Characterisation of the neural basis underlying appetitive extinction & renewal in a Cacna1c rats

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Abstract
Recent studies have revealed impairments in Cacna1c ±} heterozygous animals (a gene that encodes the Cav1.2 L-type voltage-gated calcium channels and is implicated in risk for multiple neuropsychiatric disorders) in aversive forms of learning, such as latent inhibition, reversal learning or context discrimination. However, the role of Cav 1.2 L-type voltage-gated calcium channels in extinction of appetitive associations remains under-investigated. Here, we used an appetitive Pavlovian conditioning task and evaluated extinction learning (EL) with a change of context from that of training and test (ABA) and without such a change (AAA) in Cacna1c ±} male rats versus their wild-type (WT) littermates. In addition, we used fluorescence in situ hybridization of somatic immediate early genes (IEGs) Arc and Homer1a expression to scrutinize associated changes in the medial prefrontal cortex and the amygdala. Cacna1c ±} animals successfully adapt their responses by engaging in appetitive EL and renewal. However, the regional IEG expression profile changed. For the EL occurring in the same context, Cacna1c ±} animals presented higher IEG expression in the infralimbic cortex and the central amygdala than controls. The prelimbic region presented a larger neural ensemble in Cacna1c ±} than WT animals, co-labelled for the time window of EL in the original context and prolonged exposure to the unrewarded context. With a context change, the Cacna1c ±} infralimbic region displayed higher IEG expression during renewal than controls. Taken together, our findings provide novel evidence of distinct brain activation patterns occurring in Cacna1c ±} rats after appetitive extinction and renewal despite preserved behavioral responses.

Keywords:
Cacna1c, Pavlovian extinction learning, Appetitive renewal, catFISH, Prefrontal cortex, Amygdala

Introduction

Synaptic plasticity is a fundamental property of neurons including activity-dependent changes in the efficacy and strength of synaptic transmission at preexisting synapses. Such changes are the major cellular mechanisms underlying learning and memory. Although several types of synaptic plasticity have been delineated across neuronal types and brain regions, all of them share a critical role for Ca^{2+}-mediated processes (see (Mateos-Aparicio & Rodríguez-Moreno, 2020; Rebecca Nalloor et al., 2012)). In addition, some symptoms of psychosis have been suggested to stem from alterations in associative learning related to changes in synaptic plasticity (Hall et al., 2009; Kapur, 2003). Genome-wide association studies (GWAS) have strongly related genetic variation in CACNA1C, a gene that encodes the Cav 1.2 L-type voltage-gated calcium channels, with an increased risk of psychiatric disorders (Ferreira et al., 2008; Hall et al., 2015). These studies identify single nucleotide polymorphisms (SNPs), associated with bipolar disorder, schizophrenia, major depressive disorder, and autism (Green et al., 2013; Moon et al., 2018). The effect of CACNA1C risk-associated SNPs remains unclear. They produce altered CACNA1C dosage and in some cases decrease CACNA1C expression (e.g., there is good evidence that the risk SNPs rs1006737 is associated with decreased expression in the hippocampus), but the way they impact the gene is not consistent across studies (Bigos et al., 2010; Roussos et al., 2014; Tigaret et al., 2021; Yoshimizu et al., 2015).

CACNA1C expresses the transcript Cav1.2 that plays a key role in learning and memory by modulating learning-related neural pathways. Calcium influx in post-synaptic neurons signals cascades to regulate the activity and transcription factors such as the cAMP response element-binding protein (CREB), nuclear factor of activated T cells (NFTA) pathway, and Hebbian synapse plasticity (Deisseroth et al., 2003; Moosmang et al., 2005). Critically, calcium influx via L-type voltage-gated calcium channels triggers the transcription of calcium-regulated genes including brain-derived neurotrophic factor (BDNF), which has a significant role in

learning processes (West et al., 2001). Moreover, recent studies have revealed cognitive deficits in a hemizygotic deletion model (Cacna1c ±), including reduced latent inhibition of contextual fear conditioning (Tigaret et al., 2021), impaired appetitive reversal learning (Moon et al., 2018), long-term spatial memory (White et al., 2008), context discrimination in an aversive preparation (Temme et al., 2016), and contextual fear conditioning extinction (Temme & Murphy, 2017).

Learning the relationships between events is critical in the production of adaptive responses, and in changing environments it is vital to determine when a previously learned response is no longer adaptative and, therefore, should no longer be implemented. One example is extinction learning (EL) where, after a response to a cue is acquired on the basis that it predicts aversive or appetitive outcomes, experience of the cue without those outcomes results in the removal of the previously acquired response. This gives an individual the ability to interact flexibly with the environment, a process which is impaired in some psychiatric disorders [12, 14]. While early analyses of EL suggested it was unlearning of the previously learned behavior, more recent evidence suggests that extinction is instead the learning of new relationships (Bouton, 2004). For example, despite successful extinction learning, recover of the response can occur if the individual is re-exposed, either immediately or with a delay in time, to the context in which the original experience was learned (Donoso et al., 2021; Gao et al., 2018; Lengersdorf et al., 2015; Mendez-Couz et al., 2021). This phenomenon is known as renewal (Bouton, 2004; Bouton & Ricker, 1994) and suggests that extinction is not the undoing of prior learning. In addition, extinction studies (Andrianov et al., 2015; André et al., 2015; Mendez-Couz et al., 2021) have implicated neurotransmitter systems and mediators of signaling pathways that are known to be required in other forms of learning (Seyedabadi et al., 2014), as well as the involvement of synaptic plasticity processes believed to underlie new learning (Hagena et al., 2016; Harley, 2004; Lesch & Waider, 2012). It is broadly known that extinction is highly context-specific, thus suggesting the involvement of the hippocampus (Mendez-Couz

et al., 2019). The amygdala has also proved to be essential for the extinction of Pavlovian conditioned behaviors (for a review see (Bouton et al., 2021)). Although most of the work supporting the role of the amygdala, and particularly BLA in EL, comes from fear extinction studies, its role in EL is not limited to aversive conditioning procedures. Previous studies have demonstrated that BLA lesions impair the extinction of appetitive incentive value (Lindgren et al., 2003) and treatment with NMDA antagonists in the avian amygdala impairs the encoding of appetitive EL of context-related conditioned appetitive approach (Gao et al., 2018). Moreover, extinction of an appetitive task involves the absence of a previously present reward, thus suggesting a stimulus-response modulation of the cognitive components of memory systems, for which the amygdala, tightly interconnected with the prefrontal cortex and hippocampus, might play a significant role (Ferbinteanu, 2019; McDonald & White, 1993; Vasquez et al., 2019).

It is noteworthy that most studies of extinction processes, at both behavioral and neural levels, have focused on aversive conditioning (Cammarota et al., 2007; Ernst et al., 2017; Kim & Richardson, 2009; Szapiro et al., 2003). However, it has been demonstrated that appetitive and aversive events are (at least partially) differentially processed (Niyuhire et al., 2007). Moreover, despite the known importance of Cav 1.2 mediated processes in learning and memory more generally, there has been no prior investigation of their potential role in renewal. Therefore, in the present study, we investigated the extinction and renewal of appetitive conditioned magazine approach responses in Cacna1c ± rats. In addition, we used fluorescence *in situ* hybridization (FISH) to map activity-dependent mRNA expression of Homer1a (H1a) and Arc in the prefrontal cortex and amygdaloid nuclei, following extinction and renewal of the appetitive response. Arc (activity-regulated cytoskeletal-associated protein, also known as Arg3.1) and H1a are effector IEGs, that have several cellular functions capable of modifying synaptic function (Brakeman et al., 1997; Lanahan & Worley, 1998; Link et al., 1995; Lyford et al., 1995). Their expression can be induced by neural activity (Abraham et al., 1993; Worley

et al., 1993) or behavioral stimulation (Hess et al., 1995; Sethumadhavan et al., 2020). Because both genes are activated following novel exploration, Arc and H1a are considered to function together as part of an activity-dependent genomic program to induce and stabilize long-term changes in synaptic efficacy in neural ensembles encoding specific experiences (Vazdarjanova et al., 2002). Due to the brief period of transcription of these genes and the difference in size of their primary transcripts, H1a and Arc have been proven as useful biomarkers for the regional, temporal, and functional differentiation of the contribution of specific brain regions in distinct phases of the learned behavior (R. Nalloor et al., 2012; Vazdarjanova et al., 2002).

