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Hippocampal 'zipper' slice studies reveal a necessary role for calcineurin in the increased activity of L-type Ca^{2+} channels with aging

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Abstract

Previous studies have shown that inhibition of the Ca^{2+}-/calmodulin-dependent protein phosphatase calcineurin (CN) blocks L-type voltage sensitive Ca^{2+} channel (L-VSCC) activity in cultured hippocampal neurons. However, it is not known whether CN contributes to the increase in hippocampal L-VSCC activity that occurs with aging in at least some mammalian species. It is also unclear whether CN's necessary role in VSCC activity is simply permissive or is directly enhancing. To resolve these questions, we used partially dissociated hippocampal "zipper" slices to conduct cell-attached patch recording and RT-PCR on largely intact single neurons from young-adult, mid-aged, and aged rats. Further, we tested for direct CN enhancement of L-VSCCs using virally mediated infection of cultured neurons with an activated form of CN. Similar to previous work, L-VSCC activity was elevated in CA1 neurons of mid-aged and aged rats relative to young adults. The CN inhibitor, FK-506 (5 μM) completely blocked the aging-related increase in VSCC activity, reducing the activity level in aged rat neurons to that in younger rat neurons. However, aging was not associated with an increase in neuronal CN mRNA expression, nor was CN expression correlated with VSCC activity. Delivery of activated CN to primary hippocampal cultures induced an increase in neuronal L-VSCC activity but did not elevate L-VSCC protein levels. Together, the results provide the first evidence that CN activity, but not increased expression, plays a selective and necessary role in the aging-related increase in available L-VSCCs, possibly by direct activation. Thus, these studies point to altered CN function as a novel and potentially key factor in aging-dependent neuronal Ca^{2+} dysregulation.

Keywords

L-type; Phosphatase; Hippocampus; Aging

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Conflict of interest
None.

1. Introduction

Neuronal L-type voltage sensitive Ca^{2+} channels (L-VSCCs) are potent and tightly regulated signaling molecules involved in the control of membrane excitability, Ca^{2+}-induced Ca^{2+} release (CICR), synaptic plasticity, and gene expression, among other processes (Gallin and Greenberg, 1995; Lipscombe et al., 2002; Surmeier, 2007; Thibault et al., 2007; Tsien et al., 1986). Activity of L-VSCCs is increased in hippocampal CA1 neurons during aging (Thibault and Landfield, 1996) and may underlie, in part, numerous age-related alterations in hippocampal Ca^{2+} signaling and regulation (Disterhoft and Oh, 2007; Murphy et al., 2006; Thibault et al., 2007; Toescu and Verkhratsky, 2007). Blockade of L-VSCCs reverses or ameliorates many of the most consistent bio-behavioral markers of brain aging including the enhanced Ca^{2+}-dependent slow afterhyperpolarization (AHP) and reduced neuronal excitability (Moyer et al., 1992), impaired frequency facilitation (Thibault et al., 2001), increased susceptibility to long-term depression (Norris et al., 1998b), and diminished spatial cognition (Batuecas et al., 1998; Deyo et al., 1989; Disterhoft and Oh, 2006; Veng et al., 2003). Moreover, increased L-VSCC activity may result in or interact with increased CICR with aging (Gant et al., 2006; Kumar and Foster, 2004; Thibault et al., 2007).

Despite the accumulating evidence of a role for increased L-VSCC activity in hippocampal aging, little is yet known about the mechanisms that mediate this increase. Evidence has been found for modestly elevated expression of the less abundant isoform ($Ca_V1.3$) of the pore-forming L-VSCC subunit in hippocampus of aged rats (Herman et al., 1998; Veng et al., 2003). However, these changes in expression do not appear sufficient to account fully for age-differences in L-VSCC activity (Hell et al., 1993; Davare and Hell, 2003), especially in light of recent evidence suggesting that expression of the predominant $Ca_V1.2$ isoform may be reduced with aging (Rowe et al., 2007). Moreover, an age-related increase in $Ca_V1.2$ phosphorylation by the cAMP-dependent protein kinase (PKA), which appeared initially to account for increased L-VSCC activity (Davare and Hell, 2003), was not observed in a subsequent study (Murphy et al., 2006).

