Regulation of the expression of the psychiatric risk gene *Cacna1c* during associative learning.

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Short title: $Ca_v1.2$ in hippocampal learning and memory

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Abstract

CACNA1C encodes the $Ca_v1.2$ L-type voltage gated calcium channel. Generic variation in CACNA1C has been consistently identified as associated with risk for psychiatric disorders including schizophrenia, bipolar disorder, major depressive disorder and autism. Psychiatric risk loci are also enriched for genes involved in the regulation of synaptic plasticity. Here we show that the expression of *Cacna1c* is regulated in the rat hippocampus after context exposure, contextual fear conditioning and fear memory retrieval in a manner that correlates to specific memory processes. Using quantitative in situ hybridisation the expression was down-regulated in CA1 by brief exposure to a novel context and to a conditioned context, and up-regulated in the dentate gyrus after contextual fear conditioning. No changes were measured after prolonged context exposure followed by conditioning, a procedure that retards fear conditioning (latent inhibition), nor with fear memory recall leading to extinction. These results are consistent with a selective role for $Ca_v1.2$ in the consolidation of context memory and contextual fear memory, and with processes associated with the maintenance of the fear memory after recall. The dysregulation of *CACNA1C* may thus be related to associative memory dysfunction in schizophrenia and other psychiatric disorders.

Introduction

Long-term potentiation and the synaptic plasticity underlying long-term memory formation require *de novo* gene transcription and protein synthesis[1][2][3][4][5]. Previous research has shown changes in the expression of specific genes accompanying different aspects of associative learning; identifying separable molecular signatures of distinct memory processes including consolidation, recall and extinction[6][7][8][9][10]. Notably both early and late phases of transcriptional regulation following the induction of plasticity have been recognised[11][12].

Calcium influx into the post-synaptic neuron plays a critical role in regulating the changes in gene expression which accompany synaptic plasticity [13]. This calcium signal acts via signalling cascades to regulate the activity of transcription factors such as CREB. There are multiple routes for calcium to enter the cell, including via N-methyl-D-aspartate receptors (NMDA-R), GluA2-lacking calcium permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA-R) and indirectly, metabotropic glutamate receptor (mGluR), and voltage gated calcium channels (VGCCs). The regulation of expression of subunits of NMDA-Rs and mGluRs has been investigated following long term potentiation (LTP) revealing increased NR2B and mGluR1c expression evident in a late phase from 24 hrs following the induction of LTP in dentate gyrus granule cells [14]. These results demonstrate key channels are themselves subject to activity-dependent regulation following the induction of plasticity, with a late phase profile of transcriptional regulation. However much less is known about the regulation of expression of VGCCs during learning and plasticity events.

L-Type VGCCs are known to play a central role in controlling activity-dependent synaptic plasticity[15–17]. $Ca_V1.2$ channels, the predominant form of L-Type VGCCs in the mammalian brain, are ideally situated somato-dendritically to link neuronal activation to calcium

signalling and the regulation of gene expression[15, 16]. The C-terminal tail of the α1c subunit of $Ca_v1.2$ (encoded by *CACNA1C)* directly binds calmodulin, a high affinity calcium binding protein, that through its interaction with target molecules including calcium-calmodulin kinases is key to triggering the signalling cascades which transmit the calcium signal to the nucleus, contributing to the regulation of activity-dependent genes such as BDNF.

Notably L-Type VGCCs, in particular the *CACNA1C* gene, have been strongly associated with risk for psychiatric disorders. Genome-wide association studies have shown a highly significant association between genetic variation in *CACNA1C* and risk for both bipolar disorder and schizophrenia, and cross disorder studies have suggested that genetic variation in the *CACNA1C* gene contributes risk across a range of psychiatric disorders including major depressive disorder and autism[18][19][20] [21][22] [23]. These findings are consistent with the more general observation that psychiatric risk loci are enriched for genes involved in the regulation of synaptic plasticity, including VGCCs, components of the NMDA receptor complex and the interactors of the Fragile X Mental Retardation Protein FMRP [24]. However, it is not clear whether *CACNA1C* is itself regulated transcriptionally during learning, and if so whether the regulation of *CACNA1C* is associated with specific phases of learning and memory.

Here we sought to further investigate the involvement of L-Type VGCCs in plasticity by investigating the regulation of the expression of the *Cacna1c* gene (the rodent homologue of *CACNA1C*) during associative learning. To examine learning related changes in *Cacna1c* expression we focussed on contextual fear conditioning (CFC) and memory (CFM), a form of associative learning known to depend on protein synthesis in the hippocampus [11]. In addition, we investigated the transcriptional regulation of *Cacna1c* after the recall of CFC under conditions of recall that promote the maintenance of the CFM ("reconsolidation") or

extinction, both of which are independent associative memory mechanisms that depend on hippocampal protein synthesis[25][26], and in latent inhibition (LI), an effect in which pre-exposure to neutral stimulus retards subsequent conditioning [27] that depends on L-Type VGCCs [28]. We here report the selective regulation of *Cacna1c* during specific phases of associative memory formation, results that are likely to be of relevance in understanding the contribution of genetic variation in this gene to risk for psychiatric disorders.

