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Early memory formation disrupted by atypical PKC inhibitor ZIP in the medial prefrontal cortex but not hippocampus

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Abstract

Atypical isoforms of protein kinase C (aPKCs; particularly protein kinase M zeta: PKM ζ) have been hypothesised to be necessary and sufficient for the maintenance of long-term potentiation (LTP) and long term memory by maintaining postsynaptic AMPA receptors via the GluR2 subunit. A myristoylated PKM ζ pseudosubstrate peptide (ZIP) blocks PKM ζ activity. We examined the actions of ZIP in medial prefrontal cortex (mPFC) and hippocampus in associative recognition memory in rats during early memory formation and memory maintenance. ZIP infusion in either hippocampus or mPFC impaired memory maintenance. However, early memory formation was impaired by ZIP in mPFC but not hippocampus; and blocking GluR2-dependent removal of AMPA receptors did not affect this impairment caused by ZIP in the mPFC. The findings indicate: (i) a difference in the actions of ZIP in hippocampus and medial prefrontal cortex, and (ii) a GluR2-independent target of ZIP (possibly PKC λ) in the mPFC during early memory formation.

Keywords

Recognition memory; hippocampus; medial prefrontal cortex; encoding; maintenance

INTRODUCTION

Recognising an association between a stimulus and where it was previously encountered is a fundamental explicit memory process. Lesion studies have demonstrated that the perirhinal cortex, hippocampus, medial prefrontal cortex (mPFC), and interactions between these regions are essential for successful performance of an object-in-place (OIP) associative recognition memory task (Barker et al 2007, Barker & Warburton 2011). Furthermore, localised infusion of an NMDA glutamate receptor antagonist (AP5) bilaterally into each of these three regions impairs memory acquisition in the OIP task (Barker & Warburton 2013, Barker & Warburton 2008). Together these results demonstrate that associative recognition

memory depends on the hippocampus, perirhinal cortex and medial prefrontal cortex (mPFC) operating as a functional network (Barker et al 2007, Barker & Warburton 2011, Barker & Warburton 2013, Barker & Warburton 2008) and suggest that synaptic plastic changes within this hippocampal-cortical neural circuit may underlie the learning of object-place associations. To seek further evidence for synaptic plasticity changes related to such associative recognition memory, inhibition of atypical isoforms of protein kinase C (PKC) was investigated in mPFC and hippocampus during two different stages of memory (acquisition and maintenance).

PKC comprises a family of protein kinases consisting of at least 10 isoforms divided into three subgroups: conventional, novel and atypical. The atypical PKC isoforms are PKM ζ , PKC ζ and PKC λ (Naik et al 2000, Oster et al 2004, Ren et al 2013). Both PKC λ and PKM ζ are highly expressed in the forebrain (Naik et al 2000, Oster et al 2004, Ren et al 2013). PKC λ consists of a catalytic domain and an atypical regulatory domain possessing only one of the two cysteine-rich regions present in the diacylglycerol region of the regulatory domain in novel and conventional PKC isoforms. PKM ζ consists solely of the catalytic domain and lacks the regulatory domain altogether, thus remaining constitutively active. Previous work with a myristoylated PKM ζ pseudosubstrate inhibitory peptide (ZIP) that inhibits PKM ζ has indicated that ZIP administration prevents the maintenance of long term potentiation (LTP) and long term memory. ZIP administration reverses previously established LTP in hippocampal slices (Jones 2007) and erases long-term auditory fear memory in the amygdala (Parsons & Davis 2011, Serrano et al 2008), conditioned taste aversion, learned active avoidance, morphine-associated reward memory and morphine withdrawal-associated aversive memory in the basolateral amygdala (Gamiz & Gallo 2011, He et al 2011), cocaine conditioned reward in the nucleus accumbens shell (Shabashov et al 2011) and object location memories in the hippocampus (Migues et al 2010). There is evidence that ZIP acts on PKM ζ (Yao et al 2013); however, the specificity of ZIP has recently been questioned (Lee et al 2013, Volk et al 2013, Wu-Zhang et al 2012). Higher concentrations of ZIP can impair PKC λ as well as PKM ζ (Ren et al 2013).