The Ca^{2+}-/calmodulin-dependent protein phosphatase calcineurin (CN) is another potential candidate mechanism for mediating the aging-related increase in L-VSCC activity. The relationship between CN and VSCC activity varies considerably, depending on cell type. Laboratories employing different neuronal and non-neuronal cultures have found evidence either for CN-mediated inhibition of L-VSCCs (Armstrong, 1989; Day et al., 2002; Hernandez-Lopez et al., 2000; Lukyanetz et al., 1998; Oliveria et al., 2007; Schuhmann et al., 1997), or that CN has no effect, or increases L-VSCC activity, particularly in cardiomyocytes (Branchaw et al., 1997; Frace and Hartzell, 1993; Victor et al., 1997; Wang et al., 2001; Yatani et al., 2001; Zeilhofer et al., 2000). A possible role for CN in modulating hippocampal L-VSCCs emerged from our prior studies showing that inhibition of CN function blocked L-VSCC activity in cultured hippocampal neurons (Norris et al., 2002). However, it is not clear from previous work whether CN also modulates L-VSCC activity in aged animal brain cells.

With regard to aging, several biochemical studies indicate that CN activity and CN-dependent processes are enhanced in the hippocampus of aged rats (Foster et al., 2001; Jouvenceau and Dutar, 2006; Norris et al., 1998a; but see Agbas et al., 2005). In addition, overexpression of activated CN in fore-brain neurons of adult mice precipitates aging-like alterations in hippocampal synaptic plasticity and cognition (Mansuy et al., 1998; Winder et al., 1998), whereas blockade of CN or CN-dependent signaling cascades in aged rodents restores synaptic and cognitive function to young-adult levels (Genoux et al., 2002; Norris et al., 1998a). However, CN gene expression in brain neurons may be reduced with aging (Rowe et al., 2007). Conversely, CN expression in reactive astrocytes is apparently elevated with aging and neuropathology (Norris et al., 2005).

Nevertheless, as noted, it remains unclear whether CN modulates L-VSCC activity in adult hippocampal neurons, as it does in embryonic cultures, or whether CN contributes to the aging-related increase in L-VSCC activity. Moreover, the nature of CN modulation of VSCC function in hippocampal neurons is poorly understood.

To address these questions here, we employed the partially dissociated hippocampal "zipper" slice preparation (so named for its tendency to dissociate gradually along the main cell body layers), which was developed by Gray et al. (1990) to enhance single-channel recording in adult animal brain. We have found that the preparation is highly advantageous for aging studies (Thibault and Landfield, 1996), and also allows the extraction of largely intact, physiologically characterized neurons for subsequent molecular analysis (Chen et al., 2000; Blalock et al., 2001). Blockade of CN function with FK-506 was used to determine whether CN activity contributes to increased L-VSCC activity during brain aging. Further, an activated form of CN was delivered in a viral vector (Norris et al., 2005) to cultured hippocampal neurons to test whether CN activity enhances as well as is necessary for L-VSCC activity. The results directly implicate CN activity as a candidate mechanism in the aging-related increase in L-VSCC function (and, consequently, in multiple Ca^{2+}-related biomarkers, e.g., Disterhoft and Oh, 2007; Thibault et al., 2007). Moreover, they show that activation of CN is capable of amplifying as well as permitting L-VSCC activity.

2. Materials and methods

2.1. Preparation of hippocampal "zipper" slices

Adult (6−9-month-old, mean = 8.34 months), mid-aged (15−19-month-old, mean = 17.63 months), and aged (20−29-month-old, mean = 23.44 months) male Fischer 344 rats were obtained from the National Institute on Aging's colony at Harlan. Rats were treated in accordance with the guidelines established by the Institutional Animal Care and Use Committee.

Procedures for obtaining partially dissociated hippocampal "zipper" slices from guinea pigs was developed by Gray et al., 1990 and modified by our research group for studies on aged rats and mRNA analysis of individual, largely intact neurons (Chen et al., 2000; Thibault and Landfield, 1996). Fig. 1 outlines the zipper slice protocol used in this study to obtain VSCC current activity and CN mRNA levels from CA1 neurons. Briefly, rats were euthanatized with CO_2 gas and rapidly decapitated. Brains were extracted and stored for 1−2 min in ice-cold artificial cerebrospinal fluid (ACSF) saturated with 95% O_2/5% CO_2 gas. Composition of the ACSF was (in mM): 114 NaCl, 2.5 KCl, 2 $MgCl_2$, 30 $NaHCO_3$, 10 glucose, and 0.1 $CaCl_2$. Hippocampi were dissected free and 350 μm transverse slices were prepared using a McIlwain Tissue Chopper. Slices were then transferred to prewarmed (32 °C), oxygenated ACSF containing 2 mM $CaCl_2$ and 0.7 mg/ml pronase to begin the partial dissociation process. After a 30-min incubation, pronase-containing ACSF was removed and slices were incubated for an additional 15 min in prewarmed ACSF containing 0.5 mg/ml thermolysine. Slices were washed twice in ACSF and were then bathed for 1−4 h in ACSF containing either 5 μM FK-506 (Calbiochem, EMD Chemicals, San Diego) or DMSO vehicle. In both conditions, DMSO was diluted to 0.1% of the ACSF volume.

