Surface L-type Ca\textsuperscript{2+} channel expression levels are increased in aged hippocampus

Félix Luis Núñez-Santana,\textsuperscript{1} Myongsoo Matthew Oh,\textsuperscript{1} Marcia Diana Antion,\textsuperscript{1} Amy Lee,\textsuperscript{2} Johannes Wilhelm Hell\textsuperscript{3} and John Francis Disterhoft\textsuperscript{1}

\textsuperscript{1}Department of Physiology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA
\textsuperscript{2}Departments of Molecular Physiology and Biophysics, Otolaryngology-Head and Neck Surgery, and Neurology, University of Iowa, Iowa City, IA 52242, USA
\textsuperscript{3}Department of Pharmacology, University of California, Davis, CA 95615, USA

Summary

Age-related increase in L-type Ca\textsuperscript{2+} channel (LTCC) expression in hippocampal pyramidal neurons has been hypothesized to underlie the increased Ca\textsuperscript{2+} influx and subsequent reduced intrinsic neuronal excitability of these neurons that lead to age-related cognitive deficits. Here, using specific antibodies against Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 subunits of LTCCs, we systematically re-examined the expression of these proteins in the hippocampus from young (3 to 4 month old) and aged (30 to 32 month old) F344xBN rats. Western blot analysis of the total expression levels revealed significant reductions in both Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 subunits from all three major hippocampal regions of aged rats. Despite the decreases in total expression levels, surface biotinylation experiments revealed significantly higher proportion of expression on the plasma membrane of Ca\textsubscript{v}1.2 in the CA1 and CA3 regions and of Ca\textsubscript{v}1.3 in the CA3 region from aged rats. Furthermore, the surface biotinylation results were supported by immunohistochimical analysis that revealed significant increases in Ca\textsubscript{v}1.2 immunoreactivity in the CA1 and CA3 regions of aged hippocampal pyramid neurons. In addition, we found a significant increase in the level of phosphorylated Ca\textsubscript{v}1.2 on the plasma membrane in the dentate gyrus of aged rats. Taken together, our present findings strongly suggest that age-related cognitive deficits cannot be attributed to a global change in L-type channel expression nor to the level of phosphorylation of Ca\textsubscript{v}1.2 on the plasma membrane of hippocampal neurons. Rather, increased expression and density of LTCCs on the plasma membrane may underlie the age-related increase in L-type Ca\textsuperscript{2+} channel activity in CA1 pyramid neurons.

Key words: biotinylation; Ca\textsubscript{v}1.2; Ca\textsubscript{v}1.3; calcium; phosphorylation; qRT-PCR.

Introduction

The calcium hypothesis of aging (Khachaturian, 1987; Landfield, 1987) posits that age-related cognitive deficits are mainly due to changes in cellular mechanisms that maintain and regulate intracellular Ca\textsuperscript{2+} homeostasis. Among them, change in Ca\textsuperscript{2+} channel number and/or function has been suggested to be a key factor (Khachaturian, 1994). While age-related increase in function of L-type Ca\textsuperscript{2+} channels (LTCCs) in CA1 pyramid neurons (Moyer & Disterhoft, 1994; Thibault & Landfield, 1996) and rescue of normal aging- and Alzheimer’s disease-related cognitive deficits with LTCC antagonists have been demonstrated (Deyo et al., 1989; Ban et al., 1990), there is conflicting evidence for altered number of LTCCs with aging. Increased (Herman et al., 1998; Chen et al., 2000; Veng & Browning, 2002), no change (Blaolck et al., 2003; Kadish et al., 2009), and reduced (Rowe et al., 2007) expression levels of the central pore-forming \(\alpha\)-subunits of the L-type Ca\textsuperscript{2+} channels Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 in hippocampus from aged animals have been reported. These apparently conflicting findings may be due to the level of analysis conducted: from single cell to whole hippocampus. Therefore, we systematically examined the expression levels of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 using Western blot, immunohistochemistry, and real-time quantitative PCR analysis in the three major hippocampal regions of young and aged rats.

Results

Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 protein levels are reduced in the aged hippocampus

Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 expression levels were examined in CA1, CA3, and dentate gyrus (DG) of young (\(N = 19\)) and aged (\(N = 19\)) rats using antibodies specific for the two \(\alpha\)-subunits of these LTCCs (Fig. 1, Fig. S1). We found significantly reduced expression of both Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 subunits in all three regions from aged rats (Fig. 2). Furthermore, the reductions were nearly identical for both subunits at each hippocampal region: 40% in CA3, 30% in CA1, and 10–20% in DG as compared with young adults (Fig. 2). Representative full-length blots from Western blot analyses are shown in Fig. S2.

This is the first demonstration that the protein levels of both LTCC \(\alpha\)-subunits are reduced throughout the hippocampus of aged rats. However, this raised a conundrum: How can there be increased Ca\textsuperscript{2+} conductance via LTCCs in CA1 pyramid neurons (Moyer & Disterhoft, 1994; Thibault & Landfield, 1996) with fewer pore-forming subunits? To address this question, we began by examining the level of the Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 subunits found on the plasma membrane.

Surface/total ratios of Ca\textsubscript{v}1.2 and Ca\textsubscript{v}1.3 are increased in aged hippocampus

We postulated that the relative ratios of Ca\textsubscript{v}1.2 and/or Ca\textsubscript{v}1.3 detected on the surface of cell membranes might be elevated in hippocampal tissue from aged rats. To test this hypothesis, we performed cell surface biotinylation assays (Thomas-Crusells et al., 2003) on dorsal hippocampal slices from young (\(N = 9\)) and aged (\(N = 9\)) rats.

The surface/total ratio of Ca\textsubscript{v}1.2 subunit was significantly increased in CA1 (37%) and CA3 (22%) regions of aged rats (Fig. 3A,C). A similar surface/total ratio increase was also observed for the Ca\textsubscript{v}1.3 subunit, but it was significant only in the CA3 (29%) and not in the CA1 (15%) region of aged rats (Fig. 3B,D).
While these results demonstrate that higher levels of LTCCs are present on the cell membrane, they did not provide the location of the increased surface expression. Therefore, we performed immunohistochemical analysis to identify the locus of the increase.

Cav1.2 immunoreactivity (Cav1.2-IR) is increased in somatic region of aged CA1 and CA3 neurons

CA1 pyramidal neurons from aged subjects have been shown to have increased LTCC activity (Thibault & Landfield, 1996) and enhanced calcium action potentials (Moyer & Disterhoft, 1994). Therefore, we postulated that increases in LTCC subunit expression would be observed mostly in the somatic region of aged CA1 pyramidal neurons.

We observed Cav1.2-IR within the hippocampal formation and hippocampal cell layers similar to previous reports (Hell et al., 1993; Clark et al., 2003; Hall et al., 2013). Significant increases in Cav1.2-IR were observed in the somatic regions of aged CA1 (Fig. 4) and CA3 (Fig. 5) pyramidal neurons. No change in Cav1.2-IR and expression was observed in DG granule cells (Fig. 6). Furthermore, no significant changes in Cav1.2 subunit expression were observed in stratum radiatum of CA1 (Fig. 4C) or CA3 (Fig. 5C) hippocampal region.

