore basale, p <0,01 v Rapamicina; n = 4)

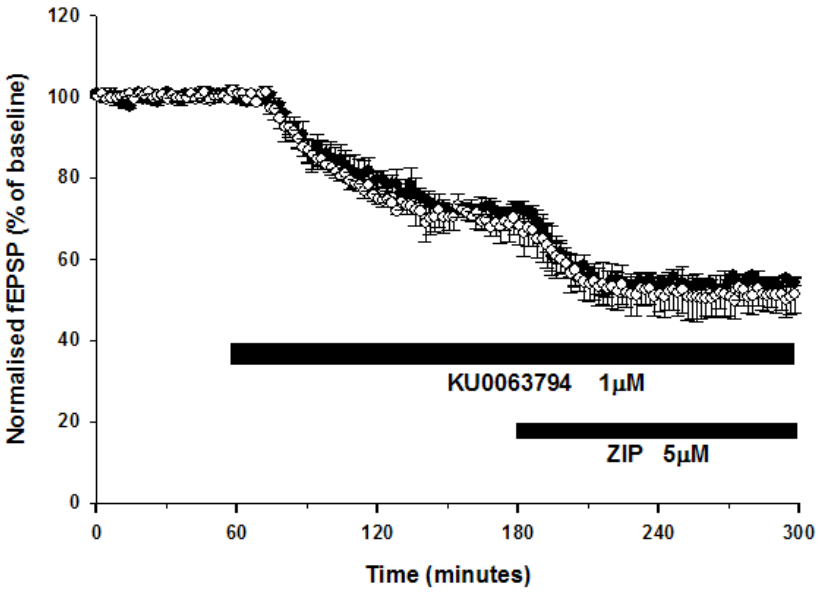

Figura 5.1.5 Effetto di KU0063794 e ZIP sulla corteccia peririnale di P14 KU0063794 KU0063794 produce una significativa riduzione della trasmissione sinaptica basale, simile a quella prodotta da rapamicina (lato temporale, cerchi neri: 72 ± 2% del valore basale, p <0.01; lato entorinale, cerchi bianchi 69 ± 2% del valore basale, p <0,01). La successiva applicazione di ZIP produce una ulteriore depressione piccola ma significativa della trasmissione sinaptica (lato temporale: ZIP 54 ± 0,6% del valore basale, p <0,01 v KU0063794; lato entorinale: ZIP 51 ± 3% del valore basale, p < 0,01 v KU0063794; n = 4)

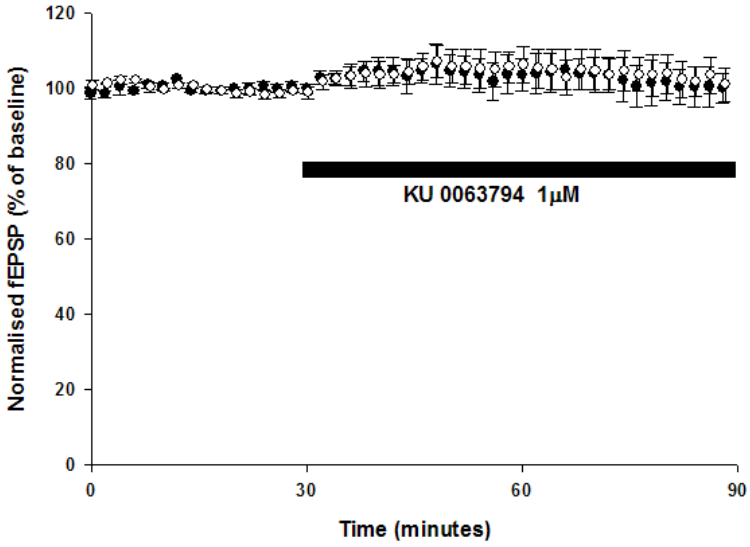

Figura 5.1.6 Effetto di KU0063794 su corteccia peririnale di adulto L'inibizione di mTOR indotta da KU0063794 non mostra nessun effetto sulla trasmissione sinaptica basale in corteccia peririnale di ratti adulti (lato temporale, cerchi neri: 100 ± 4% del valore basale, p> 0.05; lato entorinale , cerchi bianchi 102 ± 4% del valore basale, p> 0.05, n = 3)

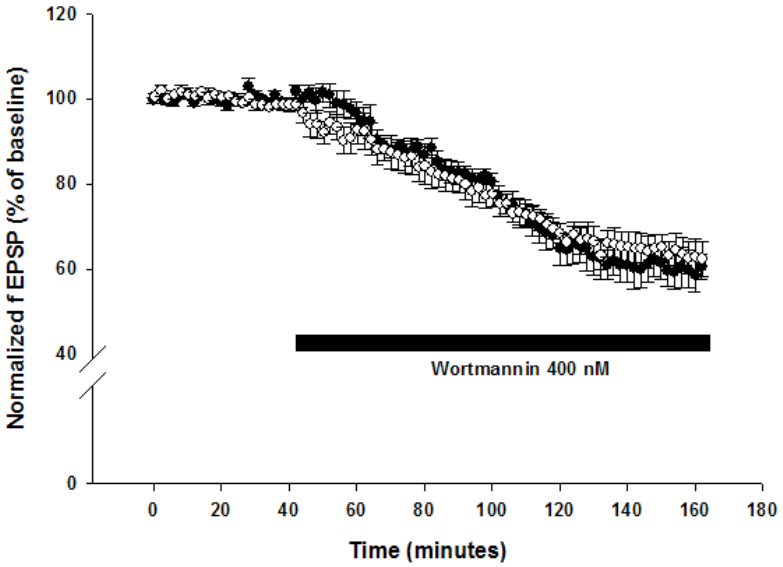

Figura 5.1.7 Effetto di Wortmannin sulla corteccia peririnale di P14 Wortmannin è in grado di diminuire significativamente la trasmissione sinaptica nella corteccia peririnale di P14 (lato temporale, cerchi neri: 61 ± 4% del valore basale, p <0,001; lato entorinale, cerchi bianchi 64 ± 3% del valore basale, p <0,001, n = 5). Nessuna differenza è stata trovata tra le due pathways (p> 0,05).

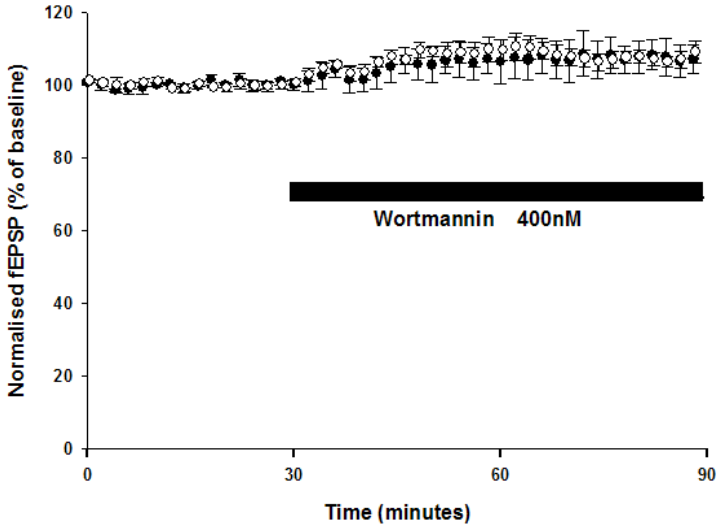

Figura 5.1.8 Effetto di Wortmannin sulla corteccia peririnale adulta L'inibizione di PI3K mediante l'applicazione di Wortmannin non mostra alcun effetto sulla trasmissione sinaptica basale in corteccia peririnale di ratti adulti (lato temporale, cerchi neri: 107 ± 4% del valore basale, p > 0,05; lato entorinale, cerchi bianchi 107 ± 2% del valore basale, p> 0.05, n = 3) Nessuna differenza é stata osservata tra le due pathways (p>0.05).