Methods

Animals

Sixty-four male Lister Hooded rats (250 – 300 g) were housed in pairs in conventional NKP RC2R cages within a holding room maintained at 21°C on a 12h reversed light/dark cycle (lights on 8:00pm) and with ad libitum access to food (Harlan 2014 global rodent diet) and water. Experiments were conducted in the dark period. Animals were sacrificed from their home cages using a rising concentration of CO_z at specific time points following each behaviour of interest. All procedures were conducted in accordance with local Cardiff University Ethical Committee approval and the United Kingdom 1986 Animals (Scientific Procedures) Act (Project license PPLs 30/2236 and 30/2722).

Behavioural procedures

Contextual fear conditioning (CFC) took place in a rat conditioning box with a metal grid floor (Standard modular test chamber for rat, Med Associates Inc., Vermont, USA). For CFC, individual animals were placed into the novel conditioning chamber for 2 mins prior to receiving a 0.5 mA scrambled footshock for 2 secs. They remained in the chamber for an additional 1 min before being returned to home cages. A *Novelty* group was exposed to the context for 2 mins without receiving a footshock and were returned to home cages. Conditioned animals were sacrificed 2, 4, 8 and 24 hrs later. Naïve home cage litter mates were sacrificed at the same time. *Novelty* animals were sacrificed 4 hrs following exposure.

A separate group of animals were individually pre-exposed to the context for 8 hrs before receiving the footshock to induce latent inhibition (*LI*) to control for gene expression associated with the experience of the footshock in the absence of CFM formation [6]. A pre-exposure (*PreExp*) group was also used to assess altered gene expression by the prolonged exposure to the context, spending 8 hrs in the box without a subsequent footshock. Both of the latter groups were sacrificed 4 hrs following return to home cages. Thus, a 4 hrs post-training to sacrifice delay is represented in all experimental groups.

A further cohort underwent CFC to measure the regulation of *Cacna1c* expression after recall and extinction. Forty-eight hrs later animals were returned to the conditioning context for either 2 mins (*Recall (2 min)*) or 10 mins (*Extinction (10 min)*). The 2 min exposure induces a cellular mechanism typically associated with reconsolidation of the recalled fear memory and which is required for the maintenance of the memory, whereas the prolonged re-exposure leads to the formation of an inhibitory associative extinction memory characterised by reduced conditioned freezing behaviour [29]. The recall groups were sacrificed 2 hrs following re-exposure to the context along with the conditioned group that were not re exposed to the training context (*No Recall*). This time was selected because our own previous data suggest that altered gene expression can be measured at this delay after recall (e.g. Trent et al. 2015) and that molecular events associated with recall occur in a shorter temporal span than after learning (Alberini et al. 2005).

n = 6 for all groups except CFC groups sacrificed 2 and 8 hrs after conditioning (n = 4)

Behavioural analysis

Freezing behaviour served as a measure of unconditioned and conditioned fear to the context during the training and recall tests and was defined as complete immobility except for respiration. The behaviour was digitally recorded and quantified by two independent scorers blind to the experimental group. One unit of freezing behaviour was defined as a

continuous absence of movement sampled 1s in every 10s. Th percentage of time spent freezing was calculated every 2 min, or 1 min immediately after the footshock (post-unconditioned stimulus (Post-US)). The data by the two scorers were concordant. The scores were expressed as mean ± SEM. Repeated measures ANOVAs using Mauchley's test for sphericity with Greenhouse-Geisser correction were used to compare freezing levels before US (Pre-US) and post-US phases during conditioning and to analyse freezing levels in the Recall (2min) and Extinction (10min) groups during the subsequent recall test.

In situ hybridisation

As described previously [30], brains were removed immediately post-mortem, fresh frozen on dry-ice and stored at – 80°C. Coronal sections (14 µm) through the dorsal hippocampus were made (Leica Microsystems CM1860UV), mounted on poly-L-lysine coated glass microscope slides and air-dried at RT. Sections were fixed in 4 % PFA solution and dehydrated before storage at 4°C in 95% ethanol until required.