In addition, as LTCCs are found in glial cells and its expression has been documented to change with astrocyte activation after brain injury, trauma, and aging (Wisniewski & Terry, 1973; Vaughan & Peters, 1974; MacVicar, 1984; Westenbroek et al., 1998; Chung et al., 2001; Djamshidian et al., 2002; Finch, 2003; Xu et al., 2007), we also examined whether the observed plasma membrane increases in LTCC subunits with aging were of glial/astrocytic origin. No detectable expression/colocalization of LTCC subunits was observed in glial cells with our antibodies (Figs 4–6).

Parallel to Cav1.2, immunohistochemical experiments were conducted to assess changes in Cav1.3 protein expression at the cellular level. However, the presence of various nonspecific, high-intensity bands in our Cav1.3 Western blots (Fig. S2B,D) prevents us from confidently reporting our Cav1.3 immunohistochemical findings, as the obtained Cav1.3 immunoreactivity might be the results of nonspecific binding of our current antibody in brain tissue. Hence, it remains to be determined whether immunohistochemical analyses of Cav1.3 expression at the cellular level are consistent with our Cav1.3 biotinylation experiments.

![Fig. 1 Characterization of antibody specificity for Cav1.2 and Cav1.3 proteins. Hipocampal lysates from wild-type (WT) and L-type-deficient (KO) mice were resolved by SDS-PAGE and immunoblotted with either CNC1 (J.W. Hell), ab144 (A.L: Amy Lee), commercially available anti-Cav1.2 (Alo: Alomone Labs, ACC-003; NM: Neuromab Antibodies Inc. L57/46,) or commercially available anti-Cav1.3 (Alo: Alomone Labs, ACC-005; NM: Neuromab Antibodies Inc. N38/8) antibodies. Blots were developed using Amersham ECL Plus and Hyperfilm ECL. Both anti-Cav1.2 and anti-Cav1.3 antibodies from commercial sources revealed nonspecific bands in hippocampal lysates from KO tissue. CNC1 and ab144 showed no cross-reactivity with either Cav1.3 or Cav1.2 proteins in hippocampal lysates. Note that this example figure is assembled from multiple blots with similar exposure time that have been aligned for illustrative purposes only. See Fig. S1 for immunoblots as loaded in gel.](image_url)

![Fig. 2 Total Cav1.2 and Cav1.3 L-type calcium channel protein levels are reduced in all three major hippocampal regions of aged rats. Homogenates from whole CA3, DG, and CA1 of dorsal hippocampus (four 1-mm-thick slices per animal) were analyzed using semi-quantitative Western blotting techniques and immunoblotted using highly specific antibodies against Cav1.2 and Cav1.3 L-type calcium channel α1 subunits. (A, B) Representative Western blots comparing expression of Cav1.2 and Cav1.3 proteins in CA3, DG, and CA1 regions from two young and two aged rats. Young and aged CA3, DG, and CA1 region samples were resolved in pairs (side by side) on the same gel. Note that a shorter exposure time was used for DG region for the purpose of illustration (See Figs S2 and S5). (C, D) Quantitation of total L-type calcium channel expression normalized to GAPDH and relative to young for each region. All results were confirmed by repeating the experiments and analysis three times. Significant reductions in Cav1.2 and Cav1.3 were observed in all three major hippocampal regions of aged animals. Unpaired t-test: *P < 0.05, **P < 0.0001. Data reported as the mean ± SEM.](image_url)
Phosphorylation of surface-expressed Ca$_{1.2}$ is increased in the DG of aged rats

Increased LTCC activity by 4-6-fold has been reported when the Ca$_{1.2}$ subunits are phosphorylated (Sculptoreanu et al., 1993; Kavalali et al., 1997). In addition, Serine 1928 (S1928) (Davare & Hell, 2003) and Serine 1700 (S1700) (Fuller et al., 2010) of Ca$_{1.2}$ can be phosphorylated, but only S1928 phosphorylation has been shown to be increased with normal aging (Davare & Hell, 2003). Therefore, we further explored the possibility that more S1928 in Ca$_{1.2}$ might be phosphorylated in the hippocampal regions from aged rats. Biotinylated plasma membrane proteins were isolated and immunoblotted with anti-CH1923-1932P (p1928), an antibody designed to specifically detect Ca$_{1.2}$ when phosphorylated at S1928 (De Jongh et al., 1996; Davare & Hell, 2003). We found a significant 1.18-fold increase in phosphorylated Ca$_{1.2}$ in DG of aged rats (Fig. 7B). No significant age-related difference in phosphorylated Ca$_{1.2}$ was detected in either CA1 or CA3.

Ca$_{1.2}$ and Ca$_{1.3}$ mRNA levels are unchanged with aging

A positive correlation between Ca$_{1.3}$ mRNA and LTCC activity has been previously demonstrated in CA1 pyramidal neurons using single cell reverse transcription PCR experiments (Chen et al., 2000). Therefore, we performed a systematic assay to assess Ca$_{1.2}$ and Ca$_{1.3}$ mRNA levels in each hippocampal region using the real-time quantitative PCR method to determine whether the mRNA levels were altered with normal aging and/or in a specific hippocampal region(s). We found no significant age-related changes in mRNA levels for both Ca$_{1.2}$ and Ca$_{1.3}$ (Table 1, Fig. S4).

Discussion

The present study is the first to demonstrate that the pore-forming α$_{1}$ subunits for the L-type voltage-gated Ca$^{2+}$ channels (Ca$_{1.2}$ and Ca$_{1.3}$) are significantly reduced in whole tissue lysates from all three major hippocampal regions of aged rats (Fig. 2). However, the biotinylation and immunohistochemical data demonstrate that age-related increases in Ca$_{1.2}$ are observed in CA1 and CA3 regions. Notably, the increase in the Ca$_{1.2}$ subunit was in the somatic region of CA1 and CA3 pyramidal neurons (Figs 4 and 5). In addition, no detectable expression/colocalization of Ca$_{1.2}$ subunits was observed in glial cells with our antibodies (Figs 4–6). Therefore, the present results support the ‘calcium hypothesis of aging’ (Khachaturian, 1987; Landfield, 1987) in that the age-related increase in surface expression of L-type voltage-gated Ca$^{2+}$ channels (Ca$_{1.2}$ and Ca$_{1.3}$) in hippocampal pyramidal neurons we demonstrate may play an important role in the cognitive deficits observed in normal aging subjects.