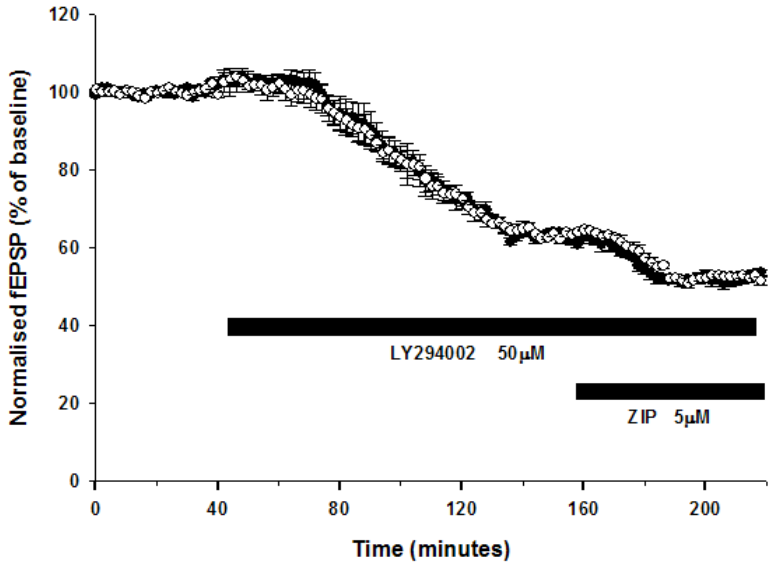

Figura 5.1.9 Effetto di LY294002 e ZIP su corteccia peririnale di P14 LY294002 produce una diminuzione significativa della trasmissione sinaptica (lato temporale, cerchi neri: 63 ± 0,5% del valore basale, p <0,001; lato entorinale, cerchi bianchi 63 ± 0,6% del valore basale, p <0,001, n = 4). ZIP, applicato dopo che la risposta sinaptica si è nuovamente stabilizzata, produce una ulteriore piccola, ma significativa, diminuzione di ampiezza fEPSP (lato temporale: 53 ± 1% del valore basale, p <0.01 v LY294002; lato entorinale 52 ± 0,6% del valore basale, p <0,01 v LY294002). Nessuna differenza è stata trovata tra le due pathways (p> 0,05).

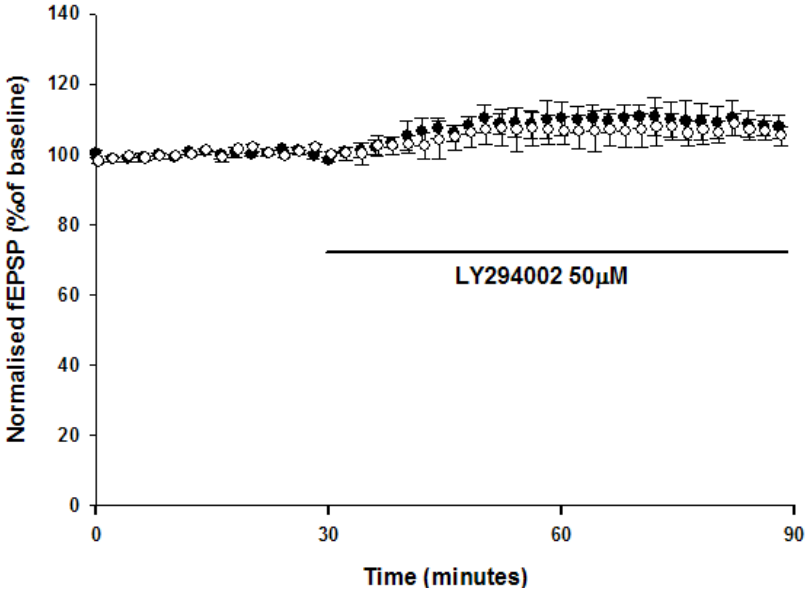

Figura 5.1.10 Effetto di LY294002 su corteccia peririnale di adulto LY294002 non mostra alcun effetto sulla trasmissione sinaptica basale in corteccia peririnale di ratti adulti (lato temporale, cerchi neri: 108 ± 3% del valore basale, p> 0.05; lato entorinale, cerchi bianchi: 106 ± 3% del valore basale, p> 0.05, n = 3). Nessuna differenza è stata trovata tra le due pathways (p>0,05).

5.1.3 Discussione

I risultati precedentemente mostrati confermano che mTOR potrebbe essere coinvolti nei processi di sintesi proteica che portano a mantenimento del potenziamento. Diversi inibitori di mTOR (rapamicina e KU0063794) mostrano lo stesso effetto sulla trasmissione sinaptica basale in corteccia peririnale di P14, producendo una depressione della risposta di pari ampiezza senza alterare la trasmissione sinaptica basale nella corteccia peririnale di soggetti adulti. Due diversi inibitori di mTOR sono stati utilizzati al fine di rafforzare i risultati preliminari e di escludere che la rapamicina potesse avere qualsiasi azione diversa su altri elementi che potrebbero influenzare i risultati. In effetti, anche se la rapamicina è considerata un inibitore di mTOR potente e selettivo, ci sono alcune evidenze che questo composto potrebbe avere qualche effetto anche su Canali per il calcio voltaggio-dipendenti (Regimbald-Dumas, Fregeau et al 2010;. Suh, Leal et al. 2010). Alcuni di questi canali sembrano essere coinvolta nell'LTP nell'amigdala (Pinard, Mascagni et al. 2005), così provare anche un altro composto che non dovrebbe avere alcun effetto su questi canali fornirebbe un controllo e rafforzerebbe i risultati. Entrambi i composti, però, hanno mostrato lo stesso effetto di depressione della risposta sinaptica, suggerendo che una continua sintesi proteica potrebbe controllare i livelli basali di trasmissione sinaptica nella corteccia peririnale di P14, ma non nell'adulto.

La depressione della risposta sinaptica ottenuta attraverso l'inibizione di mTOR, però, è più piccola di quella prodotta dalla inibizione della sola PKMζ. Quando ZIP è applicato in corteccia peririnale di P14 in seguito della depressione massima della trasmissione sinaptica prodotta dalla inibizione di mTOR, si osserva un'ulteriore diminuzione della risposta.. È interessante notare che la depressione totale della trasmissione sinaptica prodotta dalla inibizione consecutiva di mTOR prima e di PKMζ poi, è della stessa ampiezza di quella prodotta dalla inibizione della sola PKMζ. Questi risultati

suggeriscono che, anche se PKMζ mantiene la trasmissione basale attraverso meccanismi di sintesi proteica mTOR-dipendente, alcuni meccanismi aggiuntivi possono essere coinvolti. Un'ipotesi è che mTOR regola solo la nuova sintesi di PKMζ, ma non ha effetto su quella parte di enzima che è già attivo, quindi, l'inibizione totale di PKMζ potrebbe essere ottenuto solo attraverso l'inibitore selettivo ZIP. Questa ipotesi è coerente con la constatazione che la depressione della trasmissione sinaptica ottenuta come conseguenza dell'inibizione della PKMζ nella corteccia peririnale di P14 occlude ogni successiva depressione della trasmissione sinaptica che potrebbe essere prodotta dalla rapamicina.

Pertanto, la sintesi proteica, cruciale per il mantenimento di LTP e memoria (Hoeffer e Klann 2010), può essere una possibile via per il mantenimento PKMζ-dipendente della LTP (Westmark et al 2010). In base a questi risultati, la sintesi proteica mTOR-dipendente è importante anche per la regolamentazione della trasmissione sinaptica basale nella corteccia peririnale dei P14, ma non negli adulti.