For hybridisation, a cDNA antisense probe (45-mers, 3' - TCGAAGTAGGTGGAGTTGACCACG-TACCACACTTTGTACT GGTGC -5' complementary to nucleotides 3940-3896, NM_012517.2) were synthesized commercially (SigmaGenosys). The probe was 3'-end labelled with [α-thio ^{35}S] dATP (1200 Ci/mmol; Perkin Elmer-NEN) in a 30:1 molar ratio of radiolabeled ATP:oligonucleotide using terminal deoxynucleotidyl transferase (30U/µl, Promega). Specific activity of the ^{35}S-labelled probe was between 2.0×10^5 and 3.0×10^5 dpm/µL probe. To define non-specific hybridization, adjacent slide-mounted sections were incubated with radiolabeled oligonucleotide in the presence of an excess (80X) concentration of unlabelled oligonucleotide probe. After hybridization, sections and a ^{14}C ladder (American Radiolabelled Chemicals, Saint Louis, USA) for quantification, were exposed to Kodak BioMax MR X-ray film (Sigma-Aldrich Company Ltd) for 7 days before the autoradiograms were developed.

In situ hybridisation semi-quantitative analysis

Films were analysed using ImageJ software (http://rsbweb.nih.gov/ij/). Optical density values were converted to concentrations using a standard curve calculated by reference to the ^{14}C ladder. Measurements were taken from each region of interest including corresponding regions that defined the non-specific hybridisation signal. A specific signal was then calculated for each region of interest by subtracting the mean total and non-specific values for an individual rat. The mean concentration in each region for each animal was then divided by the region mean in the respective control group to give a standardized grain count (percent) for each group. Results were expressed as mean ± SEM. Standardized results were analysed by ANOVA. Dunnett's test was used for comparisons to the control Naïve group, and Bonferroni tests for other comparisons were conducted where ANOVAs were significant. Pearson correlation analysis was performed to investigate the association between freezing behaviour and *Cacna1c* expression.

Results

CFC rats exhibited an increase in freezing behaviour after footshock

Freezing behaviour was scored for all animals that were exposed to the training context. There were no differences in freezing behaviour during the first 2 mins exposure to the context between groups ($F(3,20)_{\varepsilon=1} = 1.990$, $p = 0.148$, Fig. 1.). In those animals that received a footshock (CFC and LI groups), there was a significant interaction between freezing behaviour during Pre-US and Post US-training phases and group ($F(1,10)_{\varepsilon=1} = 34.747$, $P < 0.000$). During the Post-US phase, rats in the CFC group exhibited greater freezing following the footshock compared to LI animals that experienced prolonged pre-exposure to the context ($F(1,10)_{\varepsilon=1} = 25.879$, $P < 0.001$). Thus the CFC but not LI group showed successful acquisition of CFM [31].

Basal expression of Cacna1c

ISH was conducted to determine the basal expression profile of *Cacna1c.* Basal levels of expression were compared between the prefrontal cortex (PFC), cerebellum and hippocampus (Fig. 2.). Expression of *Cacna1c* was highest in the dentate gyrus (dg) and CA3 sub-regions of the hippocampus. Relatively high levels were also seen in the granule layer of the cerebellum. While much lower, expression in the PFC was highest in the medial PFC.

Regulated expression of Cacna1c in CA1 by exposure to a novel context and in dg after CFC

There was a reduction in *Cacna1c* expression in the CA1 in the Novelty group compared to naïve controls ($t(10) = 2.933$, $P = 0.015$, though this did not survive Dunnett's correction for multiple comparisons across all control groups ($P = 0.120$)(Fig. 3.). This reduction was measured 4 hrs after novel context exposure. No differences were observed in the CA3 or dg ($F(3,23) = 1.331$, $P = 0.292$ and $F(3,23) = 1.229$, $P = 0.325$, respectively) in the *Novelty*, *PreExp* or *LI* groups.

Expression of *Cacna1c* increased following CFC specifically in the dg ($F(4,21) = 2.823$, $P = 0.048$) when compared with naïve controls. *Post-hoc* Bonferroni tests revealed a significant difference between expression at 2 hrs and 24 hrs post conditioning, with increased expression of *Cacna1c* at 24 hrs ($P = 0.046$).

There was no effect of CFC at any time point on expression in the CA1 or CA3 regions of the hippocampus ($F(4,21) = 0.149$, $P = 0.961$ and $F(4,21) = 0.666$, $P = 0.622$, respectively).

An association between freezing behaviour and gene expression selectively in the dg is further highlighted since responses during training (post-US or novel context exposure) was correlated with the expression of *Cacna1c* 4 hours later in the dg ($r(12) = 0.540$, $P = 0.046$), but not in CA3 ($r(12) = 0.191$, $P = 0.506$) or CA1 ($r(12) = 0.253$, $P = 0.384$).

