Abbreviated title: CAMKK pathway and recognition memory

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Abstract

The role of the CAMKK pathway in object recognition memory was investigated. Rats' performance in a preferential object recognition test was examined after local infusion into the perirhinal cortex of the CAMKK inhibitor STO-609. STO-609 infused either before or immediately after acquisition impaired memory tested after a 24h but not a 20min delay. Memory was not impaired when STO-609 was infused 20min after acquisition. The expression of two downstream reaction products of CAMKK was measured by immunohistochemical staining for phospho-CAMKI^{Thr177} and phospho-CAMKIV^{Thr196} at 10, 40, 70 and 100 min following the viewing of novel and familiar images of objects. Processing familiar images resulted in more pCAMKI stained neurons in the perirhinal cortex than processing novel images at the 10min and 40min delays. Perirhinal neuronal counts for pCAMKIV were lower than for pCAMKI and no differential effects of processing novel and familiar images were found for pCAMKIV. Prior infusion of STO-609 caused a reduction in pCAMKI stained neurons in response to viewing either novel or familiar images, consistent with its role as an inhibitor of CAMKK. The results establish that the CAMKK pathway within the perirhinal cortex is important for the consolidation of object recognition memory. The immunohistochemical imaging for pCAMKI indicated that CAMKI might be involved in reconsolidation mechanisms for familiar stimuli in addition to consolidation mechanisms for novel stimuli. The activation of pCAMKI after acquisition is earlier than previously reported for pCAMKII. In contrast to CAMKI and CAMKII, CAMKIV appears to be unimportant for perirhinal recognition memory processes.

Introduction

The calcium-calmodulin dependent protein kinase enzymes (CAMKs) form intracellular signalling pathways in neurons and have been implicated in memory processes in the brain (Wayman et al., 2008). The CAMK family includes CAMKI, CAMKII, CAMKIV and CAMK-kinase (CAMKK), all of which are activated by Ca^{2+}-calmodulin. CAMKII is a multisubunit enzyme with an intracellular signalling pathway that is distinct from those of CAMKK, CAMKI and CAMKIV. CAMKK is known to activate both CAMKI and CAMKIV by phosphorylation of the $Threonine^{177}$ and $Threonine^{196}$ residues respectively (Soderling, 1999). CAMKI is described as residing in the cytoplasm of cells (Picciotto et al., 1995) whereas CAMKIV has been located in the nucleus (Jensen et al., 1991). There is increasing evidence that the CAMKK pathway plays an important role in memory. CAMKKα mutant mice show impaired fear memory (Blaeser et al., 2006; Mizuno et al., 2006) and CAMKKβ mutant mice exhibit impaired spatial memory formation (Peters et al., 2003). Additionally, pharmacological inhibition of CAMKK has been shown to impair induction of long-term potentiation (LTP) in the hippocampus (Schmitt et al., 2005). However, the role of CAMKK in recognition memory has not been previously investigated.

Recognition memory is impaired by lesions of the perirhinal cortex (Zola-Morgan et al., 1989; Gaffan and Murray, 1992; Meunier et al., 1993; Mumby and Pinel, 1994; Ennaceur et al., 1996; Meunier et al., 1996; Brown and Aggleton, 2001; Brown et al., in press). Further, some neurons within the perirhinal region respond more strongly to novel than familiar visual stimuli (Brown et al., 1987; Zhu et al., 1995a; Desimone, 1996; Xiang and Brown, 1998). Immunohistochemical imaging indicates that this difference in response is paralleled by a higher Fos expression produced within perirhinal cortex by viewing novel rather than familiar images (Zhu et al., 1995; Zhu et al., 1996).

Here we establish a role and time course for CAMKK and CAMKI activity in object recognition memory within the rat perirhinal region by using localised infusion of a CAMKK inhibitor, STO-609. STO-609 inhibits both CAMKKα and CAMKKβ (Tokumitsu et al., 2002). Additionally, we establish the time course of the differential immunohistochemical staining for one of the reaction products of CAMKK, $pCAMKI^{Thre177}$ (an activated form of CAMKI),

produced by viewing novel or familiar pictures, and that this differential activation is blocked by STO-609.

Materials and Methods

Animals

Dark Agouti male rats (Bantin and Kingman, Hull, UK) underwent behavioural testing in the preferential object recognition task or underwent a paired viewing procedure (see below). Nineteen (230-300g) were used in preferential object recognition experiments, 2 in the western blotting experiment (200-220g) and 40 (200-250g) in the paired viewing experiments. The animals were housed under a 12 h light/dark cycle (light phase, 18.00 to 6.00). Experiments 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 were approved by the University of Bristol Ethical Committee.

Experiment 1 –Infusion of the CAMKK antagonist STO-609 during the preferential object recognition test

Surgery

Prior to testing in the preferential object recognition test rats underwent surgical procedures to implant guide cannulae into the perirhinal cortex. Rats were anaesthetised with isoflurane (Merial, Harlow, UK) and placed in a stereotaxic frame with the incisor bar set so as to achieve a flat skull. Craniotomies were made 5.6mm posterior and 4.5mm lateral to bregma, and 10mm length 26 gauge stainless steel guide cannulae (Plastics One, VA) were inserted 6.7mm below the surface of the skull in the coronal plane at an angle of 20 degrees to the vertical. The cannulae were attached to the surface of the skull by constructing an implant made from bone cement (DePuy, UK) and attached to the skull with stainless-steel screws (Plastics One). After surgery, rats were allowed a 2 week recovery period during which they were housed singly. Post-recovery, cannulated rats were housed in pairs in large cages. Obdurators (Plastics One) were used to keep the cannulae patent between infusions.

Pretraining

Rats were habituated to an arena (length: 100cm, depth: 100cm, arena height: 40cm) surrounded by curtains, (height: 40-160cm) in 5 min periods each day for 4 days.

Behavioural testing and infusions

During the acquisition trial each rat was initially exposed to two identical copies of an object (Object A) until it had explored the objects for more than 40s or for a maximum of 4 min. Objects were constructed from Duplo™(Lego UK, Slough, UK) (length: ~16cm, depth: ~16cm, height: ~12cm). After a delay period, of 24h, each rat was exposed to another copy of Object A and a novel object (Object B) in a choice trial lasting 3 min. Rats received bilateral infusions of CAMKK inhibitors into their perirhinal cortex using 33 gauge infusion cannulae (Plastics One, VA) inserted into the implanted guide cannula and attached to a 25µl Hamilton syringe by polyethylene tubing. An infusion pump (Harvard Bioscience, Holliston, MA) was used to inject a volume of 1µl to each hemisphere during a 2min period. Infusion cannulae were kept in place for a further 5min following the infusion. Drug infusions were made at different time-points with respect to the acquisition phase. The CAMKK inhibitor STO-609 (Tocris, Bristol, UK) was infused into both sides of the perirhinal cortex at a dose of 1ng/side, dissolved in 0.03% DMSO in 0.9% saline For each time point, the experiment was run in two parts in a cross-over design. In the first part, rats were randomly assigned an infusion of drug or vehicle (0.03% DMSO in 0.9% saline) and an object recognition experiment was performed. In the second part each rat was given the opposite infusate and another object recognition experiment was performed using new objects. There was a minimum separation of 48h between the two parts of the experiment.