2. Method

2.1 Behavioral Method

2.1.1. Subjects

Forty-eight adult male Cacna1c hemizygous (Cacna1c+/−) rats (HET) on a Sprague Dawley background (TGR16930, Horizon, Sage Research Labs, USA) and wild-type (WT) littermates were obtained and housed in mixed-genotype groups of 2–3 individuals in standard cages (38 cm × 56 cm × 22 cm). This model is a constitutive zinc finger nuclease knockout, resulting in approximately 50% and 40% decreases in mRNA and protein levels, respectively (Sykes et al., 2019). Therefore, this model accords with altered brain expression of CACNA1C in patient cohorts. Rats were bred at Cardiff University, UK, and were housed under a 12hr/12hr light/dark cycle. Animals had *ad libitum* water throughout the experimental sessions. Before the start of the experiment, rats were moved to a food deprivation schedule with rationed daily food access to maintain animals at approximately 90% of their *ad libitum* weights. All experimental manipulations took place during the light phase of the cycle. Experiments were conducted following local ethics guidelines, and in line with the UK Animals (Scientific Procedures) Act 1986 and the European Communities Council Directive (1986/609/EEC).

2.1.2. Stimulus and apparatus

Training and testing took place in two rooms containing eight identical conditioning boxes (30 cm × 24 cm × 21 cm: Height x Width x Depth; Med Associates, Georgia, VT). Each box was placed in a sound-attenuating shell that incorporated a ventilation fan, which maintained the background noise at approximately 68 dB. The side walls of the boxes were made of aluminum whereas the front, back, and ceiling panels were made of clear acrylic. The floor was made up of 19 steel rods (4.8 mm diameter, 16 mm apart) placed above a stainless-steel tray. Food pellets were delivered to a recessed food well (aperture: 5.3 cm x 5.3 cm) in the centre of the left wall at floor level. The food well was equipped with infrared detectors that allowed the presence of the rat in the well to be automatically recorded. Every time the detector was interrupted by an entry, a single response was registered.

The CS (conditioned stimulus) consisted of flashing (0.2s on/off) 2-panel lights to the left/right of the food well for 10 s. The US (unconditioned stimulus) comprised a food pellet delivered to the food well at the offset of the CS presentation (45 mg, 1811155 [5TUL], AIN-76A supplied by Sandown Scientific, Hampton, UK). MED-PC software was used to control the delivery of food pellets and flashing light stimulus, as well as to record magazine entries. In addition, the renewal procedure relies on different experimental contexts. Here, contexts consisted of two different rooms, room one with the standard operant box setting (as described above), and room two where the operant boxes were modified with a different grid flooring, all walls covered with black and white 3 cm x 3 cm squares, and a distinctive mint odor was rubbed over the walls prior to each session.

2.1.3. Procedure

An outline of the traditional ABA renewal procedure that was used can be found in Figure 1 (Panels A & B) and Table 1. Animals had one pre-training session in which 50 food pellets were delivered on a variable time VT 30 s (15-45 s) to habituate the animals to the box and food dispenser. Animals then received eight sessions of acquisition (referred as to “Session 1”

to “Session 8”), with one session delivered per day. Each session consisted of 30 trials in which a flashing light was presented for 10 s and immediately after the termination of the stimulus a food pellet was delivered. The average ITI was 70 s, ranging from 40-100 s (40 s, 55 s, 70 s, 85 s, and 100 s randomly presented), and the total duration of each session was 40 minutes. For all animals pre-training and acquisition phases occurred in Context A. The identity of Contexts A and B were counterbalanced between animals so for half of the animals, Context A was room one and for the other half was room two. After the acquisition phase, two extinction sessions were carried out (one per day). Those sessions were similar to acquisition, but no pellets were delivered at the offset of the CS presentation. For the ABA groups this phase was performed in Context B whereas for the AAA groups extinction was carried out in Context A. Finally, a single test session was performed 24 hours after the last extinction session. In this session (similarly to extinction), animals were presented with 30 trials of the 10 s flashing light, but without food pellet delivery. For all groups, the test was performed in original Context A.

FIGURE 1

2.2. Molecular procedure

2.2.1. Tissue preparation

Immediately after the final session had concluded, the brains of 6 animals per group were removed within a maximal time of 2 min, quick-frozen in ice-cold isopentane and stored at -80°C until being cut on a cryostat (Leica CM 3050S). 20 µm coronal sections of the brain were cut at −20 °C, mounted on gelatinized slides (SuperFrost®Plus, Gerhard Menzel, GmbH, Braunschweig, Germany) and stored at -80 °C until further processing. To verify the localization of regions of interest, every 12th coronal section from the series underwent Nissl staining. Regions of interest were subsequently verified using the stereotaxic atlas of Paxinos and Watson (Paxinos & Watson, 2006).

2.2.2. In situ- Hybridization

We conducted FISH to detect the somatic expression of Homer1a and Arc mRNA. Due to the brief period of transcription (<10 min) of these genes and the difference in the size of their primary transcripts, the somatic expression for Homer1a occurs 25–30 min after a novel experience while the somatic expression of Arc occurs at <10 min after the experience (Guzowski et al., 1999; Vazdarjanova et al., 2002). Animals were sacrificed immediately after the test day was completed. Thus, in the AAA groups, Homer1a acts as a biomarker for the initial neural response to an extinguished stimulus on day three, while Arc expression reflects the neural response to extended exposure to the extinguished stimulus. In the case of the ABA groups, H1a was used as a marker of the renewal phenomenon occurring in the original context A, after the extinction that had occurred in context B, whereas Arc reflected the extinction learning in the original context A after a prolonged lack of reward (Figure 1B). Digoxigenin-labelled probes were generated using the Ambion MaxiScript Kit (Invitrogen, Carlsberg). Homer1a cDNA plasmid was prepared by Entelechon (Bad Abbach, Germany) using a ~1.2 kb Homer1a transcript (Brakeman et al., 1997), and the Arc cDNA plasmid was prepared using a ~3 kb Arc transcript according to the sequence of Lyford et al. (Lyford et al., 1995). For the double fluorescence in situ hybridization, a previously established protocol was used, as described in (Hoang et al., 2018; Méndez-Couz et al., 2019). In short, tissue sections were fixed and acetylated in paraformaldehyde (PFA, 4%, ice-cold) for 10 min, washed in saline sodium citrate (SSC) twice, and placed for 10 min in acetic anhydride solution (96.96% diethyl pyro carbonate (DEPC)-water, 0.89% NaCl, 1.62% triethanolamine, 0.52% acetic anhydride). After an additional rinse with SSC, tissue sections were prehybridized in prehybridization buffer (1:1, SSC: prehybridization buffer) for 30 min at room temperature (RT) followed by a hybridization process (Grüter et al., 2015). For this purpose, 1 ng/µl of RNA probe in hybridization buffer was applied, comprising 20/1,000 µl of Homer1a-Biotin and 20/1,000 µl Arc-Digoxigenin (50:1:1) in hybridization buffer. The solution was kept at 90°C

for 5 min then chilled on ice to prevent reannealing until addition onto each glass slide. Afterwards, the diluted probe was added, and samples were incubated in the humidified hybridization chamber at 56°C until the following day.

One day after the abovementioned procedure, tissue sections underwent stringency washings to remove non-specific and repetitive RNA hybridization. The first steps comprised five rinsing steps in SSC at 56°C, followed by RNase A (50 µg/100 ml 2× SSC) at 37°C, followed by rinsing with diluted SSC for 10 min at 37°C and three washings with diluted SSC, from 37°C to 56°C, finally, an additional two washings at RT and a Tris-buffered saline (TBS) rinse were conducted to bring back the p.H to 7.5.

For the signal detection of both Homer1a-Biotin, and Arc-DIG, streptavidin was used, so the signal detection had to be performed sequentially. For Homer1a-Biotin an additional blocking step with 1% bovine serum albumin (BSA) in TBS-Tween of 70 min was carried out in a humidity chamber before the first antibody, streptavidin CY2 (Dianova, Cat# 016-220-084, RRID: AB_2337246) was applied at 1:250, 1% BSA: TBS-Tween, for 30 min. An enhancement step was included, in which sections were incubated with b-Anti-Streptavidin (Vector Laboratories Cat# BA-0500, RRID: AB_2336221) at 1:100 in 1% BSA in TBS-Tween, followed by TBS washings and a *de novo* incubation with Streptavidin CY2 in the same conditions at before. Sections were rinsed in TBS and preserved overnight at 4°C.