Immediately before electrophysiological recording, each slice was nicked with a scalpel blade at the CA1 cell body layer and transferred to a small microcentrifuge tube containing ~1 ml of Ca^{2+}-free ACSF. Slices were then shaken gently by hand and monitored periodically for dissociation along CA1 *stratum pyramidale* before transfer to a perfusion style recording chamber (Warner Instruments, Hamden, CT) containing recording medium (see below). Once a suitable neuron was identified for recording (using a 40× objective, Fig. 1B), slices were

perfused with recording media at ~4 ml/min for 1 min to wash away cellular debris and any residual Ca^{2+}-free shaking media.

2.2. Hippocampal cell cultures

Primary hippocampal cell cultures, containing a mixture of neurons and astrocytes, were prepared from Sprague–Dawley rat pups (embryonic day 18) and plated on 35 mm culture dishes using slight modifications of the Banker and Cowan method (Banker and Cowan, 1977), as previously described (Porter et al., 1997). Most experiments were conducted on cells that were between 7 and 10-days-*in vitro* (DIV) in age.

2.3. Recombinant adenoviral vectors

cDNA for the first 398 amino acids of the CN A α subunit initially was encoded in a retroviral vector pJG2 kindly provided by Dr. Grace Pavlath at Emory University, Atlanta. This CN fragment lacks a critical autoinhibitory domain and exhibits marked Ca^{2+}-/calmodulin-independent activity (Hubbard and Klee, 1989). CN was amplified from pJG2 by PCR and sub-cloned downstream of a CMV promoter in pIRES2-DsRed-Express (Clontech, Mountain View, CA). CMV-aCN-IRES-DsRed2 was then subcloned into the pAd-link adenoviral shuttle vector and recombined with wild type dl327 adenoviral DNA in HEK-293 cells to obtain Ad-aCN-DsRed2. Ad-LacZ-GFP (kind gift from Dr. Rita Balice-Gordon at the University of Pennsylvania), encodes β-galactosidase (LacZ) under the control of a CMV promoter, and green-fluorescent protein (GFP) under the control of an IRES. Ad-NFAT-Luc was a kind gift from Dr. Jeff Molkentin at the University of Cincinnati. Viruses were added to hippocampal neuronal cultures 48 h prior to recording at an MOI of 100, which is sufficient for transfection of ~70% of neurons as determined with an X-gal stain to label β-galactosidase expression in Ad-LacZ-GFP-infected cultures.

2.4. Patch-clamp electrophysiology

Recording pipettes consisted of glass capillary tubes pulled on a horizontal micropipette puller. Whole-cell pipettes were coated with polystyrene Q-dope (GC/Waldom, Inc., Rockford, IL) and had a mean tip resistance of 2.74 ± 0.06 MΩ . On-cell patch pipettes were coated with Sylgard (Dow Corning, Midland, MI, USA). For cell culture studies patch pipettes had a mean tip resistance of 4.53 ± 0.07 MΩ and for zipper studies tip resistance was 5.2 ± 0.07 MΩ . All recording pipettes were fire-polished immediately before recording.

Extracellular solution for whole-cell recordings contained (in mM): 111 NaCl, 5 $BaCl_2$, 5 CsCl, 2 $MgCl_2$, 10 glucose, 10 HEPES, 20 tetraethylammonium (TEA) Cl, 0.01 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX), and 0.001 tetrodotoxin (TTX), pH 7.35, osmolarity = 330 mOsm. Pipette solution for whole-cell recordings contained (in mM): 145 methane sulfonic acid, 10 HEPES, 3 $MgCl_2$, 11 EGTA, 1 $CaCl_2$, 5 MgATP, 13 TEA Cl, 0.1 leupeptin, pH 7.35, osmolarity = 320. On-cell patch extracellular recording solution contained (in mM): 140 K gluconate, 3 $MgCl_2$, 10 glucose, 10 EGTA, and 10 HEPES, pH 7.35, osmolarity = 300 mOsm. On-cell patch pipette solution consisted of (in mM): 20 $BaCl_2$, 90 choline Cl, 10 TEA Cl, and 10 HEPES, pH 7.35, osmolarity = 290 mOsm.