PKM ζ is thought to maintain memories by sustaining enhanced AMPA receptor (AMPA) levels in the post-synaptic-density via the GluR2 subunit of AMPARs (Migues et al 2010, Yao et al 2008). Hence, inhibition of PKM ζ by ZIP results in a reduction of enhanced AMPA receptor levels during LTP or memory maintenance (Migues et al 2010). This reduction can be blocked by GluR2 $_{3Y}$, a synthetic compound mimicking the GluR2 carboxy tail of the AMPA receptor: GluR2 $_{3Y}$ is a highly selective inhibitor of AMPA receptor endocytosis (Migues et al 2010). Conversely, PKC λ , is involved in LTP induction following activation of phosphatidylinositol 3,4,5 triphosphate (PIP $_3$). PKC λ is crucial in the insertion of AMPARs into the post-synaptic density via the GluR $_1$ subunit (Ren et al 2013).

Does ZIP administration and changes in AMPA receptor endocytosis affect earlier stages of memory formation in addition to memory maintenance in an associative recognition memory task? When two interconnected regions of the brain are necessary during the performance of a task, are there regional differences in the mechanisms of action of ZIP? To investigate these issues, ZIP or GluR2 $_{3Y}$ was infused locally into the hippocampus or mPFC to be active during different memory stages. The results reveal GluR2-independent atypical

PKC actions during early memory formation that are found in the mPFC but not the hippocampus.

MATERIALS AND METHODS

Subjects

All experiments were conducted in male Dark Agouti rats (Harlan, UK), weighing 250–300g at the start of surgery. The animals were housed under a 12 h light/dark cycle (light phase, 6:00 P.M. to 6:00 A.M.). Behavioural training and testing were conducted during the dark phase of the cycle. All animal procedures were performed in accordance with the United Kingdom Animals Scientific Procedures Act (1986) and associated guidelines and had approval from the University of Bristol Ethics Committee. All efforts were made to minimize any suffering and the number of animals used during all experimental procedures.

Surgery

Rats were anaesthetized with isoflurane (induction 4%, maintenance 2-3%) and implanted with permanent guide cannulae aimed at the mPFC and hippocampus. The stereotaxic coordinates for the mPFC were AP +3.20 mm, ML ± 0.75 mm and DV -3.5 mm (from bregma), and for the hippocampus were AP -4.82 mm, ML ± 2.6 mm and DV -3.0 mm (from dura mater). After surgery, each animal was given fluid replacement therapy (5 ml of glucose saline s.c.) and Temgesic (0.05 ml, i.m.) for pain relief. Rats were allowed to recover for at least 10 days before habituation to the testing arena began. Between infusions, the cannulae were kept closed by dummy inserts.

Apparatus

Exploration occurred in an open-topped arena (90 × 100 cm) with 50 cm wood walls and a scaffold covered with a black cloth to a height of 150 cm on one side to conceal the experimenter. For easier navigation around the arena, one of the walls of the arena was black in colour while the other three were grey. Extra maze cues were visible to the rat over the three lower walls. The floor of the arena was covered with sawdust. An overhead camera and a video recorder were used to monitor and record each animal's behaviour for later analysis. The objects presented were constructed from "Duplo" (Lego UK, Slough, UK) and varied in shape, color and size. They were too heavy to be displaced by the animal.

Habituation and behavioural procedure

After being handled for a week, the animals were habituated to the arena without stimuli for five days in total. On the first three days of habituation, animals were placed in the arena in pairs for 10-15 min and on the last two days; animals were placed in the arena for 5 min each. The object-in-place task comprised an acquisition phase and test phase separated by a 24 h delay. In the acquisition phase, animals were presented with four different objects placed in the corners of the arena 15 cm from the walls. Each animal was placed in the centre of the arena and allowed to explore the objects for 5 min. In the current study, we found that a sample phase of this duration was sufficient to elicit memory lasting for at 24h. In the test phase, two of the objects on one side of the arena were in exchanged positions and the animal was allowed to explore the objects for 3 min. The pairs of objects on each side of

the arena in the acquisition phase and the objects that changed positions in the test phase were counterbalanced between rats. Normal rats with associative recognition memory intact will spend more time exploring the two objects that have changed positions compared to the objects in the familiar configuration. This behavioural procedure is based on our previous work (Barker & Warburton 2011).