Data analysis

Time spent exploring each object was scored blind as to treatment by the experimenter using a computer program. Exploratory behaviour was defined as the rat directing its nose toward the object at a distance of <2 cm; other behaviour, such as looking around while sitting on the object, was not considered exploration. A discrimination ratio (DR) was used to measure memory performance and was calculated by dividing the difference in

time exploring the novel and the familiar object by the time taken exploring both objects. Rats that failed to complete a minimum of 10s exploration in the acquisition trial or 5s exploration in the choice trial were excluded from the analysis. Over the course of several experiments, occasionally rats had to be excluded from the analysis due to cannula failures. The following is a summary of the rats included in the statistical analysis of the inhibitor experiments. There were 2 groups of rats used in these experiments, each group consisted of 12 individuals. In the first experiment (group 1; n=9 rats) a 20min delay between the acquisition and choice trials was used and infusate was injected 15min before the acquisition trial. In the subsequent experiments there was a 24h delay between acquisition and choice trials and the time of infusion was varied from 15min before the acquisition trial (group 2; n=8 rats), immediately [<2min] after (group 1; n=9) or 20min after the acquisition trial (group 2; n=10). There was no evidence from the behaviour following control infusions that spontaneous exploration or familiarity discrimination changed across the course of the experiments. DR values were analysed using repeated measures ANOVA with factors treatment (drug/vehicle) and infusion time. The significance level was determined as p=0.05, 2-tailed.

Verification of cannulae positions

Rats were anaesthetised with Euthatal (Rhône Mérieux, Toulouse, France) and transcardially perfused with 0.1M phosphate buffer containing formal saline, pH7.4. Coronal sections (40µm) were cut on a freezing microtome and the sections were stained with cresyl violet. Cannula locations were compared to a stereotaxic atlas (Paxinos and Watson, 1998) and histological examination confirmed that the tips of the cannulae were within the perirhinal cortex (Shi and Cassell, 1999); Fig. 1A. Data from other laboratories (Martin, 1991; Izquierdo et al., 2000; Attwell et al., 2001) and our unpublished data indicate that the infused tissue extended to an approximately 0.5-1mm radius from the cannula tip. This volume includes the majority of perirhinal cortex (Shi and Cassell, 1999) but only minor parts of neighbouring entorhinal cortex or area Te2.

Experiment 2 – Western blotting of pCAMKI and pCAMKIV antibodies onto perirhinal cortex and Te2 cortical lysate.

The rats were placed in a novel cage for 20min and then anaesthetised with isoflurane and their brains removed. Perirhinal cortex and area Te2 were dissected and immediately placed into phosphate buffer. The tissue samples were then transferred into lysis buffer (50mM Tris HCl (pH 7.4), 1% triton X-100, 0.1% SDS, 1mM EDTA pH 8.0, protease inhibitors (Roche, Herfordshire, UK), phosphatase inhibitors, (Roche)). The brain tissue was then homegenised with a sonicator (around 10 strokes). Lysates were left on ice for 1h for the proteins to solubilise and were then spun at 13000rpm for 15min at 4°C. The supernatant was collected and a Bradford protein (Biorad) assay was performed to determine the protein concentration. Protein samples (50 µg) were next subjected to electrophoresis in a 10% acrylamide gel and electrophoretically transferred to a PVDF membrane (Millipore, Watford, Uk). Membranes were incubated overnight at 4°C with primary antibody to pCAMKI or pCAMKIV (1:200, Santa Cruz, CA: catalogue #s sc-28438-R and sc-28443-R). After incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody at a dilution of 1:10000 (Sigma) for 1h, immunolabeled proteins were visualized by autoradiography using chemiluminescence ECL (Roche).

Experiment 3 - Presentation of novel and familiar images followed by immunohistochemical staining for pCAMKI^{Thre177} and pCAMKIV^{Thre196}

Apparatus

Novel and familiar images were presented simultaneously to individual rats under carefully controlled conditions (Zhu et al., 1996; Warburton et al., 2005). Each rat was trained in a paired viewing chamber (30 x 30 x 35 cm); the top of the chamber was open, the side and rear panels were blackened and the front panel was made from clear perspex. In the middle of the front panel there was a 3cm observation hole located 6cm above the floor. Barriers (12cm long x 9 cm high) were placed 4.5cm on either side of the observation hole to ensure the rat's body was aligned perpendicular with respect to the two monitor screens (model 4Vlr; AOC Spectrum, Los Angeles, CA). The monitors were placed 30 cm from the

observing hole and a black barrier between the monitors and observation hole ensured that the rat's right eye could not see the left monitor and vice versa. When a rat's head was in the observing hole it interrupted an infra-red beam and its tongue could just reach a metal lick tube. A trial began once the rat's head had continuously interrupted the infra-red beam for a randomly variable interval of 3-4s. Each monitor then displayed an image (15 x 12 cm) for 4.5s. A drop of blackcurrant juice was delivered by the lick tube 0.5s before the end of the image display. Stimulus display and juice delivery were computer (Viglen, Pentium II, St Albans, UK) controlled. A camera system was used to monitor and record each rat's behaviour during experimental sessions to confirm that the head was positioned as expected during stimulus display. The positioning was satisfactory for all rats whose data are reported.

Training

Rats were allowed *ad libitum* access to water for 2h each day. Rats were housed in groups of three per cage for the duration of the experiment. Initially rats were trained in the paired viewing apparatus for 2 days. During these 2 days rats learned to go to the observation hole for juice reward and there was no presentation of images. The next phase lasted for 6 days and during this time rats saw a series of novel and familiar images. In each morning session 30 images, (FAM set) were presented simultaneously to each eye. The images were presented in a pseudo-random order to ensure that different images were simultaneously shown to each eye. After a delay of 3h the rats were placed back in the apparatus for the afternoon session, in which each eye was shown 15 images from the FAM set and 15 novel images. During each trial one eye was shown a novel image and the other eye was shown a FAM image. Each afternoon a different set of novel images was shown along with the FAM image set. Thus prior to the last afternoon each eye had seen the same number of novel and familiar images. On the afternoon of the last day 30 novel images (NOV) were shown to one eye and the 30 FAM images were shown to the other eye. The effective memory delay was at least 3h (the delay between seeing the familiar stimuli during the morning and afternoon sessions of the last day). The FAM set for half the animals was the NOV set for the other half of the animals; this counterbalanced the image sets across animals. Further, exposure to NOV or FAM images to the left or right eye was also counterbalanced.

Immunohistochemical staining for pCAMKI

Rats were transcardially perfused with 0.1M phosphate buffer (PB) containing 50mM sodium fluoride (NaF) followed by 4% paraformaldehyde in PB-NaF, pH7.4. The perfusions took place at different time points after the final images had been shown; 10 min (n=6), 40 min (n=6), 70 min (n=7) and 100 min (n=6). The brains were then removed and placed in 4% paraformaldehyde in PB-NaF for 24 h, followed by 48 h in 30% sucrose in PB. Coronal sections were cut on a freezing microtome at a thickness of 40µm and floating sections were processed for immunohistochemistry using the avidin-biotin (ABC; Vector Laboratories) method. Sections were washed in Triton Tris buffered saline with 0.2% Triton X-100, 0.08M Tris Buffer and 0.9% NaCL (TTBS); they were then blocked with TTBS containing 1% bovine serum albumin (BSA), 5% normal goat serum (NGS). Sections were then incubated in a polyclonal rabbit primary antibody to pCAMKI or pCAMKIV (1:200, Santa Cruz, CA: catalogue #s sc-28438-R and sc-28443-R) in TTBS containing 0.25% BSA and 5% NGS for 48 hours. The secondary antibody used was polyclonal goat anti-rabbit (1:500, Vector Laboratories, Peterborough, UK) and 3,3'-diaminobenzine was used for visualisation.