Phosphorylation of Ca$_{1.2}$ LTCC has been previously shown to enhance Ca$^{2+}$ influx (Sculptoreanu et al., 1993; Kavalali et al., 1997). Ca$_{1.2}$ α$_{1}$ subunit can be phosphorylated at Serine 1928 (S1928) (Davare & Hell, 2003) and at Serine 1700 (S1700) (Fuller et al., 2010), and it has been suggested that S1700 phosphorylation plays a greater modulatory role than S1928 phosphorylation (Brandmayr et al., 2012). However, only S1928 phosphorylation has been shown to be increased in the hippocampus with normal aging (Davare & Hell, 2003). Similarly, we also found significant age-related increase in S1928 phosphorylation, but only in the DG with no apparent changes in other hippocampal regions (Fig. 7). The difference with the previous report (Davare & Hell, 2003) may be due to our focus on the phosphorylation of cell surface channels in each hippocampal region of the dorsal hippocampus, whereas the previous report examined S1928 phosphorylation in the entire hippocampus. In addition, we found high level of Ca$_{1.2}$ protein in the DG as compared with CA1 and CA3 from whole tissue lysate (Fig. S5). Furthermore, no age-related changes in the Ca$^{2+}$-dependent postburst afterhyperpolarization have been observed in DG granule cells (Baskys et al., 1987; Niesen et al., 1988; Reynolds & Carlen, 1989). Therefore, S1928 phosphorylation cannot account for increased calcium influx and reduced neuronal excitability with normal
Fig. 4 Expression of Ca\textsubscript{1.2} L-type subunit in soma and radiatum of young and aged CA1 pyramidal neurons. (A) Representative confocal images of hippocampal CA1 pyramidal layer sections showing immunohistochemical labeling for Ca\textsubscript{1.2} (CNC1) of a young and an aged rat. Six regions of interest (box) with equal dimensions in both the stratum pyramidale (3) and the stratum radiatum (3) layers of CA1 were drawn to collect immunofluorescence data. (B) Quantitative analysis of integrated fluorescence intensity in soma of CA1 pyramidal neurons of young and aged rats. (C) Quantitative analysis of integrated fluorescence intensity in the stratum radiatum of young and aged rats. Significant increases in somatic expression of Ca\textsubscript{1.2} subunit were observed in aged CA1 pyramidal neurons (B) ($P < 0.05$). No significant differences in Ca\textsubscript{1.2} subunit expression were detected between stratum radiatum of young and aged rats. No colocalization of CNC1 was observed in glial cells. AutoQuant image deconvolution software (Media Cybernetics, Rockville, MD) was used to reduce background signal for the purpose of illustration. Fluorescence intensities and analyses were performed using raw, unmodified, images. Data reported as the mean ± SEM.

Fig. 5 Expression of Ca\textsubscript{1.2} L-type subunit in soma and radiatum of young and aged CA3 pyramidal neurons. (A) Representative confocal images of hippocampal CA3 pyramidal layer sections showing immunohistochemical labeling for Ca\textsubscript{1.2} (CNC1) of a young and an aged rat. Six regions of interest (box) with equal dimensions in both the stratum pyramidale (3) and the stratum radiatum (3) layers of CA3 were drawn to collect immunofluorescence data. (B) Quantitative analysis of integrated fluorescence intensity in soma of CA3 pyramidal neurons of young and aged rats. (C) Quantitative analysis of integrated fluorescence intensity in the stratum radiatum of young and aged rats. Significant increases in somatic expression of Ca\textsubscript{1.2} subunit were observed in aged CA3 pyramidal neurons (B) ($P < 0.05$). No significant differences in Ca\textsubscript{1.2} subunit expression were detected between stratum radiatum of young and aged rats. No colocalization of CNC1 was observed in glial cells. AutoQuant image deconvolution software (Media Cybernetics, Rockville, MD) was used to reduce background signal for the purpose of illustration. Fluorescence intensities and analyses were performed using raw, unmodified, images. Data reported as the mean ± SEM.
We found no age-related changes in Ca\textsubscript{1,2} or Ca\textsubscript{1,3} mRNA expression in the present study. Previous reports examining the mRNA or \textit{LTCC} \textalpha-subunits in hippocampus from aged animals have been inconsistent; with groups reporting an increase (Herman et al., 1998; Chen et al., 2000; Veng & Browning, 2002), no change (Blalock et al., 2003; Kadish et al., 2009), or reductions (Rowe et al., 2007) in mRNA or \textalpha-subunit expression with aging. The discrepancy between the findings may be due to the anatomical specificity and/or method of analysis used in the studies (i.e. differences in splice variants amplified by the different primers of different groups). At single cell resolution, a positive correlation between Cav1.3 mRNA levels and functional channel density in the adult and aged CA1 pyramidal neurons has been reported: that is, the more Cav1.3 mRNA, the greater the LTCC activity (Chen et al., 2000). Using whole hippocampus, a report using semi-quantitative RNAse protection analysis revealed that Cav1.2 and Cav1.3 mRNA expression levels are increased in aged rats (Herman et al., 1998), whereas no change in either mRNA (Blalock et al., 2003; Kadish et al., 2009) or a reduction in Cav1.2 mRNA (Rowe et al., 2007) were reported using microarray methods. Our present real-time quantitative PCR data support the lack of change in Cav1.2 or Cav1.3 mRNA expression level with normal aging in the subregions of the hippocampus (Table 1). Notably, we also observed that the level of Cav1.2 and Cav1.3 mRNA expression levels were different than that for the protein levels: for protein, DG > CA3 ≥ CA1 (Fig. S5); for mRNA, CA3 ≥ CA1 > DG (Table 1). Furthermore, a 4-fold increase in Cav1.2 mRNA relative to Cav1.3 mRNA was observed in the CA1 region, which supports the previous reports that approximately 80% of LTCCs are Cav1.2 channels (Hell et al., 1993; Clark et al., 2003).

The exact mechanism by which LTCC activity is increased with aging in CA1 neurons is as yet unclear. While our findings provide a better understanding of the processes that take place during aging, we cannot exclude the role that other processes may have in channel function, activity, and intracellular Ca\textsuperscript{2+} concentrations. For example, the activity of the pore-forming LTCC \textalpha-subunits can be regulated by...
L-type Ca\textsuperscript{2+} channels are increased with aging. F. L. Núñez-Santana et al.

Table 1  L-type calcium channel mRNA levels within the hippocampal subregions are not altered by normal aging

|        | CA1                  | CA3                  | DG       |
|--------|----------------------|----------------------|----------|
|        | \( \Delta C_{Y}^{\text{YOUNG}} \) | \( \Delta C_{Y}^{\text{OLD}} \) | \( \Delta C_{Y}^{\text{YOUNG}} \) | \( \Delta C_{Y}^{\text{OLD}} \) | \( \Delta C_{Y}^{\text{YOUNG}} \) | \( \Delta C_{Y}^{\text{OLD}} \) |
| Ca\textsubscript{1,2} RT-PCR |
| Pair 1 | 5.66 | 5.66 | 4.94 | 5.15 | 6.72 | 7.02 |
| Pair 2 | 5.68 | 5.30 | 5.04 | 5.10 | 7.15 | 6.96 |
| Pair 3 | 5.54 | 5.34 | 4.79 | 4.68 | 6.68 | 7.02 |
| Pair 4 | 5.44 | 5.73 | 4.86 | 5.20 | 7.05 | 7.27 |
| Pair 5 | 5.85 | 5.52 | 5.02 | 4.90 | 6.92 | 6.73 |
| Pair 6 | 5.69 | 5.90 | 4.95 | 4.73 | 7.03 | 6.80 |
| Pair 7 | 5.64 | 5.38 | 4.84 | 4.69 | 6.78 | 6.92 |
| Average \( \pm SD \) | 5.64 \( \pm 0.13 \) | 5.55 \( \pm 0.22 \) | 4.92 \( \pm 0.09 \) | 4.92 \( \pm 0.23 \) | 6.90 \( \pm 0.18 \) | 6.96 \( \pm 0.17 \) |
| \( \Delta \Delta C_{T} (\Delta C_{T}^{\text{OLD}} - \Delta C_{T}^{\text{YOUNG}}) \) | -0.10 \( \pm 0.26 \) | 0.00 \( \pm 0.25 \) | 0.06 \( \pm 0.25 \) |
| Fold change (2\(^{-\Delta \Delta C_{T}}\)) | 1.07 | 1.00 | 0.96 |
| Ca\textsubscript{1,3} RT-PCR |
| Pair 1 | 7.29 | 7.30 | 5.78 | 5.91 | 6.76 | 7.19 |
| Pair 2 | 7.10 | 6.96 | 5.78 | 5.77 | 7.14 | 7.44 |
| Pair 3 | 7.13 | 7.12 | 5.54 | 5.59 | 7.10 | 7.49 |
| Pair 4 | 6.85 | 7.25 | 5.59 | 5.91 | 7.24 | 7.19 |
| Pair 5 | 7.30 | 7.05 | 5.89 | 5.75 | 7.06 | 6.96 |
| Pair 6 | 7.42 | 7.30 | 5.56 | 5.57 | 7.22 | 7.20 |
| Pair 7 | 7.06 | 6.78 | 5.63 | 5.53 | 7.32 | 7.39 |
| Average \( \pm SD \) | 7.16 \( \pm 0.19 \) | 7.11 \( \pm 0.19 \) | 5.68 \( \pm 0.13 \) | 5.72 \( \pm 0.16 \) | 7.12 \( \pm 0.18 \) | 7.27 \( \pm 0.18 \) |
| \( \Delta \Delta C_{T} (\Delta C_{T}^{\text{OLD}} - \Delta C_{T}^{\text{YOUNG}}) \) | -0.05 \( \pm 0.07 \) | 0.04 \( \pm 0.21 \) | 0.15 \( \pm 0.26 \) |
| Fold change (2\(^{-\Delta \Delta C_{T}}\)) | 1.04 | 0.98 | 0.96 |

