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Sex-dependent modulation of age-related cognitive decline by the L-type calcium channel gene *Cacna1c* ($Ca_v1.2$)

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Abstract

Increased calcium influx through L-type voltage-gated calcium channels (L-VGCC) has been implicated in the neuronal dysfunction underlying age-related memory declines. In the present study we sought to test the specific role of *Cacna1c* (which encodes $Ca_v1.2$) in modulating age-related memory dysfunction. Short-term, spatial, and contextual/emotional memory was evaluated in young and aged, wild-type as well as mice with one functional copy of *Cacna1c* (haploinsufficient), using the novel object recognition, Y-maze, and passive avoidance tasks respectively. Hippocampal expression of *Cacna1c* mRNA was measured by quantitative polymerase chain reaction (qPCR). Aging was associated with object recognition and contextual/emotional memory deficits and a significant increase in hippocampal *Cacna1c* mRNA expression. *Cacna1c* haploinsufficiency was associated with decreased *Cacna1c* mRNA expression in both young and old animals. However, haploinsufficient mice did not manifest an age-related increase in expression of this gene. Behaviorally, *Cacna1c* haploinsufficiency prevented object recognition deficits during aging in both male and female mice. A significant correlation between higher *Cacna1c* levels and decreased object recognition performance was observed in both sexes. We have also observed a sex-dependent protective role of decreased *Cacna1c* levels in contextual/emotional memory loss, specifically in male mice. These data provide evidence for an association between increased hippocampal *Cacna1c* expression and age-related cognitive decline. Additionally, they indicate an interaction between the *Cacna1c* gene and sex in the modulation of age-related contextual memory declines.

Keywords

aging; memory; cognition; mice; hippocampus

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INTRODUCTION

Aging has been widely associated with cognitive impairments and greater vulnerability for the development of neurodegenerative disorders (Raz *et al.*, 1998; Hedden & Gabrieli, 2004; Mattson & Magnus, 2006). There is a need to identify novel targets for the development of pharmacotherapies to maintain cognitive integrity in the elderly. The 'calcium hypothesis of aging' proposes a key role for an altered neuronal calcium homeostasis in mediating changes in neuronal development associated with aging (Khachaturian, 1987; Landfield, 1987). Subsequent studies have shown that chronic elevation of calcium influx has been implicated in aging-associated neuronal death (Disterhoft *et al.*, 1994). L-VGCC current densities are significantly elevated in hippocampal neurons of old compared to young rats (Landfield, 1994; Campbell *et al.*, 1996; Thibault & Landfield, 1996; Disterhoft *et al.*, 2004; Wang & Mattson, 2014) and surface L-VGCCs expression levels have been found to be increased in aged hippocampus (Nunez-Santana et al., 2014). However, there is conflicting evidence for aging-induced altered L-VGCC expression levels; increased (Herman *et al.*, 1998; Chen *et al.*, 2000; Veng & Browning, 2002), no change (Blalock *et al.*, 2003; Kadish *et al.*, 2009; Nunez-Santana *et al.*, 2014) or decreased expression levels (Rowe *et al.*, 2007) have been previously observed.

The L-VGCC family consists of four distinct channels referred to as $Ca_v1.1$-$Ca_v1.4$. While $Ca_v1.2$ and $Ca_v1.3$, have both been identified in the brain, $Ca_v1.2$ accounts for about 80% in the rodent brain (Hell *et al.*, 1993; Sinnegger-Brauns *et al.*, 2009). The gene coding for the pore-forming alpha-1C subunit of $Ca_v1.2$ is *Cacna1c* (Soldatov, 1994). To date, the hypothesis that selectively decreasing $Ca_v1.2$ or *Cacna1c* improves age-related changes in cognitive performance has not been tested. Moreover, since sex differences in cognitive decline during aging have been identified (see Gur & Gur, 2002), we investigated the role of the Cacna1c gene in age-associated memory decline by comparing the effects of Cacna1c haploinsufficiency on cognitive behavioral function between male and female mice. We have previously shown that young Cacna1c haploinsufficient mice have an ~50% decreased $Ca_v1.2$ protein levels in the hippocampus as well as decreased L-VGCC current density in CA1 compared to their wild-type littermates (Dao et al., 2010). Our data reveal a correlation between increased age-related hippocampal *Cacna1c* mRNA expression and impaired short-term memory in the novel object recognition task, which were prevented in both male and female *Cacna1c* haploinsufficient mice. We also found that *Cacna1c* haploinsufficiency prevented age-related declines in emotional/contextual memory (passive avoidance) in male but not female mice. These findings suggest a relationship between hippocampal Cacna1c and age-related memory deficits and indicate a possible sex-dependent mechanism underlying this calcium channel's role in emotional-associated cognitive impairment.

METHODS AND MATERIALS

Mice

Male and female wild-type and *Cacna1c* haploinsufficient mice were obtained and generated as previously described by (Dao *et al.*, 2010). Founder male and female heterozygous *Cacna1c* knockout mice originally developed by Deltagen Inc. (San Mateo, CA) were obtained from Jackson Laboratories (Bar Harbor, ME). They were then

backcrossed to C57BL/6J for at least five generations prior to arrival, and greater than eight additional generations in our laboratory. All the mice used in the present study were bred in-house by breeding heterozygous males, generated in our own colony, and wild-type C57BL/6J females obtained from The Jackson Laboratories. At the time of initial behavioral testing the age of the animals was 4-5 months for the young groups and 17-18 months for the aged groups.