Un altro meccanismo che potrebbe giocare un ruolo nel mantenimento PKMζ-dipendente del potenziamento della risposta sinaptica nella corteccia peririnale P14 coinvolge la fosfoinositide 3-kinasi (PI3K). Come meglio descritto in precedenza, è noto che PI3K svolge un ruolo importante nella regolazione di mTOR (Sabatini 2006; Tsokas, Ma et al 2007;. Gobert, Topolnik et al 2008;. Costa-Mattioli, Sossin et al 2009). Inoltre, PI3K è coinvolto nella espressione e nel mantenimento di 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). Infine, PI3K regola l'attività di PDK1, fondamentale per l'attivazione di PKMζ (Kelly, Crary et al. 2007) Pertanto, in una pathway potenziata, come quelle che si osservano nella corteccia peririnale di P14 sembrano essere, l'inibizione della PI3K dovrebbe portare ad una depressione della trasmissione sinaptica. I risultati hanno mostrato di confermare questa ipotesi: gli inibitori della PI3K Wortmannin o

LY294002 producono una significativa depressione della trasmissione sinaptica. In questo caso, due inibitori della PI3K diversi sono stati usati per aumentare la precisione degli esperimenti. Entrambi i composti sembrano avere lo stesso effetto. Ancora una volta, però, la depressione della trasmissione sinaptica prodotta dalla inibizione di PI3K è minore della depressione prodotta dalla inibizione diretta di PKMζ. La successiva applicazione di ZIP diminuisce ulteriormente la risposta, e la depressione finale della trasmissione sinaptica è alla fine dello stessa ampiezza di quella prodotta da ZIP da solo. Questi risultati sono simili ai risultati degli esperimenti effettuati con inibitori di mTOR. Ancora una volta, l'ipotesi è che PKMζ è a valle di questa cascata di segnali, ma solo la sintesi di nuova PKMζ è sotto il controllo di PI3K/mTOR. L'inibizione degli effettori PI3K-dipendente, però, non ha effetto su PKMζ già attivo. Inoltre, PI3K controlla PDK1, cruciale per l'attivazione di PKMζ. Pertanto, non si può escludere che l'effetto osservato con l'inibizione di PI3K avvenga anche attraverso un'inibizione indiretta dell’attività PKMζ tramite l'inibizione di PDK1.

5.2 Ruolo dei Recettori Metabotropici del Glutammato di Gruppo I nella regolazione di PKMζ durante il neurosviluppo.

5.2.1 Introduzione

Come descritto nei capitoli precedenti, PKMζ sembra mantenere un elevato livello della trasmissione sinaptica nella corteccia peririnale di P14 agendo a valle di alcuni meccanismi di segnalazione intracellulare che coinvolgono PI3K e mTOR. È noto che l'attivazione di una varietà di recettori del glutammato, tra cui i recettori metabotropici del glutammato (mGluRs), può innescare cascate di segnalazione che sostengono LTP e regolano mTOR (Hoeffer e Klann 2010). Inoltre, è noto che PI3K, una chinasi coinvolta in fenomeni di memoria e apprendimento, è attivata dal complesso calcio / calmodulina (Joyal, Burks et al 1997;. Wang, Fibuch et al 2007.) e dalla subunità βγ della proteina G (Lopez Ilasaca-1998). È stato inoltre dimostrato che i recettori metabotropici del Glutammato di gruppo I (mGluR1 e mGluR5) sono accoppiati all'interno delle cellule alla proteina trimerica Gq: la subunità α di GQ induce l'idrolisi dei fosfoinositidi, con formazione di diacilglicerolo (DAG) e inositol-3-fosfato, inducendo un aumento della concentrazione intracellulare di $Ca2^{+}$. La subunità βγ attiva PI3K (Pin e Duvoisin 1995).

Ci sono numerose testimonianze dell'interazione tra i recettori mGlu e PI3K; i recettori mGlu di gruppo I attivano PI3K, inducendo una pathway di signalling intracellulare che mostra importanti effetti neuroprotettivi. Attraverso l'attivazione di PI3K, mGluRs di gruppo I prevengono l'apoptosi neuronale (Rong, Ahn et al. 2003), promuovono l'attivazione PI3K-dipendente di Akt e mTOR (Hou e Klann 2004), regolano l'attivazione della microglia (Chong, Kang et al. 2005), sono neuro protettivi nei confronti di peptidi Aβ in modelli animali di malattia di Alzheimer (Liu, Gong et al. 2005) e mostrano effetti

neuroprotettivi in modelli animali di ischemia cerebrale (Scartabelli, Gerace et al. 2008).

Infine, vi sono alcune evidenze che dimostrano che mGluRs sono coinvolti nella regolazione della sintesi proteica (Weiler e Greenough 1993;. Angenstein, Greenough et al 1998), quindi non è improbabile che essi possano in qualche modo regolare anche la sintesi di PKMζ.

Partendo dalla nozione che i recettori mGluR di gruppo I, in particolare mGlu5, sono altamente espressi nelle prime fasi del neurosviluppo (Casabona, Knöpfel et al 1997;. Copani, Casabona et al 1998), questi recettori sembravano essere dei buoni candidati per mantenere gli alti livelli di trasmissione sinaptica basale che sono stati osservati nella corteccia peririnale di P14, eventualmente attraverso meccanismi mediati da PI3K /mTOR.

Quindi, al fine di esaminare il ruolo dei recettori mGlu di gruppo I nel mantenimento la trasmissione sinaptica basale in P14, e il loro possibile ruolo nella regolazione dell'attività PKMζ, sono stati eseguiti i seguenti esperimenti.

5.2.2 Risultati

5.2.2.1 Ruolo di mGlu1 nella trasmissione sinaptica basale in corteccia peririnale

Al fine di determinare se una attivazione continua di mGlu1 può contribuire al mantenimento della trasmissione sinaptica basale in corteccia peririnale di P14, dopo 30 minuti di registrazione di base, un antagonista selettivo di mGlu1 (2μM LY456236) è stato applicato per 1 ora (Figura 5.2.1). LY456236 produce una diminuzione nella trasmissione sinaptica basale in corteccia peririnale di P14 (temporale: 76 ± 0,7% del valore basale, p <0,001; entorinale 78 ± 01% del valore basale, p <0,001 , n = 4). Nessun effetto di LY456236 si osserva nella corteccia peririnale quando il composto viene applicato su fettine di cervello adulto (2-3 settimane), come mostrato nella **Figura 5.2.2** (temporale: 101 ± 2% della baseline, p> 0,05; entorinale 99 ± 1,6% del valore basale, p> 0.05, n = 3).

Questi esperimenti sono stati ripetuti utilizzando un altro inibitore di mGluR1, il modulatore allosterico negativo(NAM) JNJ16259685. Dopo 30 minuti di registrazione di baseline, l'applicazione di JNJ16259685 10μM produce una significativa riduzione della trasmissione sinaptica basale in fettine di corteccia peririnale da P14 (**Figura 5.2.3** lato temporale 77 ± 1% del valore basale, p <0,001; lato entorinale 76 ± 1% del valore basale, p <0,001, n = 4). L'effetto sulla trasmissione sinaptica basale di JNJ16259685 è della stessa entità della diminuzione prodotta dall'altro antagonista di mGlu1 LY456236, confermando i risultati precedenti. L'applicazione di JNJ16259685 non mostra alcun effetto sulla corteccia peririnale degli adulti (**Figura 5.2.4**, lato temporale 103 ± 2% del valore basale, p> 0.05; lato entorinale 101 ± 3% del valore basale, p> 0.05, n = 3).