All recordings were obtained using an Axopatch 200A patch-clamp amplifier (Molecular Devices Corporation, Sunnyvale, CA). Data were filtered at 2 kHz and digitized at 5 kHz. Voltage commands and data acquisition were controlled by pCLAMP software. All experiments were conducted at room temperature.

Prior to recording, junction potentials were nulled in the bath using the pipette offset control on the Axopatch 200A. Seal quality was determined using the seal test feature of pCLAMP, and other membrane and recording parameters such as membrane capacitance and resistance,

access resistance, and holding current were calculated using the membrane test feature (with filter settings at 10 kHz). For whole-cell studies, the membrane potential was held at −70 mV and all voltage steps were 150 ms in duration. Series resistance was not compensated for reasons outlined in our earlier reports (Porter et al., 1997). *I*/*V* relationships for each cell were established by successively stepping the membrane potential in 10 mV increments from holding to +60 mV. Normalized current values obtained from each *I*/*V* curve were fit with a Boltzman equation to determine the half maximal activation voltage. Cells were then stepped to their maximal activation voltage a total of five times (30 s interstep interval) to generate an average maximal waveform. Current density was calculated at the maximal activation voltage (*i.e.* +10 mV) by taking the integral under the curve and dividing that value by the cell's membrane capacitance (*i.e.* pA/pF).

For cell-attached patch studies, only patches with a seal >10 GΩ were used for statistical analysis. No differences in seal quality were observed across treatment groups. Maximal current in each patch was achieved by stepping the patch membrane from its holding potential of −70 to +10 mV. A series of 45−50 steps to +10 mV (15 s interstep interval) was used to generate an average ensemble current for each patch. Currents were leak-subtracted off-line using hyperpolarizing pulses, identical in duration and opposite in polarity. Current density ($pA/\mu m^2$) for each patch was derived by dividing the average ensemble current by the patch area. Patch area, which is inversely proportional to the pipette resistance, was estimated for each recording using the equation $a = 12.6(1/R + 0.018)$, where '*a*' is the patch area and '*R*' is the pipette resistance (Sakmann and Neher, 1983).

2.5. Single cell mRNA analysis

After recording, individual cells were extracted with the recording pipette, aspirated in a collection pipette, and placed immediately into an ice-cold microfuge tube containing cell lysis buffer (1× first strand buffer, 10 U RNase inhibitor). The cell lysate was stored at −80 °C until PCR.

RNA messages of each collected single cell were analyzed by one-step real-time qRT-PCR using TaqMan qRT-PCR procedure (Applied Biosystems, CA). For an mRNA, 1 ul cell lysate was added to a 50-μl PCR reaction mix (1× PCR master mix; 1× RT mix; 0.3 μM each of the mRNA-specific forward and reverse primers and 0.2 μM TaqMan primer). The RT-PCR was run in an ABI 7700 sequence detection system, for 1 cycle at 48 °C, 30 min, then 1 cycle of 95 °C, 10 min, followed by PCR at (95 °C, 15 s and 60 °C, 1 min) per cycle for 45 cycles. Relative quantification of RNA was obtained from plotting the Ct on a standard curve in each PCR run.

2.6. Homogenization of cell culture tissue and Western blot

At 48 h after viral treatment, hippocampal cultures in 35 mm dishes were homogenized in ice-cold sucrose buffer (0.3 M sucrose, 0.75 M NaCl, 0.01 Tris–HCl pH 7.4, 0.02 M EGTA, 0.02 M EDTA) containing a complete panel of phosphatase (Calbiochem 539134) and protease (Calbiochem 524625) inhibitors, calpain 1 and II inhibitors (8 μg/ml each) (Sigma), and benzamidine hydrochloride (320 μg/ml) (Sigma). Within each treatment condition, material from six dishes was combined in sucrose buffer and spun at 3500 rpm for 5 min at 4 °C. Pellets were resuspended in 300 μl of sucrose buffer, combined with 1% SDS, and heated at 65 °C for 15 min. Samples were then combined with an SDS-based running buffer and loaded onto individual lanes of an 8% polyacrylamide gel, with protein concentrations (determined by the Lowry method) remaining constant across lanes, and resolved using SDS-PAGE. This procedure was replicated across at least five different sister cultures (*i.e.* $n \geq 5$). Samples were transferred to PVDF membranes and probed with appropriate antibodies: rabbit anti-$Ca_V1.2$ or $Ca_V1.3$ (Alomone Labs, Jerusalem, Israel) each at a 1:300 dilution; goat anti-rabbit