Ethical standards—All experimental procedures were approved by the University of Maryland Animal Care and Use Committee and were conducted in full accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Genotyping—DNA was extracted from tail clips by Proteinase K digestion followed by isopropanol precipitation. Genotype was determined through PCR amplification of a common 400bp product and knockout-specific 650bp product using three different primers:

- **(i)** a common sense primer, 5' TCTCTCCCACCTCGCACGCCGAATC 3'
- **(ii)** a wild-type specific anti-sense primer, 5' CACGACTGGCCTCTACTGCTCTTGAC 3'
- **(iii)** a knockout-specific anti-sense primer, 5' GACGAGTTCTTCTGAGGGGATCGATC 3'

Cacna1c expression

All the animals used in the behavioral studies were euthanized by decapitation five days following behavioral testing (see Fig. 1). Brains were collected and 1 mm coronal sections were cut at Bregma −2.30 using a mouse brain matrix. Dorsal hippocampi were then dissected and subsequently immediately frozen on dry ice, and stored at −80°C. The dorsal hippocampus was collected since it has been associated with cognitive functions, while the ventral hippocampus has been mainly related to stress, emotion and affective behaviors (see Fanselow & Dong, 2010). Total RNA was isolated from left hippocampus of mice by a phenol-chloroform-alcohol method using RNAzol (Molecular Research Center Inc, OH, USA) and the Directzol RNA kit (Zymo Research, CA, USA) following manufacturer protocols. Shipton et al., (2014) showed dissociation between the left and right hippocampus in the regulation of hippocampal-dependent memory and in particular, hippocampus-dependent associative spatial long-term memory has been shown to specifically require the left but not right hippocampus in mice. On-column DNase I digestion was included during the RNA extraction to eliminate potential DNA contamination. Four hundred ng of total RNA per sample were reverse transcribed into cDNA in a 20μL reaction volume using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The cDNA product was diluted in nuclease-free water (1:5) and the RT-PCR reaction was conducted using the SensiFAST SYBR LowROX kit (Bioline, USA) in 10 μL total volume. The RT-PCR reaction was run on a ViiA7 platform (Lifetechnologies Inc, USA) with a three-step cycling program as follows: an initial denaturation of 20 sec at 95° C followed by 40 cycles with a denaturation step of 1 sec at 95° C, an annealing and an extension step of 20 sec at 62°C with the optics on at this last step. A melting curve was executed at the end of the amplification step. The primers for *Cacna1c* were designed using

open source program Primer3 (Koressaar & Remm, 2007; Untergasser *et al.*, 2012) and synthesized by IDT Inc, USA corresponding to the following sequences. The primers for *Cacna1c* were designed using open source program Primer3 (Koressaar & Remm, 2007; Untergasser *et al.*, 2012) and synthesized by IDT Inc, USA corresponding to the following sequences: *Forward* TCA CCA TTG CCT CCG AAC ATT A; and *Reverse* GGG CTT TAT TGG CTG TGT CTT G. Three set of primers were used for reference genes and obtained from the PrimerTime™qPCRPrimers of IDT Tfrc: Mm.PT.56a.13036034, RPLP0: Mm.PT.56a.8566742.g and RPL13: *Mm.PT.56a.4260941.* All primer sets generated a single melting peak and were confirmed as a single amplification product by gel electrophoresis. All three reference genes were included with *Cacna1c* on each PCR run performed in triplicate. Relative expression was determined using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001), using the geometric mean CT value of the three reference genes for normalization. Values are expressed as fold change with respect to wild-type young males used as reference group.

Behavioral studies

All mice were tested in all the behavioral tests. Experiments progressed from relatively least stressful to more stressful (i.e., Y-maze, novel object recognition and then passive avoidance; **Fig. 1**). Due to the large number of mice studied, the groups were randomly assessed in three experimental cohorts that were combined for data analysis.

Y-maze (spontaneous alternation task)—To assess the effects of aging and *Cacna1c* haploinsufficiency on working memory, we used the Y-maze paradigm (Coburn-Litvak *et al.*, 2003; Dudchenko, 2004; Bannerman *et al.*, 2008; Gotz & Ittner, 2008). The Y-maze apparatus (Stoelting Co., IL, US) consists of three identical arms (5 × 35 cm) joined in the middle thus forming a “Y” shape. The Y-maze protocol was based on previously published methods (Sarnyai *et al.*, 2000). Briefly, mice were placed into the “start” arm of the maze and thereafter explored the apparatus for eight min. All the experimental trials were recorded with a digital video-camera. The videos were then scored by a trained observer blind to the experimental groups. Number of total arm entries and sequence of arm entries were measured. Percent alternations were calculated as: [the number of consecutive entries into three different compartments, divided by the total alternations (number of arm entries minus 2)] × 100.

Novel object recognition (NOR)—We assessed short-term recognition memory using the NOR task (Balderas *et al.*, 2008; Moore *et al.*, 2013). The NOR apparatus consists of a Plexiglas open-field chamber (40×9×23 cm) and was carried out under dim light conditions (10 LUX). The NOR behavioral testing consisted of three different sessions across two days: During the habituation phase (Day 1), the animals explored the NOR apparatus for 30 min in the absence of objects. Twenty-four hours later, during the familiarization session (Day 2), two identical objects were fixed on the floor of the apparatus symmetrically 8.5cm from the wall and the animals were allowed to explore the objects for 30 min. The objects were either two 50ml clear glass conical flasks (4.5 cm bottom diameter × 7 cm height) or two white-painted small glass vials (2.5 cm bottom diameter × 6 cm height). After familiarization with the “familiar” objects, mice were immediately returned to their home cages. Following a 30-min delay, mice were placed back into the NOR apparatus, in which one of the “familiar”

objects used during the familiarization session was replaced by a “novel” object (retention phase). Mice were permitted to freely explore the objects for a period of six min. During both familiarization and retention sessions the objects were used in a counterbalanced between-groups manner. All the sessions were videotaped by a digital video-camera. The retention sessions were manually scored by a trained observer blind to the experimental groups, using the AnoStar scoring software (Cleversys Inc., VA, US). Mice were considered to be interacting with the objects when their head was facing the object in a pre-set distance of ≤ 1 cm. A discrimination ratio was calculated as the time a mouse was interacting with the novel object divided by the total time of interaction with both the objects during the retention phase (Bevins & Besheer, 2006).