Prolonged exposure to the conditioned context results in reduced freezing behaviour

All animals successfully acquired CFM as observed by increased freezing following the footshock compared to the 2 mins before the US during conditioning ($F(1,15)_{\varepsilon = 1}$ = 293.515, p = 0.000; Group X Freezing Behaviour $F(2,15)_{\varepsilon = 1}$ = 1.238, p = 0.318), Fig. 4.). Forty-eight hours after CFC, animals re-exposed to the training context exhibited high levels of freezing indicative of successful recall of CFM. There were no Group X Freezing Behaviour differences between Post-US phase and the first 2 mins of recall ($F(1,10)_{\varepsilon = 1}$ = 0.237, p = 0.637). Prolonged 10 min exposure to the context resulted in reduced levels of conditioned freezing indicative of the extinction of CFM ($F(2.296, 11.479)_{\varepsilon = 0.574}$ = 14.531, p = 0.001). Freezing levels were significantly reduced in the last 2 mins of the session compared to the first 2 min after recall ($F(1, 5)_{\varepsilon = 1}$ = 93.889, p = 0.000).

Reduced expression of Cacna1c in the CA1 following brief recall

Cacna1c expression differed in the CA1 region between the Recall (2 min) and Recall (10 min) extinction groups 2 hr after recall of CFM (F (2,15) = 4.628, p = 0.027, Fig. 5.). Post-hoc Bonferroni tests revealed a significantly reduced expression in animals that underwent the short 2 min recall session compared to those that experienced the 10 min extinction session (p = 0.029). There were no differences in expression of *Cacna1c* in the CA3 or dg region of the hippocampus in any of the three groups (F(2,15) = 0.358, P = 0.705 and (F(2,15) = 0.196, P = 0.824, respectively). There was weak negative correlation between freezing behaviour in the last 2 min of the recall test and *Cacna1c* expression in CA1 (r(10) = 0.504, P= 0.094), but not in CA3 (r(10) = 0.027, P = 0.933) or dg (r(10) = 0.088, P = 0.785).

Discussion

We show that the expression of *Cacna1c* was regulated in the hippocampus in an activity-dependent manner and specifically with distinct learning and memory events. Basal expression of *Cacna1c* was found to be highest in the dg region of the hippocampus, followed by the CA3, and much lower levels measured in CA1. *In situ* hybridisation revealed that exposure to a novel context down regulated *Cacna1c* expression in the CA1. Increased expression was seen in the dg 24 hrs following CFC. Decreased expression in the hippocampal CA1 field also followed re-exposure to the conditioned context. However, the retrieval-associated decrease was seen in rats exposed for 2 min but not a 10 min context exposure, which is associated with the extinction of CFM, indicating that retrieval associated regulation of *Cacna1c* expression is not related to extinction processes.

Delayed regulated expression of Cacna1c in the dg following CFC

There was an increased expression of *Cacna1c* 24 hrs compared to levels 2 hrs following CFC. There were no changes observed in other regions of the hippocampus, or at other time points investigated. This indicates that the expression of *Cacna1c* is related to the consolidation of CFM, selectively in the dg.

This increase in expression 24 hrs following CFC suggests a role for $Ca_v1.2$ channels in a long-lasting alteration in the synapse as a result of plasticity. Previous studies have found delayed upregulation of gene expression in subunits of glutamate receptors in the dg following the induction of LTP in hippocampus [14, 32]. Thomas et al., (1996)[14] found an increase in the GluRN2B subunit of NMDA receptors evident from 24 hrs, peaking at 48 hrs, along with increases in mGluR1c which only became evident at 96 hrs following induction of LTP. It was proposed that these late-phase profiles of expression may relate to cascades of events that are required for the maintenance of LTP. Persistence of long-term memory has also been found to be related to delayed protein synthesis of BDNF at around 12 hrs following CFC

[33]. The increase of expression in *Cacna1c* observed here may indicate a delayed role in consolidation and long-term maintenance of CFM. Increases in *Cacna1c* expression in the dg with CFC are consistent with a role for this hippocampal region in CFM acquisition[34][35]. As the changes were specifically seen in the dg, it is also possible that this increase in *Cacna1c* in relation to learning could contribute to the recently reported role of α1c subunit-containing $Ca_v1.2$ channels in regulating neurogenesis in the dg[36][37].

Selective reduced expression of Cacna1c in CA1 following context exposure

We show that both unconditioned and conditioned context exposure leads to a decrease in *Cacna1c* expression in the CA1. This regulation in CA1 may not be directly linked to novelty processing *per se*, a function co-ordinated by the dg and CA3 regions [38][39], but with the role of the hippocampus in the formation and storage of the conjunctive representation of the context necessary for the consolidation and reconsolidation of CFC[40][41][42]. The regulation of *Cacna1c* expression in CA1 is therefore consistent with its role as a key region for the consolidation and reconsolidation of contextual fear (CS-US) memory[43][44][45]. The regulation of *Cacna1c* expression in both dg and CA1 after acquisition but not recall (CA1 only) is consistent with the differential contributions of these two hippocampal regions to CFM acquisition and retrieval [46].

