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Constitutively active group I mGluRs and PKMzeta regulate synaptic transmission in developing perirhinal cortex

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Abstract

Synaptic transmission is essential for early development of the central nervous system. However, the mechanisms that regulate early synaptic transmission in the cerebral cortex are unclear. PKM ζ is a kinase essential for the maintenance of LTP. We show for the first time that inhibition of PKM ζ produces a profound depression of basal synaptic transmission in neonatal, but not adult, rat perirhinal cortex. This suggests that synapses in early development are in a constitutive LTP-like state. Furthermore, basal synaptic transmission in immature, but not mature, perirhinal cortex relies on persistent activity of metabotropic glutamate (mGlu) receptor, PI3Kinase and mammalian target of rapamycin (mTOR). Thus early in development, cortical synapses exist in an LTP-like state maintained by tonically active mGlu receptor-, mTOR- and PKM ζ - dependent cascades. These results provide new understanding of the molecular mechanisms that control synapses during development and may aid our understanding of developmental disorders such as autism and schizophrenia.

Keywords

LTP; PKM ζ ; group I mGluR; cerebral cortex; development; mTOR; protein translation

1. Introduction

Normal brain development requires formation of appropriate and precise synaptic connections, which may occur through activity-dependent plasticity mechanisms that occur at precisely timed stages. There is evidence that at or around the time of birth intrinsic neuronal activity can drive synchronised oscillations and intracellular calcium waves in both neocortex and hippocampus (Garaschuk et al 2000; Allene and Crossart 2010). Postnatal synaptic activity is then triggered mainly by depolarising GABAergic mechanisms that

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develop prior to glutamate transmission (Ben Ari et al 2007). However, glutamate transmission is now also known to drive activity that is important for normal postnatal cortical development (Allene and Crossart 2010). For example, the stabilisation of immature synapses may occur through synchronised glutamatergic transmission, which drives AMPA receptor insertion into the postsynaptic neuronal membrane (Rajan et al 1999; Hanse et al 2009; Haas et al 2006), thus unsilencing silent synapses. In addition, critical periods of cortical development are associated with silent AMPA receptor-lacking synapses being converted into AMPA receptor-containing synapses (Groc et al 2006).

Some of the mechanisms of synapse stabilisation and development are similar to those that operate in the expression/maintenance of LTP. Therefore stabilisation of, and transmission at, developing synapses is potentially under the control of LTP-like induction, expression and maintenance mechanisms. PKM ζ is a kinase essential for the maintenance of LTP (Hrabetova and Sacktor 1996; Ling et al 2002) and also plays critical roles in learning and memory (Drier et al 2002; Pastalkova et al 2006) and posttraumatic stress disorder (Cohen et al 2010). Previous work has shown that whilst inhibition of PKM ζ reverses experimentally induced LTP there is no effect of PKM ζ inhibition on basal synaptic transmission (Ling et al 2002; Serrano et al 2005). The role of LTP in synapse stabilisation during development raises the intriguing possibility that PKM ζ may be important in development. Interestingly, it has been shown recently that PKM ζ confers dendritic stabilisation during development of xenopus retinotectal pathway (Liu et al 2009). However, whether PKM ζ regulates synaptic transmission during development is not known.

We now show that PKM ζ , and other mechanisms that are important in the maintenance of LTP, critically regulate basal synaptic transmission in neonatal perirhinal cortex. Thus, LTP was readily induced in adult perirhinal cortex but could not be induced in P14 perirhinal cortex. Surprisingly, inhibition of PKM ζ depressed basal synaptic transmission in immature (P12-14) but not in adult perirhinal cortex. Furthermore, mTOR-dependent protein translation also maintains basal transmission in P14 but not in adult cortex. Finally, basal synaptic transmission in neonatal cortex also relied on PI3kinase and group I mGluR activity. These results provide the first evidence that basal synaptic transmission in immature perirhinal cortex relies on tonically active PKM ζ -, mTOR-dependent and PI3kinase/group I mGluR mechanisms. This suggests that basal synaptic transmission is in a fully potentiated state early in development and that normal synaptic and cortical development most likely rely on sustained LTP-like mechanisms.