Double-labelling of pCAMKI and pCAMKIV

To determine whether pCAMKI and pCAMKIV were co-localised, some sections from a rat perfused 10min after viewing novel and familiar images (Experiment 3), were also double labelled for pCAMKI and pCAMKIV. This required first single labelling pCAMKI stained neurons with the same protocol as detailed above except that the primary antibody was applied at a dilution of 1:100 and incubation was for 24h; visualisation was with DAB. After blocking with excess avidin and biotin, sections were then labelled with the pCAMKIV antibody at 1:100 dilution for 24h and visualised with the purple coloured stain Vector VIP (Vector laboratories).

Data analysis

All processing and counting were performed blind as to treatment. The pCAMKI-labelled cells were counted using an automated image analysis system using in-house software (CellCountMainv.3.1, J. Leendertz) that counted oval or irregular objects within a given size range 10-22µm and that were above a contrast threshold determined against a

smoothed surrounding background level. The threshold was preset in preliminary experiments so that clearly stained neurons were counted. Sections were viewed using a Leica microscope (DM5000B, Leica, Germany) and images (768 x 512 pixels) were shown on a screen and were captured at an effective magnification of x240 with 256 grey level resolution. Automated counting of neurons was performed in counting frames on both the left and right hemispheres for rectangular areas (0.94 x 0.62mm) that included all cortical cell layers from two sections from each brain. The counting frames were positioned over perirhinal cortex, visual association cortex (here termed Te2), lateral entorhinal cortex, auditory cortex and primary visual cortex (Fig. 1C, D). Counts of pCAMKIstained neuronal soma were compared between the hemisphere primarily processing novel and that primarily processing familiar information.

Counts of pCAMKIV-stained neurons were made with a different particle counting program that was able to count ring-shaped and filled neuronal outlines (*Leica Qwin* image analysis program, Leica, Germany). Particles composed of defined number of pixels were counted provided they were of resticted length, this ensured that soma with and without apical dendrites were counted but isolated apical dendrite profiles were not. Counting frames were the same as those used for the pCAMKI counts. Test images of pCAMKI-stained neurons were counted with both types of image counting program and the programs produced equivalent counts.

Stereological corrections were not made because only relative measures were sought. To reduce variance across rats and different immunohistochemical batches of processed material, counts were normalised: for each rat the mean count for each region in a particular hemisphere was divided by the mean count for both hemispheres for that region. These data were analysed using a repeated-measures ANOVA with factors stimulus repetition (NOV/FAM), area and time (10, 40, 70 or 100 min). The significance level was determined as $p=0.05$, 2-tailed.

In the CA1 region of the hippocampus cell counts of pCAMKIV could not be made due to an unclear separation between neighbouring stained neurons. To obtain a measure of staining intensity within this region the strength of staining across the entire counting frame

was averaged. These measurements were performed on the same sized counting frames as used in the cell counts.

Experiment 4 – Prior infusion of STO-609 followed by presentation of novel or familiar images and by immunohistochemical staining for pCAMKI

To determine the effect of the CAMKK antagonist STO-609 on the level of pCAMKI^{Thre177}-stained neurons in the perirhinal region we infused the antagonist prior to viewing novel or familiar images in two separate experiments. In both experiments rats with implanted guide cannulae directed at the perirhinal cortex (as described in Experiment 1) were trained in the paired viewing procedure (as described in Experiment 2) and presented with familiar and novel images over 6 days. In each morning session 30 images (FAM set) were presented simultaneously to each eye. After a delay of 3h the rats were placed back in the apparatus for the afternoon session, in which each eye was shown 15 images from the FAM set and 15 novel images. For the *novel* experiment, on the afternoon of the last day one set of 30 novel images (NOV1) was shown to one eye and another set of novel images (NOV2) was shown to the other eye. Each animal received an infusion of vehicle to one side of the brain and an infusion of STO-609 (as in Experiment 1) to the other side of the brain. The use of NOV1 set and NOV2 was counterbalanced with respect to viewing by the left or right eye and drug or vehicle infusion side across animals. Infusions of STO-609 (same dose as in Experiment 1) were made 15min before viewing of novel images in the afternoon session of the last day. Rats were then transcardially perfused 40 min after viewing novel images and their brains processed for counting of pCAMKI^{Thre177} stained neurons (as described in Experiment 2). For the familiar experiment, on the afternoon of the last day one a familiar set of 30 images was shown to each eye. The same infusion time and perfusion times were used as in the *novel* experiment. For verification of cannulae location in this experiment see Fig. 1B.

Results

Experiment 1: Object recognition memory following infusion with the CAMKK inhibitor STO-609

To establish the role of CAMKK in the consolidation of recognition memory, the effects of local infusion of CAMKK inhibitor STO-609 into the perirhinal cortex were determined on rats' preferential exploration of a novel compared to a familiar object. Memory was tested after a 20min or a 24h delay. The mean of the time spent exploring the novel object minus that exploring the familiar object divided by the total exploration time at test (discrimination ratio: DR) was calculated for vehicle or inhibitor infusions.

The behavioural results established that the CAMKK inhibitor STO-609 impaired object recognition memory after a 24h delay when infused 15min before or immediately following the acquisition trial; see Fig. 2. No impairment was found when the inhibitor was infused 20min after acquisition. Object recognition memory was unaffected over a 20 min delay when STO-609 was infused 15min before acquisition.

In detail, the ***vehicle controls*** showed significant preferential exploration of the novel rather than the familiar object at both 20min and 24h time intervals (t-tests, $p \leq 0.05$) in all tests. Additionally, the total time exploring the objects in the acquisition or choice phases showed no significant effect of STO-609 compared to vehicle during any of the experiments (see Table 1).

For the ***STO-609 condition with a 20min delay***, a one factor ANOVA (treatment [vehicle/STO-609]) revealed no significant effect of treatment ($F_{(1,8)}=5.90$, $p=0.4$). The rats receiving STO-609 before acquisition discriminated between novel and familiar objects at the 20min delay (DR>0: t-test, $p=0.05$).

For the ***STO-609 condition with a 24h delay***, two factor ANOVA (treatment, infusion time[-15, 0, +20min]) revealed a significant main effect of treatment ($F_{(1,24)}= 7.17$, $p=0.01$) and a significant interaction of treatment by time ($F_{(2,24)}= 4.80$, $p=0.02$). When 2 factor ANOVA was applied to the data for infusions 15min before and immediately after acquisition there was a significant main effect of treatment ($F_{(1,15)}= 9.51$, $p=0.01$) but no significant interaction of treatment with time ($F_{(1,15)}= 1.36$, $p=0.3$). Rats receiving STO-609 15min before and 0min after acquisition did not discriminate novel from familiar (DR>0: t-test, $p=0.9$, $p=0.2$). There was a significant effect of treatment (STO-609 or vehicle) when the animals received infusion

15min before (ANOVA: p=0.03) but not immediately after or 20min after acquisition (ANOVAs: p=0.2 for each). Post hoc analyses with Tukey's test showed significant differences between the memory of animals infused with STO-609 at different times: rats infused 15min before (p=0.01) and immediately (0min) after the acquisition trial showed significantly (p=0.03) lower DR values when compared to those infused 20min after the acquisition trial.

These findings indicate that CAMKK is necessary for long-term (24h) memory and is active during the acquisition trial and/or shortly afterwards. Based on this finding we decided to investigate the activation of the enzymes immediately downstream of CAMKK (CAMKI and CAMKIV) following the viewing of novel or familiar images. CAMKK activates CAMKI and CAMKIV through phosphorylation of their Threonine^{177} and Threonine^{196} residues respectively. We used immumohistochemistry to label these residues and by doing so obtain a measure of CAMKI and CAMKIV activity. Initially pilot experiments were performed to determine the cytological distribution of pCAMKI^{Thre177} and pCAMKIV^{Thre196}.