Total RNA was isolated from major hippocampal regions of young and aged rats. Equivalent amounts were converted into cDNA. Real-time quantitative PCR was performed on triplicates of subject for Ca\textsubscript{1,2}, Ca\textsubscript{1,3}, and GAPDH. \( \Delta C_{T} \) is calculated by subtracting threshold fluorescence of internal housekeeping gene GAPDH, for example, \( (C_{T,\text{GAPDH}}^{\text{OLD}} - C_{T,\text{GAPDH}}^{\text{YOUNG}}) \).

protein–protein interactions, a number of which can enhance L-type channel function (Catterall, 2000; Calin-Jageman & Lee, 2008). Second, calcineurin expression levels and activity have also been shown to be increased in hippocampus from aged animals (Foster et al., 2001; Norris et al., 2005; Eto et al., 2008) and inhibiting it reduces LTCC activity (Norris et al., 2002, 2010) and the Ca\textsuperscript{2+}-dependent postburst afterhyperpolarization (AHP) (Vogalis et al., 2004). Third, post-translational modifications of the Ca\textsubscript{1,2} LTCC protein by proteolytic cleavage of the C-terminus region can significantly impact voltage-dependent activation and activity of the channel (Wei et al., 1994; Hulme et al., 2006). Finally, Ca\textsubscript{1,2} and Ca\textsubscript{1,3} channels are regulated by proteosomal degradation (Altier et al., 2011; Gregory et al., 2011) and oxidative stress through the action of reactive oxygen species, which can lead to increased accumulation of Ca\textsuperscript{2+} inside hippocampal neurons. (Kouroie, 1998; Fusi et al., 2001).

Age-related increase in LTCC function, specifically in CA1 pyramidal neurons, has been a popular hypothesis to explain age-related cognitive deficits (Disterhoft & Oh, 2006; Foster, 2007; Thibault et al., 2007). Previous reports have demonstrated that Ca\textsuperscript{2+} influx into neurons is significantly increased in CA1 pyramidal neurons from aged animals (Moyer & Disterhoft, 1994; Thibault & Landfield, 1996) due to increased density of LTCCs (Thibault & Landfield, 1996), which leads to reduced intrinsic excitability (Landfield & Pitler, 1984; Thompson et al., 1990; Moyer et al., 1992) and synaptic plasticity (Norris et al., 1998). Rescue of age-related cognitive deficits (Deyo et al., 1989) and restoration of intrinsic neuronal excitability (Moyer et al., 1992; Norris et al., 1998) and synaptic plasticity (Norris et al., 1998) with the use of LTCC blockers (e.g., nimodipine and nifedipine) have further provided support for the previously held viewpoint. In addition to the enhanced Ca\textsuperscript{2+} influx via LTCCs, Ca\textsuperscript{2+} released from the endoplasmic reticulum through ryano-
Materials and methods

Subjects

Young adults (3–4 month old) and aged (30–32 month old) male F1 hybrid Fischer 344 X Brown Norway rats (F344xBN; Harlan, Indianapolis, IN, USA) were used in this study. Rats were group housed with ad libitum access to food and water and maintained in a climate-controlled room with a 14:10 h light/dark cycle. The F344xBN rats are long-lived with >50% survival rate at 34 months of age (National Institute on Aging, Aged Rodent Colonies Handbook, www.nia.nih.gov/research/dab/aged-rodent-colonies-handbook/strain-survival-information) and have significantly less pathological complications with normal aging as compared with the Fischer 344 (F344) rats (Bronson, 1990; Lipman et al., 1996). Every effort was made to ensure that only healthy rats were included in the experiments. Rats with palpable tumors, skin ulcers, infections, or difficulty moving were excluded from the studies. All experimental procedures were approved by the Northwestern University Animal Care and Use Committee and conformed to NIH standards (NIH Publications No. 80-23). All efforts were made to minimize animals’ discomfort and the number of animals used.

Antibodies

The previously characterized rabbit anti-Ca$_{v}$1.2 (CNC1) antibody (provided by J.W. Hell) was raised against a peptide covering residues 818–835 within the cytoplasmic loop between domains II and III of the Ca$_{v}$1.2 protein (Hellt et al., 1993; Hall et al., 2013). The rabbit anti-Ca$_{v}$1.3 (Ab144) antibody (provided by A. Lee) was raised against a synthetic peptide corresponding to Ca$_{v}$1.3 N-terminal sequence (MQHQRQQQED-HANEANYARGTRKC; Covance Research Products, Denver, PA, USA) (Jenkins et al., 2010; Gregory et al., 2011). Specificity is further demonstrated in Fig. S1. The rabbit anti-CH1923-1932P (p1928) antibody, which specifically binds to phosphorylated Ca$_{v}$1.2 when phosphorylated at Serine 1928, was raised against a phosphopeptide corresponding to Cav1.3 N-terminal sequence (MQHQRQQQED-HANEANYARGTRKC; Covance Research Products, Denver, PA, USA) (Jenkins et al., 2010; Gregory et al., 2011). Specificity is further demonstrated in Fig. S1. The rabbit anti-CH1923-1932P (p1928) antibody, which specifically binds to phosphorylated Ca$_{v}$1.2 when phosphorylated at Serine 1928, was raised against a phosphopeptide consisting of residues 1923–1932 (De Jongh et al., 1996; Davare & Hell, 2003). Rabbit polyclonal anticalpain antibody was obtained from Abcam (Cambridge, MA, USA) and chicken anti-glia fibrillary acidic protein (GFAP) antibody was obtained from Millipore (Temecula, CA, USA).