One day later, the somatic Arc signal was detected by Arc-Dig immunohistochemistry. In order to reduce unspecific background staining, endogenous peroxidase was blocked by 0.3% H_2O_2 and after that, endogenous biotin and electrostatic loading of proteins were reduced by 20% avidin (Vector Labs, Cat# SP2001). Afterwards, the primary antibody for Anti-Digoxigenin was applied at 1:400 (Roche, Cat #11207733910, RRID: AB_514500) in 1% BSA (Sigma Aldrich, St. Louis, MO, USA) in TBS-Tween 20% biotin (Avidin-Biotin Blocking Kit) for 90 min at RT. The sections were newly washed in TBS and a biotinylated Tyramid (bT)-enhancement step was performed for 20 min, consisting of 1% bT and 0.3% H_2O_2 in TBS. The

second antibody was applied after new rinsing in TBS, Steptavidin Cy5 (Jackson ImmunoResearch Labs Cat# 016-170-084, RRID: AB_2337245) 1:2,000 in 1% BSA TBS-Tween. To label the cell nuclei, 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsberg, CA, USA) was added in a concentration of 1:10,000. Slides were finally rinsed in TBS and distilled water, air-dried in absence of light and coverslipped with a fluorescence-specific medium (Dianova SCR-38447).

2.2.3 Quantification

For the in-situ hybridization, we analyzed representative small areas within the regions of interest of the prelimbic (PL), infralimbic (IL) and cingulate (Cing) regions of the prefrontal cortex, all of them measured at ±3.70 mm from Bregma, each measuring 436 x 87µm. In all cases, placement of the sampling rectangles commenced in layer II. The medial (MeA), lateral (LaA), basal (BaA) and central (CeA) nuclei of the amygdala were sampled at ±3.14 mm from Bregma (See Figure 1C). To avoid quantification bias, the IEG´s corresponding color channels were deactivated and only the corresponding DAPI color channel was left active for orientation to choose the regions of interest. In addition, Nissl staining using 1% toluidine blue was performed for surveillance of tissue quality and spatial orientation. Furthermore, negative controls were prepared for assessment of specificity, in which the probe was omitted. Intranuclear staining was not detected in the negative controls, indicating that the staining observed in the test slides was specific. Images were acquired using a slide scanner confocal microscope, regions of interest were identified using the ImageJ image software (Rueden et al.) and positive cell results were manually counted and expressed as a percentage of the total neuronal nuclei analyzed per subfield and animal. For the ISH analysis, the experimenter was unaware of the behavioral experimental group related to each image.

2.3 Data analysis

2.3.1. Behavioral analysis

The frequency of magazine entries (nosepokes) was recorded for 30 s before the presentation of the CS (preCS period), the CS period (10 s), and the 10 s immediately after the US was delivered (US period) for all 30 trials in each session. The number of nosepokes was recorded, and the dependent variable submitted to analysis was the score during the 10 s CS period minus the average score per 10 s across the 30 s preCS period. Mixed analyses of variance (ANOVAs) were used to examine the within-subject factor of acquisition session, with between-subject factors of group (AAA or ABA) and genotype (HET or WT). A similar analysis was performed on the data from the two extinction sessions and the final test session. As can be seen in section 3.1.1 there was an unexpected session × genotype × group interaction during acquisition (reflecting pre-manipulation AAA vs ABA differences in the HET groups). Thus, to confirm that the analysis of renewal was not affected by these random pre-manipulation differences, a supplementary analysis of the extinction and test phase data was performed with the data normalized by subtracting response levels at the end of training (i.e. in training session 8). All null hypothesis statistical tests reported here used a significance value of $p = .05$. When sphericity was violated, Greenhouse-Geisser correction was used.

2.3.2. Immediate Early Genes expression analysis

For the analysis of mRNA Arc and H1a expression, complete nuclei were chosen and Arc and H1a positive signals were identified by examining each overview using ImageJ software (Lima et al., 2016). During "experimenter-blind" analysis, nuclei that contain Arc and H1a as well as double-labelled cells containing the mRNA signal were counted and the percentage of Arc, H1a and double labelled "both" positive nuclei from all nuclei were calculated separately. Averages were calculated by using three consecutive slides per region and animal. CeA, LaA, MeA, and BaA areas of the amygdala; and prelimbic, infralimbic and cingulate regions on the prefrontal cortex were averaged per animal and per sample region. Differences in somatic expression of Arc and Homer1a were analyzed using a two-way ANOVA for each region of interest, with between-subject factors of the group (AAA or ABA) and genotype (HET or WT).

FIGURE 2

3. Results

3.1. Behavioral results

3.1.1. Acquisition of the Pavlovian task in context A successfully occurs in both Cacna1c ± and WT groups.

Figure 2 (Panel A - ABA, Panel B - AAA) shows the nosepoke rates from Session 1 to Session 8 of training. While it is clear that all groups displayed acquisition of responding to the light CS, there was an indication of differences between groups, with group HET-AAA appearing to respond more frequently during the CS than group HET-ABA while the WT AAA and ABA groups did not differ. This description was confirmed with a mixed ANOVA with a within-subject factor of session (acquisition sessions 1-8) and between-subject factors of group (AAA or ABA) and genotype (HET or WT). Note, due to a recording failure, there was no data from two HET animals (one each from the AAA and ABA groups) for session 5, thus these animals were omitted from the training phases analysis (but an analysis including the animals with the missing data replaced by the mean of their session 4 and 6 responses did not differ from that reported here). There was a significant main effect of session $F(3.40, 142.7) = 41.43$, $MSE = 577.87$, $p < .001$, $\eta_p^2 = .50$, and also a significant session × genotype × group interaction $F(3.40, 142.7) = 2.60$, $MSE = 577.87$, $p = .047$, $\eta_p^2 = .05$. However, there was no main effect of genotype, $F(1,42) = 1.04$, $MSE = 5006.04$, $p = .313$, $\eta_p^2 = .02$; or group $F(1,42) = 3.65$, $MSE = 5006.04$, $p = .063$, $\eta_p^2 = .08$; nor any other significant interaction [largest F for the genotype × group interaction, $F(1,42) = 3.65$, $MSE = 5006.04$, $p = .129$, $\eta_p^2 = .05$]. Returning to the significant triple interaction, simple effect analysis revealed that the HET-AAA group displayed significantly more nosepokes on sessions 4, 7, and 8, than the HET-ABA group [$F(1,42) = 4.09$, $MSE = 1171.47$, $p = .049$, $\eta_p^2 = .09$; $F(1,42) = 7.46$, $MSE = 1807.14$, $p = .009$, $\eta_p^2 = .15$; $F(1,42)$

= 4.09, *MSE* = 2159.19, p = .020, η_p^2 = .12 respectively], but there were no differences between WT-AAA and WT-ABA [largest F for session 3, *F*(1,42) = 1.18, *MSE* = 2159.19, p = .280, η_p^2 = .02]. Since the ABA vs AAA manipulation had not yet been applied, the differences between groups HET-AAA and HET-ABA must be random. Thus, the inferential statistical analysis of the Extinction and Renewal test sessions was performed on both the raw responses and on the data normalized by subtracting response rates at the end of training.

3.1.2 Extinction and renewal are present across both WT and Cacna1c animals.

Figure 2 shows the mean number of magazine entries during the two extinction sessions and the renewal test session (2C for the ABA groups and 2D for the AAA groups). As can be seen from the figure, all groups showed reduced responding during extinction (albeit with the HET-AAA group starting from a higher baseline reflecting their higher levels of responding at the end of training). Most importantly, the AAA groups showed numerically lower response levels during the test session than extinction session 2, while the ABA groups showed an increase from extinction session 2 to the renewal test session.

This description of the results was confirmed by a mixed ANOVA with a within-subject factor of session (extinction 1, extinction2, test) plus between-subject factors of group (AAA or ABA) and genotype (HET or WT). There was a significant main effect of session *F*(2, 88) = 4.36, *MSE* = 147.54, p = .016, η_p^2 = .090, and critically a significant session × group interaction *F*(2, 88) = 10.01, *MSE* = 147.54, p < .001, η_p^2 = .185, but importantly no session × genotype *F*(2, 88) = 1.48, *MSE* = 147.54, p = .233, η_p^2 = .033, or session × genotype × group interaction *F*(2, 88) = 2.11, *MSE* = 147.54, p = .128, η_p^2 = .046. There was no main effect of genotype *F*(1, 44) = 2.12, *MSE* = 231.77, p = .152, η_p^2 = .046, but there was a main effect of group *F*(1, 44) = 9.09, *MSE* = 231.77, p = .004, η_p^2 = .171, and a genotype × group interaction *F*(1, 44) = 4.10, *MSE*

= 231.77, p = .049, η_p^2 = .085. This interaction reflected the initially higher rate of responding in the HET-AAA group (following from their higher response rates at the end of training) and simple main effect analysis revealed that the across the extinction and test period responses were higher in group HET-AAA than group HET-ABA $F(1,44) = 11.72$, $MSE = 77.09$, $p < .001$, η_p^2 = .21, with no difference between groups WT-AAA and WT-ABA $F(1,44) < 1$. Returning to the critical session × group interaction, simple effects analyses revealed a significant increase in response rates reflecting a renewal effect for the ABA groups $F(1,44) = 4.71$, $p = .035$, η_p^2 = .097, with the decrease in responses for the AAA groups not reaching significance $F(1,44) = 2.35$, $p = .133$, η_p^2 = .051.