Experiment 2: Immunohistochemical imaging and Western blotting of pCAMKI and pCAMKIV antibodies within perirhinal cortex and Te2

Immunohistochemistry for pCAMKI^{Thre177} revealed staining of neuronal somal cytoplasm and apical dendrites. In many cases the stained neurons were identifiably pyramidal cells and the staining extended to the apical dendrites of approximately 30% of these neurons (Fig. 3). This distribution is consistent with a cytoplasmic occurrence within neurons (Picciotto et al., 1995). Further, another commercially available antibody showed a similar distribution with staining occurring in the cytoplasm (Abcam, UK: catalog # ab62215). Phospho-CAMKIV^{Thre196} staining was principally in pyramidal cells. The staining occurred peripherally within the somal cytoplasm and extended to the apical dendrites of approximately 80% of these pyramidal neurons (Fig. 3). A similar distribution was found with a different commercially available antibody for pCAMKIV$^{thr196\text{-}200}$: labelling occurred in apical dendrites and cytoplasm (Abcam, UK: catalog # ab59424). This distribution for p*CAMKIV* differs from the occurrence within neuronal nuclei reported by (Jensen et al., 1991).

Because of this discrepancy with the expected *CAMKIV* localisation, western blots were run to check for the specificity of the antibodies used (Fig. 4). The western blot for the

pCAMKIV antibody revealed bands at 60kDa and 70kDa. The 60kDa is in close agreement with the CAMKIV molecular weight of 60kDa; however the presence of the weaker band at 70kDa probably reflects nonspecific binding. The western blot for the *pCAMKI* antibody revealed bands at 42kDa and 84kDa. The 42kDa is in close agreement with the CAMKI molecular weight of 45kDa. The presence of the weaker band at 84kDa probably reflects dimmers of the CAMKI molecule (Kambe et al., 2010). These results indicated that the pCAMKI antibody was more selective than the pCAMKIV antibody.

Experiment 3: Quantification of pCAMKI^{Thre177} and pCAMKIV^{Thre196} -stained neurons in the brain following exposure to novel or familiar images

We quantified levels of pCAMKI and pCAMKIV expression at different times following the viewing or novel or familiar images by counting immunohistochemically labelled neurons in the perihinal cortex and in control regions. Using a paired viewing procedure (Zhu et al., 1996; Warburton et al., 2005), novel and familiar images were presented simultaneously on computer monitors positioned in the left and right-hand visual hemifields, so as to restrict initial visual processing to the opposite hemisphere of the brain. Immunohistochemical staining for the activated forms of pCAMKI^{Thr177} and pCAMKIV^{Thr196} was performed in hemispheres primarily processing novel and those primarily processing familiar images ('novel' and 'familiar' hemispheres) from brains perfused at different times following the viewing of the images. To compare differences in the relative expression of pCAMKI and pCAMKIV, neurons were counted in novel compared to familiar hemispheres.

PhosphoCAMKI^{Thre177} counts:

Labelling of pCAMKI labelled neurons was strong in all areas of the brain studied and occurred through layers II to VI. Phospho-CAMKI counts were made of approximately oval shaped somata with continuous staining through the cytoplasm (Fig. 5). The overall 3 factor ANOVA of raw counts (novelty[novel,familiar], area[perirhinal and Te2, control regions (auditory, entorhinal cortex, V1 and hippocampus)], time) showed a significant interaction of novelty by time ($F_{3,21}$ =3.62, p=0.03) but no significant interaction of novelty by area ($F_{15,21}$ <1,

p=0.8) or novelty by area by time ($F_{1,21}$ <1, p=0.1) and no significant main effect of novelty ($F_{1,21}$ =3.26, p=0.09).

For the designed comparison seeking effects within perirhinal cortex, 2 factor ANOVA showed a significant interaction of novelty by time ($F_{3,21}$ =3.15, p<0.05) and a significant main effect of novelty ($F_{1,21}$ =6.04, p=0.02). Post hoc ANOVA tests revealed that counts in the perirhinal cortex for the familiar hemisphere were significantly higher than those for the novel hemisphere at 10min (p=0.03) and 40min (p=0.05) but not at 70 (p=0.4) or 100min (p=0.07) (Fig. 5). A post hoc Tukey test on the differences between novel and familiar counts revealed a significantly greater difference in counts at 40min than at 70min (p=0.02).

The other regions showed no significant effect of novelty (auditory, lateral entorhinal and visual cortices, area Te2 and hippocampus: p=0.9, 0.8, 0.9, 0.3 and 0.2, respectively) and no significant interaction of novelty by time (p=0.4, 0.1, >0.9, 0.3 and >0.9).

PhosphoCAMKIV^{Thre196} counts:

Labelling of pCAMKIV labelled neurons was strong in some cortical regions such as the auditory cortex but was weaker in the perirhinal cortex and area Te2. Staining occurred through layers II-VI. Phospho-CAMKIV counts were made of somata (Table 2). Perirhinal counts were in the region of 30 per counting frame compared to ~200 for pCAMKI (see also Fig. 7). The overall 3 factor ANOVA (novelty [novel,familiar], area[auditory, entorhinal cortex, perirhinal cortex, Te2 and V1], time) showed a significant interaction of novelty by time ($F_{3,80}$ =3.20, p=0.05) but no significant interaction of novelty by time by area ($F_{12,80}$ <1, p=0.7) and no significant main effect of novelty ($F_{1,80}$ <1, p=0.9). There was no significant effect of novelty for any individual area. For the designed comparison seeking effects within perirhinal cortex, 2 factor ANOVA showed no significant effects (see Table 2 for means and SEMs of the counts).

Staining of pCAMKIV in the hippocampus appeared qualitatively greater than that seen in the perirhinal cortex. It formed a continuous band within the pyramidal layer of CA1, preventing the counting of individual neurons. Therefore a measurement of average staining intensity was made for each counting frame. A two factor ANOVA (novelty [novel,familiar], time) for these CA1 measurements of staining showed no significant effect of novelty ($F_{1,20}$ <1, p=0.4) and no significant interaction of novelty by time ($F_{3,20}$ <1, p=0.5).

Double-labelling of pCAMKI and pCAMKIV

The immunohistochemical experiments (Experiment 2) showed that both pCAMKI and pCAMKIV labelled neurons occurred in the perirhinal cortex. To determine whether pCAMKI and pCAMKIV were co-localised within neurons, sections from a rat perfused 10min after viewing novel and familiar images, were doubly-labelled for the two activated enzymes (Fig. 6). The double-labelling revealed that a small proportion of neurons (~25%) within the perirhinal cortex were co-labelled for both pCAMKI and pCAMKIV (Fig. 6C). Fig. 6 also illustrates that many more perirhinal neurons were labelled for pCAMKI than for pCAMKIV.

Experiment 4: Effect of prior infusion of STO-609 on levels of pCAMKI-stained neurons in the brain following exposure to novel or familiar images

The behavioural experiments established that inhibition of CAMKK impaired object recognition memory (Experiment 1) and the immunohistochemical experiments established that the activation of CAMKI was differentially regulated by viewing novel or familiar images (Experiment 3). To determine whether CAMKK inhibition disrupted the differential expression of pCAMKI produced by viewing novel or familiar images, counts of pCAMKI-stained neurons were made following the infusion of the CAMKK inhibitor STO-609 into the perirhinal cortex. Effects of STO-609 on counts of pCAMKIV stained perirhinal neurons were not sought because these did not differ significantly following the viewing of novel or familiar images.