2. Materials and Methods

2.1 In vitro electrophysiology

Adult rats (2-3 months) were anesthetized with an isoflurane/oxygen mixture and decapitated, and the brain was rapidly removed. Neonatal rats (P12-14) were decapitated without anaesthesia and the brain rapidly removed. The brain was placed in ice-cold artificial CSF (aCSF) (bubbled with 95% O₂/5% CO₂) which comprised the following (in mM): 124 NaCl, 3 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgSO₄, 10 D-glucose. Perirhinal slices: A midsagittal section was made, the rostral and caudal parts of the brain were removed by single scalpel cuts at 45° to the dorsoventral axis, and each hemisphere

glued by its caudal end to a vibroslice stage (Campden Instruments). Slices (400 μ m) of PRH were taken in the region 4 mm behind bregma. Parasagittal hippocampal slices (400 μ m) were prepared using standard procedures. Slices were stored submerged in aCSF (20–25°C) for 1–6 h before transferring to the recording chamber. A single slice was placed in a submerged recording chamber (28–30°C; flow rate, 2 ml/min) when required. Standard *in vitro* extracellular field recordings were made from the perirhinal cortex or hippocampus (Ziakopoulos et al., 1999; Massey et al., 2004). Evoked field EPSPs (fEPSPs) in cortex were recorded from layers II/III from directly below the rhinal sulcus (area 35). 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. Stimuli (constant voltage) were delivered alternately to the two stimulating electrodes (each electrode, 0.033 Hz). fEPSPs were reduced to 60–70% of maximum amplitude and a baseline of synaptic transmission established before induction of synaptic plasticity. High frequency stimulation (HFS) or theta burst stimulation (TBS) was delivered to induce LTP. (HFS: one or four trains of 100 Hz, 1 s. Repeated every 30 s when 4 trains delivered. TBS: bursts of four stimuli at 100 Hz. Each burst repeated four or six times at intervals of 200ms. This sequence is repeated four times at intervals of 10s, thus giving four repetitions of four bursts or four repetitions of 6 bursts). To induce LTD, LFS (900 stimuli, 1 Hz) was delivered. fEPSPs were monitored and reanalyzed off-line using the acquisition and analysis software WinLTP (Anderson and Collingridge, 2007). In perirhinal cortex fEPSPs can be composed of multiple components, the earliest of which are non-synaptic and equivalent to the fibre volley as seen in hippocampal slice recordings (Ziakopoulos et al., 1999). To ensure that only synaptically evoked responses were measured calcium-free aCSF was perfused onto perirhinal cortex slices at the end of all experiments. This allows assessment of the non-synaptic/synaptic components of the evoked response and allows a true analysis of the fEPSP. The peak amplitude of evoked fEPSPs was measured and expressed relative to the preconditioning baseline. Drugs were made up as stock solutions and added to the perfusate at a final concentration as indicated in the figures and text.

2.2. Immunoblot analysis

Perirhinal cortex was dissected from adult and P14 rats and stored frozen at –80°C. 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, and supplemented with a protease inhibitor cocktail (Calbiochem, Gibbstown, NJ, USA). Protein concentration was determined via Bio-Rad Assay and 50 μ g 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-PKC ζ 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).

2.3. Measurement of polyphosphoinositide hydrolysis in cortical slices

Receptor agonist-stimulated PI hydrolysis was measured in perirhinal cortical slices, as described by Nicoletti et al. (1986). In brief, 14 day old or adult rats were killed by decapitation, and perirhinal cortices were sliced (350×350 µm) using a Mc Ilwain tissue chopper. Slices were incubated at 37°C under constant oxygenation for 30-45 min in Krebs-Hensleit buffer equilibrated with 95% O₂, 5% CO₂ to pH 7.4. Forty µl of gravity packed slices were then incubated for 60 min in 250 µl buffer containing 1 µCi of myo-[³H]inositol (specific activity 18 Ci/mmol, GE Healthcare, Milano, Italy). Slices were incubated with LiCl (10 mM, for 10 min) followed by the indicated concentrations of MPEP or JNJ16259685. One h later, the incubation was stopped by the addition of 900 µl of methanol:chloroform (2:1), after washing the slices with ice-cold buffer. After further addition of 300 µl chloroform and 600 µl water, the samples were centrifuged at low speed to facilitate phase separation. After centrifugation at 2,000 g for 20 min, the [³H]InsP present in the supernatant was separated by anion exchange chromatography in 10-ml columns containing 1.5 ml of Dowex 1-X-8 resin (formate form, 100-200 mesh, BioRad, Milan, Italy). Columns were washed twice with water, once with a solution of 5 mM sodium tetraborate and 40 mM sodium formate to elute cyclic InsP and glycerophosphoinositols, and then with 6.5 ml of 0.2 M ammonium formate and 0.1 M formic acid for the elution of InsP (see Nicoletti et al., 1986). Total radioactivity in the perirhinal cortex was determined by counting a 100 µl aliquot of each phase.