La depressione prodotta da entrambi gli antagonisti di mGlu1 è più piccola di quella prodotta dall' inibizione di PKMζ di mTOR, indicando che, sebbene l'attivazione continua di mGlu1 recettori probabilmente contribuisce a mantenere la trasmissione basale nella corteccia peririnale di P14, altri meccanismi potrebbero essere coinvolti in questo fenomeno. Al fine di confermare che la diminuzione osservata sulla trasmissione sinaptica basale in corteccia peririnale di P14 conseguente al blocco della mGlu1 avviene attraverso un meccanismo mTOR-dipendente, dopo 60 minuti di registrazione di base, inibitore di mTOR 1μM KU0063794 è stato applicato, seguito dal antagonista di mGlu1 LY456236 (**Figura 5.2.5**). I dati mostrano che l'inibizione di mTOR deprime la trasmissione sinaptica basale, come osservato in precedenza (**capitolo 5.1.2.2**) (lato temporale: 67 ± 1% del valore basale, $p < 0.01$; entorinale 68 ± 1% del valore basale, $p < 0.01$, $n = 3$). Nessuna ulteriore riduzione della trasmissione sinaptica, si osserva in seguito alla successiva applicazione di LY456236 (Temporale 65 ± 2% del valore basale, $p > 0.05$; entorinale laterale 66 ± 1% del valore basale, $p > 0.05$, $n = 3$). Pertanto, questi risultati suggeriscono che mGlu1 mantiene la trasmissione sinaptica basale nella corteccia peririnale p14 attraverso un meccanismo mTOR-dipendente.

5.2.2.2 Ruolo di mGlu5 nella trasmissione sinaptica basale in corteccia peririnale

Per valutare se mGlu5, così come mGlu1, contribuisce al mantenimento di elevati livelli basali di trasmissione sinaptica in corteccia peririnale di P14, l'antagonista di mGlu5 MPEP 10μM è stato applicato dopo 30 minuti di registrazione di baseline (**Figura 5.2.6**). Il blocco di mGlu5 produce una piccola ma significativa riduzione della trasmissione sinaptica nella corteccia peririnale di P14 (lato temporale 87 ± 1% del valore basale, $p < 0{,}001$; lato entorinale 83 ± 2% del valore basale , $p < 0{,}001$, $n = 7$). Questi risultati confermano il ruolo di mGlu5 nel mantenimento della trasmissione sinaptica

basale in corteccia peririnale di P14. Nessun effetto dell' inibizione di mGlu5 si osserva su fette di corteccia peririnale adulta (**Figura 5.2.7,** lato temporale 97 ± 2% del valore basale, p> 0.05; lato entorinale 99 ± 2% del valore basale, p> 0,05, n = 5).

Al fine di confermare che il calo osservato sulla trasmissione sinaptica basale in corteccia peririnale di P14 avviene attraverso un meccanismo mTOR-dipendente, dopo 60 minuti di registrazione di base, inibitore di mTOR rapamicina 5μ è stata perfusa sulla fettina, seguita dall'antagonista di mGlu5 MPEP (**Figura 5.2.8**). L'inibizione di mTOR deprime la trasmissione sinaptica basale, come osservato in precedenza (capitolo 5.1.2.2) (lato temporale: 67 ± 2% della baseline, p<0,001; entorinale 68 ± 1% della baseline, p <0,001, n = 6). Nessuna ulteriore riduzione della trasmissione sinaptica si osserva in seguito alla successiva applicazione di MPEP (lato temporale 63 ± 1% del valore basale, p> 0.05; lato entorinale 64 ± 1% del valore basale, p> 0.05, n = 3).
Pertanto, la depressione della trasmissione basale indotta da MPEP nella corteccia peririnale P14 non è stato osservato dopo depotenziamento rapamicina-indotto. Questi risultati suggeriscono che cascate di signalling mGlu5/mTOR-dipendenti mantengono la trasmissione sinaptica basale nella corteccia neonatale.

5.2.2.3 Effetto dell'antagonismo combinato di mGlu1 e mGlu5 sul mantenimento della trasmissione sinaptica basale in corteccia peririnale di P14

Dal momento che i nostri risultati precedenti dimostrano che l'antagonismo selettivo di mGluR1 o mGlu5 in corteccia peririnale di P14 produce una depressione nella trasmissione sinaptica di più o meno il 20% ciascuno, è stato interessante scoprire se questo effetto è additivo, e se è si verifica attraverso l'inibizione dell'attività di PKMζ. Per verificare questa ipotesi, dopo

una registrazione di baseline di 30 minuti, il selettivo mGlu1 NAM, JNJ16259685, è stato applicato sulla fetta di corteccia peririnale di ratto P14, seguito dall'antagonista selettivo di mGlu5, MPEP (**Figura 5.2.9**). L'inibitore selettivo di PKMζ, ZIP, è stato applicato dopo MPEP. Il blocco di mGlu1 produce una significativa riduzione della trasmissione sinaptica nella corteccia peririnale di P14 (lato temporale: 77 ± 1% del valore basale, p <0,001; lato entorinale: 80 ±0,8% del valore basale, p <0,001; n = 4). L'applicazione successiva di MPEP in corteccia P14 diminuisce ulteriormente la trasmissione sinaptica basale in modo additivo (lato temporale: 59 ± 0,6% del valore basale, p <0,001 v JNJ16259685, lato entorinale: 59 ± 0,6% del valore basale, p <0,001 v JNJ16259685; n = 4). La successive applicazione di ZIP, però, produce un'ulteriore piccola, ma significativa, depressione della trasmissione sinaptica (lato temporale: 51 + 0,7% del valore basale, p <0,01 v MPEP; lato entorinale: 51 +1% del valore basale, p> 0,01 v MPEP; n = 4). Pertanto, il depotenziamento ZIP-indotto della baseline non è completamente occluso dal blocco di mGluR1 e mGluR5, questo suggerisce ancora una volta che l'attività di PKMζ è probabilmente regolata dai recettori metabotropici di Gruppo I, anche se non completamente.

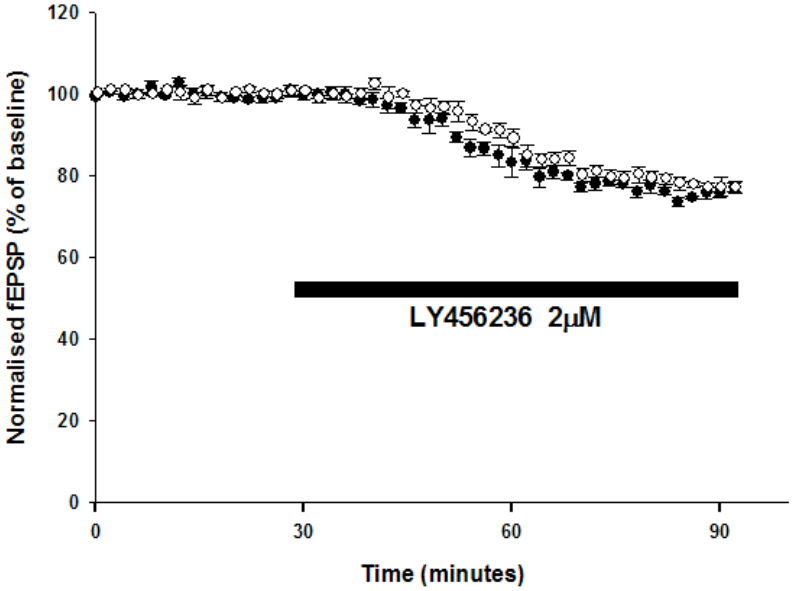

Figura 5.2.1 Effetto di LY456236 sulla corteccia peririnale di P14 L'antagonista selettivo di mGlu1The selective mGlu1, LY456236, produce una diminuzione della risposta sinaptica basale (lato temporale, cerchi neri: 76 ± 0.7% della baseline, p<0.001; lato entorinale, cerchi bianchi 78 ± 01% della baseline, p<0.001, n=4).