In order to confirm that these results were not affected by the presence of pre-manipulation differences in response rates at the end of the training, a second analysis (using the same factors as above) was performed on the data normalized by subtracting response rates in acquisition session 8 from those in extinction session 1, extinction session 2 and the test session (normalized descriptive data not shown in Figure 2). This secondary analysis also revealed a significant main effect of session $F(2, 88) = 4.36$, $MSE = 147.54$, p = .016, η_p^2 = .090, as well as a significant session × group interaction $F(2, 88) = 10.01$, $MSE = 147.54$, p < .001, η_p^2 = .185, but importantly no session × genotype $F(2, 88) = 1.48$, $MSE = 147.54$, p = .233, η_p^2 = .033, or session × genotype × group interaction $F(2, 88) = 2.11$, $MSE = 147.54$, p = .128, η_p^2 = .046. There was again no main effect of genotype $F(1, 44) < 1$, and in contrast to the analysis of raw response rates there was also no main effect of group $F(1, 44) = 1.81$, $MSE = 5817.32$, p = .186, η_p^2 = .039, nor genotype × group interaction $F(1, 44) = 2.74$, $MSE = 5817.32$, p = .105, η_p^2 = .059. Thus, normalization removed the overall group and genotype by group interactions stemming from the elevated pre-manipulation response rates in group HET-AAA but the critical group by session interaction reflecting the renewal effect remained the same.

3.1.3 Cacna1c ± can engage in the extinction of an appetitive task in a different context (B) and display a renewal effect after returning to the original context (A), in an ABA procedure.

Critically, the ABA groups showed increased response during the test session (in the original training context A) compared to the end of extinction (in the separate context B) - this exemplifies the standard ABA renewal effect, that is, animals recover their magazine responses after extinction when they are re-exposed to the acquisition context. In contrast, the AAA groups showed a continued decrease in responding during the test session (occurring in context A, the same as the extinction sessions). Moreover, this pattern of results was clear for both HET and WT animals. Analysis of the last extinction session and the test session using the raw data revealed a significant session × group interaction $F(1,44) = 6.85$, $MSE = 113.15$, $p = .012$, $\eta_p^2 = .13$. Follow-up of the interaction revealed that there was a difference between sessions for the ABA groups, $F(1,44) = 4.71$, $p = .035$, $\eta_p^2 = .10$, but not for the AAA groups, $F(1,44) = 2.35$, $p = .133$, $\eta_p^2 = .05$. Importantly, there was no main effect of genotype $F(1,44) = .30$, $MSE = 130.71$, $p = .589$, $\eta_p^2 = .01$, and no interactions including genotype were significant (highest $F(1,44) = 1.02$, $MSE = 130.71$, $p = .318$, $\eta_p^2 = .02$, for genotype × group interaction). These results showed an increase in the number of magazine entries when animals were placed in the original context (ABA groups) regardless of the genotype. Both Cacna1c ± and WTs were successful at renewing the extinguished conditioned response when returned to the acquisition context. This is important as renewal is a key phenomenon to understanding the role that contextual cues play in retrieving previously extinguished responses and giving us information about the process of extinction in itself.

3.2. Molecular results

3.2.1. In the AAA procedure, the PL in Cacna1c animals displays a higher number of co-labelled Arc and H1a neurons as compared to WT controls, accounting for a common network activated both during extinction and with extended exposure to the extinction context

Figures 3 shows the prefrontal cortex; prelimbic, infralimbic and cingulate regions analyzed. Figure 3A and B represent the percentage of immunoreactive cells for Homer1, Arc, and both IEGs in the prelimbic region. ANOVA analysis of the percentage of double labelled cells for both IEGs revealed a significant main effect of genotype, $F(1, 20) = 5.39$, $MSE = 5.87$, $p = .031$, $\eta_p^2 = .21$, group $F(1, 20) = 5.39$, $MSE = 5.87$, $p = .031$, $\eta_p^2 = .21$, and a significant genotype × group interaction, $F(1, 20) = 9.08$, $MSE = 5.89$, $p = .007$, $\eta_p^2 = .31$. Simple analysis of the interaction revealed a higher percentage of active cells in HET-AAA than WT-AAA, $F(1, 20) = 14.23$, $MSE = 5.87$, $p = .001$, $\eta_p^2 = .41$, but no significant differences between genotypes in the ABA groups, $F(1, 20) = .24$, $MSE = 5.87$, $p = .630$, $\eta_p^2 = .01$. Results showed no significant effect of genotype, group, and genotype × group interaction for H1a [highest $F(1, 20) = 1.15$, $MSE = 57.72$ $p = .189$, $\eta_p^2 = .08$, for the main effect of genotype], or Arc [highest $F(1, 20) = 3.80$, $MSE = 69.94$, $p = .065$, $\eta_p^2 = .16$., for the genotype × group interaction].

Figure 3

3.2.2 Cacna1c ± displays higher activation of the IL region after the prolonged exposure to the original context A in extinction (in AAA) and in the extinction of the original context A after renewal (in ABA).

Figures 3 C and D represent the percentage of immunoreactive cells for Homer1, Arc, and both IEGs in the Infralimbic area. ANOVA analysis for Arc expression revealed a significant main effect of genotype, $F(1, 20) = 4.50$, $MSE = 154.24$, $p = .047$, $\eta_p^2 = .18$, but no significant effect of group $F(1, 20) = 1.01$, $MSE = 154.24$, $p = .328$, $\eta_p^2 = .05$, or genotype × group interaction, $F(1, 20) = .132$, $MSE = 154.24$, $p = .720$, $\eta_p^2 = .01$. Results for H1a do not reveal effects of

genotype, group, or the genotype × group interaction [highest $F(1, 20) = 3.25$, $MSE = 192.13$, $p = .087, \eta_p^2 = .14$, for the main effect of genotype]. The same was true for the analysis of both IEGs [highest $F(1, 20) = 2.75$, $MSE = 5.38$, $p = .113, \eta_p^2 = .12$, for the genotype × group interaction].

3.2.3 The cingulate cortex is equally engaged in EL and Renewal in Cacna1c ± compared to WT littermates.

Figures 3 E and F represent the percentage of immunoreactive cells for Homer1, Arc, and both IEGs in the Cingulate area. ANOVA analysis of the double-labelled cells IEGs revealed no significant effect of genotype, group, or genotype × group interaction [highest $F(1, 20) = .66$, $MSE = 54.05$, $p = .425, \eta_p^2 = .13$, for the main effect of genotype]. The same was true for the analysis of H1a, [highest $F(1, 20) = 1.52$, $MSE = 48.86$, $p = .231, \eta_p^2 = .07$, for the main effect of group] and Arc [highest $F(1, 20) = 2.20$, $MSE = 1.38$, $p = .153, \eta_p^2 = .10$, for the main effect of genotype].

3.2.4. Extinction of a Pavlovian appetitive task, either in the same context (AAA) or after renewal (ABA) results in higher activation of the central nucleus of the amygdala in Cacna1c animals

Figure 4 shows the results from the medial, lateral, basal and central nuclei of the amygdala. Panel A represents the percentage of immunoreactive cells for Homer1, Arc, and both IEGs in the CeA. ANOVA analysis for Arc revealed a significant main effect of genotype, $F(1, 20) = 6.81$, $MSE = 94.17$, $p = .017, \eta_p^2 = .25$, but no main effect of group, $F(1, 20) = .11$, $MSE = 94.17$, $p = .106, \eta_p^2 = .12$, or genotype × group interaction $F(1, 20) = 1.19$, $MSE = 94.17$, $p = .288$, $\eta_p^2 = .06$. HETs showed a higher number of positive Arc cells than WT regardless of AAA or ABA

group. There was no significant effect of genotype, group, or genotype × group interaction for H1a [highest $F(1, 20) = .36$, $MSE = 54.91$, $p = .553$, $\eta_p^2 = .02$, for the main effect of group], and both IEGs [highest $F(1, 20) = 3.04$, $MSE = 3.66$, $p = .096$, $\eta_p^2 = .13$, for the main effect of group].