Drug delivery

The drugs used were ZIP and scrambled ZIP (sZIP; Tocris Bioscience), and GluR2_{3Y} and scrambled GluR2_{3Y} (sGluR2_{3Y}; Cambridge Bioscience). ZIP and sZIP were dissolved in physiological saline and infused at a concentration of 10mM in accordance with published work (Serrano et al 2008, Yao et al 2013) (but see (Lisman 2012)); GluR2_{3Y} and sGluR2_{3Y} were diluted in physiological saline and infused at a concentration of 30μM (Ge et al 2010). Infusions were made into each region using a 33-gauge infusion cannula inserted into the implanted guide cannula and attached via polythene tubing to a 5μl Hamilton syringe. Drugs were infused into the mPFC at a rate of 0.5μl/min and into the hippocampus at a rate of 0.25μl/min over a period of 2 minutes. These volumes of infusate and rates of infusion have been used extensively previously (Akirav & Maroun 2006, Barker & Warburton 2013) and have been shown to result in drug spread of 1-1.5 mm³ (Martin 1991). The infusion cannulae remained in place for an additional 5 min following the infusion.

Experimental design

Experiment 1: To study the involvement of PKM ζ or GluR2-dependent AMPA receptor endocytosis in early memory formation, the infusion (ZIP or GluR2_{3Y}) was given into either the mPFC or hippocampus 15 min before the acquisition phase. To study the involvement of PKM ζ or GluR2-dependent AMPA receptor endocytosis in memory maintenance, the infusion was given into either the mPFC or hippocampus 19 h after the acquisition phase. (Unpublished observations indicate that ZIP is not present in the brain 4 h after infusion). Experiment 2: To test whether the effect of ZIP on early memory formation were achieved through AMPA receptor endocytosis, GluR2_{3Y} (or sGluR2_{3Y} as a control) was infused into the mPFC 1 h before ZIP, which was in turn infused 15 min before the acquisition phase. Similarly, to test the effect of blocking GluR2 dependent AMPA receptor endocytosis on subsequent PKM ζ inhibition during memory maintenance, GluR2_{3Y} or scrambled GluR2_{3Y} was infused into the mPFC or hippocampus 18 h after the acquisition phase, with ZIP infused 1 h later (i.e. 19 hours after the acquisition phase).

Experiment 3: This experiment examined the effects of simultaneously co-infusing GluR2_{3Y} and ZIP (or sZIP), GluR2_{3Y} and ZIP or sZIP (at the same concentrations and volumes as above) into the mPFC 15 min before the start of the acquisition phase. This experiment therefore examined whether the lack of effect of GluR2_{3Y} was because two separate infusions. In Experiment 4, GluR2_{3Y} was infused 1 h before GluR2_{3Y} plus ZIP (or sZIP), which was in turn infused into the mPFC 1 h before the acquisition phase. This experiment replicated Experiment 2 but with sZIP (not sGluR2_{3Y}) as the control condition. This experiment also used a double infusion protocol so as to ensure that GluR2_{3Y} was present in the brain prior to the infusion of ZIP and remained present in the brain with ZIP throughout the period of early memory formation. In Experiments 1-4, all rats underwent each infusion

protocol with an interval of seven days. For example, in Experiment 1, an animal that received a ZIP infusion in the mPFC prior to memory acquisition (encoding) in the first experimental session, received a ZIP infusion during maintenance, a sZIP infusion during encoding or a sZIP infusion during maintenance seven days later. This cross-over design allowed for within-subjects statistical analysis. Two cohorts of animals were used in these experiments. Experiment 1 and the hippocampal infusions of Experiment 2 were carried out with one cohort of animals; Experiments 3 and 4 and the mPFC infusions in Experiment 2 were carried out with a second cohort.