Passive avoidance—Passive avoidance is a memory task based on emotional/contextual learning (Ogren, 1985). The passive avoidance task was performed as previously described (Yamada *et al.*, 2003), with minor modifications. The apparatus consists of a two-chambered light-dark shuttle box (34 cm height × 37 cm width × 18 cm depth; Coulbourn Instruments, PA, USA) interconnected with a guillotine door. The experimental protocol consisted of two sessions; the training and the retention session, conducted 24 hours apart. During the training session, the animals were individually placed into the light compartment (800 LUX) of the apparatus and explored the light compartment for a 30-sec adaptation period with the door between the light and dark compartments closed. After the adaptation period, the door between the two compartments was automatically raised and mice were given a further five min to explore the compartment during which the latency of crossing to the dark compartment was measured. Upon entering the dark compartment, the guillotine door was closed and three sec later an inescapable foot-shock (0.32 mA, 2 sec duration) was delivered through the grid floor. Thirty sec after the foot-shock animals were immediately returned to their home cage. On the retention day (24 hours following training) mice were placed into the light compartment of the apparatus and after a 30-sec delay the door between the two compartments was raised. The latency to enter the dark compartment was automatically measured by the Coulbourn Instruments software. The trial was terminated after five min when the animal did not cross into the dark compartment.

Statistical Analysis

All values are expressed as the mean ± SEM. All data were analyzed by three-way analysis of variance (ANOVA) for factors ‘sex’, ‘age’ and genotype. For the behavioral studies, where three-way ANOVA did not reveal an effect of ‘sex’, data from both male and female mice were combined and a two-way ANOVA was then performed. ANOVAs were followed by LSD post-hoc comparison when statistical significance was reached (i.e., $p<0.05$). Values outside of the group mean ±2 SD were considered statistical outliers and excluded from all the analyses. All statistical analyses were performed using Statistica v10 (StatSoft Inc., USA).

RESULTS

Spatial memory was not altered in the Y-maze in aged mice

We assessed the effects of aging on both spontaneous alternation behaviors (percent alternation and total arm entries) in the Y-maze (WT male young: n=13; WT male old: n=13; HET male young: n=12; HET male old: n=11; WT female young: n=14; WT female old: n=12; HET female young: n=10; HET female old: n=7). A three-way ANOVA did not reveal any effect of 'age' ($F_{[1,86]}$=0.10; p=0.75), 'genotype' ($F_{[1,86]}$=0.22; p=0.64), 'sex' ($F_{[1,86]}$=0.89; p=0.35), or an 'age' x 'genotype' x 'sex' interaction ($F_{[1,86]}$=0.10; p=0.75) in spontaneous % alternation of mice. None of the other interactions were significant. Because there was no significant effect of sex we combined data from the males and females. Two-way ANOVA for percent alterations for both male and female mice combined (Fig. 2A) similarly did not show any significant effect of 'age' ($F_{[1,89]}$=0.63; *p=0.43*), 'genotype' ($F_{[1,89]}$=0.27; *p=0.60*), or a 'age' x 'genotype' interaction ($F_{[1,89]}$=0.18; *p=0.68*). However, three-way ANOVA showed that aging was associated with a significant decrease in the total arm entries (age effect: $F_{[1,86]}$=36.68; p<0.001), irrespective of 'genotype' ($F_{[1,86]}$=3.13; p=0.08), 'sex' ($F_{[1,86]}$=3.02; p=0.09), 'age' x 'genotype' x 'sex' interaction ($F_{[1,86]}$=2.80; p=0.1), or any of the other interactions. Two-way ANOVA analysis of total arm entries on the combined data from both male and female mice showed a significant effect of 'age' ($F_{[1,89]}$=36.97; p<0.001), but not 'genotype' ($F_{[1,89]}$=3.12; p=0.08) or an 'age' x 'genotype' interaction ($F_{[1,89]}$=0.0003; p=0.99) (Fig. 2B).

Aging-related decrease in novel object recognition was prevented in *Cacna1c* haploinsufficient mice

In the NOR task a three-way ANOVA revealed a significant effect of 'genotype' ($F_{[1,82]}$=11.21; *p<0.01*) and an 'age' x 'genotype' interaction ($F_{[1,82]}$=5.30; p<0.05), but no significant effect of 'age' ($F_{[1,82]}$=1.66; p=0.20), 'sex' ($F_{[1,82]}$=0.56; p=0.46), or any of the other interactions (WT male young: n=12; WT male old: n=13; HET male young: n=13; HET male old: n=10; WT female young: n=13; WT female old: n=12; HET female young: n=10; HET female old: n=7). In order to examine *Cacna1c* haploinsufficiency effects on aging and genotype, we performed a two-way ANOVA by combining data from male and female mice (Fig. 3A). A two-way ANOVA showed a significant effect of 'genotype' ($F_{[1,86]}$=10.24; p<0.01) and a significant 'age' x 'genotype' interaction ($F_{[1,86]}$=4.25; p<0.05). *Post-hoc* comparison indicated a significant decrease in the discrimination ratio of WT old mice compared with WT young mice (p<0.01). In contrast, in *Cacna1c* haploinsufficient mice there was no significant effect of age on the discrimination ratio (p=0.74).