Figure 4

Panel B represents the percentage of immunoreactive cells for Homer1, Arc, and both IEGs in the BaA. Results showed no significant effect of genotype, group, or genotype × group interaction for Arc [highest $F(1, 20) = 1.32$, $MSE = 99.66$, $p = .264$, $\eta_p^2 = .06$, for the genotype × group interaction], H1a [highest $F(1, 20) = .74$, $MSE = 51.23$, $p = .399$, $\eta_p^2 = .04$, for the main effect of genotype], and both IEGs [highest $F(1, 20) = 1.48$, $MSE = 5.12$, $p = .239$, $\eta_p^2 = .07$, for the main effect of genotype]. The same pattern of results is also present for the LaA (Panel C); there was no significant effect of genotype, group, or genotype × group interaction for Arc [highest $F(1, 20) = 2.91$, $MSE = 78.02$, $p = .103$, $\eta_p^2 = .13$, for the main effect of genotype], H1a [highest $F(1, 20) = 1.01$, $MSE = 28.81$, $p = .327$, $\eta_p^2 = .05$, for the main effect of group], and both IEGs [highest $F(1, 20) = 2.25$, $MSE = 4.01$, $p = .149$, $\eta_p^2 = .10$, for the genotype × group interaction]. In addition, the results for MeA (Panel D) showed no significant effect of genotype, group, or genotype × group interaction for Arc [highest $F(1, 20) = 1.14$, $MSE = 33.46$, $p = .299$, $\eta_p^2 = .05$, for the genotype × group interaction], H1a [highest $F(1, 20) = .101$, $MSE = 21.53$, $p = .757$, $\eta_p^2 = .01$, for the main effect of group], and both IEGs [highest $F(1, 20) = 2.88$, $MSE = 1.18$, $p = .105$, $\eta_p^2 = .13$, for the main effect of genotype].

3. Discussion

The present study aimed to further investigate non-fear-based Pavlovian extinction and renewal processes in WT and Cacna1c ± rats. Moreover, we aimed to characterize the neural basis of Pavlovian appetitive extinction in this model by performing fluorescence in situ hybridization to detect the experience-dependent nuclear encoding of Homer1a and Arc in sub-areas of the medial prefrontal cortex and amygdaloid nucleus. In terms of behavior, the results showed that animals from both genotype groups were able to learn the association between the CS and the outcome. Moreover, they showed a similar pattern of responses over the extinction phase, that is, a progressive decrease in the number of magazine entries in the presence of the CS when the outcome was no longer presented. When extinction occurred without a context change, both groups decreased the conditioned responses to the CS in a slower fashion than animals receiving extinction in a novel context, in other words, the change of context was recognized independently of the genotype, and extinction progressed faster in a different context for all groups. Critically, when extinction occurred in a novel context, Cacna1c ± animals were able to renew the conditioned responses in the same way as their WT littermates when they were re-exposed to the CS in the original acquisition context.

After extinction and renewal of the appetitive Pavlovian task, our results show specific regional elevations of the IEGs studied in the Cacna1c ± animals in the PL and IL of the mPFC and the CeA of the amygdala, as compared to their WT littermates. Specifically, the prefrontal cortex of the Cacna1c ± groups showed an increased number of neuronal nuclei simultaneously labelled for Arc and H1a IEGs, which suggests a common neural ensemble codifying for extinction session three, and the extended exposure to the same context A, once extinction learning had been settled and consolidated. Arc expression was higher in Cacna1c ± animals for both the IL region and Ce Amygdaloid nucleus, reflecting a differential engagement of these structures in the prolonged exposure to the context in which extinction took place, when this occurs in the same context as acquisition (AAA procedure) and the extinction of context A in the ABA procedure after renewal.

Cacna1c ± can engage in extinction and renewal of a Pavlovian appetitive task

Despite the well-known role of Ca2+-mediated processes in synaptic plasticity underlying learning and memory, the results from the present study suggest that reduced Cacna1c expression is not sufficient to prevent the acquisition and extinction of an appetitive cue-outcome association. These results contrast with previous literature showing impaired fear extinction in a Cav1.2 conditional knockout mice model (Temme & Murphy, 2017). Most of the current literature on the neurobiological basis of Pavlovian extinction learning foccuses on fear-conditioned learning (Bouton et al., 2021; Maren, 2000), leaving a scarcity of studies on appetitive forms of extinction learning such as the one used in the current study. In the case of aversive learning, it has been previously shown that deletion of Cav1.2 produced a significant deficit in fear extinction without altering acquisition or fear consolidation to a tone or a context, suggesting that Cacna1c is necessary for updating information about an aversive association. This process seems to be mediated by the synaptic regulation of the lateral amygdala; the calcium influx through Ca_v 1.2 channels in the lateral amygdala is believed to mediate associative long term potentiation during fear conditioning (Bauer et al., 2002), and Ca_v 1.2 blockade using verapamil impacted long-term fear memory without affecting acquisition (Bauer et al., 2002; Davis & Bauer, 2012). Similarly, nifedipine and nimodipine (L-type voltage-gated calcium channels inhibitors) prevent the extinction of cue-fear conditioning in a dose-dependent manner without affecting the acquisition or expression of fear conditioning (Cain et al., 2002). Despite several studies demonstrating the role of L-type voltage-gated calcium channels in mediating fear extinction, the specific conditions in which these impairments are seen remains controversial. For example, a study by Mckinney et al. (McKinney et al., 2008) found that mice lacking Ca_v 1.2 in the forebrain did not show any impairment in the acquisition or extinction of fear memory. In addition, the deletion of Ca_v 1.2 and Ca_v 1.3 might not always influence fear extinction (Schafe, 2008). Therefore, the impact of

CACNA1C on extinction might be dependent on the specificity of the channels blockaded, might be mitigated using animal models where the expression of Cacna1c is only decreased, and might be specific to aversive preparations where recall of previous memories is crucial for an animal's survival (or have a strongly adaptative function in general). Here, Cacna1c+/− animals appeared to successfully learn and update information about an appetitive cue-outcome association, either because the decreased level of Cacna1c expression did not impact this learning process, or perhaps because of some compensatory mechanism allowed them to reach the same level of performance as their WT littermates. A study by Koppe et al. (Koppe et al., 2017) demonstrate that Cacna1c knockout animals used a different strategy on a cue discrimination task, despite standard behavioral analysis showing no gross differences between those animals compared to controls. In that study, knockout animals did not learn the cue-discrimination rule but instead adopted a reward-increasing strategy (outcome rule). This potential difference in the strategy used, or the presence of a compensatory mechanism, would impact the neurological mechanisms underpinning the task.

It should also be noted that as the current study only included males, one could not rule out the possibility of deficits in extinction occurring in a sex-specific manner, as it was shown in other genetically modified models tested in Pavlovian paradigms where only females, (but not males), showed differences in extinction learning (Bengoetxea et al., 2021).

Higher activation of the PL, IL and CeA areas across extinction occurring in the same context as acquisition (AAA) suggests different processing of the inhibition of the original association, and the inhibition of the conditioned response, between Cacna1c and wild-type animals.

In our study, functional differences in the prefrontal cortex and the central amygdaloid nucleus arise between Cacna1c animals and their wild-type littermates for the encoding of these experiences. We analyzed the IEGs Homer1a and Arc nuclear expression, which have been

repeatedly used to precisely establish the time in which a region is activated following a behavioral experience. Due to the brief period of transcription of these genes, somatic expression gives a very precise read-out of to the neurons that engaged in encoding or processing the experience (R. Nalloor et al., 2012). Homer1a achieves its maximum somatic expression 25–30 min after an experience, whereas Arc expression occurs within 5–6 min of neuronal activation (Guzowski et al., 1999), dispersing into the cytoplasm after somatic expression (Guzowski & Worley, 2001; Guzowski et al., 1999). Importantly, recent ex-vivo studies demonstrate that Arc transcription displays distinct temporal kinetics depending on the input, opening up the possibility of different Arc transcription patterns depending on the region and cell type (Lituma et al., 2022). However, in-vivo experiments after an associative learning stimulus, similar to the present study, have shown intranuclear localization of the mRNA transcript in the basolateral amygdala (Barot et al., 2008; Zelikowsky et al., 2014), and the mPFC (Zelikowsky et al., 2014) in response to stimulation occurring 5 min before killing, whereas stimuli occurring 30-35 min previously elicited extranuclear labelling of Arc transcription.

To determine how Cacna1c animals process extinction in the same context (AAA) or renewal after extinction in a different context (ABA) as compared to WT controls, we conducted fluorescence in situ hybridization of Homer1a and Arc. Specifically, in the AAA conditions Homer1a served as a biomarker for the engaged extinction learning, while Arc indicated the somatic location of the extended exposure to the same context once extinction learning has been settled and consolidated. In the ABA procedure, the H1a indicated activity related to renewal occurring after extinction in a different context (B), whereas the Arc indicated the extinction of the original context A.