Using the paired viewing procedure, novel images (*Novel experiment*) or familiar images (*Familiar experiment*) were presented simultaneously to both eyes on computer monitors positioned in the left and right visual hemifields. Fifteen minutes before the final viewing of the images rats underwent an infusion of vehicle into perirhinal cortex in one hemisphere and of STO-609 in the other. Infused at this time, STO-609 impaired object recognition memory (Experiment 1: see Fig.2). Rats were perfused 40min following the final viewing of novel/familiar images, the time at which were found different counts of pCAMKI stained neurons following viewing of novel compared to familiar images (Experiment 3: see Fig. 5).

Overall analysis of the combined data for the *novel* and *familiar* experiments by three factor ANOVA (novelty [novel or familiar stimuli], treatment [vehicle/STO-609], area[auditory

cortex, entorhinal cortex, perirhinal cortex, Te2, hippocampus, V1]) of raw pCAMKI counts revealed no significant main effect of treatment ($F_{1,13}$ = 1.1, p=0.3), nor interaction of treatment by area by novelty ($F_{1,9}$ = 2.8, p=0.09) but the interaction of treatment by area was significant ($F_{1,9}$ = 4.9, p=0.02); Fig. 7.

For the designed comparison seeking effects within perirhinal cortex, two factor ANOVA showed a significant effect of treatment for perirhinal cortex (p=0.001). For the novel pictures, STO-609 infusion significantly reduced pCAMKI counts in the perirhinal cortex by 23% compared to vehicle (ANOVA; p=0.03). For the familiar pictures, STO-609 infusion significantly reduced pCAMKI counts in the perirhinal cortex by 16% compared to vehicle (ANOVA; p=0.02). The reduction in counts was not significantly different for the novel and familiar pictures (ANOVA; p=0.6).

Within the neighbouring, non-infused regions, there were significant effects of treatment for area Te2 (ANOVA; p=0.03) and lateral entorhinal cortex (ANOVA; p=0.05) but no significant differences between the reductions for novel and familiar pictures. The reduction reached significance in lateral entorhinal cortex (p=0.02) for the familiar but not the novel pictures, and approached significance for the familiar pictures in Te2 (p=0.07); Fig. 7.

These results show that perirhinal infusion of STO-609 produces a reduction in counts of neurons expressing its reaction product, pCAMKI, following both viewing of novel and familiar images, in consistency with the role of STO-609 as an inhibitor of CAMKK. These reductions are found in perirhinal cortex but also in neighbouring lateral entorhinal cortex and area Te2.

Discussion

The effect of the CAMKK inhibitor STO-609 on recognition memory

The results demonstrate that long-term (24h) object recognition memory is impaired when the CAMKK inhibitor, STO-609, is infused into perirhinal cortex prior to or immediately after acquisition. To our knowledge this is the first study to show STO-609 impairs memory. As impairment was found for an infusion immediately after acquisition, the action of STO-609 is most readily explained as being on consolidation mechanisms rather than on acquisition. As no impairment was found for infusions 20 min after acquisition, the effect must be on early consolidation. These results therefore indicate a role for CAMKK in the early consolidation of

long-term recognition memory. Recognition memory measured after a 20 min delay was unimpaired. A long-term but not shorter-term memory impairment has also been found for mutant mice with a deleted CAMKKβ gene where the impairment was in memory for social transmission of food preferences (Peters et al., 2003).

Immunohistochemical imaging of pCAMKI and pCAMKIV following viewing of novel or familiar images

In support of a mnemonic role for CAMKK in perirhinal cortex, the processing of familiar pictures resulted in an enhanced level of immunoreactivity of the CAMKK reaction product, pCAMKI^{thre177}, compared to the processing of novel pictures. The direction of this differential change (familiar > novel) is the opposite to that found for Fos (Zhu et al., 1995; Zhu et al., 1996; Wan et al., 1999), phosphorylated CREB (Warburton et al., 2005) and pCAMKIIα (Tinsley et al., 2009). Moreover, counts of pCAMKI stained neurons were reduced following infusion of STO-609 into the perirhinal cortex after the viewing of familiar as well as novel pictures. The reductions are consistent with the role of STO-609 in inhibiting CAMKK, which in turn phosphorylates the threonine^{177} residue present on pCAMKI. The immunohistochemical results establish a differential role for CAMKK in the processing of familiar compared to novel stimuli, but do not exclude a role in processing both novel and familiar stimuli. They raise the possibility that CAMKK might be involved in reconsolidation mechanisms related to re-exposure to the familiar stimuli in addition to consolidation mechanisms for novel stimuli. As the perceptual demands of the object recognition memory task were low and behavioural impairment was found when the infusion was after acquisition, it is implausible that the impairment was produced by effects upon visual perceptual rather than mnemonic processing. The absence of an effect on recognition memory at a 20min delay additionally suggests that the major effect of STO-609 was not on perceptual processes.

Differential pCAMKI activation occurred 10-40min after viewing images, a time course consistent with that of the impairment of object recognition memory produced by STO-609 infusion. STO-609 needed to be active during and immediately after acquisition; infusion 20min after acquisition (resulting in CAMKK inhibition >~ 30min after acquisition) was ineffective. This time course of pCAMKI activation is also consistent with that (up to 60min) produced by LTP in hippocampal cell cultures, that activation also being blocked by STO-609

(Schmitt et al., 2005). CAMKK and pCAMKI activation begins and ends earlier after recognition memory acquisition (~10-40min) than does activation of pCAMKII (~20-100min) (Tinsley et al., 2009).

The STO-609 infusion in perirhinal cortex produced significant reductions in pCAMKI activation in the neighbouring regions of area Te2 and lateral entorhinal cortex, the reductions being of comparable magnitude to those in perirhinal cortex and not differing significantly for novel and familiar images. It is unlikely that these reductions are primarily due to spread of the infusate. Data from others (Martin, 1991; Izquierdo et al., 2000; Attwell et al., 2001) and our own findings indicate that perirhinal infusions reach most of perirhinal cortex as previously defined (Shi and Cassell, 1999); however, the spread into entorhinal cortex and area Te2 is limited (<10%). Accordingly, it seems that inhibition of CAMKK in perirhinal cortex reduces pCAMK1 activation in area Te2 and lateral entorhinal cortex. Correspondingly, there may be a contribution to the impairment of recognition memory produced by the infusion of STO-609 into perirhinal cortex from such transmitted influences to area Te2 and/or entorhinal cortex.

Counts of pCAMKIV stained neurons did not differ significantly in response to viewing novel compared to familiar images. It is possible that the failure to find a difference was because counts for pCAMKIV were lower than for pCAMKI or because of variance introduced by the antibody's non-specific staining. However, in interpreting the effects of CAMKII inhibitors, Tinsley et al (2009) argued that pCAMKIV activation is insufficient to support recognition memory. The low levels of pCAMKIV staining (even including non-specific staining) and lack of differential effect produced by viewing novel and familiar stimuli in the current experiments suggest that perirhinal CAMKIV activation is unimportant for object recognition memory. The impairment in fear conditioning seen in CAMKIV knockout mice (Wei et al., 2002) might be due to loss of CAMKIV in regions other than perirhinal cortex, such as the hippocampus or amygdala.

How does CAMKK exert its actions?