Cacna1c haploinsufficiency prevented aging-related reductions in contextual/emotional memory in passive avoidance learning in male but not female mice

During the training session of the passive avoidance task (Fig. 4A), a three-way ANOVA revealed a significant effect of 'age' ($F_{[1,84]}$=8.06; p<0.01) but not 'genotype' ($F_{[1,84]}$=0.08; p=0.77), 'sex' ($F_{[1,84]}$=0.47; p=0.50), or an 'age' x 'genotype' x 'sex' interaction ($F_{[1,84]}$=0.01; p=0.90). None of the other interactions were significant. In contrast, during the retention phase (Fig. 4B), a three-way ANOVA revealed significant effects of 'age'

($F_{[1,84]}$=6.28; p<0.05), 'sex' ($F_{[1,84]}$=7.40; p<0.01), and a 'sex' x 'genotype' interaction ($F_{[1,84]}$=7.03; p<0.01) in the latency for the animals to cross in the previously footshock-conditioned dark compartment of the passive avoidance apparatus during the retention phase (Fig. 4B) (WT male young: n=13; WT male old: n=13; HET male young: n=13; HET male old: n=10; WT female young: n=14; WT female old: n=13; HET female young: n=10; HET female old: n=6). There was no overall significant effect of genotype ($F_{[1,84]}$=0.49; p=0.49) and the other interactions were not significant. *Post-hoc* analysis indicated a significant decrease in light-dark latency in WT old male mice compared to WT young male controls (p<0.05). *Cacna1c* haploinsufficiency prevented aging-induced contextual/emotional memory impairment as indicated by the prolonged light-dark latency of HET old male mice in the passive avoidance learning task compared with WT old male mice (p<0.05).

Effects of ageing on hippocampal *Cacna1c* expression

Relative *Cacna1c* expression was measured in hippocampal sections (Fig. 5). A three-way ANOVA showed a significant effect of 'genotype' ($F_{[1,73]}$=38.70; p<0.001), 'age' ($F_{[1,73]}$=17.40; p<0.001) and a 'genotype' x 'age' interaction ($F_{[1,73]}$=4.46; p<0.05), but no significant effect of 'sex' ($F_{[1,73]}$=0.05; p=0.82). None of the other interactions were significant (WT male young: n=10; WT male old: n=12; HET male young: n=12; HET male old: n=9; WT female young: n=12; WT female old: n=11; HET female young: n=9; HET female old: n=6). Two-way ANOVA analysis of relative Cacna1c expression on combined data from male and female mice combined revealed significant effect of 'age' ($F_{[1,77]}$=17.60; p<0.001), 'genotype' ($F_{[1,77]}$=41.73; p<0.001) and an 'age' x 'genotype' interaction ($F_{[1,77]}$=5.41; p<0.05). Post-hoc analysis demonstrated a significant aging-induced increase in relative hippocampal *Cacna1c* expression specifically in WT mice (p<0.001). In *Cacna1c* haploinsufficient mice, *Cacna1c* levels were significantly lower compared to WT in young mice (p<0.01). Relative *Cacna1c* levels were significantly lower in *Cacna1c* haploinsufficient old mice compared to their WT old counterparts (p<0.001).

Correlation analysis between *Cacna1c* expression and object recognition discrimination ratio revealed that object recognition memory and hippocampal *Cacna1c* levels are negatively correlated (Fig 3B). Importantly, This was similarly true in both male (r = −0.58, *p<0.001*) and female (r = −0.49, *p<0.01*) mice and within-group correlation analyses were also significant (data not shown).

Correlation analyses between Cacna1c expression and the ratio of retention/training latency time supported a negative correlation between emotional/contextual memory and hippocampal Cacna1c levels in male (r= −0.37, p=0.01; Fig. 4C), but not female (r= 0.03, p=0.86; Fig. 4D), mice. Similarly, correlation analysis between Cacna1c expression and passive avoidance retention latency time, uncorrected for training latency, also revealed that emotional/contextual memory and hippocampal Cacna1c levels are negatively correlated in male (r = −0.35, p<0.05; data not shown), but not female (r = 0.14, p=0.40; data not shown), mice.

DISCUSSION

Our data indicate age-related declines in object recognition memory in both male and female wild-type aged mice, which were prevented by genetic deletion of a single *Cacna1c* allele. Importantly, we also observed a robust negative correlation between *Cacna1c* expression in the hippocampus and short-term object recognition memory. We also showed that *Cacna1c* haploinsufficiency had a protective role in age-related emotional/contextual memory declines in male but not female mice. NOR (Burke *et al.*, 2010), and passive avoidance (Zanotti *et al.*, 1989; Knauber & Muller, 2000; Fiore *et al.*, 2002) tasks have been previously used to assess memory declines during aging in rodents. While the hippocampus is primarily known for its involvement in spatial memory (Broadbent *et al.*, 2004), it has been also shown to have a role in object recognition memory (O'Brien *et al.*, 2006), as well as memory associated with aversive tasks (i.e., passive avoidance here) (e.g. Izquierdo *et al.*, 1992). Our findings support the involvement of the *Cacna1c* gene in the hippocampus to at least partly underlie short-term object recognition memory and sex-dependently contextual/emotional memory. On the other hand, our data do not support an effect of aging on Y-maze spatial memory. This is not surprising as Y-maze has not been associated with age-related memory declines (Arendash *et al.*, 2001).

Pre-existing evidence reveals robust alternations in neuronal calcium homeostasis during aging. In particular, influx of calcium through L-VGCC is significantly elevated in the hippocampus of aged rodents (Campbell *et al.*, 1996; Thibault & Landfield, 1996; Wang & Mattson, 2014). An increase in calcium voltage-activated currents has been also found to occur in hippocampal CA1 neurons of aged rodents (Landfield & Pitler, 1984; Pitler & Landfield, 1990; Kerr & Abraham, 1993; Campbell *et al.*, 1996) and rabbits (Moyer *et al.*, 1992; Disterhoft *et al.*, 1993). In addition, increased age-dependent cell death has also been associated with an increased density of L-VGCCs in long-term hippocampal cell cultures (Porter *et al.*, 1997). Moreover, Davare and Hell, (2003) demonstrated increased cAMP-dependent protein kinase phosphorylation-induced activation of L-VGCC $Ca_v1.2$ (S1928) in the hippocampus of aged rats. While Nunex-Santana *et al.* have recently found a decrease of L-VGCCs in whole hippocampal tissue lysates, they also showed an age-related increase of S1928 phosphorylation in the dentate gyrus (Nunez-Santana *et al.*, 2014). Our findings also support a role of L-VGCC during aging, since we have observed significant increases of *Cacna1c* mRNA levels in the hippocampus of aged mice. While previous reports have similarly found age-related increased hippocampal *Cacna1c* expression levels (Herman *et al.*, 1998; Chen *et al.*, 2000), there are other reports of either no change (Blalock *et al.*, 2003; Kadish *et al.*, 2009; Nunez-Santana *et al.*, 2014) or decreased *Cacna1c* levels (Rowe *et al.*, 2007). These discrepancies between the findings might be due to differences in analysis and/or methods used. Indeed, some of the aforementioned studies relied on gene array analysis and did not confirm the *Cacna1c* findings by other methods. Whether in our animals age-related *Cacna1c* expression alternations are region- and/or cell-specific remain to be determined. Furthermore, a limitation of our study is that we measured only Cacna1c mRNA concentrations, and not $Ca_v1.2$ protein levels or electrophysiological function of channels. We focused on mRNA because it is more readily quantifiable in the >90 samples between eight experimental groups utilized in our study. The significant correlations