Most of the ISH differences found in Cacna1c animals correspond with extinction in the original context A, be that across the test session in the case of the AAA conditions, or the extinction of the original context after renewal in the ABA procedure (i.e. late in the test

session). Extinction without a change in context is more difficult to achieve, as there is no contextual cue to discriminate between the rewarded and non-rewarded (i.e., extinction) phases, and so animals needs to learn that there was a change of rules in the previously rewarded context (Méndez-Couz et al., 2021). The IEG expression associated with the renewal read-out remained similar between Cacna1c and WT animals. For extinction learning of a previously formed association to occur, a flexible behavioral response is needed, for which the mPFC is reported as a key structure (Torres-Berrío et al., 2019). In non-aversive forms of learning, such as spatial navigation, the mPFC and its functional interactions with the hippocampus are required for successful extinction (Avigan et al., 2020). In this regard, we have previously reported that the PL and IL regions display c-Fos IEG elevations when the late phases of extinction take place in the same context as the original association formation (Méndez-Couz et al., 2019). In addition, temporary inactivation of the PL region before extinction did not impair the process, but a functional reorganization in the brain network underlying extinction occurred (Méndez-Couz et al., 2015). Apart from changes in the behavioral flexibility accounting for the delayed extinction of the original context in the Cacna1c rats, we cannot rule out the possibility of an increased persistence of the original association. In this regard, the IL cortex in Cacna1c animals shows higher activation for the extinction of context A in both AAA and ABA conditions. The role of the mPFC- amygdala associations in behavioral persistence, conditioned fear and extinction of drug-seeking has been extensively described (Delamater, 2004; Herry et al., 2010). Although less is known in the case of non-pathological appetitive forms of Pavlovian conditioning, extinction of fear memories requires plasticity in the IL and BDNF, a key mediator of synaptic plasticity, prevents persistence and extinction failures when directly infused in the mPFC (Peters et al., 2010). Considered together with the fact that behavioral extinction (and renewal) was preserved in the Cacna1c animals, the differences in PL and IL activation, could suggest that WT and Cacna1c animals engage different neural mechanisms

underpinning the behavioural flexibility needed for extinction learning and/or the persistence of the original memory trace in Cacna1c animals.

However, the extinction of a previously reinforced conditioned response comprises not only cognitive processing but also involves emotional components of the memory systems (Bouton, 2011; Goodman & Packard, 2019). In this light, it is interesting that we observed elevated CeA Arc activation in the Cacna1c animals for both AAA and ABA conditions, indicating differential involvement of the CeA in both extended extinction without a context shift and extinction in the original training context after renewal. The role of the CeA is especially intriguing in appetitive extinction. We have previously shown c-Fos activation in the CeA in the late stages of early (Méndez-Couz et al., 2016) and late spatial preference in the same context, compared to no-extinction controls. The central amygdala is primarily GABAergic, thus inhibitory (McDonald, 1982). Studies in humans show the CeA functions as an integrative hub, converting emotionally relevant sensory information about the environment into behavioral and physiological responses (Gilpin et al., 2015). The extended amygdala structure, which includes other limbic forebrain structures such as the accumbens or stria terminalis, mediates both negative affective states associated with stress but also with appetitive stimuli, such as alcohol dependency (Koob, 2008). This structure is densely populated by pro-and anti-stress neuropeptides, as Neuropeptide Y, associated with context-dependent acquisition and retrieval (Méndez-Couz et al., 2022; Méndez-López Couz et al., 2021). Thus one possibility would be that the CeA modulation of dorsal hippocampal NPY, co-released by GABA in the hippocampus, would temper long-term depression (Kemp & Manahan-Vaughan, 2007, 2008) and long-term-term potentiation mechanisms of synaptic plasticity associated with environmental features (Kemp & Manahan-Vaughan, 2008). Thus, although the CeA in aversive fear learning and aversive EL has been associated with anxiety, its role as the relay between the BLA and the hippocampus might underlie its context-dependent activation during extinction of Pavlovian associations in the Cacna1c, in the case of non-aversive EL.

Conclusions

Here, we demonstrate that Cacna1c+/− animals can successfully engage in appetitive extinction and renewal of a Pavlovian task, contrasting with previous findings suggesting a key role of Ca_v 1.2 in fear extinction processes, and suggesting a differentiated role between appetitive and aversive learning and extinction. However, the higher activation in the mPFC and central amygdaloid nucleus found in the Cacna1c model suggest differences in the perception of the context or the inhibition of the conditioned response during extinction learning. Thus there is a possibility that the preserved behavioral responses are underpinned by different neural mechanisms and that there is compensation for impaired Ca_v 1.2 function. Moreover, in light of the fact that the association between variations in CACNA1C and symptoms of psychosis is one of the most robust findings in GWAS studies and mental health (Moon et al., 2018), the current findings also suggest that understanding the contribution of L-type voltage-gated calcium channels to the extinction of appetitively-based associations may also be clinically informative.

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Conflict of interest

The authors declare that they have no conflict of interest, financial or otherwise, related to this work.

Table 1. Behavioural Procedure details

Groups	N	Acquisition	Extinction	Renewal/test
Het-ABA	11		Light Context B	
Het-AAA	11	Light → Pellet	Light Context A	Light
WT-ABA	13	Context A	Light Context B	Context A
WT-AAA	13		Light Context A	

Notes:

Light = 10s flashing (0.2s on/off) of 2-panel lights to the left/right of the magazine
ITI = average 70s, range 40-100s (4 * each of 40s, 55s, 70s, 85s, 100s). 30 trials per session. US presented at CS Offset - data (number and duration of nosepokes) recorded for 30s PreCS, 10s CS, and 10s PostCS. Context A in room 1 with standard operant boxes. Context B in room 2 with grid floor and walls covered with black and white squares (Contexts A/B counterbalanced across animals). Rats under food restriction procedure

Figure 1

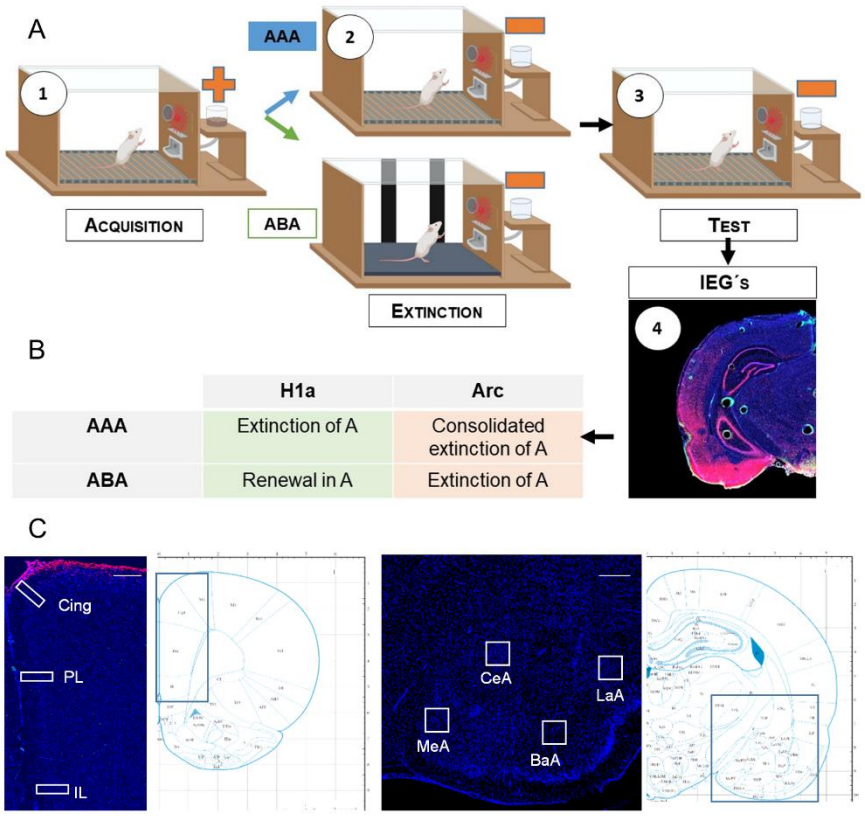
A
AAA
ABA
1
2
3
4
ACQUISITION
EXTINCTION
TEST
IEG´S
B
H1a
Arc
AAA
Extinction of A
Consolidated extinction of A
ABA
Renewal in A
Extinction of A
C
Cing
PL
IL
CeA
LaA
MeA
BaA