3. Results

3.1. PKM ζ maintains basal transmission in neonatal perirhinal cortex

Activation of the atypical kinase PKM ζ is essential in the maintenance of LTP, as demonstrated by the extensive use of the PKM ζ inhibitor ZIP. Thus, application of ZIP reverses LTP but has no effect on non-potentiated synapses (Hrabetova and Sacktor, 1996; Ling et al 2002). However, we found in P14 perirhinal cortex the PKM ζ inhibitor ZIP depressed synaptic transmission under control conditions (filled circles: $53 \pm 4\%$ of baseline, $P < 0.001$; $n = 7$; Fig 1A). This surprising effect on baseline transmission suggests the intriguing hypothesis that synapses in P14 perirhinal cortex are in an LTP-like state under basal conditions. If these neonatal synapses are indeed basally potentiated then it should be possible to induce 'depotentialization' of baseline transmission. Importantly, it follows that the activity dependent reversal of LTP should prevent any subsequent depression by ZIP. To test these ideas, LTD was saturated in one input by delivering 3 periods of LFS (open circles: $54 \pm 4\%$ of baseline 30 min after last LFS, $P < 0.001$, $n = 7$; Fig 1A). Subsequent application of ZIP had no effect on the input in which LTD had been previously induced ($94 \pm 9\%$ compared to pre-ZIP level; $P > 0.05$, $n = 7$; Fig 1A). Application of scrambled ZIP, that has no effect on LTP, had no effect on basal transmission (Fig 1B). These results suggest that constitutive activation of PKM ζ maintains basal transmission in an LTP like state.

3.2. Lack of experimentally induced LTP in neonatal perirhinal cortex

If synapses in P14 perirhinal cortex are in a basally potentiated state then this should reduce the likelihood of experimentally inducing LTP. Indeed, we were completely unable to induce LTP in neonatal (P12-14) rat perirhinal cortex slices: high frequency stimulation (HFS; 100Hz, 1s), that produces LTP in a variety of brain regions across a range of different ages, did not induce LTP in neonatal perirhinal cortex ($95 \pm 3 \%$, 30 min post HFS, $P > 0.05$, $n = 6$, **data not shown**). Similarly, 4 HFS trains, that induce LTP in adult perirhinal cortex (Ziakopoulos et al 1999; Massey et al 2004), failed to induce LTP in P14 perirhinal cortex ($98 \pm 2 \%$, 30 min post HFS, $P > 0.05$, $n = 5$, **data not shown**). In addition, two different theta burst stimulation protocols (see methods) also failed to produce LTP in neonatal perirhinal cortex ($105 \pm 3 \%$, $P > 0.05$, $n = 7$; $103 \pm 6 \%$, $P > 0.05$, $n=8$; **data not shown**). Whilst LTP was not induced by these induction protocols PTP and decaying short-term potentiation were observed in these experiments.

Our explanation for the inability to induced LTP in neonatal perirhinal cortex is that LTP mechanisms are fully saturated and therefore the induction of experimentally-induced LTP is occluded. To examine this possibility we ‘unsaturated’ LTP by induction of LTD (or ‘depotentialisation’). Low frequency stimulation (LFS; 1 Hz, 900 s) induced LTD ($74 \pm 2 \%$ of baseline, $P < 0.05$, $n = 4$, Fig 1C) and subsequent HFS resulted in lasting potentiation (to $99 \pm 8 \%$ of original pre-LTD baseline, 60 min post HFS; $P < 0.05$, $n = 4$; Fig 1C). We next investigated whether PKM ζ activity is involved in the long-term potentiation induced following LTD. After LTP, the application of ZIP depressed synaptic transmission in both the potentiated pathway ($50 \pm 5 \%$ of baseline; $P < 0.01$; Fig 1C) and the control pathway ($48 \pm 3 \%$ of baseline, $P < 0.01$; Fig 1C). Therefore, the maintenance of LTP (induced following LTD) and the maintenance of basal synaptic transmission both rely on activation of PKM ζ . This further indicates that basal synaptic transmission in immature perirhinal cortex is in a fully potentiated LTP-like state and that this LTP-like state relies on PKM ζ activity.