between Cacan1c levels and cognitive performance suggests that hippocampal mRNA Cacna1c levels predict function in our mouse line.

Increased age-related calcium influx through L-VGCC has been hypothesized to be detrimental to memory processes (e.g. Lynch, 2004). In the present study we provide further evidence that elevated hippocampal L-VGCC mRNA levels is associated with cognitive declines during aging. Findings from previous studies in Alzheimer's disease patients as well as animal models of cognitive dysfunction suggest that elevated hippocampal calcium influx is associated with synaptic dysfunction and neuronal degeneration, which might lead to cognitive impairments (Sandin *et al.*, 1990; Mattson & Magnus, 2006). Enhanced L-VGCC function during aging has been shown to be at least partly responsible for the increase in after-hyperpolarization in the hippocampus, in particular the CA1 region, leading to robust alternations in synaptic plasticity (Norris *et al.*, 1998b; a; Thibault *et al.*, 2001). Several studies have demonstrated that L-type channel antagonists were able to reverse learning deficits in aged rodents (Levere & Walker, 1992; Ingram *et al.*, 1994; Riekkinen *et al.*, 1997; Batuecas *et al.*, 1998; Veng *et al.*, 2003), rabbits (Deyo *et al.*, 1989a; Deyo *et al.*, 1989b; Kowalska & Disterhoft, 1994; Solomon *et al.*, 1995; Woodruff-Pak *et al.*, 1997; Rose *et al.*, 2007; Hopp *et al.*, 2014), non-human primates (Sandin *et al.*, 1990) and humans (Ban *et al.*, 1990). These findings have been suggested to at least partly involve a compensatory action of L-VGCC antagonism over age-induced altered calcium metabolism (Gibson & Peterson, 1987). In contrast, in young intact animals, L-VGCC antagonism revealed contradictory effects. For instance, nimodipine administration enhanced memory performance in young chicks at low doses, while it induced an amnesic effect at high doses in a visual discrimination task (Deyo *et al.*, 1990). Nifedipine also impaired retention in the same task (Deyo *et al.*, 1992). Nimodipine administration was shown to improve spatial working memory in the 8-arm radial maze in your rats (Levy *et al.*, 1991). Clements *et al.*, (1995) showed no effects of nimodipine, nifedipine or amlodipine on passive avoidance or visual discrimination learning tasks in young chicks. Moreover, Quartermain *et al.*, (1993) showed improvements of memory on an emotional-response learning task in amlodipine-treated young mice. Finally, in the inhibitory avoidance task, administration of nifedipine was found to be beneficial on memory retention in young rats (Quevedo *et al.*, 1998). Importantly, selectively targeting L-***VGCC*** blockage to the hippocampus is sufficient to enhance spatial memory (Quevedo *et al.*, 1998; Kim *et al.*, 2011). However, since existing L-type channel blockers inhibit all L-VGCC subtypes, none of these previous studies showed specificity to a particular LVGCC. Also, whether L-VGCC involvement in learning and memory processes is age-specific is not clear.

As such, the present study is the first, to our knowledge, to report beneficial effects of decreased hippocampal *Cacna1c* expression on age-associated memory impairment, as well as sex-dependent effects in emotional/contextual memory. In particular, we observed prevention of age-related memory decline in male, but not female, *Cacna1c* haploinsufficient mice in the passive avoidance memory task, and a significant correlation between increased *Cacna1c* expression and impaired passive avoidance learning specifically in male mice. Sex differences in learning and memory retention have been attributed to hormone-induced differences in hippocampal morphology and function (McEwen, 1997;

Romeo *et al.*, 2004; Hajszan *et al.*, 2007). Notably, in the present study, we did not observe differences in *Cacna1c* expression in the two sexes, suggesting that a possible interaction between gonadal hormones and *Cacna1c* gene might have driven the observed sex-dependent behavioral effects in the passive avoidance task. This is consistent with the finding that β-estradiol directly potentiates calcium influx via $Ca_v1.2$ via the dihydropyridine binding site (Sarkar et al., 2008) and that $Ca_v1.2$ is a target of estradiol in vivo (Brewer et al., 2009).

In contrast with the novel object recognition and passive avoidance tasks, we demonstrated that aged mice did not manifest spatial working deficits in the Y-maze task, regardless of sex or genotype. These results are in line with previous findings by Arendash *et al.*, (2001), who did not observe any impairment in Y-maze alternations between young (5-7 months) and old mice (15-17 months). However, we have observed aging-related decreases in total arm entries in both male and female mice, irrespective of genotype. Decreased total arm entries in the Y-maze paradigm indicates decreased spontaneous locomotor activity (e.g. Luszczki *et al.*, 2005). Impaired prefrontal cortex activation has been implicated in hypo-locomotor and decreased gait speed effects of aging in humans (Andreescu *et al.*, 2011).