We also noted that the regulation in *Cacna1c* expression was sensitive to the duration of context exposure with reductions in mRNA levels seen after short exposures (*Novelty and Recall (2 min) groups*), but not longer periods (*PreExp and Recall (10 min) groups*). This observation may relate to a selective role for $Ca_v1.2$ in context memory processing during consolidation & reconsolidation rather than pavlovian CS-US and CS-no US events related to CFM encoding, LI and extinction.

In summary, the regulation of *Cacna1c* transcription in the hippocampus after conditioned or unconditioned context exposure and following CFC indicate a role for $Ca_v1.2$ in specific

memory processes; down-regulation of expression correlated with CA1-associated contextual memory and upregulation of expression in the dg associated with CFM encoding. To determine a causal role for $Ca_v1.2$, in these distinct memory processes by region requires more refined genetic or molecular rodent models.

Implications for the role of $Ca_v1.2$ in hippocampal-dependent learning and memory

There has been long standing pharmacological evidence for the role of LVCCs in the consolidation and extinction of fear memory[28][25]. Genetic models have indicated that there may be differential contribution of the major brain isoforms $Ca_v1.2$ and $Ca_v1.3$ (encoded by the *CACNA1D* gene linked to risk for bipolar disorder[47] to different components of associative memory. Mice with forebrain knock out of *Cacna1c* show no deficits in consolidation and extinction of CFC[48] and the consolidation of cued fear memory[49], while *Cacna1d* null mutants show impaired consolidation, but not extinction [50]. These data may indicate a specific role for $Ca_v1.3$ with fear memory formation. However, these models show compensatory adaptations in activity-dependent neuronal signalling[49][51], which make these data difficult to interpret. The role of $Ca_v1.2$ with associative memory processing is indicated in a gain-of-function model that shows enhanced cued and contextual fear memory via altered consolidation, strengthening and/or extinction[52]. The causal role of $Ca_v1.2$, selectively, in fear memory and behaviour remains to be determined using better genetic or molecular animal models.

Implications for psychiatric disorders

The *CACNA1C* gene has been strongly associated with risk for psychiatric disorders including schizophrenia and bipolar disorder in genome-wide association studies [23][21][22][19][20]. These disorders are known to be associated with cognitive alterations that include alterations in learning, memory and affective processing. The current results show that *Cacna1c* expression is regulated in distinct regions of the hippocampus at the transcriptional

level and which correlate with context processing required for specific components of associative CFM formation and maintenance. Genetic variation in *Cacna1c* may impact on the plasticity during key phases of associative learning. It is likely that any distinction in the role of *Cacna1c* in these aspects of learning will be further revealed by investigating the functional regulation of calcium influx through LVGCCs. Our results thus provide additional evidence that a link exists between $Ca_v1.2$ and distinct behavioural domains associated with common behavioural phenotypic features of psychiatric disorders including schizophrenia, ASD and BD.

Fig. 1. Freezing behaviour (% time spent freezing) during Pre-US and Post-US phases of CFC. Freezing behaviour in the fear conditioned *CFC* group was greater than in the *LI* group that underwent prolonged pre-exposure to the context before footshock. Bars represent average freezing behaviour for each group. Rats in the *Novelty* and *PreExp* groups were exposed to the context for 3 min and 8 hours respectively and did not receive a footshock. n = 6 for all groups. *** P < 0.001.