Behavioural measures and statistical analysis

All measures of exploration were made with the experimenter blind to the drug infusion status of each animal. Object exploration was defined as the animal facing and actively exploring the object. Other behaviours, such as sitting or leaning on the object were not considered as exploration. For inclusion in the analysis, animals were required to explore all the objects for more than 15 s in the acquisition phase and 10 s in the test phase. In Experiment 2, two animals were excluded from analysis for failing to explore the objects for more than 10 s in the test phase. There was no significant effect of any of the drug treatment protocols on total exploration during the sample or test phases in any of the experiments (Table 1c). Preference for the novel configuration of objects was determined using a discrimination ratio; the difference in the time spent exploring the novel and the familiar configurations of objects divided by the total time spent exploring both pairs of objects, thereby taking into account, individual differences in total exploration (Barker et al 2007, Dix & Aggleton 1999, Ennaceur & Delacour 1988). Comparisons were made using multifactor ANOVA (followed by *post hoc* pairwise comparisons) or paired *t* tests. Additional analyses examined whether the animals had discriminated between the pairs of objects, using a one-sample *t* test against no preference ($DR = 0$). All statistical analyses used a significance level of 0.05.

Histology

At the end of the experiment, each rat was anaesthetized with Euthatal (Rhone Merieux, Toulouse, France) and perfused transcardially with phosphate buffered distilled water followed by 4% formal saline. The brain was postfixed in formal saline. The brain was transferred to sucrose in 0.2 M phosphate buffer 48 h prior to sectioning. Coronal sections were cut at 40 μ m on a cryostat and stained with cresyl violet. Cannulae locations were checked against standardized sections of the rat brain. In all rats, needle tips were located in or close to the prelimbic region of the mPFC and in the dorsal hippocampus, such that the intra-mPFC infusions would affect more than 50% of the ventral portion of the prelimbic region in the mPFC, and the intra-hippocampal infusions would affect the CA1 region of the dorsal hippocampus, as shown by our previous studies using fluorophore conjugated muscimol (Barker & Warburton 2013).

RESULTS

Experiment 1: Effects of ZIP on memory formation and maintenance

Experiment 1 investigated the effects of ZIP in mPFC or hippocampus upon memory in the object-in-place (OIP) task (Figure 1a). Rats were infused bilaterally with ZIP, or a scrambled version of ZIP (sZIP) as a control, into the hippocampus (Figure 1b) or mPFC (Figure 1c), either 15min before the acquisition phase (to investigate effects during early memory formation) or 19h after acquisition (to investigate effects on memory maintenance). Infusion of ZIP into the hippocampus impaired maintenance but not acquisition or early consolidation of the OIP task (ANOVA: drug by time of infusion interaction: $F_{1,25} = 5.95$, $p = 0.02^*$; main effect of drug: $F_{1,25} = 8.00$, $p = 0.01^{**}$; main effect of time of infusion: $F_{1,25} = 0.75$, $p = 0.4$; Figure 1d), in line with previous reports for other types of memory (Hardt et al 2010, Miguez et al 2010, Pastalkova et al 2006, Serrano et al 2008, Yao et al 2008). However, unexpectedly, ZIP infusion into the mPFC disrupted early memory formation as well as maintenance of OIP recognition memory (ANOVA: drug by time of infusion interaction: $F_{1,21} < 1$, $p = 0.5$; main effect of drug: $F_{1,21} = 14.32$, $p = 0.001^{**}$; main effect of time of infusion: $F_{1,21} < 1$, $p = 0.5$; Figure 1e). Animals infused with sZIP in either region showed significant discrimination when infused during either early memory formation or memory maintenance: one-sample t tests against no preference ($DR = 0$); hippocampus, encoding; $t_{13} = 4.35$, $p < 0.005^{\dagger\dagger}$; hippocampus, maintenance; $t_{12} = 5.52$; $p < 0.0005^{\dagger\dagger\dagger}$; mPFC, encoding; $t_{12} = 2.96$, $p < 0.05^{\dagger}$; mPFC, maintenance $t_9 = 2.27$; $p < 0.05^{\dagger}$; Figures 1d,e). ZIP infusion into the mPFC impaired discrimination when infused during either early memory formation ($t_{12} = 1.98$; $p = 0.07$) or memory maintenance ($t_{12} = 0.32$; $p = 0.8$). Infusion of ZIP into the hippocampus during early memory formation did not impair discrimination between novel and familiar configurations (one-sample t test against $DR = 0$; $t_{13} = 4.17$; $p < 0.005$), whereas ZIP infusion into the hippocampus during memory maintenance led to discrimination not dissimilar from chance ($t_{12} = 0.19$; $p = 0.9$). Accordingly, the actions of ZIP upon early memory formation differed between hippocampus and mPFC.