Sample preparation and Immunoblotting

Whole dorsal hippocampi from 19 young and 19 aged rats were dissected out and immediately placed in cold (approximately 0 °C) oxygenated (95% O$_2$/5% CO$_2$) aCSF (in mM: 124 NaCl, 1.25 NaH$_2$PO$_4$, 2.5 KCl, 26 NaHCO$_3$, 1.25 glucose, 2.4 CaCl$_2$, and 2.0 MgSO$_4$; pH 7.4) before transverse dorsal hippocampal slices (400 μm) were made using a manual tissue slicer (Stoelting Co., Wood Dale, IL, USA). The hippocampal slices were then transferred to fresh oxygenated ice-cold aCSF containing several protease and phosphatase inhibitors (1 μg mL$^{-1}$ pepstatin A, 10 μg mL$^{-1}$ leupeptin, 20 μg mL$^{-1}$ aprotonin, 200 μM phenylmethylsulfonyl fluoride, 8 μg mL$^{-1}$ calpain inhibitor I, 8 μg mL$^{-1}$ calpain inhibitor II, 1 mM p-nitrophenyl phosphate, 50 mM NaF, 20 mM sodium pyrophosphate, and 4 μM microcystin LR) and immediately microdissected under a microscope (Zeiss Stemi DV4) to yield the three major hippocampal subdivisions (CA1, CA3, and Dentate Gyrus) (Coultrap et al., 2005). Each microdissected region was individually homogenized and sonicated in 1% Triton X-100 lysis buffer containing protease and phosphatase inhibitors and cleared by ultracentrifugation. Protein concentration was determined by the BCA assay using bovine serum albumin (BSA) as a standard (Pierce, Rockford, IL, USA).

For quantification of total L-type Ca$^{2+}$ channel expression in the three major hippocampal regions of young and aged rats, samples containing equal amounts of proteins (20 μg) were resolved by SDS-PAGE and analyzed by immunoblotting with either anti-CNC1 or Ab144. Fresh blots were used for each channel of interest, and each blot was reprobed with GAPDH for normalization and to control for variability during sample loading. Immunoreactive bands were visualized using a ChemiDoc XR+ Molecular Image System with Image Lab™ Software (Bio-Rad Laboratories, Hercules, CA, USA), and only signals doubling with increasing exposure times were used for quantification and analysis. All immunoblots were measured and quantified by densitometry using NIH ImageJ image analysis software (rsb.info.nih.gov/ij).

Whole slice cell surface biotinylation

To assess the relative cell surface expression of L-type Ca$^{2+}$ channels in hippocampal pyramidal neurons, cell surface biotinylation experiments were conducted on acute hippocampal slices (n = 8, 250 μm slices per rat) from nine young and nine aged rats. This technique has been successfully shown to reach all layers of acute hippocampal slices of up to 400 μm in thickness using Sulfo-NHS–SS–biotin as labeling reagent with very low to no labeling of intracellular proteins (Thomas-Crusells et al., 2003). Alternate slices from the dorsal half of both left and right hippocampi were exposed to 1 mg mL$^{-1}$ Sulfo-NHS–SS–biotin-labeling reagent (Pierce) for 30 min before separating each hippocampal region for processing and isolation of surface proteins using streptavidin magnetic beads (Pierce).

Immunoblotting with p1928, CNC1, and Ab144 antibody was performed after SDS-PAGE separation of total region lysates (input) and biotinylated (surface) fraction proteins. GAPDH was used as both loading and internal protein control to confirm the success of the biotinylation procedures (Fig. S3).

L-type Ca$^{2+}$ channel surface expression and phosphorylation of Ca$_{v}$1.2 measurements

Following Western blot analysis, optical density values for total lysate (input) and biotinylated (surface) fraction proteins. GAPDH was used as both loading and internal protein control to confirm the success of the biotinylation procedures (Fig. S3).

RNA isolation and cDNA synthesis

CA1, CA3, and DG regions were isolated from young and aged rats in pairs, homogenized in RPLT-Plus Lysis Buffer (Qiagen, Valencia, CA, USA).

© 2013 The Authors. Aging Cell published by the Anatomical Society and John Wiley & Sons Ltd.
and stored at −80 °C until RNA isolation. Samples were further dissociated with QiaShredder columns, and the total RNA was isolated via Qiagen RNEasy Plus Kit according to manufacturer’s directions. RNA was dissolved into 60 μL RNase-free water, stored on ice, and the yield was determined with a nanodrop spectrophotometer (Thermo-Scientific, Rockford, IL, USA). 650 ng (CA1, CA3) or 240 ng (DG) of total RNA was converted into cDNA with reverse transcriptase and multiple primers (qScript™ cDNA SuperMx; Quanta Biosciences, Gaithersburg, MD, USA) on a thermocycler per manufacturer’s instructions and stored at −20 °C until use. Synthesis of cDNA was commenced within 2 h of RNA isolation.

**Immunohistochemistry**

Young (3–4 month old) and aged (30–32 month old) male F344xBN rats were anesthetized and intracardially perfused with ice-cold sodium phosphate buffer (0.1 m PB [pH 7.4]) supplemented with several protease and phosphatase inhibitors (PPI; cComplete and PhosSTOP Inhibitor Cocktail Tablets; Roche, Indianapolis, IN, USA) and followed by ice-cold 4% paraformaldehyde in PB (supplemented with PPI). Brains were removed, postfixed (overnight), and cryoprotected by successively sinking in 10% (w/v) and 30% (w/v) sucrose in PB at 4 °C for 72 h (Marshall et al., 2011). Forty-micrometer-thick coronal sections containing the hippocampus were made, hippocampus dissected out, and stored at −20 °C in cryoprotectant solution (0.1 m PB, pH 7.4, 30% (w/v) sucrose, 30% (w/v) ethylene glycol and 1% (w/v) polyvinylpyrrolidone) (Watson et al., 1986) until processed for immunohistochemistry.

Immunohistochemistry (IHC) was performed as previously described (Ferraguti et al., 2004; Wu et al., 2008) with some modifications. The tissue processing and data collection and analysis were performed blind to the age of the animals. Five hippocampal slices from each animal were systemically and randomly selected for double immunolabeling with antibodies against glial fibrillary acidic protein (GFAP) and Cav1.2 or Cav1.3 and stored at −80 °C until RNA isolation. Samples were anesthetized and intracardially perfused with ice-cold sodium phosphate buffer (0.1 m PB [pH 7.4]) supplemented with several protease and phosphatase inhibitors (PPI; cComplete and PhosSTOP Inhibitor Cocktail Tablets; Roche, Indianapolis, IN, USA) and followed by ice-cold 4% paraformaldehyde in PB (supplemented with PPI). Brains were removed, postfixed (overnight), and cryoprotected by successively sinking in 10% (w/v) and 30% (w/v) sucrose in PB at 4 °C for 72 h (Marshall et al., 2011). Forty-micrometer-thick coronal sections containing the hippocampus were made, hippocampus dissected out, and stored at −20 °C in cryoprotectant solution (0.1 m PB, pH 7.4, 30% (w/v) sucrose, 30% (w/v) ethylene glycol and 1% (w/v) polyvinylpyrrolidone) (Watson et al., 1986) until processed for immunohistochemistry.