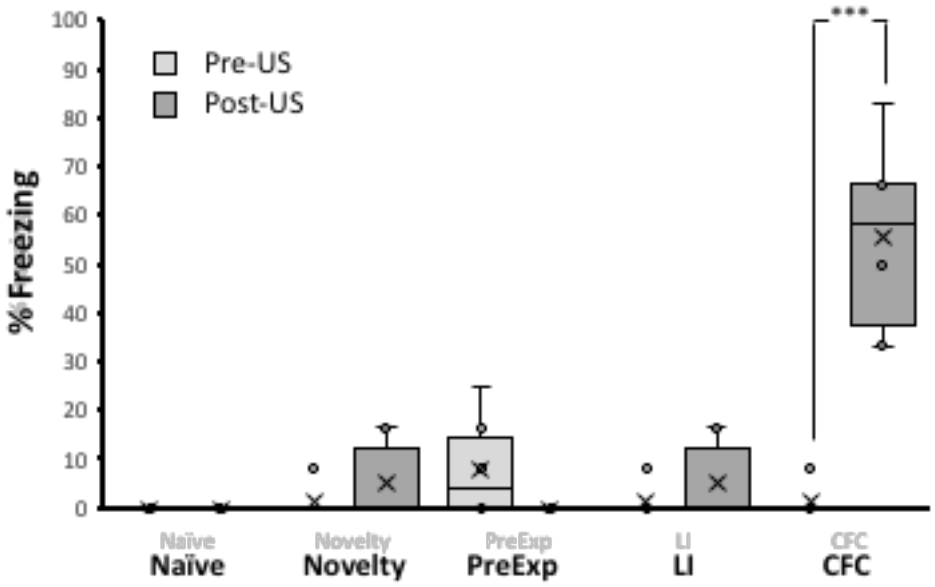


Fig. 2. Basal expression of *Cacna1c* mRNA. **a** Quantification of relative *in situ* hybridisation (ISH) density values. Specific hybridization was calculated by subtracting the "non-specific" signal, defined by excess unlabelled oligonucleotide probe, from "total" values normalised to the specific signal measured in the medial PFC (mPFC). Greatest expression was observed in the dentate gyrus (dg). Bars represent mean of the specific hybridisation values. Error bars are +/- SEM. n = 4. **b** Representative ISH autoradiogram images of *Cacna1c* expression in the PFC, cerebellar and hippocampal subregions. Total hybridization of the probe is shown on the left-hand side and non-specific hybridization on the right.

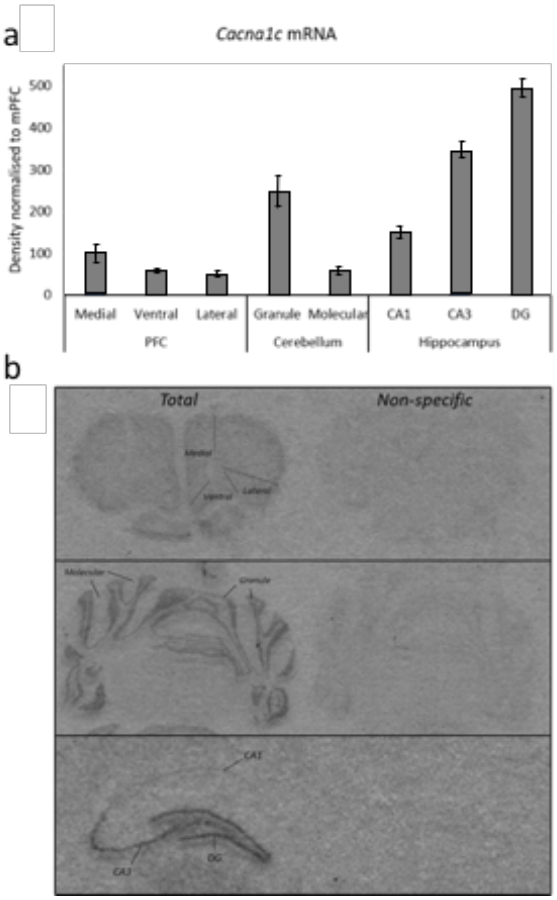
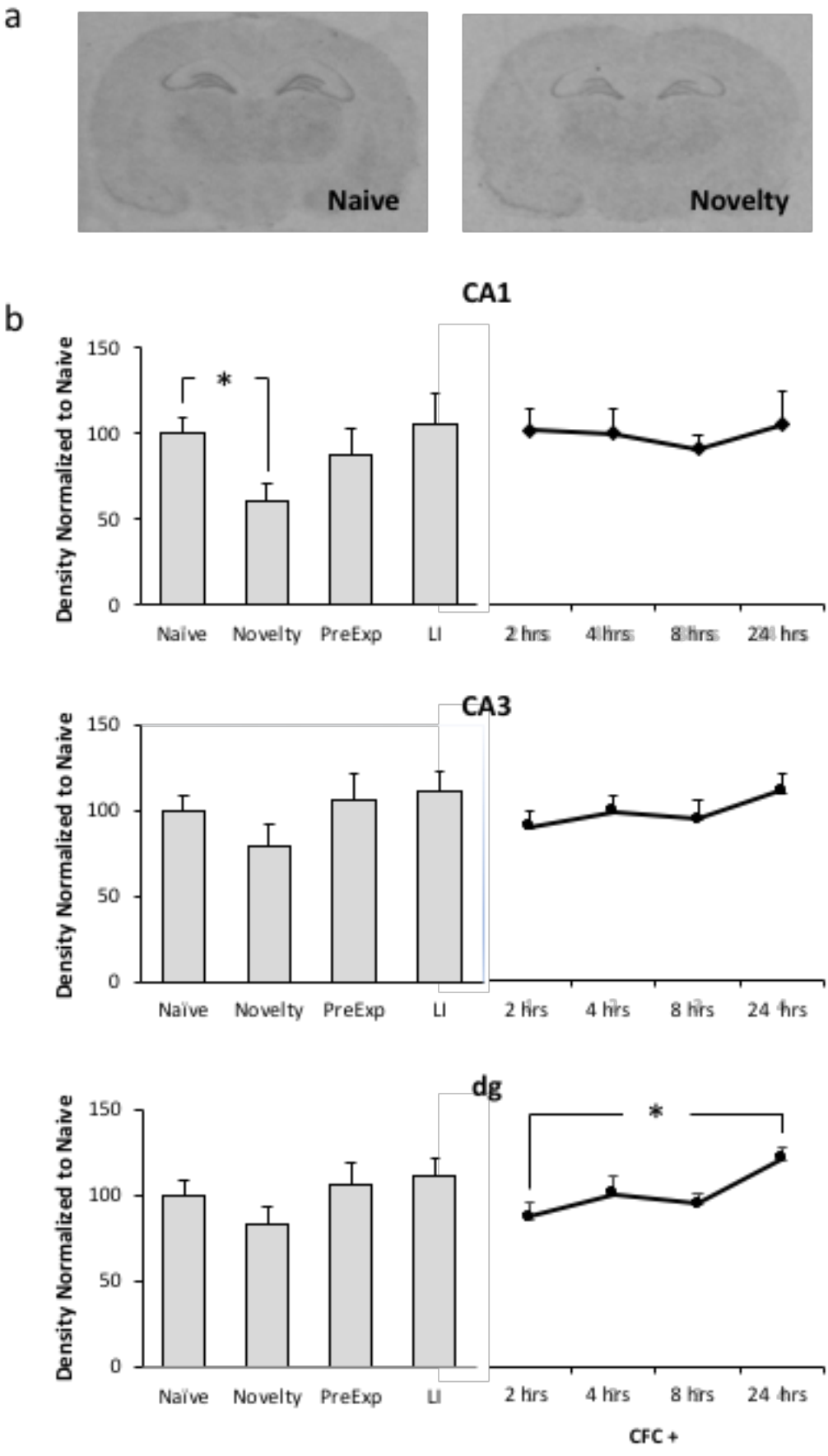