Experiment 2: Does ZIP act through AMPA receptor endocytosis?

In Experiment 2 GluR2_{3Y} was infused 1h before ZIP infusions to block AMPA receptor endocytosis. It was hypothesised that administration of GluR2_{3Y} would prevent ZIP-induced amnesia produced by inhibition of PKM ζ and the consequent endocytosis of AMPA receptors. The ZIP infusions were made into hippocampus or mPFC 19h after acquisition in the OIP task (Figure 1a), the time point at which they had produced memory impairment in Experiment 1. ZIP infusions were also made into mPFC 15 min before acquisition as such an infusion had caused memory impairment in Experiment 1. An inactive scrambled version of the GluR2_{3Y} peptide (sGluR2_{3Y}) was used as a control treatment. When infusions of GluR2_{3Y} were made into either the mPFC or hippocampus during maintenance and 1 h before ZIP, the impairment of memory maintenance caused by ZIP infusion was blocked: one-sample t tests, significant preference ($DR > 0$); mPFC, $t_{10} = 6.24$; $p < 0.0001^{\dagger\dagger\dagger}$; hippocampus; $t_8 = 3.35$; $p = 0.01^{\dagger\dagger}$; (Figures 2a, b). In contrast, when sGluR2_{3Y} was similarly infused into either region after acquisition and 1h prior to ZIP, memory impairment was not blocked: one-sample t tests against no preference ($DR = 0$); mPFC, t_{10}

= 0.10; $p > 0.05$; hippocampus; $t_8 = 0.99$; $p > 0.05$; (Figures. 2a, b). The difference in effects on memory maintenance of GluR2_{3Y} and sGluR2_{3Y} infused into the hippocampus were significant ($t_8 = 4.42$, $**P = 0.002$). When infusions were made into mPFC prior to acquisition, neither GluR2_{3Y} nor sGluR2_{3Y} blocked the impairment of early memory formation produced by ZIP, however GluR2_{3Y} did block the effects of ZIP during maintenance. (ANOVA: main effect of drug: $F_{1, 10} = 20.12$, $p < 0.001^{***}$; main effect of time of infusion: $F_{1, 10} = 34.97$, $p < 0.001$; drug by time interaction, $F_{1, 10} = 17.36$, $p < 0.01^{**}$; Figure 2a). Accordingly, the action of ZIP on memory maintenance could be explained as arising from prevention of AMPA receptor endocytosis, but its action on early memory formation in mPFC could not.

Control experiments for possible effects of double infusions or of GluR2_{3Y} itself

In Experiment 2 impairment in early memory formation was produced when there were two separate, successive infusions into mPFC. To check that the lack of effect of GluR2_{3Y} was not because two separate infusions were made, in Experiment 3 GluR2_{3Y} and ZIP or GluR2_{3Y} and sZIP were co-administered in a single infusion. An impairment in early memory formation was produced by pre-acquisition co-infusion into mPFC of GluR2_{3Y} and ZIP but not GluR2_{3Y} and sZIP; the effects differed significantly (paired t test: $n = 11$, $t_{10} = 4.31$, $p = 0.002$; Figure 2c, d). The co-infusion of GluR2_{3Y} and ZIP resulted in chance exploration (DR=0; $t_{10} = 1.05$, $p > 0.05$), whereas the novel configuration was preferred after co-infusion of GluR2_{3Y} and sZIP (DR > 0; $t_{10} = 4.19$, $p = 0.002^{††}$; Figure 2c). As a further control (and replication), infusions were made into the mPFC of GluR2_{3Y} followed 1 h later by co-infusion of GluR2_{3Y} and ZIP or sZIP 15 min before acquisition. Infusions of GluR2_{3Y} and ZIP, but not sZIP, significantly impaired early memory formation (difference between groups, $F_{1,9} = 21.44$, $P < 0.005^{**}$; differential exploration after ZIP (DR =0) $t_9 = 1.78$, $p > 0.05$; differential exploration after sZIP (DR > 0) $t_9 = 4.05$, $p < 0.005^{††}$; Figure 2d); Thus the lack of blocking effect of GluR2_{3Y} on ZIP at early maintenance was not due to the use of double infusions.