secondary antibody (1:100,000) coupled to horseradish peroxidase. Bands were detected using the ECL-Plus detection kit (Amersham Biosciences, Piscataway) and quantified using a Storm 860 molecular imager.

2.7. Statistics

VSCC current records were analyzed quantitatively using pClamp software (v.8 and higher) (Molecular Devices). All statistical analyses were performed using Statview 5.01. Effects of age, drug treatment, viral treatment, and all interactions, were determined by analysis of variance. *Post hoc* analyses were performed using Scheffe's *F*-test. Significance for all statistical comparisons was set at $p \leq 0.05$.

3. Results

3.1. Acute blockade of CN suppresses the age-related increase in VSCC activity

Hippocampal "zipper" slices (Fig. 1) prepared from adult, middle-aged, and aged rats, were exposed to 5 μM FK-506 or 0.1% DMSO vehicle for 1−3 h. VSCC activity then was measured in cell-attached patches from partially dissociated CA1 pyramidal neurons. Patch activity from 110 cells were recorded and the *n* for each treatment group was as follows: adult control 26, adult FK-506 21, mid-aged control 24, mid-aged FK-506 14, aged control 14 and aged FK-506 11. Representative ensemble currents recorded during step depolarizations from −70 to +10 mV are shown in Fig. 2A for adult and aged rat slices treated with and without FK-506. Results of this study are shown in Figure 2B. A two-way ANOVA revealed a significant age × drug treatment interaction [$F(2, 104) = 3.89$, $p < 0.05$]. *Post hoc* analyses showed that VSCC activity was significantly greater in the aged group, relative to the adult group ($p < 0.05$), but did not differ significantly between the adult and mid-aged groups ($p = 0.11$), or the mid-aged and aged ($p = 0.78$) groups. These results are highly similar to previous findings from our laboratory and confirm that aging in associated with an increase in VSCC activity (Campbell et al., 1996;Thibault and Landfield, 1996). Addition of FK-506 did not alter VSCC activity in the adult group, but it reduced maximal current density in both the mid-aged and aged groups. However, this reduction reached significance only for the aged group [$F(1, 23) = 6.7$, $p < 0.05$]. The results demonstrate that increased VSCC activity with aging depends critically on the expression and/or activity of CN.

To determine whether increased VSCC activity in the older groups was associated with increased expression of CN, a subset of recorded neurons (adult $n = 10$, aged $n = 9$) were extracted using the patch recording pipette and collected for subsequent mRNA analysis (Fig. 3A). For the aged group, neurons were extracted from mid-aged and aged rats. In this subset of cells, VSCC activity was significantly greater in the aged group [$F(1, 17) = 4.63$, $p < 0.05$] (Fig. 3B). However, mRNA levels for the CN A isoform were slightly, though insignificantly, reduced with aging (Fig. 3C), and within-cell correlational analysis across all cells also failed to find a significant relationship between CN mRNA levels and VSCC activity (Fig. 3D). Thus, while CN activity appears necessary for the aging-related increase in VSCC activity, this increase apparently does not require elevated expression levels for the CN A isoform.

3.2. Increased CN activity is sufficient to drive the upregulation of L-VSCC activity

To determine whether increased CN activity could drive the upregulation of VSCC activity in hippocampal neurons, we infected hippocampal neuronal cultures with adenovirus encoding a constitutively active form of CN (Ad-aCN-DsRed2). A representative NFAT-reporter assay for cultures treated with Ad-aCN-DsRed2, or with control adenovirus that encodes β-galactosidase (Ad-LacZ-GFP), is provided in Fig. 4A. The luciferase construct in this experiment, delivered via adenovirus, is encoded downstream of multiple copies of an NFAT binding site from the IL-4 promoter (Wilkins et al., 2004). NFAT-driven luciferase expression,

which is highly sensitive to CN activity, is markedly increased in CN-treated cultures, and this increase is blocked by potent antagonists of CN and NFATs (CsA and VIVIT, respectively).