Figure 1. Timeline of the experiment and areas of study. **A)** Timeline of the experiment. Cacna1c ± rats and their wild-type littermates underwent Pavlovian training in context A, in which a pellet followed the cue light (for details of intervals please refer to table 1). After the association had been acquired, rats underwent extinction of the previously reinforced response in the same context in which acquisition took place (AAA groups) or in a different context (ABA groups). Finally, the four groups of animals returned to the original context for testing. On each day, animals spent 40 min in the cages, one session per day for all phases. **B)** Read-out of the Homer1a and Arc IEG´s somatic expression according to the behavioural phenomena occurring on the test day. C) DAPI stained section of the prefrontal cortex and amygdala showing the regions of interest scrutinized in the infralimbic (IL), prelimbic (PL) and cingulate cortex (Cing), and medial (MeA), central (CeA), lateral (LaA) and basal (BaA) nuclei of the amygdala, respectively. Atlas figures modified from (Paxinos and Watson, 2006) Scale bar: 500 μm. Figure A created with biorender.com.,

Figure 2

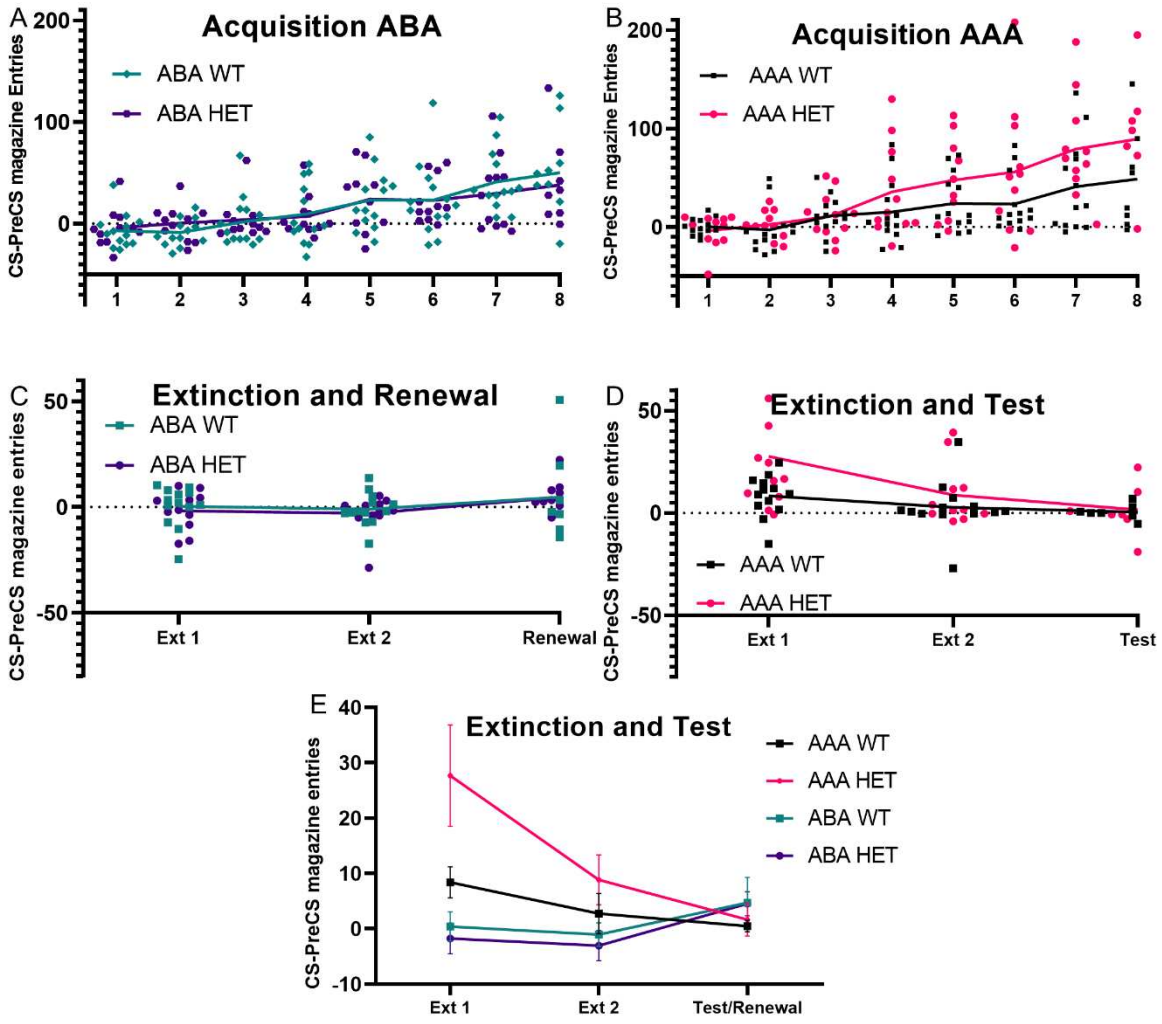
A
Acquisition ABA
ABA WT
ABA HET
CS-PreCS magazine Entries
200
100
0
1
2
3
4
5
6
7
8
B
Acquisition AAA
AAA WT
AAA HET
CS-PreCS magazine Entries
C
Extinction and Renewal
ABA WT
ABA HET
CS-PreCS magazine entries
50
0
-50
Ext 1
Ext 2
Renewal
D
Extinction and Test
AAA WT
AAA HET
Test
E
Extinction and Test
AAA WT
AAA HET
ABA WT
ABA HET
40
30
20
10
-10
Test/Renewal

Figure 2. Cacna1c ± animals acquired the appetitive Pavlovian task and adapted their behavioural response during extinction. Renewal after extinction with context change is unaffected. A, B) Mean number of magazine entries across acquisition during CS presentation (minus the average of the three 10 seconds periods before the CS) **C)** Extinction in a different context B and renewal in the original context A (ABA groups). Contrary to the literature on fear-conditioned tasks Cacna1c ± animals showed no differences with respect to their wild type control littermates, engaging in extinction and renewal of the appetitive Pavlovian conditioned response when tested in the original context. **D)** Extinction without context change (AAA groups). Cacna1c ± and wild-type animals were equally able to engage in extinction of the Pavlovian appetitive task. Compared to the ABA groups, extinction was slower in the AAA groups, and extended into the test session. **E**) Extinction and Test comparison for all groups, showing clear renewal for the ABA groups.