Image analysis

Confocal images from CA1 region of hippocampus were obtained at a magnification of 40× using a Nikon Eclipse C1si Spectral Imaging Confocal Microscope System at the Nikon Imaging Center and Cell Imaging Facility at Northwestern University. Exposure parameters for Ca1.2, Ca1.3, and GFAP were standardized across all captured images and maintained throughout image acquisition for both young and aged hippocampal slices. Images were analyzed using MetaMorph™ imaging software (Molecular Devices, Sunnyvale, CA, USA), and statistical analyses were performed using StatView software. To study age-related changes in L-type subunit expression in CA1 and CA3, data were collected from raw, unmodified, images by drawing three rectangular regions of interest (ROI) with equal dimensions in both the stratum pyramidale and the stratum radiatum layers of CA1 and CA3 regions. For DG, ROIs were placed atop the granular cell layer. The fluorescence intensity for each ROI was then averaged to calculate the integrated fluorescent intensity for each hippocampal slice. Averaged values from all 5 hippocampal slices from each animal were then averaged to collect the animal’s integrated fluorescent intensity used for plotting and statistical analysis. Significant group differences in protein expression were evaluated using analysis of variance (ANOVA) with statistical significance set to P < 0.05. All data are reported as the means ± SEM.

**Real-time PCR of mRNA levels**

Primer sequences compatible with rat and mouse were a gift from C. Savio Chan (Northwestern University). The primers were designed to bridge exons of cDNA to eliminate concern of genomic DNA contamination and previously tested for comparable efficiency during PCR. Primers include Cacna1c, bridging exons 7–8 [sense primer-GGCATCACCACAATTGCA, antisense Primer- TACACCGGGCCACACTATA], Cacna1d, bridging exons 41–42 [sense primer-TGACATTGGGCCAGAAATCC, antisense primer- GGTGGTATTGTGTGCTGGAA, and GAPDH, bridging exons 3–4 [sense primer- GCTGAGTATGTCGTGGAGTCTA, antisense primer- TTCCTGGTGTTACACCCAT]. For real-time quantitative PCR, 1 pair of young and aged cDNA (1 μL) triplicate samples from each of CA1, CA3, and DG were measured in parallel for threshold fluorescence accumulation (Ct) of each gene target (Cacna1c, Cacna1d, and GAPDH) in a 96-well tray with a Step One Plus™ Applied Biosystems QPCR Machine using SYBR Green as a reporter and ROX™ dye as a passive reference control. After PCR, a melt curve analysis was done for each sample to ensure that the primers were specific. The threshold Ct was manually set to be 1.1 ±Rn (reporter-reference control baseline fluorescence) for all targets and fell within the exponential phase of amplification. The comparative ΔCt method described by Livak and Schmittgen (Livak & Schmittgen, 2001) was used to compare young and aged samples with GAPDH, which was used as internal housekeeping control. ΔCt for each sample target gene was calculated as follows: ΔCt = (mean CtTarget – mean CtGAPDH). To obtain fold change due to age, ΔΔCt was calculated with the following equation: (ΔCtAGED - ΔCtYOUNG) and presented in Table 1.

**Statistical analysis**

All statistical analyses were performed using StatView analysis software, and significant group differences in protein expression were evaluated using analysis of variance (ANOVA) with statistical significance set to P < 0.05. All data are reported as the mean ± SEM. Duplicates were performed on all reported phosphorylation and surface expression assays.

**Acknowledgments**

The authors thank Dr. Geoffrey G. Murphy (University of Michigan, Ann Arbor, MI) for generously providing us with Ca1.2 and Ca1.3 KO
tissue; Dr C. Savio Chan and Vivian Hernandez for technical assistance, reagents, and use of real-time PCR equipment; Drs. Johannes W. Hell and Amy Lee for their contributions to conceptual discussions and practical input; Drs. Geoffrey T. Swanson, Bryan A. Copits, and Jeffery Burgdorf for discussions regarding biotinylation experiments; Drs. Murali Prakriya, Robert Vassar, and Peter Penzes for valuable discussions during the study, and Dr. Dina Simkin for critical review of the manuscript. Immunohistochemical data were collected and analyzed at the Northwestern University Cell Imaging Facility generously supported by NCI CCSG P30 CA060553 awarded to the Robert H Lurie Comprehensive Cancer Center. This work was supported by National Institutes of Health Grants AG008796 (J.F.D.), AG017139 (J.F.D.), AG017502 (J.W.H.), DC009433 (A.L.), and HL087120 (A.L.), and a Carver Research Program of Excellence Award (A.L.).

Author contributions
F.L.N., M.M.O. and J.F.D. designed research; F.L.N. and M.D.A. performed research; F.L.N. and M.D.A. analyzed data; J.W.H. and A.L. contributed reagents; M.M.O., F.L.N. and J.F.D. drafted the manuscript; All authors wrote/edited the paper.

Conflict of interest
None.