For electrophysiology experiments, whole-cell VSCC activity was recorded 48 h after adenoviral infection. Untreated cultures were examined in parallel to determine the effects of adenovirus on membrane properties and VSCC activity. A total of 58 cells were analyzed (untreated, $n = 19$; Ad-LacZ-GFP, $n = 18$, Ad-aCN-DsRed2, $n = 21$). Adenovirus treatment had no effects on several membrane and/or recording parameters including access resistance (untreated, 6.24 ± 0.42 MΩ ; Ad-LacZ-GFP, 5.77 ± 0.41 MΩ ; Ad-aCNDsRed2, 6.29 ± 0.35 MΩ), and membrane capacitance (untreated, 51.72 ± 2.37 pF; Ad-LacZ-GFP, 48.95 ± 2.96 Ad-aCN-DsRed2, 52.67 2.5 ± pF). Adenoviral treatment did, however, affect maximal VSCC current density [$F(2, 55) = 6.99$, $p < 0.01$]. *Post hoc* analyses indicated that VSCC current was increased in the Ad-aCN-DsRed2 group relative to both the Ad-LacZ-GFP ($p < 0.01$) and untreated groups ($p < 0.05$) (Fig. 4C). Conversely, adenovirus alone had only minimal effects on VSCC activity as evidenced by the slight non-significant reduction in VSCC current density in Ad-LacZ-GFP-treated cells relative to untreated cells. Active CN did not appear to alter the voltage dependency of VSCCs, as *I*/*V* curves amongst all treatment groups were qualitatively similar (Fig. 4D). Overall, the half-maximal activation voltage for these studies was −12.5 mV. And while active CN was associated with increased VSCC activity, protein levels for $Ca_V1.2$ and $Ca_V1.3$ (Fig. 4E) were not significantly different between Ad-aCN-DsRed2- and Ad-LacZ-GFP-treated cultures ($n = 5$ per group).

In a previous study, we showed that potent CN inhibitors, such as FK-506, affected L-type VSCC activity in hippocampal neurons, without altering the activity of N-, P-, and Q-type VSCCs (Norris et al., 2002). In the present study, two approaches were used to determine whether activated CN selectively modulated L-VSCC current. In the first approach, Ad-LacZ-GFP ($n = 18$) and Ad-aCN-DsRed2-treated neurons ($n = 23$) were recorded in cell-attached patch mode, and L-VSCC activity was maximized by including the L-VSCC activator Bay K 8644 in the patch pipette to augment the fraction of L-VSCC current. As shown in Fig. 5A and B, L-VSCC current was significantly greater in Ad-aCN-DsRed2-relative to Ad-LacZ-GFP-treated cells [$F(1, 39) = 4.19$, $p < 0.05$] and this increase in patch VSCC activity (~182%) was proportionally larger than the increase observed for whole-cell recordings (~146%) when L-VSCC activity was present, but not maximized (see Fig. 4C).

In the second approach, cultures were bathed in recording medium containing the L-type VSCC antagonist nimodipine (10 μM), and non-L-type VSCC current was recorded in whole-cell mode. As shown in Fig. 5C, when the contribution of L-type channels was eliminated, Ad-aCNDsRed2-infected neurons ($n = 9$) showed a slight (~10%), but nonsignificant reduction in maximal VSCC current density relative to Ad-LacZ-GFP-treated neurons ($n = 9$), consistent with a selective action of CN on L-type VSCCs.

4. Discussion

The present work provides the first evidence that CN function is necessary for the increase in hippocampal L-VSCC activity that develops over the course of aging in rats. Moreover, these studies show that overexpression of activated CN selectively increases L-VSCC activity, but not expression in cultured hippocampal neurons. Together with previous work showing that L-VSCCs drive activation of CN in hippocampal neurons (Foster et al., 2001; Graef et al., 1999), the present findings suggest that CN may amplify its own actions by enhancing hippocampal L-VSCC activity. Thus, a positive feedback cycle between CN and L-VSCCs may develop with aging and disrupt the balance of numerous Ca^{2+}-dependent processes in the hippocampus, leading to impaired cognitive function and increased susceptibility to neurodegeneration.