3.3. In adult perirhinal cortex PKM ζ regulates LTP but not basal transmission

LTP is readily induced in adult (2-3 month) rat perirhinal cortex (Ziakopoulos et al 1999; Massey et al 2004) and the application of ZIP completely reversed LTP (LTP: $131 \pm 5 \%$, 60 min post HFS, $P < 0.01$. After ZIP: $98 \pm 5 \%$ of baseline, $P < 0.01$, $n = 7$, Fig 1D) with only a small non-significant effect on the control input ($93 \pm 4 \%$ of baseline, $P > 0.05$, $n = 7$, Fig 1D). Therefore in the adult perirhinal cortex PKM ζ -dependent mechanisms are not active during basal transmission but become active following LTP. We next assayed levels of PKM ζ and found that PKM ζ levels were significantly higher ($P < 0.01$) in perirhinal cortex from P14 animals compared to adult animals (adults: $100 \pm 7 \%$, $n = 8$; P14: $154 \pm 9 \%$, $n = 9$, Fig 1E). These results suggest that in neonatal cortex, in contrast to adult cortex, there is constitutive basal activity of PKM ζ that maintains synapses in a tonically potentiated state.

3.4. PKM ζ regulates LTP but not basal transmission in P14 hippocampus

LTP was readily induced by HFS in CA1 region of neonatal hippocampus ($141 \pm 5 \%$ of baseline; $P < 0.01$; $n = 4$). In P14 hippocampus ZIP fully reversed LTP ($100 \pm 7 \%$ of baseline, $P < 0.01$) but had no effect on basal transmission in the control, non-tetanised input ($92 \pm 6 \%$ of baseline, $P > 0.05$, $n = 4$; Fig 1F). Therefore, there are differences between the

mechanisms that maintain basal synaptic transmission in P14 perirhinal cortex and P14 hippocampus.

3.5. mTOR activity controls basal transmission in neonatal cortex

Our results suggest that PKM ζ maintains basal transmission in P14 but not in adult perirhinal cortex. Whilst there is a good deal of evidence that PKM ζ maintains enhanced synaptic transmission in LTP, the underlying mechanisms by which this occurs are still not known. One possible mechanism involves PKM ζ regulation of protein synthesis through local dendritic translation (Westmark et al 2010). Translation initiation relies on the activation of mammalian target of rapamycin (mTOR), the inhibition of which prevents LTP (Hoeffer and Klann, 2010), and so we examined any role of mTOR in regulation of synaptic transmission. The mTOR inhibitor rapamycin produced a substantial depression of basal transmission in P14 perirhinal cortex ($73 \pm 3\%$ of baseline, $P < 0.001$, $n = 6$, **open circles**, Fig 2A) but had no effect on basal transmission in adult (2-3 month) perirhinal slices ($99 \pm 3\%$ of baseline, $P > 0.05$, $n = 3$; **closed circles**, Fig 2A). KU0063794, an alternative inhibitor of mTOR, also produced a substantial depression of synaptic transmission in P14 perirhinal cortex ($70 \pm 2\%$ of baseline, $n = 4$; $P < 0.01$, Fig 2C). This suggests that ongoing protein translation is important in controlling basal levels of synaptic transmission in P14 but not in adult perirhinal cortex.

3.6. Occlusion between PKM ζ and mTOR regulation of basal transmission

mTOR enables protein translation by phosphorylation of 4E-BPs that normally suppress initiation of translation (Hoeffer and Klann, 2010). It has been suggested (Westmark et al, 2010) that PKM ζ may trigger protein translation through a signalling cascade involving Pin1 – a protein that interacts with and switches off 4E-BP suppression of translation. If PKM ζ maintains elevated transmission through protein translation then this is likely to operate through the same 4E-BP initiation cascade as the mTOR-dependent translation. In keeping with this hypothesis we find that the depression of transmission by inhibition of PKM ζ in P14 perirhinal cortex occludes any subsequent depression of synaptic transmission by rapamycin (ZIP $55 \pm 3\%$ of baseline, $P < 0.001$; rapamycin $52 \pm 3\%$ of baseline, $P > 0.05$ v ZIP; $n = 4$, Fig 2B). Together these results suggest that protein translation regulated by PKM ζ maintains basal synaptic transmission in neonatal perirhinal cortex.