References
Altem, C., Garcia-Caballero, A., Simms, B., You, H., Chen, L., Walcher, J., Tedford, H.W., Hermosilla, T., Zamponi, G.W. (2011) The Cavbeta subunit prevents RPF2-mediated ubiquitination and proteasomal degradation of L-type channels. Nat. Neurosci. 14, 173-180.
Ban TA, Morey L, Anguilla E, Azzarelli O, Balsano F, Marigliano V, Caglieris N, et al. (2003) Gene microarrays in hippocampal aging: statistical profiling identifies novel mechanisms of old age dementia. Prog. Neuropsychopharmacol. Biol. Psychiatry 27, 525-551.
Baskys A, Niesen CE, Carlisle PL (1987) Altered modulatory actions of serotonin on neuronal L-type voltage-dependent Ca2+ channels. Neuroscience 23, 587-589.
Barrows RT (1990) Genetic Effects of Aging. II. Caldwell, NJ: Telford Press.
Cai Y, Chen KC, Sharrow K, Herman JP, Porter NM, Foster TC, Landfield PW (2003) Gene microarrays in hippocampal aging: statistical profiling identifies novel mechanisms of old age dementia. Prog. Neuropsychopharmacol. Biol. Psychiatry 27, 525-551.
Bronson RT (1990) Genetic Effects of Aging II. Caldwell, NJ: Telford Press.
Cai Y, Chen KC, Sharrow K, Herman JP, Porter NM, Foster TC, Landfield PW (2003) Gene microarrays in hippocampal aging: statistical profiling identifies novel mechanisms of old age dementia. Prog. Neuropsychopharmacol. Biol. Psychiatry 27, 525-551.
Chen KC, Blalock EM, Thibault O, Kaminker P, Landfield PW (2000) Expression of alpha 1D subunit mRNA is correlated with L-type Ca2+ channel activity in single neurons of hippocampal “zipper” slices. Proc. Natl Acad. Sci. USA 97, 4357-4362.
Chung YH, Shin CM, Kim MJ, Chi CI (2001) Enhanced expression of L-type Ca2+ channels in reactive astrocytes after ischemic injury in rats. Neurosci. Lett. 302, 93-96.
Clark NC, Nagano N, Kuenzi FM, Jarolmek W, Huber I, Walter D, Wietzerbel R, Boyce S, Kullmann DM, Stresing J, Seabrook GR (2003) Neurological phenotype and synthetic function in mice lacking the Cav1.3 alpha subunit of neuronal L-type voltage-dependent Ca2+ channels. Neuroscience 120, 435-442.
Coultrap SJ, Nixon KM, Alvestad RM, Valenzuela CF, Browning MD (2005) Differential expression of NMDA receptor subunits and splice variants among the CA1, CA3 and dentate gyrus of the adult rat. Brain Res. Mol. Brain Res. 135, 104-111.
Davies MA, Hell JW (2003) Increased phosphorylation of the neuronal L-type Ca2+ (2+)-channel upon aging. Proc. Natl Acad. Sci. USA 100, 16018-16023.
De Jongh KS, Murphy BJ, Colvin AA, Hell JW, Takashashi M, Catterall WA (1996) Specific phosphorylation of a site in the full-length form of the alpha 1 subunit of the cardiac L-type calcium channel by adenosine 3',5'-cyclic monophosphate-dependent protein kinase. Biochemistry 35, 10392-10402.
Deyo RA, Straube KT, Disterhoft JF (1988) Nimodipine facilitates associative learning in aging rabbits. Science 243, 809-811.
Disterhoft JF, Oh MM (2006) Learning, aging and intrinsic neuronal plasticity. Trends Neurosci. 29, 587-599.
Djoumbio B, Grassl R, Seltenhammer M, Czech T, Baumgartner C, Schmidtmaier M, Ulrich W, Zimprich F (2002) Altered expression of voltage-dependent calcium channel alpha(1) subunits in temporal lobe epilepsy with Ammon's horn sclerosis. Neuroscience 111, 57-69.
Etro R, Abe M, Hayakawa N, Kato H, Araki T (2008) Age-related changes of calcineurin and Akt1/protein kinase Balb (Akt1/PKBalpha) immunoreactivity in the mouse hippocampal CA1 sector: an immunohistochemical study. Metab. Brain Dis. 23, 399-409.
Ferragu F, Cobioli P, Pollard M, Cope D, Ishigemoto R, Watanabe M, Somogyi P (2000) Immunolocalization of metabotropic glutamate receptor 1alpha (mGluR1alpha) in distinct classes of interneuron in the CA1 region of the rat hippocampus. hippocampus. Hippocampus 14, 193-215.
Finch CE (2003) Neurons, glia, and plasticity in normal brain aging. Neurobiol. Aging 24(Suppl. 1), S123–S127; discussion S131.
Foster TC (2007) Calcium homeostasis and modulation of synaptic plasticity in the aged brain. Aging Cell 6, 319-325.
Foster TC, Sharrow KM, Masse JR, Norris CM, Kumar A (2001) Calcineurin links Ca2+ dysregulation with brain aging. J. Neurosci. 21, 4066-4073.
Fuller MD, Emrick MA, Sadleik M, Scheuer T, Catterall WA (2010) Molecular mechanism of calcium channel regulation in the fight-or-flight response. Sci. Signal. 3, ra 70.
Fusi F, Saponara S, Gagov H, Spargari G (2001) 2,5-Di-t-butyl-1,4-benzohydroquinone (BHQ) inhibits vascular L-type Ca2+ channel via superoxide anion generation. Br. J. Pharmacol. 133, 988-996.
Gallagher M, Burwell R, Burchinal M (1993) Severity of spatial learning impairment in aging: development of a learning index for performance in the Morris water maze. Behav. Neurosci. 107, 618-626.
Gant JC, Sama MM, Landfield PW, Thibault O (2006) Early and simultaneous emergence of multiple hippocampal biomarkers of aging is mediated by Ca2+ -induced Ca2+ release. J. Neurosci. 26, 3482-3490.
Gregory FD, Bryan KE, Pangris T, Calin-Jageman IE, Moser T, Lee A (2011) Harmonin inhibits preyspecific Cid/1.3 Cx(2+) channels in mouse inner hair cells. Nat. Neurosci. 14, 1109-1111.
Hall DD, Dari S, Tseng PY, Malik Z, Nguyen M, Matt L, Schnizler K, Shephard A, Mohapatra DP, Tsuruta F, Dolschenk RE, Christel CJ, Lee A, Burette A, Weinberg RJ, Hell JW (2013) Competition between alpha-actinin and Ca2+-calmodulin controls surface retention of the L-type Ca(2+)-channel Cav1.2. Neuron 78, 483-497.
Hell JW, Westenbroek RE, Warner C, Ahljanian MK, Prystay W, Gilbert MM, Snutch TP, Catterall WA (1993) Identification and differential subcellular localization of the neuronal class C and class D L-type calcium channel alpha 1 subunits. J. Cell Biol. 123, 949-962.
Herman JP, Chen KC, Boone R, Landfield PW (1998) Up-regulation of alpha1D Ca2+ channel subunit mRNA expression in the hippocampus of aged F344 rats. Neurobiol. Aging. 19, 581-587.
Hulme JT, Yarov-Yarovoy V, Lin TW, Scheuer T, Catterall WA (2006) Autoinhibitory control of the Cav1.2 channel by its proteolytically processed distal C-terminal domain. J. Physiol. 576, 87-102.
Jenkins MA, Christel CJ, Jiao Y, Abiria S, Kim KY, Usachev YM, Obermair GJ, Colbran RJ, Lee A (2010) Ca2+-dependent facilitation of Cav1.3 Ca2+ channels by densin and Ca2+-calmodulin-dependent protein kinase II. J. Neurosci. 30, 5125-5135.
Kadish I, Thibault O, Blalock EM, Chen KC, Gant JC, Porter NM, Landfield PW (2009) Hippocampal and cognitive aging across the lifespan: a bioenergetic shift precedes and increased cholesterol trafficking parallels memory impairment. J. Neurosci. 29, 1805-1816.
Kavalali ET, Hwang KS, Plummer MR (1997) cAMP-dependent enhancement of dihydropyridine-sensitive calcium channel availability in hippocampal neurons. J. Neurosci. 17, 5334-5348.
Khachatryan ZS (1987) Hypothesis on the regulation of cytosol calcium concentration and the aging brain. Neurobiol. Aging 8, 345–346.

Khachatryan ZS (1987) Calcium hypothesis of Alzheimer’s disease and brain aging. Ann. N. Y. Acad. Sci. 747, 1–11.

Kim S, Yun HM, Baik JH, Chung KC, Nah SY, Rhim H (2007) Functional interaction of neuronal Cav.3 L-type calcium channel with ryanoxin receptor type 2 in the rat hippocampus. J. Biol. Chem. 282, 32877–32889.

Knusten MG, Gammelli AE, Weiss C, Power JM, Disterhoft JF (2001) Age-related effects on eyeblink conditioning in the F344 × BN F1 hybrid rat. Neurobiol. Aging 22, 1–8.

Kourie JE (1998) Interaction of reactive oxygen species with ion transport mechanisms. Am. J. Physiol. 275, C1–C24.

Kumar A, Foster TC (2004) Enhanced long-term potentiation during aging is masked by processes involving intracellular calcium stores. J. Neurophysiol. 91, 2437–2444.

Landfield PW (1987) “Increased calcium-current” hypothesis of brain aging. Neurobiol. Aging 8, 346–347.

Landfield PW, Pitler TA (1984) Prolonged Ca2+–dependent afterhyperpolarization in hippocampal neurons of aged rats. Science 226, 1089–1092.

Lipman RD, Chrisp CE, Hazzard DG, Bronson RT (1996) Pathologic characterization of brown Norway, brown Norway × Fischer 344, and Fischer 344 × brown Norway rats with relation to age. J. Gerontol. A. Biol. Sci. Med. Sci. 51, 854–859.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

MacVicar BA (1984) Voltage-dependent calcium channels in glial cells. Science 226, 1345–1347.

Marshall MR, Clark JP III, Westenbroek R, Yu FH, Scheuer T, Catterall WA (2011) Functional roles of a C-terminal signaling complex of Cav1 channels and A-kinase anchoring protein 15 in brain neurons. J. Biol. Chem. 286, 12627–12639.