4.1. Role for CN in regulating the age-related increase in VSCC activity

Previously, we found that several structurally distinct CN inhibitors, including FK-506, selectively reduced L-VSCC currents in hippocampal culture (Norris et al., 2002). These findings contrasted with those of studies in other cell types indicating that CN activity limited L-VSCC function (Armstrong, 1989; Lukyanetz et al., 1998; Schuhmann et al., 1997; Day et al., 2002; Hernandez-Lopez et al., 2000; Oliveria et al., 2007). In our studies, CN inhibitors reduced VSCC activity whether applied minutes or hours prior to recording, whether Ba^{2+} or Ca^{2+} was used as charge carrier, or whether intracellular Ca^{2+} was strongly buffered. In addition, FK-506 effects on L-VSCCs were more pronounced in older embryonic neurons (4 weeks *in vitro*), which are characterized by increased L-VSCC activity relative to younger embryonic neurons (Porter et al., 1997). The contribution of CN function, therefore, may be elevated under conditions of increasing L-VSCC activity. Consistent with this possibility are studies on cardiomyocytes, which show that CN inhibitors minimally affect L-VSCC activity in normal cells, but markedly limit the increase in L-VSCC activity that arises with pathologic hypertrophy (Wang et al., 2001; Yatani et al., 2001).

However, studies in cell culture do not directly address whether CN modulation occurs or changes in aged animal neurons. Consequently, we tested here whether increased L-VSCC activity with aging *in vivo* was dependent on CN function. Use of the hippocampal zipper slice preparation was advantageous for this purpose because it offers excellent access to largely intact neurons in adult brain tissue for single-channel patch-clamp recording. The present study observed a marked aging-related increase in VSCC activity in vehicle-treated slices and therefore confirmed earlier findings on Ca^{2+} channel activity in aged rats (Thibault and Landfield, 1996). Moreover, FK-506 selectively and fully blocked the aging-dependent increase in VSCC activity. That is, FK-506-treated slices did not exhibit increased VSCC activity with aging (see Fig. 2B), and VSCC activity in the FK-506 group was remarkably similar to levels in control slices from the young-adult group. These results are consistent with the hypothesis that CN exerts greater influence over the component of VSCC current that is increased with aging.

One possible explanation for the age-dependent actions of CN inhibition is that CN expression increases with aging. In prior studies of hippocampal cultures, for instance, increased VSCC activity as well as the increased efficacy of FK-506 was associated with an increase in CN mRNA expression (Norris et al., 2002). To test this possibility here for *in vivo* aging, a subset of recorded neurons were collected for mRNA amplification and analysis (Fig. 3), an approach used in earlier studies to determine whether L-VSCC activity and mRNA levels for the $Ca_V1.3$ subunit were correlated within the same cells (Blalock et al., 2001; Chen et al., 2000). However, CN Aα mRNA levels were not greater in the aged group, nor were CN A levels significantly correlated with VSCC activity levels. Thus, increased VSCC activity and sensitivity to CN inhibition in hippocampal neurons with aging is not driven by the level of CN expression (at least not by mRNA expression for the CN Aα isoform).

4.2. Overexpression of activated CN increases L-VSCC activity in hippocampal neurons

The dependence of L-VSCC function on CN activity in hippocampal neurons has been established primarily with CN inhibitors. Thus, it is possible that the CN modulatory effect is primarily permissive and does not involve direct enhancement. Therefore, the capacity of activated CN to directly stimulate increased VSCC activity was investigated in primary hippocampal neuronal cultures using adenoviral mediated transfer of an activated form of CN (Fig. 4). In a previous gene microarray study, we found that delivery of activated CN to hippocampal cultures did not increase mRNA levels for any of the major L-VSCC subunits (Norris et al., 2005). Similarly, protein levels for $Ca_V1.2$ and $Ca_V1.3$ were not changed in the present study following treatment with activated CN (Fig. 4E). However, VSCC activity was

significantly greater in CN-treated cultures relative to uninfected and LacZ-treated control cultures (Fig. 4C). Moreover, active CN effects on VSCCs were likely limited to L-type isoforms, as CN's effects were augmented in the presence of the L-VSCC activator Bay K 8644, and inhibited by the L-VSCC antagonist, nimodipine (Fig. 5). The results indicate that overexpression of activated CN in hippocampal cultures is associated with increased neuronal L-VSCC activity. Similar effects of activated CN on L-VSCC activity have been noted in hypertrophic cardiomyocytes as well (Yatani et al., 2001). However, it will be important in future studies to determine whether activated CN can also increase L-VSCC activity in adult neurons.