CAMKK activates its substrates via phosphorylation. CAMKK is known to activate the MAPKinase pathway as well as CAMKI and CAMKIV (Soderling, 1999). LTP induction in hippocampal cell cultures activated CAMKI, Ras-GRF1 (Ras-guanyl-nucleotide releasing factor) and ERK (extracellular signal-regulated kinase), the activations all being blocked by

STO-609 (Schmitt et al., 2005). At the dose used by Schmitt and colleagues and in the present study, STO-609 inhibits CAMKK but does not directly inhibit CAMKI, CAMKIV or MAPkinase (Tokumitsu et al., 2002). However, the inhibition of CAMKK (Enslen et al., 1996) effects activation not only of CAMKI (Schmitt et al., 2004); and the present findings) but also MAPkinase and other kinases (Yano et al., 1998; Soderling, 1999). In particular, CAMKI has numerous other substrates such as Synapsin I (Ishida et al., 2003), CREB (Sheng et al., 1991) and ATF1 (activating transcription factor) (Sun et al., 1996). Accordingly, inhibition of CAMKK may produce effects on recognition memory processes via a number of down-stream mechanisms.

In sum, the results establish that the CAMKK pathway within the perirhinal cortex is important for the consolidation of object recognition memory. Immunohistochemical imaging for pCAMKI indicated that CAMKI might be involved in reconsolidation mechanisms for familiar stimuli in addition to consolidation mechanisms for novel stimuli. The time course of activation of pCAMKI after acquisition (~10-40min) is earlier than found (Tinsley et al., 2009) for pCAMKII. In contrast to CAMKI and CAMKII, CAMKIV appears to be unimportant for perirhinal recognition memory processes.

Acknowledgements

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Figure Legends

Fig 1. (**A**) Reconstructed individual infusion sites shown on a representative brain section (Paxinos and Watson, 1998) for the 25 animals included in Experiment 1 (filled square indicates position of the tip of a guide cannula; note some symbols may overlap). (**B**) Infusion sites from the 15 animals included in Experiment 4 (filled squares indicate the tips of the guide cannulae from the *novel* experiment (n=9), filled squares indicate the tips of the guide cannulae from the *familiar* experiment (n=6)). All cannulae were accurately located in perirhinal cortex. **C** and **D**, Regions within which pCAMKI and pCAMKIV stained cell counts were made, shown on sections -5.2 (**C**) and -6.3 (**D**) relative to bregma (Paxinos and Watson, 1998): auditory cortex (AUD), temporal association cortex (Te2), perirhinal cortex (PRH) lateral entorhinal cortex (ENT), primary visual cortex (V1) and the CA1 region of the hippocampus (HIPP).

Fig 2. Effect of perirhinal infusions of the CAMKK inhibitor STO-609 on object recognition memory in the rat. Discrimination ratios (DRs) were used as an index of memory performance in the preferential object recognition test. Results of infusing (**A**) vehicle or STO-609 15 min before the acquisition trial of the 20 min delay experiment, (**B**) vehicle or STO-609 at differing times relative to acquisition in a 24h delay test. STO-609 infusions 15min before and 0min after the acquisition trial produced significantly lower DR values than infusions 20min after the acquisition trial (*). **#** DR >0, $p<0.05$.

Fig 3. Immunohistochemical staining for $pCAMKI^{Thre177}$ and $pCAMKIV^{Thre196}$. **A-B**, photomicrographs showing pCAMKI staining in perirhinal cortex (**A**) and area Te2 (**B**). Phospho-CAMKI staining is seen principally in the somal cytoplasm and apical dendrites. **C-D**, photomicrographs showing pCAMKIV staining in perirhinal cortex (**C**) and area Te2 (**D**). Phospho-CAMKIV staining is seen principally in the somal cytoplasm and apical dendrites. Scale bar = 100µm.

Fig 4. Western blots of perirhinal cortex (PRH) and area Te2 (TE) for: (**A**) pCAMKI, revealing a strong band at 42kDa (CAMKI molecular weight = 45kDa) and a weaker band at 84kDa

probably due to pCAMKI dimmers; and (**B**) pCAMKIV, revealing a strong band at approximately 60kDa (CAMKIV molecular weight = 60kDa) and a weaker band at approximately 70kDa due to non-specific binding.

Fig 5. Graphs of raw counts of pCAMKIThre177 stained neurons in areas: (**A**) perirhinal cortex (PRH), (**B**) area Te2 (TE), (**C**) entorhinal cortex (ENT), (**D**) auditory cortex (AUD), (**E**) visual cortex (V1), and (**F**) CA1 of the hippocampus (Hipp) at indicated times after viewing novel and familiar stimuli. Note significant effect of novelty (*) at 10min ($p=0.03$) and 40min ($p=0.05$) but not at 70min or 100min within the perirhinal region.

Fig 6. Double labelling for pCAMKI and pCAMKIV in the perirhinal cortex. Photomicrographs of adjacent sections stained for: (**A**) pCAMKI visualised with DAB, (**B**) pCAMKIV visualised with DAB, and (**C**) double stained for pCAMKI visualised with DAB (brown) and pCAMKI visualised with Vector VIP (purple). In (**C**) note neurons stained for pCAMKI (black arrow), pCAMKIV (green arrow) or pCAMKI and pCAMKIV (blue arrow). Photomicrographs were taken at the same magnification. Scale bar = 100µm.

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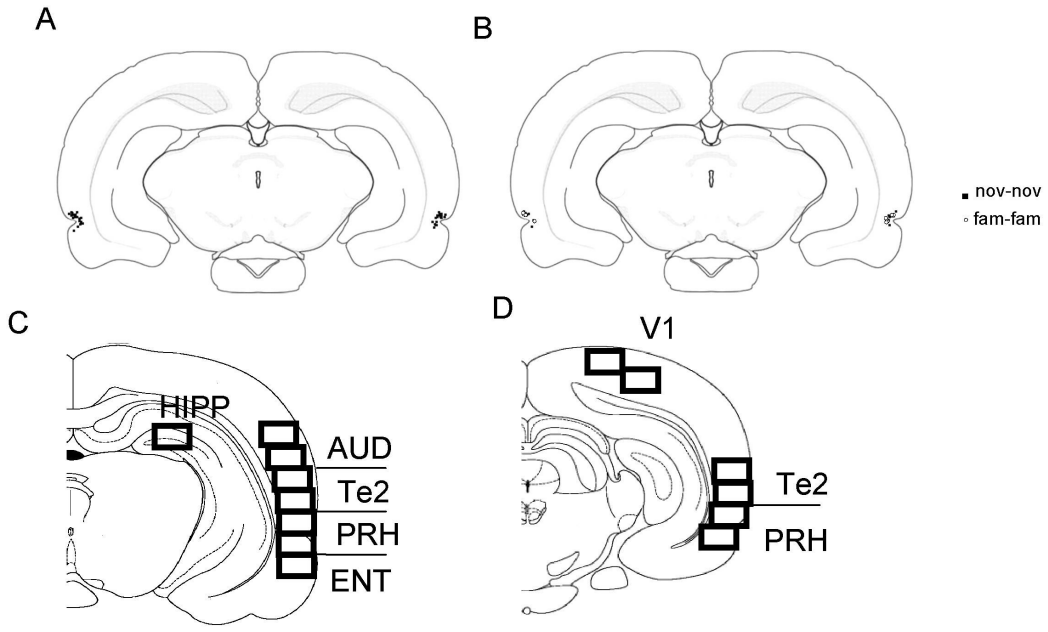
Table 1. Exploration of animals (in seconds) in the acquisition and choice trials. Values shown are the mean time exploring both objects in seconds (S.E.M) for vehicle or drug at each of the time-points investigated. For acquisition or choice trials groups receiving STO-609 did not display significantly different exploration times from their control groups, (ANOVAs, $p \leq 0.05$). For the 24 h experiments 2 –factor ANOVA(treatment, time) showed no significant effect of

treatment on exploration times for acquisition ($F_{1,24}$ = <1, p=0.9) or choice trials ($F_{1,24}$ = <1, p=0.6).