Of translational relevance, an intronic single nucleotide polymorphism (SNP; rs1006737) in human *CACNA1C* has been associated with a number of brain functional changes including hippocampal performance (Bhat *et al.*, 2012). These data include impaired working memory as well as decreased fractional anisotropy values within the hippocampus, which was directly associated with poorer learning performance in healthy individuals (Zhang *et al.*, 2012; Dietsche *et al.*, 2014; Heck *et al.*, 2014). Healthy individuals carrying the *CACNA1C* rs1006737 risk allele showed reduced bilateral activation in the hippocampus during episodic memory recall and decreased functional coupling between the right and left hippocampus (Erk *et al.*, 2010). Similarly, in a recent study, healthy carriers of *CACNA1C* risk allele showed lower activation of the right hippocampus during episodic memory encoding or retrieval (Krug *et al.*, 2014). Notably, Erk *et al.*, (2014) showed that during an episodic memory task there was a significant reduction of hippocampal activation in healthy first-degree relatives of *CACNA1C* risk allele carriers with major depression or bipolar disorder, indicating a possible familial risk. Moreover, Paulus *et al.*, (2014) using functional magnetic resonance imaging to measure neural activation during a working memory task, reported a positive association of fronto-hippocampal connectivity in healthy risk allele carriers. This same SNP has also been associated with differences in expression of *CACNA1C* in both human postmortem brain and in induced neurons (Bigos *et al.*, 2010; Gershon *et al.*, 2014; Roussos *et al.*, 2014; Yoshimizu *et al.*, 2014), though the direction of effect and functional relevance has been reported to be both increased and decreased depending upon the study and brain region assessed. Together, these studies support the *CACNA1C* gene as a key modulator of hippocampal-related learning and memory processes. However, the effects of rs1006737 on age-associated learning in humans have yet to be studied.

Our data provide evidence that decreased *Cacna1c* expression has a protective role in the modulation of age-related cognitive declines and support an interaction between *Cacna1c*,

sex, and memory impairment. Overall, our data provide additional support for the ‘calcium hypothesis’ of aging (Khachaturian, 1987; Landfield, 1987).

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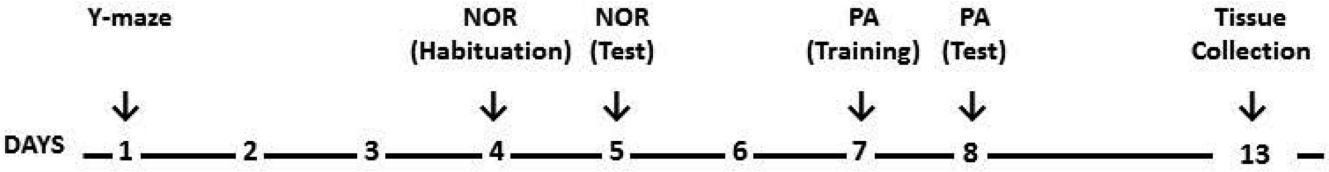

Figure 1. Behavioral and tissue collection sequence

Novel object recognition (NOR); Passive avoidance (PA).

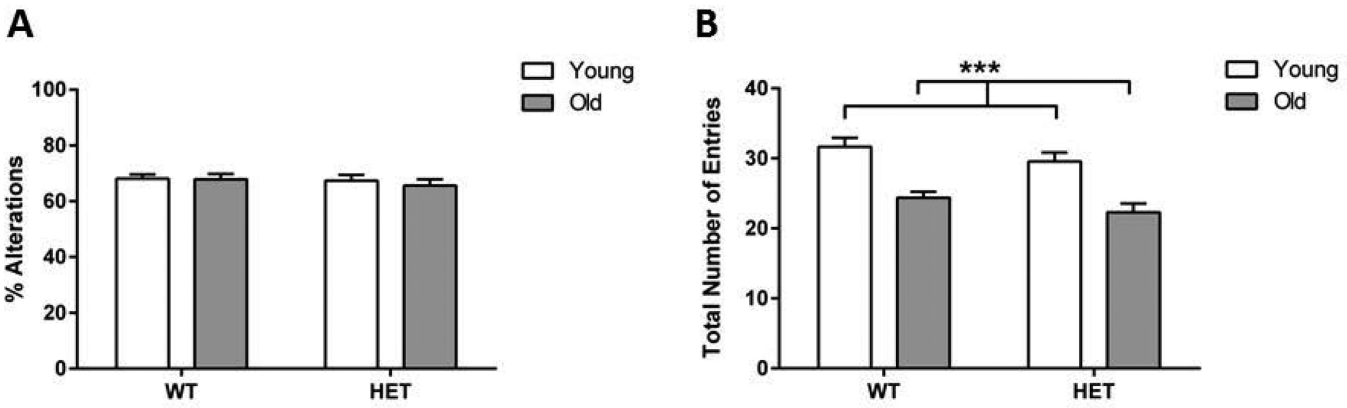

Figure 2. Y-maze performance

(A) Percent alternations in the Y-maze were measured in both wild-type (WT) and *Cacna1c* haploinsufficient (HET) mice. Aging did not induce any significant changes in percent alternations in mice. **(B)** Total number of entries into the arms of the Y-maze apparatus showed a significant decrease in exploratory activity in aged WT and *Cacna1c* HET mice irrespective of their genotype. Data represent mean ± SEM (n = 7-14/group); *** $p<0.001$.