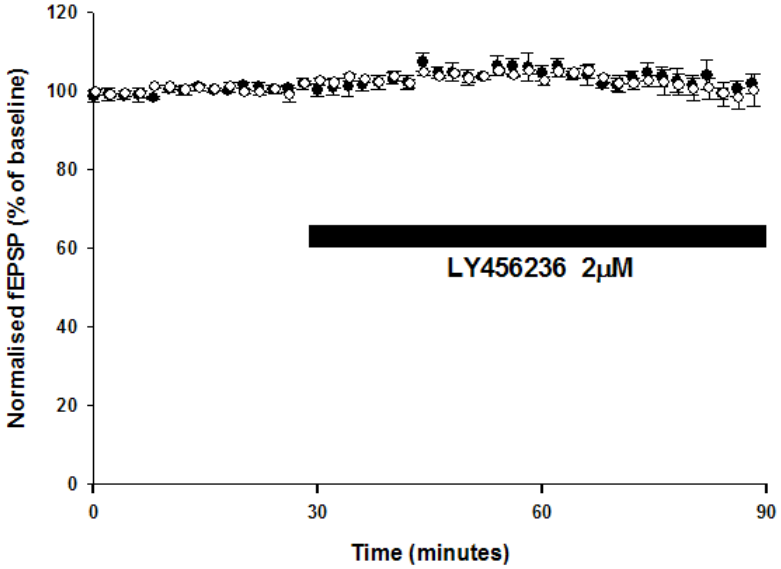

Figure 5.2.2 Effetto di LY456236 su corteccia peririnale di adulto L'antagonista di mGlu1 LY456236 non mostra alcun effetto sulla trasmissione sinaptica basale in corteccia peririnale di adulto (Temporale, cerchi neri: 101 ± 2% della baseline, p>0.05; entorinale, cerchi bianchi 99 ± 1.6% della baseline, p>0.05, n=3).

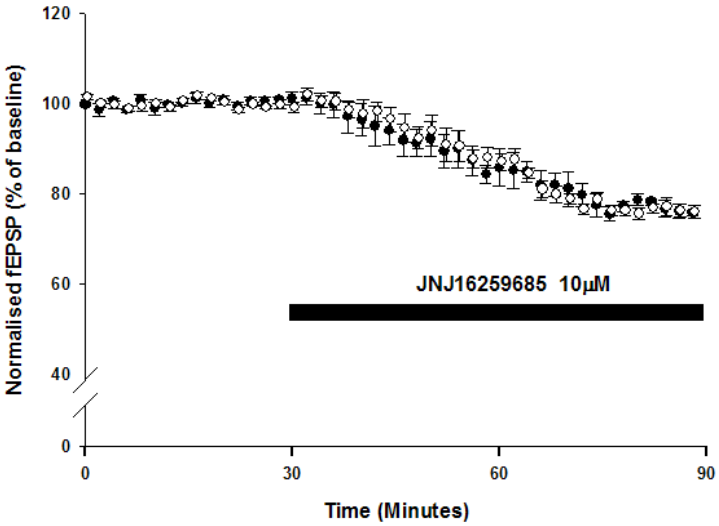

Figura 5.2.3 Effetto di JNJ16259685 su corteccia peririnale di P14 L'applicazione del NAM di mGlu1, JNJ16259685, produce una depressione significativa della trasmissione sinaptica (lato temporale, cerchi neri: 77 ± 1% del valore basale, p <0,001; lato entorinale, cerchi aperti: 76 ±1% del valore basale, p <0,001, n = 4). L'effetto sulla trasmissione sinaptica basale di JNJ16259685 è della stessa entità del calo prodotto dall'altro antagonista di mGlu1 LY456236.

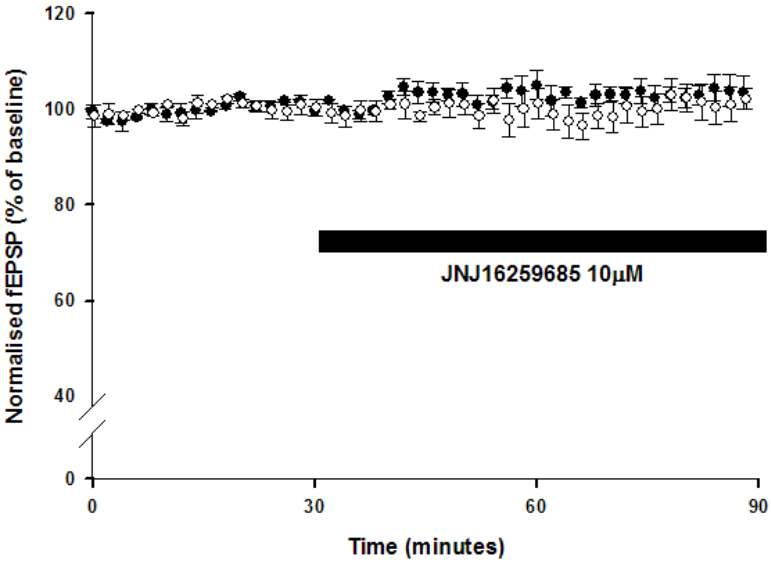

Figura 5.2.4 Effetto di JNJ16259685 su corteccia peririnale di adulto L'applicazione di JNJ16259685 non mostra alcun effetto sulla corteccia peririnale di adulti (lato temporale, cerchi neri: 103 ± 2% della baseline, p> 0.05; lato entorinale, cerchi bianchi: 101 ± 3% della baseline, p> 0.05, n = 3) . Nessuna differenza è stata trovata tra le due pathways (p> 0,05).

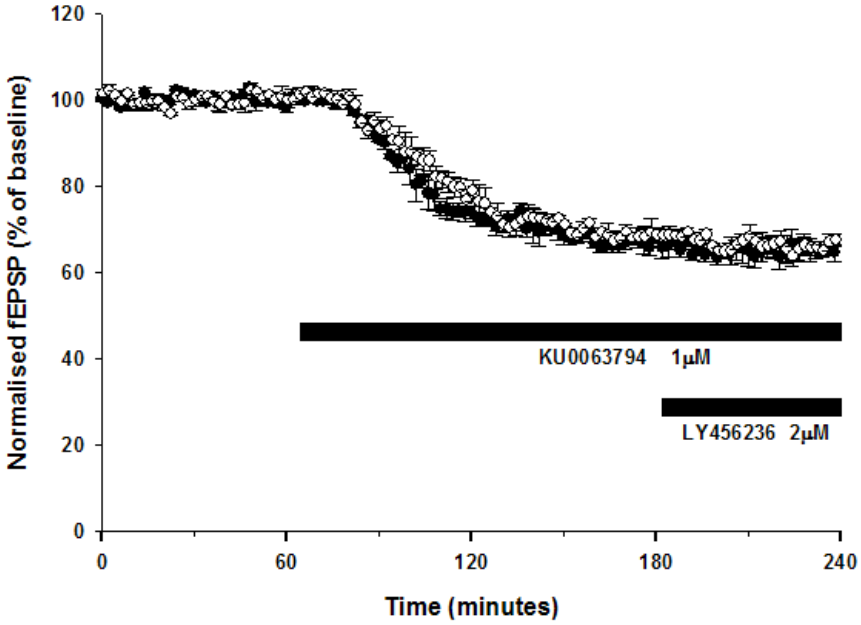

Figura 5.2.5 Effetto di KU0063794 e LY456236 su corteccia peririnale di P14 L'inibizione di mTOR da KU0063794 deprime la trasmissione sinaptica basale (lato temporale, cerchi neri: 67 ± 1% del valore basale, p <0.01; lato entorinale, cerchi aperti 68 + 1% del valore basale, p < 0.01, n = 3). Nessuna ulteriore riduzione della trasmissione sinaptica si osserva in seguito alla successiva applicazione di LY456236 (Temporale 65 +2% del valore basale, p> 0.05; entorinale 66 +1% del valore basale, p> 0.05, n = 3)