3.7. PI3kinase controls basal transmission in P14 cortex

What are the mechanisms upstream of protein translation and PKM ζ that may be involved in maintaining an elevated level of synaptic transmission in P14 perirhinal cortex? It is known that mTOR-dependent translation can be triggered by PI3kinase. The PI3kinase inhibitors wortmannin or LY294002 produced a substantial depression of transmission in P14 perirhinal cortex (wortmannin: $62 \pm 4\%$; LY294002: $62 \pm 1\%$ of baseline, $P < 0.01$ for both, $n = 4$, Fig 2C). Following depression by the mTOR inhibitor KU0063794 there was no further depression induced by inhibition of PI3K by LY294002 (KU0063794: $62 \pm 2\%$, LY294002: $61 \pm 1\%$, $n = 4$, Fig 2C). Inhibition of mTOR or PI3K had no effect on basal transmission in adult (2-3 month) cortex (KU0063794: $101 \pm 4\%$; wortmannin: $106 \pm 5\%$; LY294002: 107

$\pm 6\%$; all $n = 3$, Fig 2C). Together these results indicate that PI3K regulates basal transmission in neonatal but not adult perirhinal cortex.

3.8. Group-I mGlu receptors control basal transmission in P14 cortex

Activation of group I mGlu receptors can trigger PI3K and mTOR signalling cascades that underpin LTP (Hoeffer and Klann, 2010). Interestingly, pharmacological activation of group I mGlu receptors has been shown to enhance the production of PKM ζ in the adult hippocampus (Sajikumar and Korte, 2011). We find that the mGlu5 receptor antagonist MPEP produced a small but significant depression of basal transmission in P14 ($87 \pm 2\%$ of baseline, $P < 0.01$; $n = 7$, Fig 2D) but not in adult cortex ($98 \pm 2\%$ of baseline, $P > 0.05$; $n = 5$, Fig 2D). In addition, the mGlu1 antagonist LY456236 and the mGlu1 negative allosteric modulator JNJ16259685 each produced a depression of basal transmission in P14 cortex (LY456236: $77 \pm 3\%$, $P < 0.001$, $n = 4$; JNJ16259685: $78 \pm 2\%$; $P < 0.001$, $n = 4$, Fig 2D) but not in adult cortex (LY456236: $100 \pm 1\%$; JNJ16259685: $102 \pm 3\%$; $n = 3$, Fig 2D).

The depression of transmission induced by inhibition of group I mGlu receptors in P14 perirhinal cortex was not observed following rapamycin-induced depression (rapamycin $67 \pm 2\%$ of baseline, $P < 0.001$; MPEP $63 \pm 1\%$ of baseline, $P > 0.05$ v rapamycin; $n = 6$, Fig 2E). The same result also occurred when either of the mGlu1 antagonists JNJ16259685 or LY456236 was applied after the mTOR inhibitor KU0063794 (KU0063794 to $62 \pm 2\%$; JNJ16259685 to $61 \pm 2\%$, $P > 0.05$ v KU0063794. LY456236: to $65 \pm 2\%$, $P > 0.05$ v KU0063794, $n = 4$; Fig 2E). Furthermore, following depression of basal transmission by ZIP (to $55 \pm 2\%$, $P < 0.01$, $n = 4$), the mGlu1 negative allosteric modulator JNJ16259685 and the mGlu5 antagonist MPEP did not result in any further depression (to $56 \pm 1\%$ of baseline, $n = 4$, **Data not shown**).

3.9. Endogenous mGlu receptor activation supports the expression of PKM ζ in P14 cortex

P14 perirhinal cortex slices were treated with MPEP and JNJ16259685 alone or in combination and then used for immunoblot analysis of PKM ζ . MPEP ($10\ \mu\text{M}$) did not produce a significant reduction in PKM ζ expression (Fig. 3A) but the mGlu1 negative allosteric modulator JNJ16259685 ($10\ \mu\text{M}$) significantly reduced PKM ζ expression (Fig. 3A). In agreement with our electrophysiology data, JNJ16259685 and MPEP failed to reduce PKM ζ expression in slices from adult (2-3 month) perirhinal cortex (Fig. 3B). These data indicate that endogenous activation of mGlu1 receptors supports the expression of basal PKM ζ in P14 but not in adult perirhinal cortex.