Table 2. Counts of pCAMKIV^{Thre196} stained neurons within brain regions (auditory, entorhinal, perirhinal, TE and V1 cortices) at 10, 40, 70 and 100min after viewing novel or familiar images. The values given for hippocampus (CA1) region were provided by an analysis of the average staining intensity on counting frames. Statistics for raw counts: a repeated-measures 3 factor ANOVA (area, novelty, time) showed a significant interaction of novelty by time ($F_{3,80}$ =3.20, p=0.05). Two factor ANOVA (novelty, area) at 70min showed a significant effect of novelty (p=0.05) however this effect was not significant within individual regions. Statistics for hippocampal staining: a two factor ANOVA (Novelty, time) showed no significant effect of novelty or interaction of novelty by time. Values shown are mean (S.E.M).



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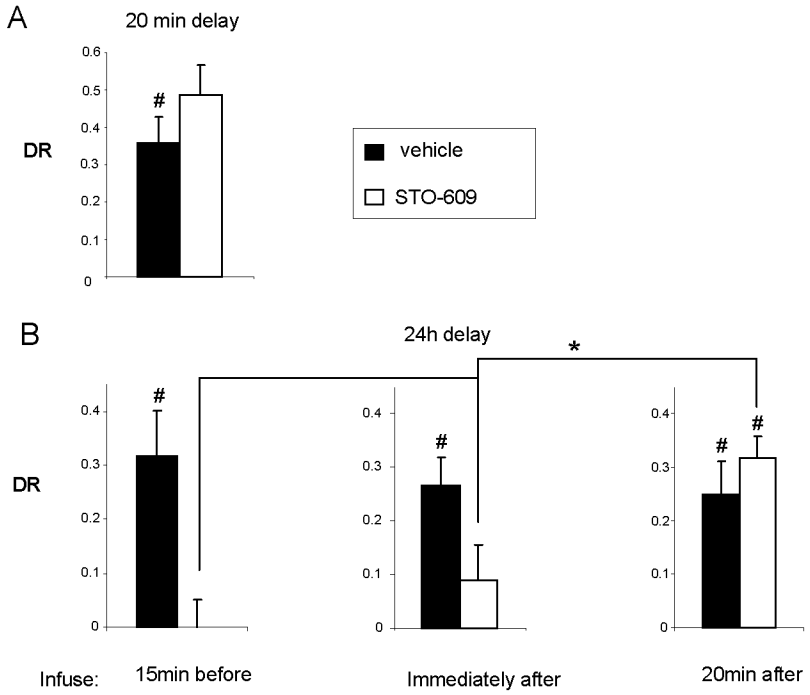

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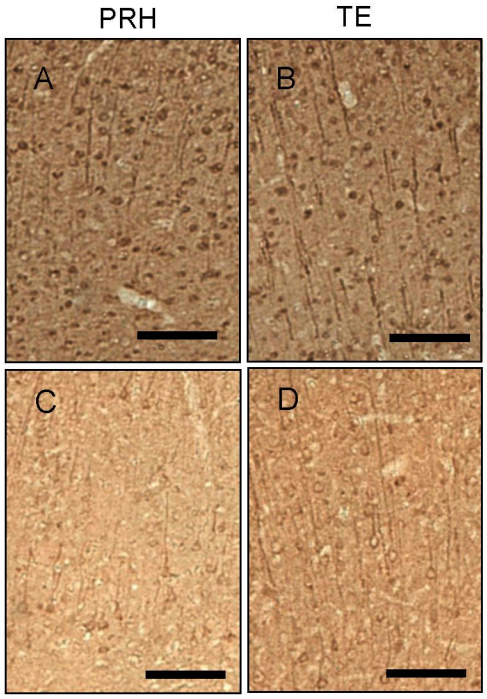

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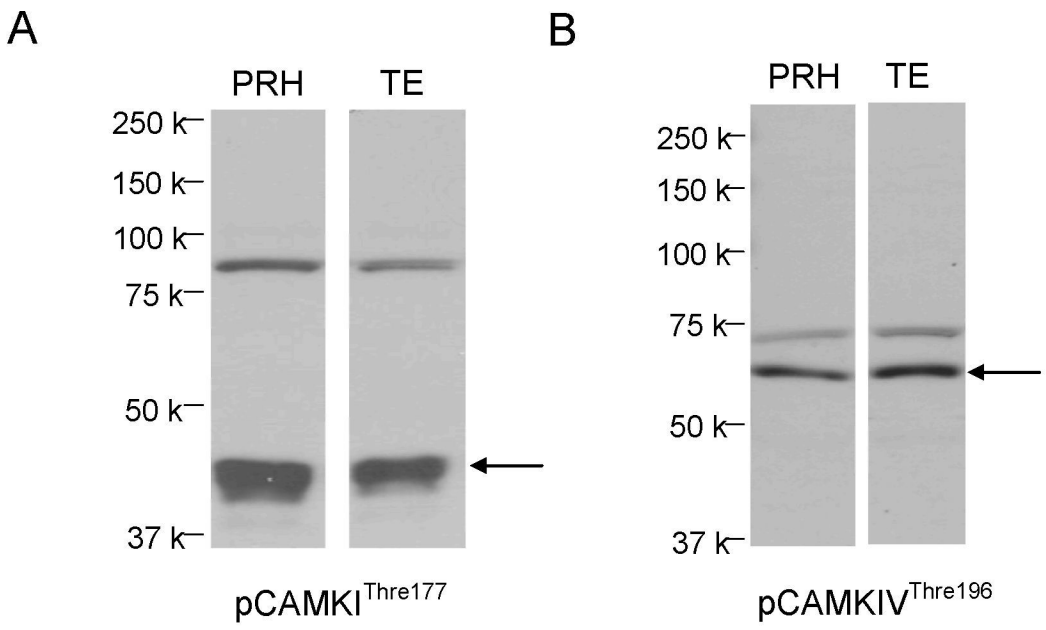

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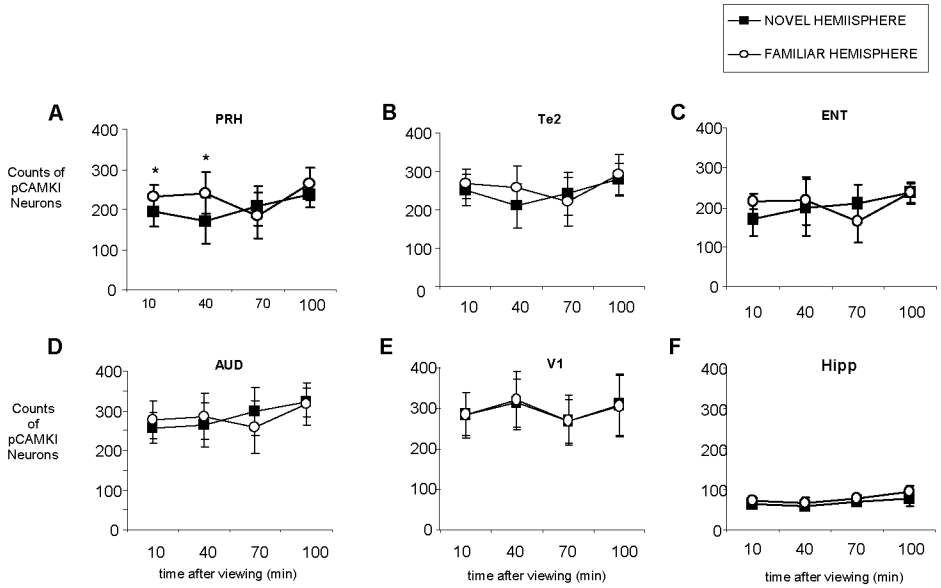