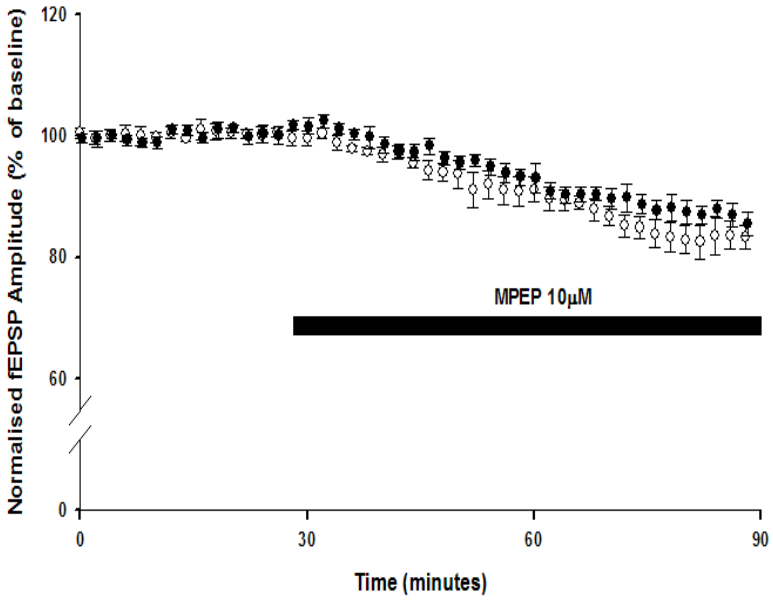

Figura 5.2.6 Effetto di MPEP su corteccia peririnale di P14 L'applicazione dell'antagonista selettivo di mGlu5 MPEP produce una piccola ma significativa riduzione della trasmissione sinaptica nella corteccia peririnale di P14 (lato temporale, cerchi neri: 87 ± 1% del valore basale, p <0,001; lato entorinale, cerchi aperti: 83 ± 2% del valore basale, p <0,001, n = 7).

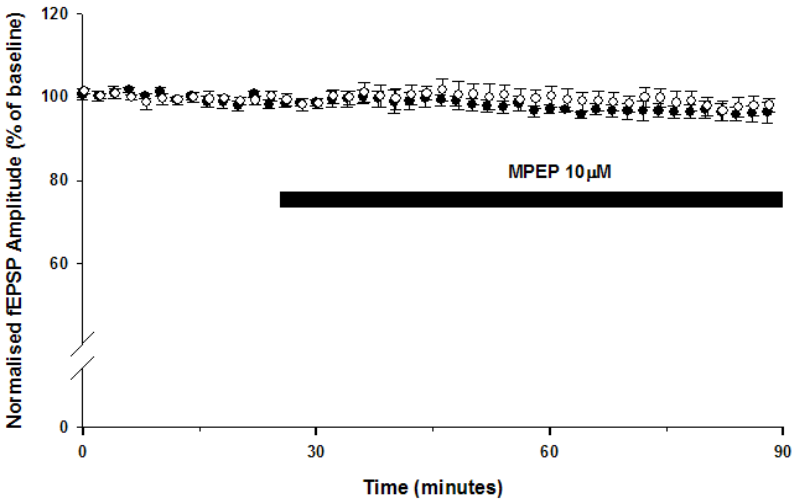

Figura 5.2.7 Effetto di MPEP su corteccia peririnale di adulto L'applicazione dell'antagonista selettivo di mGlu5 MPEP non mostra alcun effetto sulla trasmissione sinaptica basale nella corteccia peririnale di adulto (lato temporale, cerchi neri: 97 ± 2% del valore basale, p <0.05; lato entorinale, cerchi aperti: 99 ± 2% del valore basale, p >0.05, n = 7).

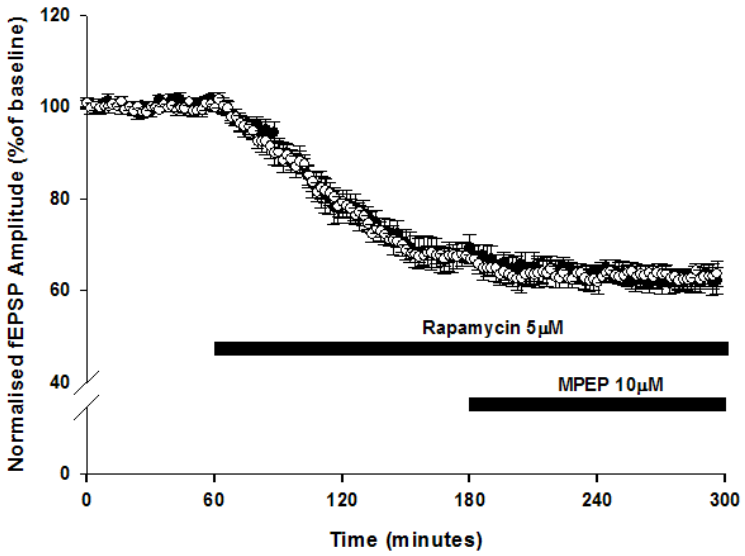

Figura 5.2.8 Effetto di Rapamicina ed MPEP su corteccia peririnale di P14
L'applicazione dell'inibitore di mTOR rapamicina deprime la trasmissione sinaptica basale, come osservato in precedenza (temporale, cerchi neri: 67 ± 2% del valore basale, p <0.001; entorinale, cerchi bianchi: 68 ± 1% del valore basale, p <0.001, n = 6). Nessuna ulteriore riduzione della trasmissione sinaptica, si osserva in seguito all'applicazione di MPEP (temporale 63 ± 1% del valore basale, p> 0.05; lato entorinale 64 ± 1% del valore basale, p> 0.05, n = 3).

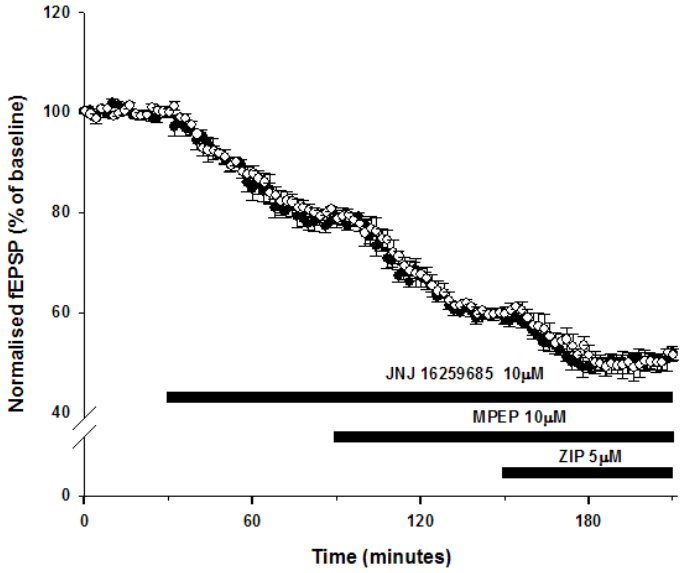

Figura 5.2.9 Effetto di JNJ16259685, MPEP e ZIP su corteccia peririnale di P14
L'applicazione di JNJ16259685 produce una significativa riduzione della trasmissione sinaptica (temporale, cerchi neri: 77 ± 1% del valore basale, p <0.001; entorinale, cerchi bianchi: 80 ± 0,8% del valore basale, p<0.001; n = 4). L'applicazione successiva di MPEP diminuisce ulteriormente la trasmissione sinaptica in modo additivo (temporale: 59 ± 0,6% del valore basale, p <0.001 v JNJ16259685; entorinale: 59 ± 0,6% del valore basale, p<0.001 v JNJ16259685). La successiva applicazione di ZIP produce una ulteriore piccola, ma significativa, depressione della trasmissione sinaptica (lato temporale: 51±0,7% del valore basale, p<0.01 v MPEP; entorinale: 51±1% del valore basale, p>0.01 v MPEP)

5.2.3 Discussione

I precedenti risultati dimostrano che è possibile che un alto livello di trasmissione sinaptica nella corteccia peririnale di P14 sia regolato da PKMζ, che è a sua volta controllata da processi PI3K- e mTOR-dipendenti. Ma cosa mantiene un alto livello di attività di PKMζ? Un possibile candidato è stato identificato nei recettori metabotropici del glutammato (mGluRs), in particolare del gruppo I

.