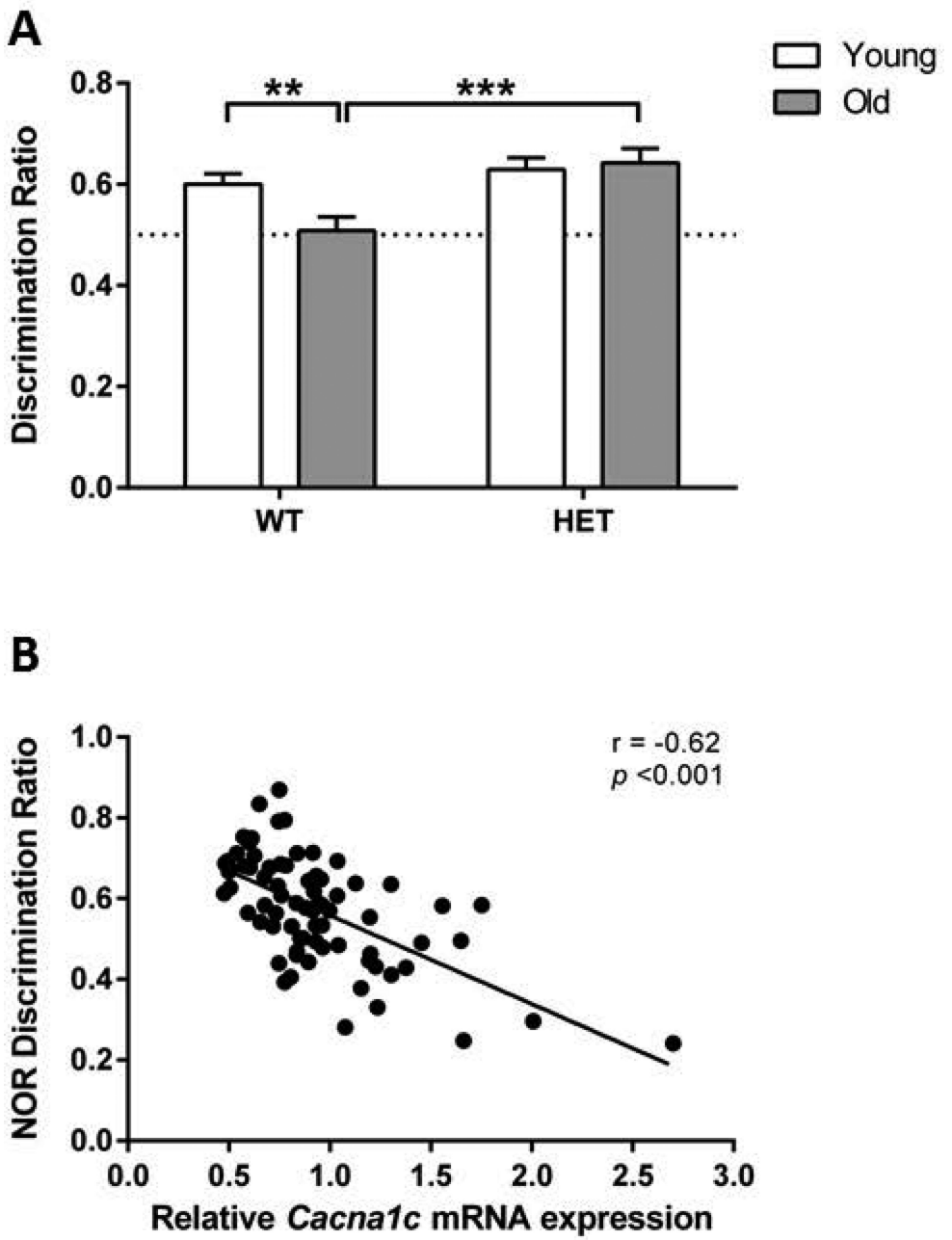

Figure 3. Novel object recognition memory performance

(A) Novel object recognition (NOR) discrimination ratio in young as well as old wild-type (WT) and *Cacna1c* haploinsufficient (HET) mice. Aging induced novel object recognition impairment in WT but not *Cacna1c* haploinsufficient (HET) mice, as indicated by a decreased discrimination ratio specifically in aged WT mice. **(B)** Correlation analyses between NOR discrimination ratio and relative *Cacna1c* levels in the hippocampus indicated a significant negative correlation. Data represent mean ± SEM (n = 7-14/group); ** *p<0.01*, *** *p<0.001*.