There was no effect of intra-hippocampal or intra-mPFC infusion of GluR2_{3Y} compared to sGluR2_{3Y} on either early memory formation or maintenance: hippocampus ($F_{1, 27} < 1$, $p > 0.1$); mPFC ($F_{1, 20} < 1$, $p > 0.1$) (Figure 3). GluR2_{3Y} did not impair early memory formation or memory maintenance when infused into the hippocampus ($p < 0.005^{††}$) or mPFC ($p < 0.05^{†}$). (Figure 3).

Exploration levels

There were no significant differences between control and experimental groups in total exploration time during either sample or choice phases of any of the experiments using the OiP task (Table 1). Accordingly, there was no evidence that the intra-hippocampal or intra-mPFC infusions produced gross changes in exploratory behaviour.

DISCUSSION

The findings of the current study are five-fold: 1) ZIP has a previously unreported role in early memory formation in the mPFC, 2) There is a regional difference between the mPFC

and the hippocampus in the molecular mechanisms of early memory formation as targeted by ZIP, 3) The action of ZIP in impairing memory maintenance, whatever its substrate, is blocked when AMPA receptor recycling is blocked by GluR2_{3Y}, 4) In contrast, the action of ZIP on early memory formation in mPFC is not dependent on AMPA receptor recycling and, 5). There is no difference in ZIP's action on memory maintenance in hippocampus and mPFC.

Infusion of ZIP into the hippocampus or mPFC impaired the maintenance of associative (OIP) recognition memory, consistent with previous findings for other types of memory and infusions in various other brain regions (Jones 2007, Miguez et al 2010, Parsons & Davis 2011, Shabashov et al 2011, Shao et al 2007). This impairment of OIP memory maintenance was found to be blocked by prior infusion of GluR2_{3Y}. Accordingly, during maintenance of associative recognition memory it is likely that the action of ZIP depends upon the endocytosis of AMPA receptors in both the mPFC and hippocampus. The findings provide support for the supposition of previous work concerning PKM ζ 's presumed role in memory maintenance and its inhibition by ZIP. PKM ζ has been shown to act through an *N*-ethylmaleimide-sensitive factor (NSF)/GluR2 dependent pathway that leads to enhanced trafficking of GluR2 containing AMPA receptors from extrasynaptic sites to the postsynaptic density (Yao et al 2008) and, moreover, PKM ζ maintains GluR2 containing AMPA receptors in the postsynaptic density (Miguez et al 2010). Hence, GluR2_{3Y}'s effect finds possible explanation: by blocking internalisation of synaptic AMPA receptors, it prevents the ZIP-induced memory maintenance impairment hypothesised to arise from AMPA receptor numbers dropping when PKM ζ is inhibited.

Interestingly infusion of ZIP into the mPFC prior to acquisition so as to be active during early memory formation also caused an impairment of associative recognition memory. In contrast, no such action on early memory formation was found following intra-hippocampal infusions of ZIP. These findings suggests either a role for PKM ζ in early memory processes in the mPFC, but not in the hippocampus or that ZIP has a target other than PKM ζ in the mPFC. The possibility of a role in early memory formation was raised by Shema et al (2011) who found that viral induced overexpression of PKM ζ in the insular cortex enhanced conditioned taste aversion memory. However, as pre-acquisition administration of ZIP in the hippocampus had no effect on OIP memory, the present study also establishes that PKM ζ is not important for early memory formation in every brain region.

When GluR2_{3Y} was infused into the mPFC before ZIP and prior to memory acquisition, OIP memory remained impaired; i.e. treatment with GluR2_{3Y} did not rescue the ZIP-induced impairment of early memory formation. A similar lack of memory impairment was found when ZIP and GluR2_{3Y} were co-infused, establishing that the lack of effect of GluR2_{3Y} was not because animals had been subjected to two infusions rather than one. The ineffectiveness of the GluR2_{3Y} rescue establishes a different action for ZIP during early memory formation. ZIP is a pseudosubstrate peptide mimicking the amino-acid sequence of the autoinhibitory domain of atypical PKCs; as such, ZIP may also inhibit another atypical PKC, PKC λ (Jiang et al 2006, Ren et al 2013). Given that PKC λ is the only other atypical PKC that is highly expressed in the forebrain (Oster et al 2004), it stands as a leading candidate substrate of the action of ZIP on early memory formation demonstrated in the current study. PKC λ has been