Come descritto in precedenza, è ampiamente noto che i recettori metabotropici del glutammato sono fondamentali nella plasticità neuronale, in particolare in processi di apprendimento e memoria. Il Gruppo I degli mGluRs, accoppiato con Gq, ha come effetto finale l'idrolisi del fosfatidilinositolo 4,5-bisfosfato (PIP2) a diacil glicerolo (DAG) e inositolo trifosfato (IP3) e tramite vie di signalling intracellulare portano ad un aumento della concentrazione intracellulare di Ca^{2+}. I recettori del Gruppo I sono anche noti per regolare l'attività di mTOR e PI3K, pertanto apparivano come un soggetto interessante da indagare, al fine di chiarire ulteriormente i meccanismi che portano alla attività sostenuta di PKMζ.

Quando l'attività di mGlu1 o mGlu5 è bloccata mediante l'applicazione di antagonisti selettivi, si osserva una piccola ma significativa depressione della trasmissione sinaptica in corteccia peririnale di P14. Il blocco di questi recettori non mostra alcun effetto sulla trasmissione sinaptica nella corteccia peririnale dell'adulto. Di conseguenza, questi recettori sembrano essere coinvolti nel mantenimento degli alti livelli di trasmissione sinaptica nella corteccia peririnale di P14, anche se i meccanismi responsabili di questo effetto non sono noti. Seguendo l'ipotesi che la trasmissione sinaptica basale è mantenuta da PKMζ forse attraverso sintesi proteica PI3K/mTOR-dipendente, l'idea era che il gruppo I degli mGluRs, regolando l'attività di mTOR e PI3K, possa essere a monte dell'intero percorso di signalling. Infatti,

quando gli antagonisti di mGlu1 o mGlu5 vengono applicati sulla fettinaa dopo la depressione della trasmissione sinaptica prodotta dalla inibizione di mTOR, non si osserva nessuna ulteriore diminuzione della risposta. Questi risultati supportano l'ipotesi che la regolazione da parte di mTOR della trasmissione sinaptica si basa, almeno in parte, sull'attivazione di mGlu1 e mGlu5. È importante ricordare che la traduzione mTOR-dipendente ha già dimostrato di essere coinvolta nella plasticità sinaptica e si sa che il gruppo I degli mGluRs è in grado di regolare la traduzione proteica mTOR-dipendente (Hoeffer e Klann 2010). Inoltre, l'inibizione di entrambi mGlu1 e mGlu5 sembra deprimere la trasmissione sinaptica in modo additivo. Ancora una volta, la depressione prodotta da entrambe gli antagonisti di mGlu1 e mGlu5 è più piccola di quella prodotta dalla inibizione diretta di PKMζ, e la successiva applicazione di ZIP produce un' ulteriore piccola diminuzione nella trasmissione sinaptica. La depressione totale della trasmissione sinaptica ottenuta con l'applicazione dei tre composti è della stessa entità di quella prodotta da ZIP da solo. Ancora una volta, quindi, questi risultati suggeriscono che, anche se la continua attivazione del gruppo I dei recettori mGlu contribuisce in grande parte a mantenere la trasmissione basale nella corteccia peririnale di P14, probabilmente esistono altri meccanismi coinvolti in questo processo.

Considerati insieme, questi risultati suggeriscono che una cascata di signalling mGlu1/5-PKMζ-mTOR-dipendente mantiene la trasmissione sinaptica basale nella corteccia neonatale.

6 DISCUSSIONE GENERALE

6.1 Discussione generale

Vi è un crescente interesse nella comunità scientifica nei riguardi di PKMζ. È stato ben dimostrato nel corso degli ultimi anni che PKMζ è cruciale per il mantenimento della LTP in differenti aree del cervello (in particolare l'ippocampo e la corteccia insulare). L'inibizione della PKMζ annulla la LTP sia in vitro che in vivo, e cancella completamente i ricordi codificati in esperimenti comportamentali di vario genere (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). Gli esperimenti eseguiti in corteccia peririnale di adulto confermano il ruolo di PKMζ nel mantenere il potenziamento anche in questa zona del cervello, cruciale per la memoria di riconoscimento. Inoltre, l'attività di PKMζ è down-regolata dalla somministrazione di protocolli di LFS, quando sono in grado di condurre ad una vera e propria, stabile LTD (o de-depressione). Per questi motivi, PKMζ non mostra alcun effetto sulla induzione di LTD, cosi' come non mostra alcun effetto sulla induzione di LTP, in cui non è coinvolta. In generale, i risultati confermano ciò che è noto su PKMζ, come è stato descritto in studi precedenti, anche se questa è la prima volta che tali studi sono svolti nella corteccia peririnale. Inoltre, gli esperimenti sulla inibizione PDK1 sembrano confermare il ruolo cruciale di questa proteina nella regolazione dell'attività PKMζ, anche se, come spiegato prima, è impossibile affermare con certezza che l'effetto osservato sul mantenimento della LTP si verifica solo attraverso una inibizione PDK1-dipendente dell'attività di PKMζ. PDK1, infatti, regola molte differenti processi cellulari, la maggior parte dei quali coinvolti nella proliferazione cellulare. Tuttavia, i risultati sono incoraggianti e potrebbero fornire una base per ulteriori lavori volti a chiarire meglio il signalling intracellulare coinvolto nella regolazione del PKMζ. La regolazione delle attività PKMζ è, infatti, un aspetto molto affascinante, perché questa proteina sembra da sola essere sufficiente per il mantenimento di LTP (Ling, Benardo et al. 2002), ma i meccanismi che portano alla sua attivazione non sono ancora chiari. È piuttosto sorprendente,

in primo luogo, che una sola proteina sia responsabile del mantenimento di un processo cosi' importante e complicato, come il mantenimento della memoria. Inoltre, l'up-regulation di PKMζ in seguito a LTP e la sua down-regulation in seguito a LTD (o viceversa: LTP conseguente ad up-regulation di PKMζ e LTD conseguente alla down-regulation di PKMζ) sembrano essere fenomeni di una semplicità sorprendente. Per quanto riguarda i meccanismi intracellulari che portano all'attivazione di PKMζ, è stato ipotizzato che PKMζ è attivato da varie chinasi, come PI3K, CaMKII e chinasi associate, che sono up-regolate entro 10 minuti post-HFS (Osten, Valsamis et al. 1996; Sacktor 2008). I livelli di PKMζ, invece, aumentano solo dopo 10 minuti post-HFS (Sacktor 2008). Pertanto, PKMζ è idoneo a costituire una via finale comune per l'induzione delle chinasi, in grado di mantenere il potenziamento sinaptico attraverso persistente attività della chinasi. I meccanismi con cui PKMζ mantiene la sua persistente attività chinasica vanno ancora chiariti. In realtà, il tempo di dimezzamento di PKMζ è di un paio d'ore al massimo, a nchese i suoi effetti possono durare per un tempo molto più lungo (forse più di un mese) (Sacktor 2008). È stato suggerito che, una volta attivata, PKMζ auto-mantiene la sua sintesi attraverso un ciclo di feedback positivo (Kelly, Crary et al. 2007), ma i fattori di trascrizione effettivamente responsabili di questa upregulation di cosi' lunga durata devono essere ancora identificati. CREB sembra essere coinvolto (Muslimov, Nimmrich et al. 2004), ma ulteriori studi saranno necessari per ottenere evidenze conclusive.