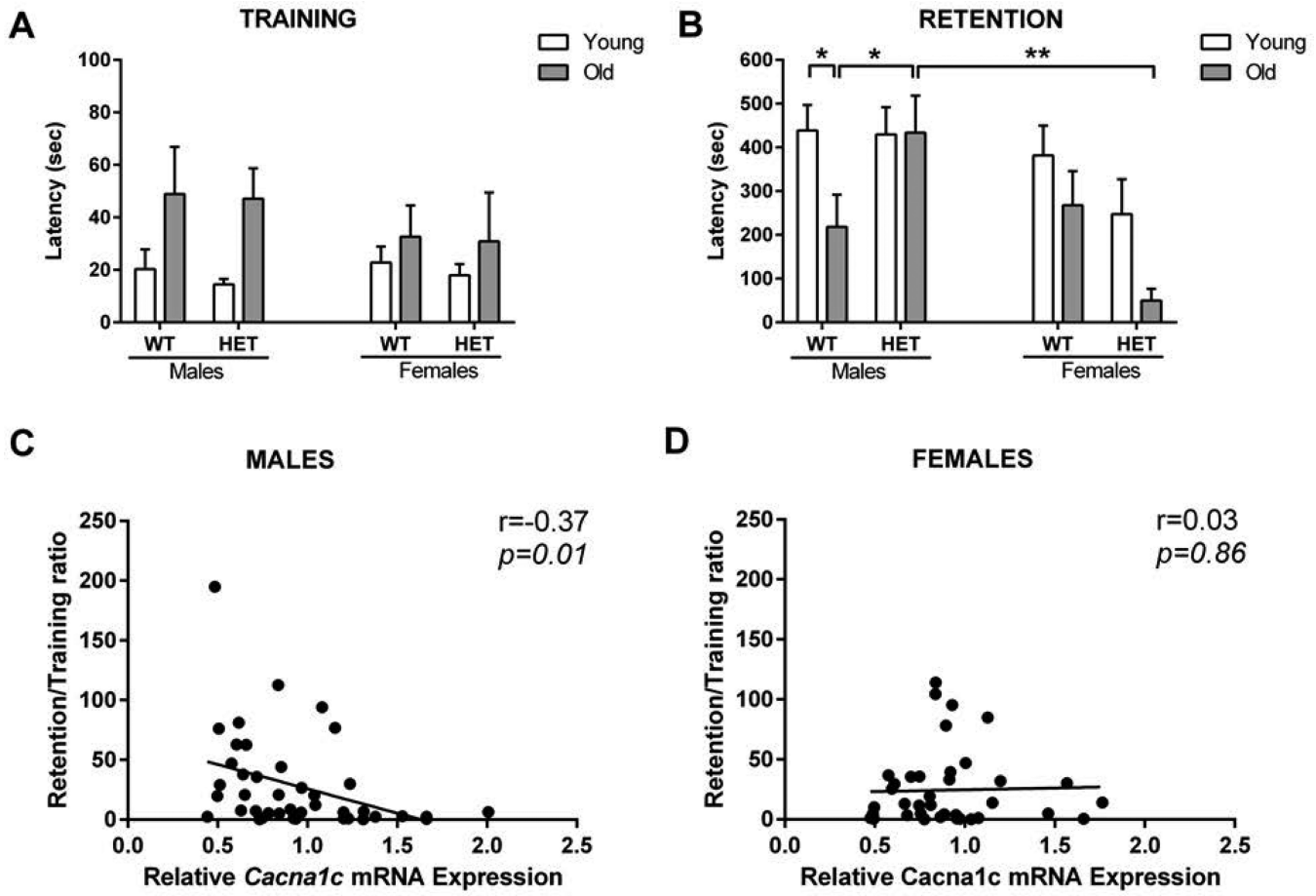

Figure 4. Passive avoidance task performance

(A) Aging increased the baseline latency to cross from the light to the dark compartment in both wild-type (WT) and *Cacna1c* haploinsufficient (HET) mice during the training session of the passive avoidance task (age effect: $p < 0.01$). **(B)** Aged WT, but not *Cacna1c* HET mice manifested impairment in passive avoidance learning, as indicated by decreased retention latency. In contrast, *Cacna1c* haploinsufficiency was not protective in the passive avoidance learning in aged female mice. Correlation analyses between ratio retention/training latency in the passive avoidance task and relative hippocampal *Cacna1c* levels indicated a negative correlation in **(C)** male but not **(D)** female mice. Data represent mean ± SEM (n = 7-14/group); * $p<0.05$, ** $p<0.01$.

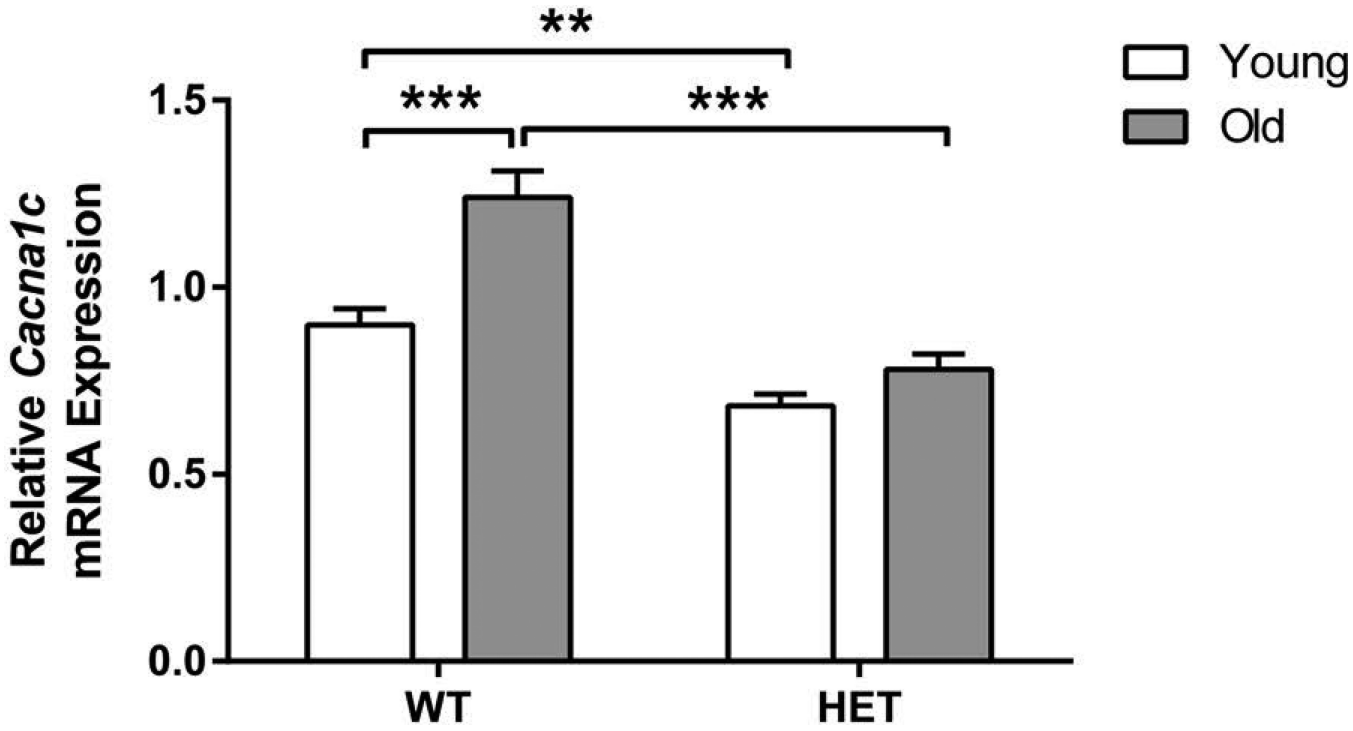

Figure 5. Hippocampal *Cacna1c* expression

Aging induced a significant increase in relative *Cacna1c* expression in the hippocampus of wild-type (WT) but not *Cacna1c* haploinsufficient (HET) mice. Relative *Cacna1c* levels did not change during aging in HET mice. Data represent mean ± SEM (n = 6-13/group); * *p<0.05*, ** *p<0.01*, *** *p<0.001*.