A greater activity of group I mGlu receptors during early postnatal development (Nicoletti et al, 1986) may explain the difference between P14 and adult perirhinal cortex. We examined this possibility by measuring PI hydrolysis, that is the canonical transduction pathway activated by mGlu1/5 receptors (Nicoletti et al 2011). Basal PI hydrolysis (which reflects the activity of all receptors coupled to $G_{q/11}$) did not differ between P14 and adult (2-3 month) perirhinal cortex (Fig 3C,D). In line with the above PKM ζ expression data, MPEP ($10\ \mu\text{M}$) did not affect basal PI hydrolysis (Fig 3C,D) but the mGlu1 negative allosteric modulator JNJ16259685 ($10\ \mu\text{M}$) significantly reduced basal PI hydrolysis in P14 (Fig 3C), but not in adult perirhinal cortex (Fig 3D). These results suggest the existence of endogenous mGlu1

receptor tone that maintains elevated levels of PI hydrolysis in developing cortex. This activity via PI3K, mTOR and PKM ζ may be responsible for the basal LTP state of synapses in immature perirhinal cortex.

4. Discussion

Our results show for the first time that mechanisms that rely on PKM ζ play a critical role in maintenance of synaptic function in the neonatal mammalian perirhinal cortex. The maintenance of basal synaptic activity during early development is driven by tonically active group I mGluRs acting through a PI3K/PKM ζ /mTOR cascade. Given the central function of PKM ζ in the maintenance of LTP (Ling et al 2002; Serrano et al 2005) we suggest that LTP is crucial for normal synaptic transmission in developing neonatal perirhinal cortex.

Our observation on the lack of LTP in neonatal perirhinal cortex was initially surprising given that LTP is readily induced in adult perirhinal cortex (Ziakopoulos et al 1999; Massey et al 2004) and is also readily induced early in development in other brain regions, such as in hippocampus (Malenka and Bear 2004). However, our results using ZIP to inhibit PKM ζ suggest that this lack of LTP in neonatal perirhinal cortex can be explained by constitutive LTP-like mechanisms that maintain basal transmission in a potentiated state during early development. ZIP is a pharmacological tool that has been used extensively as an inhibitor of PKM ζ (eg Pastalkova et al 2006; Madroñal et al 2010; Sacktor, 2011). That ZIP produced a depression of transmission only under certain conditions (eg on basal transmission but not following LTD in P14 slices; following LTP but not on basal transmission in adult slices) and that a scrambled version of ZIP had no effect on basal synaptic transmission in P14 perirhinal cortex increases confidence that the depression due to ZIP is not through generalised non selective effects on, for example, neuronal excitability. Given these results and previous data using ZIP (eg Pastalkova et al 2006; Madroñal et al 2011; Sacktor, 2011) we conclude that the effects of ZIP in this study are most likely through inhibition of PKM ζ . However, we cannot completely rule out the possibility that ZIP may cause its effects through actions on some other protein kinase.

It is likely that in early development synaptic transmission is maintained in an enhanced state to promote or stabilise synaptic connections in the immature cerebral cortex (Hanse et al 2009; Hua and Smith 2004; Cline and Haas 2008). The mechanisms by which PKM ζ maintains LTP are still a matter of debate but may involve AMPA receptor insertion/stabilisation in the synaptic membrane (Yao et al 2008). The insertion of AMPA receptors is critical for the stabilisation of synaptic connections in the immature CNS (Hanse et al 2009; Hua and Smith 2004; Cline and Haas 2008) and interestingly in this context PKM ζ has recently been shown to be important for synapse stabilisation in the retino-tectal pathway in developing xenopus (Liu et al 2009). Together these results suggest that early in development most CNS synapses may have high levels of PKM ζ that produce and or stabilise dendritic growth through LTP-like mechanisms. However, our results indicate that there is likely a depotentiation-like phenomenon that occurs during development and this may well occur across the CNS. The mechanisms that reduce the role of LTP and PKM ζ during development are not known but in perirhinal cortex might arise from LTD-like processes that underlie visual recognition memory (Brown and Bashir 2004; Griffiths et al

2008) or in other regions might be due to other developmental processes such as the end of critical periods or pruning of synapses.

Protein translation, that is known to be required for the maintenance of LTP, growth of synapses during development, and memory (Hoeffer and Klann 2010), may be a possible route for PKM ζ maintenance of LTP (Westmark et al 2010). In keeping with this we demonstrate that mTOR-dependent mechanisms are also critical for the regulation of basal transmission in immature but not adult perirhinal cortex suggesting that there is constitutive mTOR-dependent translation in early development. The occlusion between the effects of inhibition of PKM ζ and inhibition of mTOR suggests that their regulation of transmission in developing cortex may occur through a common mechanism. It is intriguing to note that in a recent study it was reported that rapamycin was able to reverse LTP and memory in adult mice with a constitutively active form of mTOR (Ehninger et al 2008). These results suggest that constitutively active mTOR maintains LTP, and provides further support for our hypothesis that LTP may be maintained by a PKM ζ /mTOR-dependent mechanism.