Esperimenti condotti su animali P14 offrono uno scenario diverso. A differenza di quanto osservato negli adulti, non solo non è possibile indurre LTP in corteccia P14 peririnale, ma l'inibizione di PKMζ riduce significativamente la risposta sinaptica, sia nella baseline sia in una pathway de-depressa. Ulteriori esperimenti hanno dimostrato che PKMζ mantiene questi livelli LTP-simili della risposta sinaptica attraverso una cascata di signalling PI3K/mTOR-dipendente. Inoltre, questi processi sembrano essere sotto il controllo del gruppo I dei recettori metabotropici del glutammato. mGluRs di gruppo I, PI3K e mTOR sono tutti fattori cruciali nella plasticità

sinaptica e i loro ruoli sono stati ampiamente discussi nei capitoli 5.1.1, 5.1.3, 5.2.1 e 5.2.3. Ora, è interessante esaminare il significato di queste osservazioni considerate nel loro insieme. Senza dubbio, un tale comportamento peculiare della corteccia peririnale solleva molti interrogativi, in quanto finora non ci sono prove di un comportamento simile in nessun'altra regione del cervello. PKMζ ora è etichettata come "la proteina che mantiene la LTP" (Hrabetova e Sacktor 1996;. Drier, Tello et al 2002; Ling, Benardo et al 2002; Pastalkova, Serrano et al 2006), quindi un effetto così grande della sua inibizione sulla baseline è abbastanza sorprendente, in un primo momento. Tuttavia, i risultati mostrati nel capitolo 4.2 forniscono un forte supporto all'ipotesi che nella corteccia peririnale di P14 la trasmissione sinaptica basale sia già potenziato da sola, come se fosse in uno "stato permanente di LTP". In quest'ottica, l'effetto di inibizione della PKMζ ha più senso. A quanto pare, in questa fase di sviluppo neurologico nella corteccia peririnale, il ruolo di PKMζ nel mantenere alti livelli di trasmissione sinaptica dovrebbe essere letto in modo diverso, cioè ezzere considerato come un processo di stabilizzazione delle sinapsi immature. Un possibile coinvolgimento di PKMζ nella stabilizzazione delle sinapsi immature nel corso dello sviluppo neurologico è stato recentemente dimostrato in Xenopus (Liu, Tari et al. 2009). Inoltre, anche se i motivi per cui la trasmissione sinaptica viene mantenuta in uno stato potenziato non sono noti, è possibile che questi meccanismi sinao finalizzati a promuovere o stabilizzare connessioni sinaptiche nella corteccia immatura cerebrale (Hua e Smith 2004; Cline e Haas 2008; Hanse, Taira et al. 2009). Questa stabilizzazione delle sinapsi immature può avvenire attraverso un meccanismo che si basa sulla trasmissione glutammatergica e l'inserimento dei recettori AMPA nella membrana neuronale (Rajan, Witte et al 1999;. Haas, Li et al 2006;. Hanse, Taira et al 2009). Poiché vi è un crescente accordo sulle prove che PKMζ mantiene LTP attraverso la regolamentazione del traffico dei recettori AMPA (Ling, Benardo et al 2006;. Yao, Kelly et al 2008; Migues, Hardt et al 2010), non è azzardato dire che questi meccanismi di stabilizzazione sinaptica sono essenzialmente gli stessi che operano nell'espressione/ mantenimento del

potenziamento a lungo termine (LTP). Pertanto la stabilizzazione della trasmissione nelle sinapsi immature è potenzialmente sotto il controllo di meccanismi di induzione, espressione e di mantenimento di processi LTP-simili.

Più tardi durante lo sviluppo, il potenziamento "di base" viene gradualmente perduto, finché non è possibile cominciare ad indurre LTP in corteccia peririnale. Ciò è già evidente a PND35, e persiste in età adulta.

I meccanismi che riducono il ruolo della LTP e PKMζ durante lo sviluppo della corteccia peririnale non sono noti, ma potrebbero derivare da processi LTD-simili che sono alla base della memoria di riconoscimento visivo (Brown e Bashir 2002; Griffiths, Scott et al 2008). L'apertura degli occhi potrebbe essere un elemento importante coinvolto in questo fenomeno. La corteccia peririnale è strettamente connessa alla corteccia visiva, per cui una ipotesi molto interessante è che i processi LTD-simili potrebbero avvenire in corteccia peririnale come conseguenza di nuovi input provenienti dalla corteccia visiva. Vale la pena sottolineare ancora una volta che a PND14 i ratti hanno gli occhi ancora chiusi. L'ipotesi che le prime esperienze visive possano produrre una qualche forma di plasticità della corteccia visiva, che a sua volta modifica le connessioni sinaptiche nella corteccia peririnale, è senza dubbio affascinante, ma che richiederebbe ulteriori indagini. Un modo per farlo potrebbe essere cercare di indurre LTP in ratti adulti in deprivazione visiva. Se i meccanismi che portano alla depressione della trasmissione sinaptica basale, che consente alla sinapsi di essere potenziata in seguito a stimolazione adeguata, dipendono da input visivi, gli animali adulti allevati al buio non dovrebbero presentare LTP, esattamente come avviene nei P14.

Un'altra possibilità interessante è che la stabilizzazione neuronale mantenuta da PKMζ si verifichi al fine di prevenire disturbi dello sviluppo. Una regolazione aberrante della sintesi proteica è importante nei disturbi dello sviluppo, come autismo e ritardo mentale (Hoeffer e Klann 2010). È possibile

che PKMζ, mantenendo una sorta di stabilità nelle sinapsi, impedisca anche il verificarsi di una sorta di sintesi proteica aberrante a livello dendritico che potrebbe produrre anomalie nella trasmissione sinaptica e nelle connessioni durante le prime fasi dello sviluppo. Pertanto meccanismi PKMζ / mTOR-dipendenti potrebbe essere essenziale anche per il normale sviluppo del sistema nervoso centrale.

Finora, non c'è lavoro svolto sugli esseri umani, ma ovviamente questa proteina è di grande interesse soprattutto in patologie che coinvolgono la memoria. Alcuni lavori preliminari dimostrano che PKMζ forma aggregati con grovigli neurofibrillari e recettori AMPA nella malattia di Alzheimer (Crary, Shao et al. 2006). Inoltre, ci sono evidenze che una disregolazione della segnalazione di mTOR sia coinvolta in molte patologie, come il morbo di Alzheimer, la sindrome da X-fragile, sclerosi tuberosa e così via (Pei e Hugon 2008;. Swiech, Perycz et al 2008; Ehninger, de Vries et al . 2009; Hoeffer e Klann 2010; Ma, Hoeffer et al 2010;. Malter, Ray et al 2010;. Sharma, Hoeffer et al 2010), e gli esperimenti descritti nel capitolo 5.1 mostrano un legame tra l'attività di mTOR e PKMζ. Infine, PKMζ potrebbe giocare un ruolo anche nelle malattie psichiatriche, come disturbo post-traumatico da stress (PSTD). Lavori preliminari hanno dimostrato, infatti, (Cohen, Kozlovsky et al. 2010) che l'inattivazione di PKMζ in varie zone cerebrali riduca i comportamenti PSTD-simil.

Pertanto, PKMζ è un argomento molto interessante da sviluppare, e una conoscenza più completa della sua attività potrebbe produrre strumenti incredibilmente utili per comprendere meglio i meccanismi alla base della stabilizzazione sinaptica nel neuro sviluppo e del mantenimento della memoria nella vita adulta.

7 Bibliografia

Bibliografia

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