shown to insert AMPARs into the post-synaptic density during LTP induction via an interaction with p62 and the GluR1 AMPAR subunit in response to PI3K activation (Ren et al 2013). This action could explain the observed lack of effect of GluR2_{3Y} on the inhibitory effect of ZIP in the mPFC during memory formation in the present study: inhibition of PKC λ disrupts LTP induction via a GluR1, rather than GluR2-dependent pathway. In vitro, the concentration of ZIP required to inhibit PKC λ is four times higher than that for PKM ζ (Ren et al 2013). The concentration of ZIP in vivo within the infused regions is not known (the doses conformed to those used by others (He et al 2011, Serrano et al 2008) in previous publications), and it is plausible that the concentration achieved was sufficient to inhibit PKC λ as well as PKM ζ . Infusions for hippocampus and mPFC were chosen to achieve equivalent concentrations within the respective regions, the differences in the volumes infused reflected differences in the spread of the drug within the two structures (Barker & Warburton 2013), so that the difference in effect of ZIP on early memory formation is not readily explicable by a difference in drug concentration: it is more plausible that there is a regional difference in mechanisms of early memory formation, though confirmation of this contention must await further investigation.

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Figure 1.

PKM ζ activity is required in mPFC but not in hippocampus during associative recognition memory formation. **(a)** Diagram of experimental arrangements for OIP task: infusion of ZIP/sZIP prior to memory acquisition (i) or during memory maintenance (ii). **(b, c)** Positions of cannulae in the hippocampus **(b)** and mPFC **(c)**. Effects on associative recognition memory of ZIP or sZIP infusion into the hippocampus **(d)** or mPFC **(e)** during either 'encoding' (i.e. early memory formation) or maintenance of OIP task. Data are mean $\pm$ s.e.m. DRs ($n = 10-14$). In hippocampus **(d)**, there was no effect of ZIP during early memory formation ('encoding') but impairment of maintenance. In mPFC **(e)**, ZIP but not sZIP significantly impaired memory at both times. †One sample t test: (DR = 0; † $p < 0.05$; †† $p < 0.005$; ††† $p < 0.0001$). *Two-way ANOVA (Drug by time of infusion interaction or Bonferonni post-hoc test): (* $p < 0.05$; ** $p < 0.01$).

Figure 2.

AMPA receptor endocytosis is required during maintenance, but not encoding in mPFC and during maintenance in hippocampus. Effects of infusing GluR2_{3Y} or sGluR2_{3Y} followed by ZIP into the mPFC or hippocampus. **(a)** In the mPFC, GluR2_{3Y} did not block the memory impairment produced by ZIP during encoding but did during maintenance. †One sample *t* test: (DR = 0; ††† *p* < 0.0001); *Two-way ANOVA (Drug by time of infusion interaction or effect of drugs): (***) *p* < 0.0001). **(b)** In hippocampus, GluR2_{3Y} but not sGluR2_{3Y} blocked the action of ZIP during maintenance. †One sample *t* test: (DR = 0; †† *p* = 0.01); * Paired *t* test: (** *p* < 0.005) **(c)** Co-infusion of GluR2_{3Y} and ZIP but not GluR2_{3Y} and sZIP into mPFC impairs early memory formation. A combination of GluR2_{3Y} and ZIP resulted in a DR not significantly different from chance exploration whereas co-infusion of GluR2_{3Y} and sZIP led to significant exploration of novelty. †One sample *t* test: (DR = 0; †† *p* < 0.005); * Paired *t* test: (** *p* < 0.005) **(d)** Infusion into the mPFC of GluR2_{3Y} followed by co-infusion of GluR2_{3Y} and ZIP 1 h later 15 min before acquisition significantly impaired the early formation of associative recognition memory. When animals were infused with GluR2_{3Y} followed by a co-infusion of GluR2_{3Y} and sZIP 1 h later, they showed significant

discrimination between the novel and familiar configurations compared to chance. Infusion of GluR2_{3Y} followed by a co-infusion of GluR2_{3Y} and ZIP 1 h later led to exploration of both pairs of objects that was not significantly different from chance. †One sample *t* test: (DR = 0; †† $p < 0.005$); * Paired *t* test: (** $p < 0.005$)

Figure 3.