Fig. 1. Expression of *Cacna1c* in CA1, CA3 and dg sub-regions of the hippocampus following different learning events. **a** Representative ISH autoradiogram images of *Cacna1c* expression in a *Naive* (left) and novel context (*Novelty*) exposed (right) rat. **b** A reduction in expression was measured in CA1 following exposure to novel context compared to naïve animals. Expression in the dg following CFC showed an increase at 24 hrs compared to levels at 2 hrs. Bars represent mean specific hybridisation values normalised to Naïve control. Error bars are +/- SEM. n = 6 for all groups except 2 and 8 hrs (n = 4). * $P < 0.05$.


a
Naive
Novelty
b
CA1
Density Normalized to Naive
150
100
50
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*
Naive
Novelty
PreExp
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2 hrs
4 hrs
8 hrs
24 hrs
CA3
Density Normalized to Naive
150
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Fig. 2. Freezing behaviour during CFC and recall. There was an increase in freezing following US presentation in all groups (t(17) = -16.898, P =< 0.001) indicating acquisition of CFM. The reduction in conditioned freezing following prolonged 10 min re-exposure indicates acquisition of extinction in the Extinction group. Data points represent the average time spent freezing (%) for each group during each training and testing epoch. *** P < 0.001 n = 6 for all groups.