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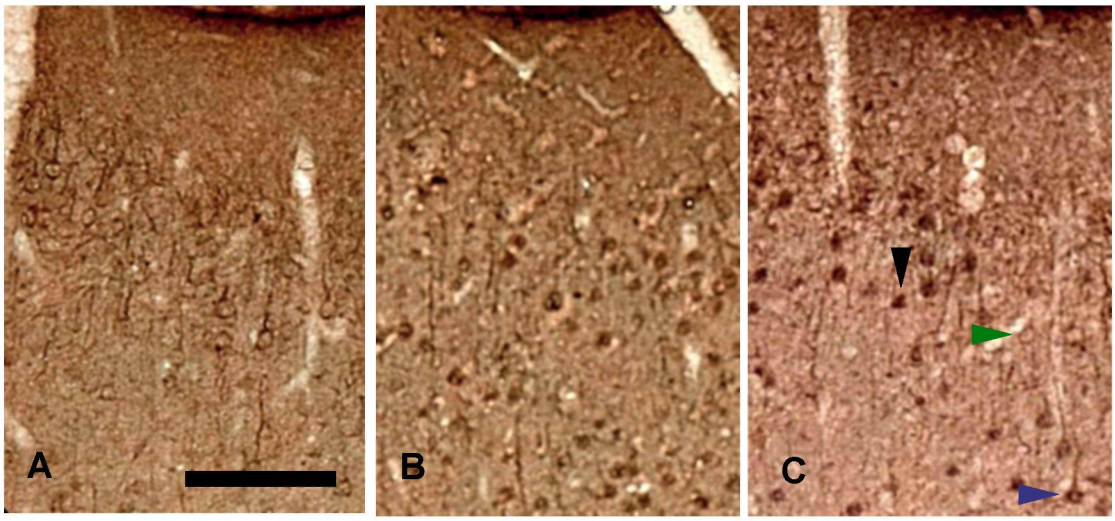

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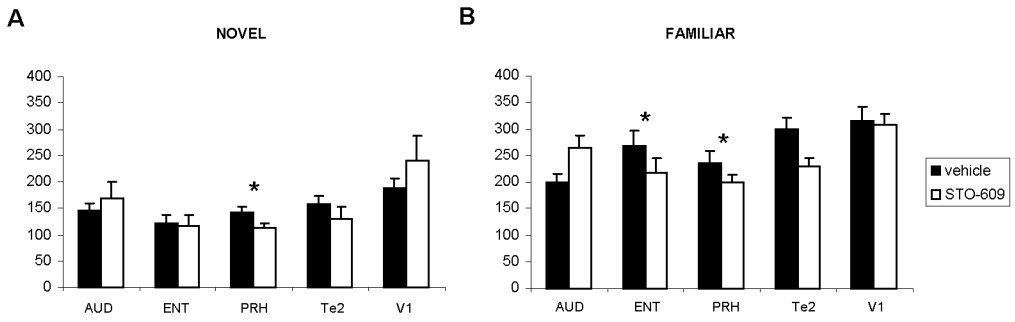

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delay	infusate	trial	Infusion time relative to the end of the acquisition trial		
			-19	0	20
20min	vehicle	acquisition	29.3(2.4)		
	STO-609	**acquisition**	**30.7(1.9)**		
	Vehicle	choice	27.6(4.0)		
	STO-609	**choice**	**28.8(4.8)**		
24h	vehicle	acquisition	36.8(1.4)	33.9(2.9)	28.2(2.1)
	STO-609	**acquisition**	**29.7(3.9)**	**35.7(1.8)**	**30.9(1.4)**
	Vehicle	choice	22.6(3.2)	23.3(2.6)	15.9(1.7)
	STO-609	**choice**	**22.7(3.7)**	**23.0(2.1)**	**21.9(2.6)**

Table 1. Exploration of animals (in seconds) in the acquisition and choice trials. Values shown are the mean time exploring both objects in seconds (S.E.M) for vehicle or drug at each of the time-points investigated. For acquisition or choice trials groups receiving STO-609 did not display significantly different exploration times from their control groups, (ANOVAs, $p \leq 0.05$). For the 24 h experiments 2 –factor ANOVA(treatment, time) showed no significant effect of treatment on exploration times for acquisition ($F_{1,24} = <1$, $p=0.9$) or choice trials ($F_{1,24} = <1$, $p=0.6$).

Perfusion time	Image type	Auditory	Entorhinal	Perirhinal	TE	V1	Hipp (CA1)
10min	**Novel**	**64.8** (*25.7*)	**33.0** (*12.3*)	**44.0** (*12.4*)	**43.7** (*14.2*)	**31.6** (*8.7*)	**103.6** (*5.2*)
	Familiar	**64.8** (*29.9*)	**41.7** (*13.9*)	**43.6** (*16.8*)	**51.0** (*19.7*)	**58.1** (*18.0*)	**104.7** (*23.1*)
40mim	**Novel**	**17.3** (*11.3*)	**7.7** (*6.3*)	**8.8** (*4.1*)	**10.3** (*5.5*)	**20.1** (*10.6*)	**107.5** (*7.3*)
	Familiar	**26.5** (*15.7*)	**22.5** (*18.0*)	**16.5** (*11.9*)	**15.5** (*10.3*)	**18.8** (*10.4*)	**109.5** (*4.5*)
70min	**Novel**	**74.3** (*47.8*)	**48.3** (*17.1*)	**45.1** (*15.9*)	**50.5** (*16.5*)	**68.8** (*22.6*)	**116.8** (*8.2*)
	Familiar	**56.3** (*19.0*)	**27.0** (*15.9*)	**33.3** (*9.9*)	**42.7** (*13.7*)	**48.1** (*18.2*)	**112.8** (*7.8*)
100min	**Novel**	**16.1** (*14.3*)	**37.0** (*8.5*)	**31.6** (*7.0*)	**42.6** (*13.9*)	**53.8** (*24.3*)	**104.8** (*3.4*)
	Familiar	**55.6** (*20.6*)	**54.7** (*16.5*)	**31.5** (*7.4*)	**37.6** (*10.5*)	**37.8** (*18.3*)	**96.8** (*4.7*)

Table 2. Counts of pCAMKIV^{Thre196} stained neurons within brain regions (auditory, entorhinal, perirhinal, TE and V1 cortices) at 10, 40, 70 and 100min after viewing novel or familiar images. The values given for hippocampus (CA1) region were provided by an analysis of the average staining intensity on counting frames. Statistics for raw counts: a repeated-measures 3 factor ANOVA (area, novelty, time) showed a significant interaction of novelty by time ($F_{3,80}$ =3.20, p=0.05). Two factor ANOVA (novelty, area) at 70min showed a significant effect of novelty (p=0.05) however this effect was not significant within individual regions. Statistics for hippocampal staining: a two factor ANOVA (Novelty, time) showed no significant effect of novelty or interaction of novelty by time. Values shown are mean (S.E.M).