Control for action of GluR2_{3Y} itself. There was no effect of GluR2_{3Y} on early memory formation ('encoding') or maintenance in (a) hippocampus or (b) mPFC. There was no effect of GluR2_{3Y} on either encoding or maintenance of memory when infused into either the hippocampus or mPFC compared to sGluR2_{3Y}. †One sample *t* test: (DR = 0; † *p* < 0.05; †† *p* < 0.005; ††† *p* < 0.0005).

Table 1

Mean exploration times $\pm$ s.e.m during the sample phase or test phase in **(a)** experiment 1 **(b)** experiment 2 and **(c)** multiple infusion control experiments (experiments 3 and 4). There was no significant effect of any of the drug treatment protocols on total exploration during the sample or test phases.

a)				
Region	Time of Infusion	Drug treatment	Total exploration in sample phase (s)	Total exploration in test phase (s)
Hippocampus	Encoding	ZIP	54.7 $\pm$ 3.4	33.8 $\pm$ 1.7
		sZIP	56.7 $\pm$ 2.7	33.2 $\pm$ 2.0
		GluR2 _{3Y}	67.2 $\pm$ 3.4	36.7 $\pm$ 2.9
		sGluR2 _{3Y}	69.2 $\pm$ 4.3	36.3 $\pm$ 2.6
	Maintenance	ZIP	53.4 $\pm$ 4.7	28.1 $\pm$ 2.3
		sZIP	50.1 $\pm$ 3.7	27.5 $\pm$ 2.4
		GluR2 _{3Y}	46.7 $\pm$ 3.6	31.4 $\pm$ 2.5
		sGluR2 _{3Y}	49.3 $\pm$ 3.2	27.4 $\pm$ 1.5
mPFC	Encoding	ZIP	53.4 $\pm$ 5.4	42.5 $\pm$ 3.1
		sZIP	64.9 $\pm$ 8.8	39.7 $\pm$ 3.3
		GluR2 _{3Y}	39.2 $\pm$ 4.0	28.9 $\pm$ 3.9
		sGluR2 _{3Y}	33.2 $\pm$ 3.1	27.5 $\pm$ 2.7
	Maintenance	ZIP	35.5 $\pm$ 3.7	27.0 $\pm$ 2.1
		sZIP	42.4 $\pm$ 3.2	26.5 $\pm$ 2.2
		GluR2 _{3Y}	34.9 $\pm$ 3.5	33.1 $\pm$ 3.7
		sGluR2 _{3Y}	38.2 $\pm$ 2.5	30.1 $\pm$ 2.2

b)				
Region	Time of Infusion	Drug treatment	Total exploration in sample phase (s)	Total exploration in test phase (s)
Hippocampus	Maintenance	GluR2 _{3Y} + ZIP	60.9 $\pm$ 4.2	36.8 $\pm$ 6.8
		sGluR2 _{3Y} + ZIP	72.9 $\pm$ 9.5	36.2 $\pm$ 5.3
mPFC	Encoding	GluR2 _{3Y} + ZIP	53.4 $\pm$ 5.8	33.7 $\pm$ 3.3
		sGluR2 _{3Y} + ZIP	56.9 $\pm$ 6.5	35.7 $\pm$ 3.9
	Maintenance	GluR2 _{3Y} + ZIP	61.8 $\pm$ 7.0	29.7 $\pm$ 3.4
		sGluR2 _{3Y} + ZIP	59.4 $\pm$ 3.9	31.7 $\pm$ 2.6

c)		
Drug treatment	Total exploration in sample phase (s)	Total exploration in test phase (s)
GluR2 _{3Y} & ZIP	65.8 $\pm$ 5.2	38.6 $\pm$ 3.8
GluR2 _{3Y} & sZIP	69.4 $\pm$ 5.6	40.4 $\pm$ 2.9
GluR2 _{3Y} + GluR23Y & ZIP	74.7 $\pm$ 5.8	36.9 $\pm$ 2.6
GluR2 _{3Y} + GluR23Y & sZIP	70.0 $\pm$ 5.0	38.8 $\pm$ 2.8