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

Isabella Panaccione [a,b,d], Rachel King [a], Gemma Molinaro [c], Barbara Riozzi [c], Giuseppe Battaglia [c], Ferdinando Nicoletti [b,c], Zafar I. Bashir [a,*]

[a] MRC Centre for Synaptic Plasticity, Department of Anatomy, University of Bristol, BS8 1TD, United Kingdom
[b] Department of Physiology and Pharmacology, University of Roma "Sapienza", Italy
[c] IRCCS Neuromed, Pozzilli, Italy
[d] NESMOS Department, School of Medicine and Psychology, Sant'Andrea Hospital, University of Rome "Sapienza", Italy

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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. PKMζ is a kinase essential for the maintenance of LTP. We show for the first time that inhibition of PKMζ 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 PKMζ- 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.

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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 Cossart, 2010). Postnatal synaptic activity is then triggered mainly by depolarising GABAergic mechanisms that 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 Cossart, 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. PKMζ 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 PKMζ reverses experimentally-induced LTP there is no effect of PKMζ 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 PKMζ may be important in development. Interestingly, it has been shown recently that PKMζ confers dendritic stabilisation during development of xenopus retino-tectal pathway (Liu et al., 2009). However, whether PKMζ regulates synaptic transmission during development is not known.

* Corresponding author. Tel.: +44 (0) 117 331 1957.
E-mail address: z.i.bashir@bristol.ac.uk (Z.I. Bashir).

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We now show that PKMζ, 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 peririnal cortex. Surprisingly, inhibition of PKMζ 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 mGlu receptor activity. These results provide the first evidence that basal synaptic transmission in immature perirhinal cortex relies on tonically active PKMζ-, mTOR-dependent and PI3kinase/group I mGlu receptor 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% O_2/5% CO_2) which comprised the following (in mM): 124 NaCl, 3 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgSO_4$, 10 D-glucose. Perirhinal slices: A mid-sagittal section was made, the rostral and caudal parts of the brain were removed by single scalpel cuts at 45° to the dorso-ventral axis, and each hemisphere glued by its caudal end to a vibroslice stage (Campden Instruments). Slices (400 um) of PRH were taken in the region 4 mm behind bregma. Parasagittal hippocampal slices (400 um) 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 200 ms. This sequence is repeated four times at intervals of 10 s, 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 reanalysed 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 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM 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 μg 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 1 h with 5% non-fat dry milk in TTBS buffer (100 mM 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 1 h 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% O_2, 5% CO_2 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- [^{3}H]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 2000 *g* for 20 min, the [^{3}H]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, Bio-Rad, 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. PKMζ maintains basal transmission in neonatal perirhinal cortex

Activation of the atypical kinase PKMζ is essential in the maintenance of LTP, as demonstrated by the extensive use of the PKMζ 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 PKMζ inhibitor ZIP depressed synaptic transmission under control conditions (filled circles: 53 ± 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 'depotentiation' 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 ± 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 ± 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 PKMζ 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; 100 Hz, 1 s), that produces LTP in a variety of brain regions across a range of different ages, did not induce LTP in neonatal perirhinal cortex (95 ± 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 ± 2%,

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