Figure 3

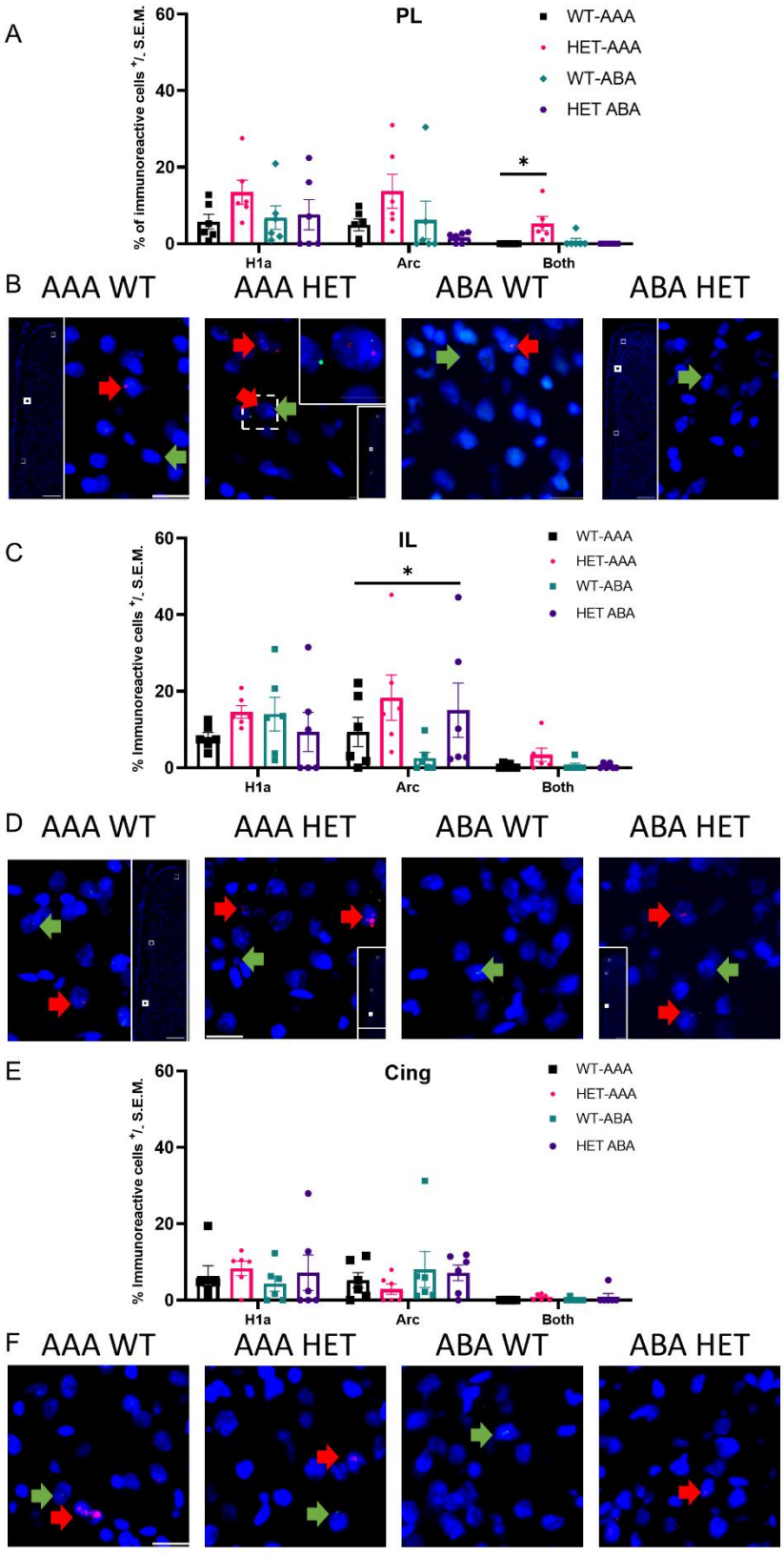
A
PL
WT-AAA
HET-AAA
WT-ABA
HET ABA
% of immunoreactive cells +/- S.E.M.
60
40
20
0
*
H1a
Arc
Both
B AAA WT
AAA HET
ABA WT
ABA HET
C
IL
WT-AAA
HET-AAA
WT-ABA
HET ABA
% Immunoreactive cells +/- S.E.M.
60
40
20
0
*
H1a
Arc
Both
D AAA WT
AAA HET
ABA WT
ABA HET
E
Cing
WT-AAA
HET-AAA
WT-ABA
HET ABA
% Immunoreactive cells +/- S.E.M.
60
40
20
0
H1a
Arc
Both
F AAA WT
AAA HET
ABA WT
ABA HET

Figure 3. A, B) In Cacna1c ± animals, a neural ensemble is simultaneously active during the initial extinction session and extended exposure to the context occurring without a context change (AAA). A) No differences between groups were evident in the amount of Homer1a or Arc, however, in the AAA procedure, Cacna1c ± animals showed a higher percentage of double-labelled nuclei ("Both") that indicate IEG activation triggered by extinction and the prolonged exposure to the same context occurring without a context change. B) Microphotographs showing Arc mRNA expression (red dots, and arrows) and H1a (green dots and arrows) in the prelimbic region for all groups Scale bar 20 µm. In AAA HET, a crop of a co-labelled nucleus for H1a and Arc is depicted. Scale bar 10 µm. On the small microphotographs, the whole section from which the sample was taken is shown. Scale bar 500 µm. Blue: Nuclear staining with DAPI. Images were taken using a 20X objective.

C, D) Cacna1c ± model displays higher activation of the IL region with extended exposure to context A in which extinction occurred (AAA), and in the extinction of the original context A after renewal in the ABA procedure**.** C) Cacna1c ± displayed a higher percentage of Arc-labelled neuronal nuclei in both AAA and ABA conditions. No significant changes were found in the percentage of Homer1a or the number of cells co-expressing both immediate early genes. D) Same as Figure B, but for the Infralimbic region. Scale bar 20 µm. $*p < 0.05$.

E, F) IEG expression in the Cingulate region following extinction learning and renewal. E) No differences were found between groups after extinction learning in the AAA procedure (H1a) or extended exposure to the context A in which extinction occurred (Arc), similarly, a lack of differences between groups occurred in the ABA procedure (H1a, renewal, Arc extinction of context A). Neither difference between groups was evident in the percentage of co-labelled nuclei in both procedure. F) Same as D, but for the cingulate region.

Figure 4

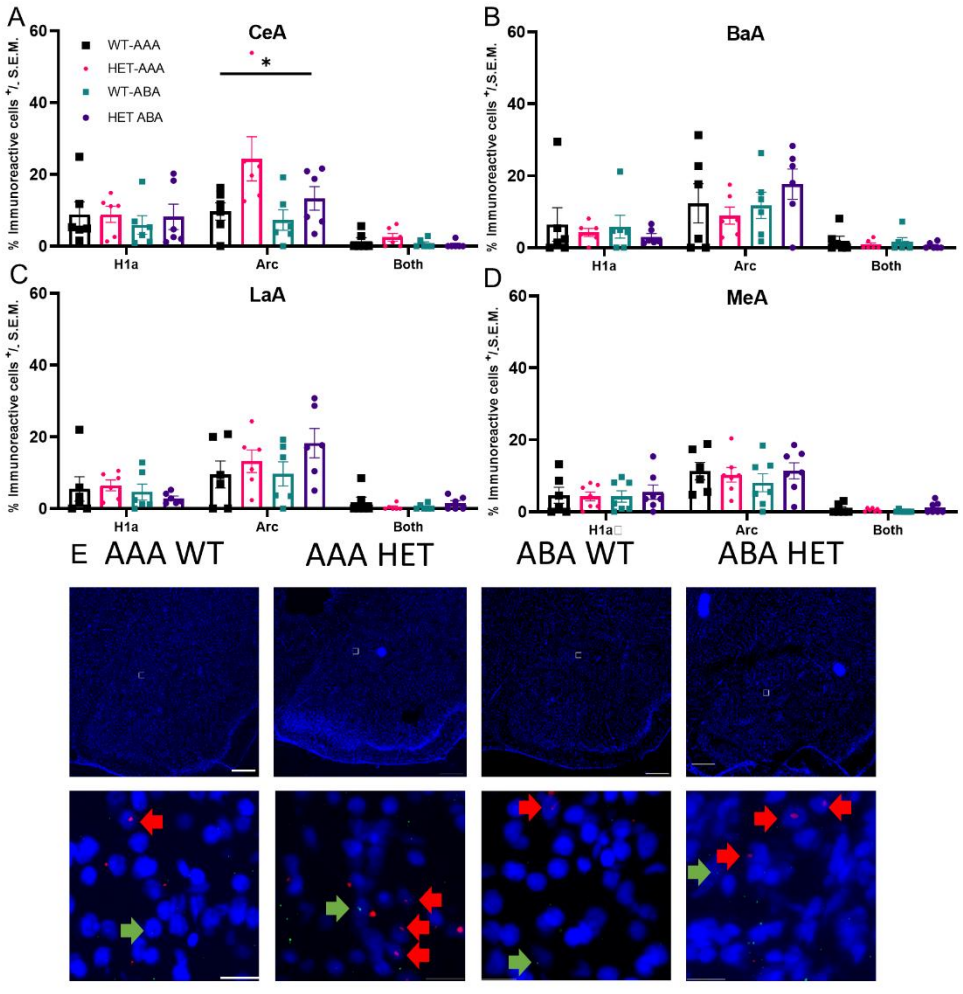
A
CeA
WT-AAA
HET-AAA
WT-ABA
HET ABA
*
% Immunoreactive cells +/- S.E.M.
60
40
20
0
H1a
Arc
Both
B
BaA
C
LaA
D
MeA
E
AAA WT
AAA HET
ABA WT
ABA HET

Figure 4. In Cacna1c ± animals, the extinction of an appetitive Pavlovian task occurring in the same context results in higher activation of the central nucleus of the amygdala, which is also evident during the extinction of the original context A after renewal in ABA. A) IEG expression in the central amygdala following extinction and renewal in the AAA or ABA procedure. The percentage of Arc-labelled neurons was higher in the Cacna1c group compared to wild-type animals in the AAA condition (reflecting the extended exposure to context A when extinction happened without context change) and ABA condition (reflecting extinction of the original context A after renewal). B, C and D) IEG expression in the basal amygdala, lateral nucleus and basal amygdaloid nucleus, respectively. No significant differences were found between the groups. D) Upper microphotographs of the regions of interest for the central amygdala. Lower, microphotographs showing Arc mRNA expression (red dots, and arrows) and H1a (green dots and arrows) in the prelimbic region for all groups Scale bar 20 µm. Nuclei were revealed with DAPI (in blue). *$p < 0.05$

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