There is emerging evidence that the stabilisation of immature synapses may occur through synchronised glutamatergic transmission, which drives AMPA receptor insertion into the postsynaptic neuronal membrane (Rajan et al 1999; Hanse et al 2009; Haas and Cline 2006), thus unsilencing silent synapses. It is known that group I mGluRs can regulate mTOR-dependent protein translation via activation of PI3 kinase and that mTOR-dependent translation and PI3kinase are involved in synaptic plasticity (Hoeffer and Klann 2010). Our results suggest that the LTP-like mechanisms that are important in early development are driven by glutamate transmission through tonic group I mGluR activation and that group I mGluRs may mediate these effects via PI3kinase and mTOR signalling cascades. Together these results suggest that there is a constant turnover of PKM ζ in neonatal slices. This is in contrast to mature hippocampus in which an increase in PKM ζ was not observed until at least 10 minutes after LTP induction (Osten et al 1996).

In further support of the above hypothesis we were able to confirm that mGlu receptor-stimulated PI hydrolysis was much greater in P14 compared to adult perirhinal cortex and importantly that basal PI hydrolysis was reduced by mGlu receptor antagonists in P14 but not adult perirhinal cortex. Critically, inhibition of group I mGlu receptors also produced a reduction in basal PKM ζ levels in P14 perirhinal cortex. Pharmacological activation of group I mGlu receptors with DHPG did not affect PKM ζ expression in either P14 or adult perirhinal cortex. A possible explanation is that the greater PKM ζ expression at P14 is fully supported by endogenous activation of mGlu1/5 receptors, which occludes any further increase by DHPG. In contrast, the extent of stimulation by DHPG in adult cortex may be too low (see Fig. 5) to increase PKM ζ expression. mGlu1/5 receptor agonists are more efficacious in stimulating PI hydrolysis in adult hippocampal slices than in adult cortical slices (Nicoletti et al., 1986; Casabona et al., 1991), which may explain the ability of DHPG to increase PKM ζ levels in adult hippocampus (Sajikumar and Korte, 2011) but not in adult perirhinal cortex. Together these results strongly suggest that tonic group I mGlu receptor activity maintains elevated PKM ζ in P14 perirhinal cortex. Tonic activity of group I mGluRs could occur through either constitutive agonist-independent activation due to intracellular proteins such as homer (Ango et al 2001) or could be due to the presence of the endogenous

agonist glutamate. Our results with the mGlu1 negative allosteric modulator JNJ16259685 (Lavreysen et al 2004) or the non-competitive antagonist LY456236 do not allow us to make a definitive distinction between the possible mechanisms of tonic mGluR activity. Wherever possible we have used more than one pharmacological ligand in our investigation of each signalling target to try to ensure as far as possible that any observed effects are not simply due to non-specific pharmacological effects. In each of these results we find that different ligands produced the same effects, thus increasing our confidence about the roles of group I mGluRs, PI3kinase and mTOR signalling in regulation of basal transmission in neonatal perirhinal cortex.

The induction of LTP has been demonstrated early in development in a number of brain regions including in hippocampus, visual and somatosensory cortex (Malenka and Bear 2004). However, an intriguing study suggested that transmission at naive neonatal CA3-CA1 synapses may also be in a potentiated state but that these synapses are very labile; basal synaptic stimulation very rapidly reversing any potentiated synapses (Abrahamson et al 2008). Therefore, LTP in perirhinal cortex synapses during neonatal development may only be different compared to that in developing hippocampal synapses by virtue of its stability. However, whether mechanisms involving PKM ζ operate to maintain synaptic transmission in other brain regions such as visual or somatosensory cortex during different stages of development will require further investigation.