Matthews EA, Linardakis JM, Disterhoft JF (2009) The fast and slow afterhyperpolarizations are differentially modulated in hippocampal neurons by aging and learning. J. Neurosci. 29, 4750–4755.

McKinney BC, Murphy GG (2008) L-type voltage-gated calcium channel Cav1.3 mediates consolidation, but not extinction, of contextually conditioned remote spatial memories in mice. Learn. Mem. 15, 326–334.

Moyer JR Jr, Disterhoft JF (1994) Nimodipine decreases calcium action potentials in rabbit hippocampal CA1 neurons in an age-dependent and concentration-dependent manner. Hippocampus 4, 11–17.

Moyer JR Jr, Thompson LT, Black JP, Disterhoft JF (1992) Nimodipine increases excitability of rabbit CA1 pyramidal neurons in an age- and concentration-dependent manner. J. Neurophysiol. 68, 2100–2109.

Moyer JR Jr, Power JM, Thompson LT, Disterhoft JF (2000) Increased excitability of aged rabbit CA1 neurons after trace eyeblink conditioning. J. Neurosci. 20, 5476–5482.

Niesen CE, Baskys A, Carlen PL (1988) Reversed ethanol effects on potassium conductances in aged hippocampal dentate granule neurons. Brain Res. 445, 137–141.

Norris CM, Halpain S, Foster TC (1998) Reversal of age-related alterations in synaptic plasticity by blockade of L-type Ca2+ channels. J. Neurosci. 18, 3171–3179.

Norris CM, Blalock EM, Chen KC, Porter NM, Landfield PW (2002) Calciuminurin enhances L-type Ca2+ channel activity in hippocampal neurons: increased effect with age in culture. Neuroscience 110, 213–225.

Norris CM, Kadish I, Blalock EM, Chen KC, Thibault V, Porter NM, Landfield PW, Kranner SD (2005) Calciuminurin triggers reactive/inflammatory processes in astrocytes and is upregulated in aging and Alzheimer’s models. J. Neurosci. 25, 4649–4658.

Norris CM, Blalock EM, Chen KC, Porter NM, Thibault O, Kranner SD, Landfield PW (2010) Hippocampal ‘zipper’ slice studies reveal a necessary role for calciuminurin in the increased activity of L-type Ca2+ channels with aging. Neurobiol. Aging 31, 328–338.

Platzer J, Engel J, Schrott-Fischer A, Stephan K, Bova S, Chen H, Zheng H, Striessnig J (2000) Congenital deafness and sinalatroal node dysfunction in mice lacking class D L-type Ca2+ channels. Cell 102, 89–97.

Reynolds JN, Carlen PL (1989) The effects of midazolam on hippocampal dentate gyrus granule neurons from young and old Fischer 344 rats. Can. J. Physiol. Pharmacol. 67, 359–362.

Rowe WB, Blalock EM, Chen KC, Kadish I, Wang D, Barrett JE, Thibault O, Porter NM, Rose GM, Landfield PW (2007) Hippocampal expression analyses reveal selective association of immediate-early, neuroenergetic, and myelogenic pathways with cognitive impairment in aged rats. J. Neurosci. 27, 3098–3110.

Sculptoreanu A, Rotman E, Takahashi M, Scheuer T, Catterall WA (1993) Voltage-dependent potentiation of the activity of cardiac L-type calcium channel alpha 1 subunits due to phosphorylation by CAM-dependent protein kinase. Proc. Natl Acad. Sci. USA 90, 10135–10139.

Thibault O, Landfield PW (1996) Increase in single L-type calcium channels in hippocampal neurons during aging. Science 272, 1017–1020.

Thibault O, Gant JC, Landfield PW (2007) Expansion of the calcium hypothesis of brain aging and Alzheimer’s disease: minding the store. Aging Cell 6, 307–317.

Thomas-Crusell J, Vieira A, Saarma M, Rivera C (2003) A novel method for monitoring surface membrane trafficking on hippocampal acute slice preparation. J. Neurosci. Methods 125, 159–166.

Thompson LT, Deyo RA, Disterhoft JF (1990) Nimodipine enhances spontaneous activity of hippocampal pyramidal neurons in aging rabbits at a dose that facilitates associative learning. Brain Res. 535, 119–130.

Thompson LT, Moyer JR Jr, Disterhoft JF (1996) Trace eyelink conditioning in rabbits demonstrates heterogeneity of learning ability both between and within age groups. Neurobiol. Aging 17, 619–629.

Tombaugh GC, Rowe WB, Rose GM (2005) The slow afterhyperpolarization in hippocampal CA1 neurons covaries with spatial learning ability in aged Fisher 344 rats. J. Neurosci. 25, 2609–2616.

Vaughan DW, Peters A (1974) Neuroglial cells in the cerebral cortex of rats from young adulthood to old age: an electron microscope study. J. Neurocytol. 3, 405–429.

Veng LM, Browning MD (2002) Regionally selective alterations in expression of the alpha(1D) subunit (Cav1.3) of L-type calcium channels in the hippocampus of aged rats. Brain Res. Mol. Brain Res. 107, 120–127.

Vogalis F, Harvey JR, Furness JB (2004) Suppression of a slow post-spike afterhyperpolarization by calciuminurin inhibitors. Eur. J. Neurosci. 19, 2650–2658.

Watson RE Jr, Wiegand SJ, Clough RW, Hoffman GE (1986) Use of cryptoprotectant to maintain long-term peptide immunoreactivity and tissue morphology. Peptides 7, 155–159.

Wei X, Neely A, Lacerda AE, Olcese R, Stefani E, Perez-Reyes E, Birnbaumer L (1994) Modification of Ca2+ channel activity by deletions at the carboxyl terminus of the cardiac alpha 1 subunit. J. Biol. Chem. 269, 1635–1640.

Westenbroek RE, Bausch SB, Lin RC, Franck JE, Noebels JL, Catterall WA (1998) Upregulation of L-type Ca2+ channels in reactive astrocytes after brain injury, hypomyelination, and ischemia. J. Neurosci. 18, 2321–2334.

White JA, McKinney BC, John MC, Powers PA, Kamp TJ, Murphy GG (2008) Conditional forebrain deletion of the L-type calcium channel Ca V 1.2 disrupts remote spatial memories in mice. Learn. Mem. 15, 1–5.

Wisniewski HM, Terry RD (1973) Morphology of the aging brain, human and animal. Prog. Brain Res. 40, 167–186.

Wu WW, Chan CS, Surneer DJ, Disterhoft JF (2008) Coupling of L-type Ca2+ channels to KV7/KCNQ channels creates a novel, activity-dependent, homeostatic intrinsic plasticity. J. Neurophysiol. 100, 1897–1908.

Xu JH, Long L, Tang YC, Hu HT, Tang FR (2007) Cav1.2, Cav1.3, and Cav2.1.1 in the mouse hippocampus during and after pilocarpine-induced status epilepticus. Hippocampus 17, 235–251.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

Fig. S1 Depiction of Immunoblots from CNC1 and ab144 antibody specificity on hippocampal and cerebellar tissue lysates.

Fig. S2 Examples depicting full length Immunoblots from Western blot analyses.

Fig. S3 Selective isolation of surface-expressed proteins.

Fig. S4 Examples depicting qRT-PCR amplification plots for Ca.1.2 and Ca.1.3 gene expression analyses.

Fig. S5 Regional expression of Ca.1.2 and Ca.1.3 L-type calcium channel proteins in dorsal hippocampus.