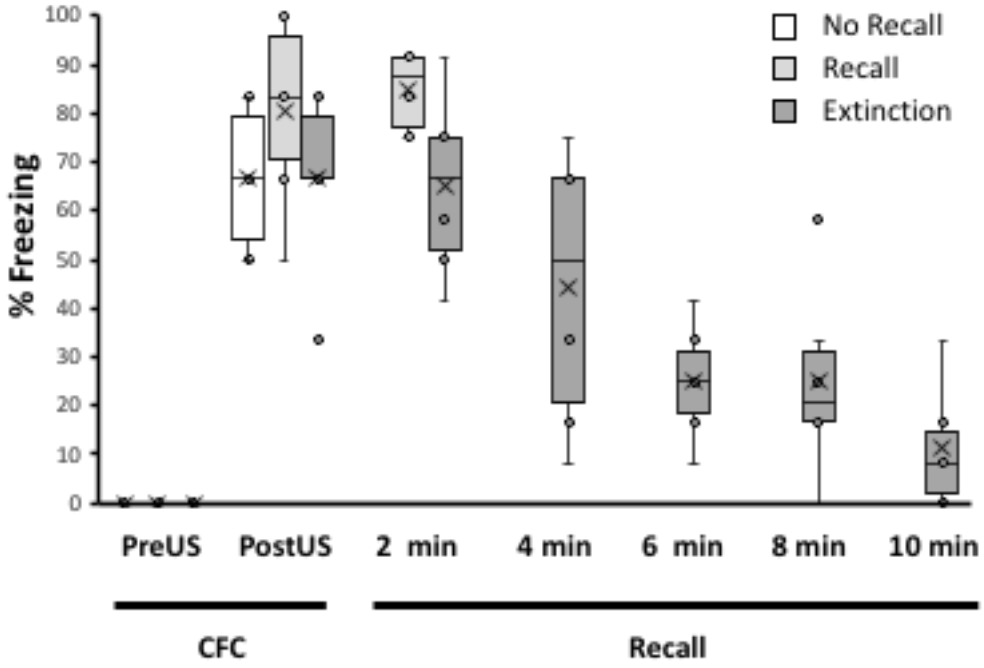
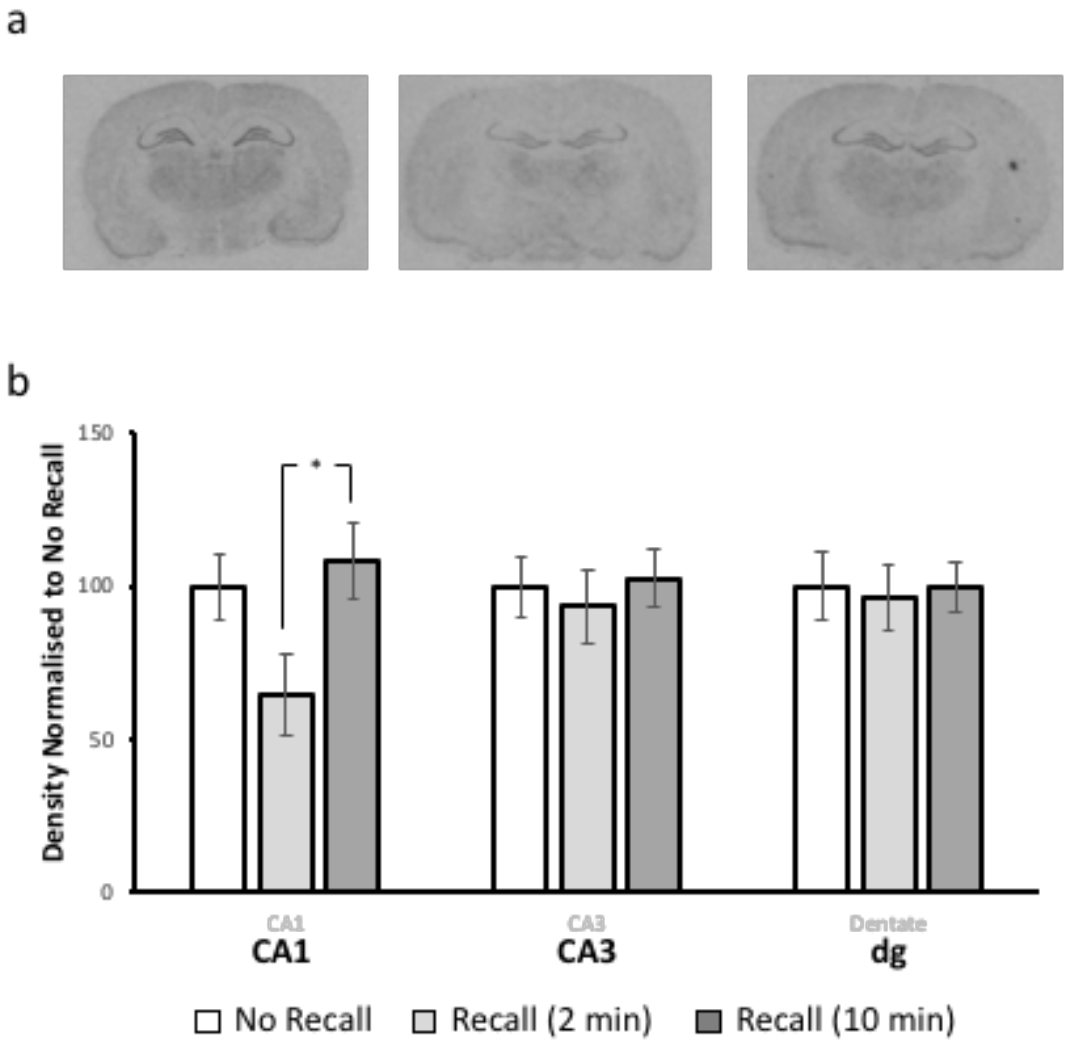


Fig. 3. Expression of *Cacna1c* 2 hrs following Recall (2 min) or Recall (10 min) session normalised to animals that underwent CFC alone (*No Recall group*). **a** Representative ISH autoradiograms of *Cacna1c* expression in a *No recall* (left), *Recall (2 min)* (middle) and *Recall (10 min)* (*Novelty*) exposed (right) rat. **b** There was a reduction in expression in the CA1 in the Recall (2 min) compared to the Recall (10 min) groups (* P < 0.05 Bonferroni corrected). Error bars are +/-SEM. * P < 0.05. n = 6 for all groups.



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