There is some evidence that mechanisms underlying LTP and de-depression may not be identical. Whilst this study does not directly address this issue we do find that de-depression is reversed by inhibition of PKM ζ . This suggests that whatever the induction mechanisms, the maintenance of basal transmission and de-depression in P14 perirhinal cortex and maintenance of LTP in adult perirhinal cortex are all dependent on PKM ζ . Mis-regulated protein synthesis is increasingly recognised as being important in developmental disorders such as autism and mental retardation (Hoeffer and Klann 2010). Disruption of dendritic protein translation, such as occurs in fragile X mental retardation and is studied in *fmr1* knockouts (Bear 2004; Dolen et al 2007; Harlow et al 2010; Till et al 2010), has attracted a lot of attention, especially concerning the relationship with mGluR-LTD (Huber et al 2002; Park et al 2008). However, LTP mechanisms are also altered in *fmr1* knockout mice (Desai et al 2006; Wilson et al 2007) and it is therefore also possible that disruption of LTP-like mechanisms produces abnormal synaptic transmission and connectivity during early development and hence results in developmental disorders associated with fragile X mental retardation.

In conclusion, signalling cascades relying on mGluR/PI3K/mTOR/PKM ζ dependent mechanisms are essential not only for learning and memory, as previously described (Hrabetova and Sacktor 1996; Ling et al 2002) but our results suggest that these same cascades are also critical for normal synaptic transmission during development of perirhinal cortex.

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Fig. 1.

(A) PKM ζ inhibition, by application of ZIP, depresses basal synaptic transmission (filled circles) in P14 perirhinal cortex. However, ZIP has no effect on the input in which LTD was first induced (open circles). (B) Control, scrambled ZIP has no effect on synaptic transmission. (C) In P14 perirhinal cortex LTP induced by HFS (following induction of LTD) is dependent on PKM ζ since potentiation is reversed by the PKM ζ inhibitor ZIP. In one input LTD is first induced (open circles). Subsequent HFS results in potentiation of transmission back to baseline levels. The other input serves as a control (closed circles).

Application of ZIP depresses transmission in both the potentiated and the control inputs. **(D)** LTP is readily induced in adult perirhinal cortex and application of the inhibitor of PKM ζ reversed LTP to baseline levels (open circles) without any significant effect on control baseline transmission (filled circles). **(E)** Levels of PKM ζ in P14 perirhinal cortex are higher than in adult perirhinal cortex; single example and pooled western blot data (normalised to adult levels) illustrated in the histogram. **(F)** LTP is readily induced in P14 hippocampal slices and is reversed by ZIP (open circles) with no effect on baseline transmission (closed circles).

In this and subsequent figures, except where stated, separate pathways in the same slice are shown by open and filled symbols. LTP inducing stimulation (HFS) is indicated by upward arrows and LTD inducing stimulation (LFS) by two upward arrows joined by a bar.

Example representative field EPSPs are shown above the graphs from the times points as indicated.

Fig. 2.

Role of mTOR-dependent translation and PI3K in PKM ζ regulation of basal synaptic transmission. (A) Application of the mTOR inhibitor rapamycin has no effect on synaptic transmission in adult (filled circles) but produces depression of synaptic transmission in P14 cortex (open circles). (B) Following depression by ZIP in P14 perirhinal cortex the application of rapamycin produces no further depression. (C) The mTOR inhibitor KU0063794 does not produce a depression of transmission in adult perirhinal cortex but depresses transmission in P14. The PI3K inhibitors wortmannin and LY294002 produce a

depression of transmission in P14 but not in adult cortex. Furthermore, there is occlusion between the effects of mTOR and PI3K since LY294002 has no further effect following the depression induced by KU0063794. **(D)** Application of the mGluR5 antagonist MPEP, the mGluR1 inhibitor LY456236 and the mGluR1 negative allosteric modulator JNJ16259685 all depressed transmission in P14 perirhinal cortex but had no effect on transmission in adult cortex. **(E)** The normal depression induced by MPEP or **(F)** by LY456236 or JNJ16259685 did not occur following the depression induced by inhibition of mTOR.

Fig. 3.

PKMζ levels are regulated by endogenous mGluR activation in P14 but not adult perirhinal cortex. Expression of PKMζ in slices from (A) P14 or (B) adult perirhinal cortex treated with mGlu receptor ligands. Representative immunoblots are shown. Densitometric data are means ± S.E.M. of 5-6 determinations. Endogenous mGluR activation maintains PI hydrolysis in P14 but not adult perirhinal cortex. Stimulation of PI hydrolysis in slices from (C) P14 or (D) adult perirhinal cortex treated with mGlu1 (JNJ16259685) or mGlu5 receptor antagonists (MPEP). Values are means ± S.E.M. of 6-9 determinations. $P < 0.05$ (One-way ANOVA + Fisher's PLSD) vs. the respective basal values (*).