4.3. Possible mechanistic basis for CN/L-VSCC interactions

The mechanistic basis for L-VSCC regulation by CN in hippocampal neurons is not presently clear. Although the $Ca_V1.2$ subunit contains NFAT binding elements near its promoter region (Dai et al., 2002), a transcriptional mechanism for regulation in neurons can probably be ruled out because of the lack of effect of activated CN on L-VSCC protein levels in this study (Fig. 4E) and others (Yatani et al., 2001) and the absence of increased Ca_V 1.2 expression with aging (Rowe et al., 2007). Moreover, FK-506 actions in the present study, and in an earlier study on hippocampal cultures (Norris et al., 2002), only required a few minutes to a few hours of drug exposure, suggestive of a posttranslational mechanism. L-VSCC β subunits, in particular, contain numerous phosphorylation sites that are targeted by CN *in vitro* (Lai et al., 1993). Whether phosphorylation of these sites is altered during aging has not been investigated.

Changes in the phosphorylation state of L-VSCCs and other Ca^{2+} channels can be achieved, in part, by protein kinases and phosphatases that are anchored to, or close to, Ca^{2+} channel subunits (Beene and Scott, 2007). Although several studies (Davare et al., 2000; Hall et al., 2006) have found no evidence for a physical association between L-VSCC subunits and CN, a recent study did detect an association (Oliveria et al., 2007). In contrast to the present study and our previous results, however, CN was found to negatively regulate VSCC activity. Further, aging appears to be associated with a shift in the subcellular distribution of CN to the cytosol (Foster et al., 2001), where direct access to membrane bound proteins may be limited. A subcellular shift in CN expression could lead to a corresponding shift in the substrates targeted by CN, some of which could provide positive regulation over L-VSCC function. Clearly, further research will be necessary to test this possibility.

4.4. Implications for the Ca^{2+} hypothesis of brain aging

The hypothesis that altered Ca^{2+} regulation and signaling during the lifespan leads to impaired neural and cognitive function with aging, and possibly neurodegenerative disease, has garnered extensive support from many molecular, electrophysiological, and behavioral studies (Murchison and Griffith, 2007; Thibault et al., 2007; Disterhoft and Oh, 2007; Foster, 2007; Mattson, 2007; Toescu and Verkhratsky, 2007). CN may be critical to altered Ca^{2+} signaling as it responds rapidly and robustly to local changes in Ca^{2+}. Moreover, CN provides strong transcriptional and/or post-translational regulation of numerous molecules involved in Ca^{2+} signaling and homeostasis in neural tissue, including IP3 receptors, NFATs, Ca^{2+}ATPases, calmodulin, and cytoskeletal elements, to name a few (Carafoli et al., 1999; Graef et al., 1999; Norris et al., 2005; Cameron et al., 1995). L-VSCCs provide a potent, and in some cases selective, source of Ca^{2+} for CN activation in hippocampal neurons (Graef et al., 1999). The capacity for CN to provide positive regulation of L-VSCCs during aging, as indicated by the present study, therefore could initiate a positive feedback loop with repercussions felt through multiple Ca^{2+} signaling pathways.

Feedback interactions between L-VSCCs and CN, if not properly regulated, may eventually lead to degenerative processes and/or hasten the progression of neurodegenerative disease.

Although these interactions may be particularly damaging in neurons, similar feedback loops may occur in other CNS cell types as well. Astrocyte activation due to brain injury, aging, or extracellular amyloid deposition is associated with increased expression of both L-VSCCs and CN (Hashimoto et al., 1998; Norris et al., 2005; Westenbroek et al., 1998; Djamshidian et al., 2002; Xu et al., 2007). Our recent work shows that activation of CN in astrocytes is strongly dependent on L-VSCCs (Sama et al., 2006) and appears to be a critical regulator of immune/inflammatory signaling cascades linked to aging and the early stages of Alzheimer's disease (Norris et al., 2005). Thus, the CN/L-VSCC interaction may represent a novel and promising target for therapeutic interventions in brain aging and neuropathology. In this regard, it is interesting to note that the CN inhibitor, tacrolimus (*i.e.* FK-506) was recently shown to improve cognitive function in amyloidogenic mice (Dineley et al., 2007), as well as to reduce neuroinflammation and extend the lifespan of mice with tau pathology (Yoshiyama et al., 2007).

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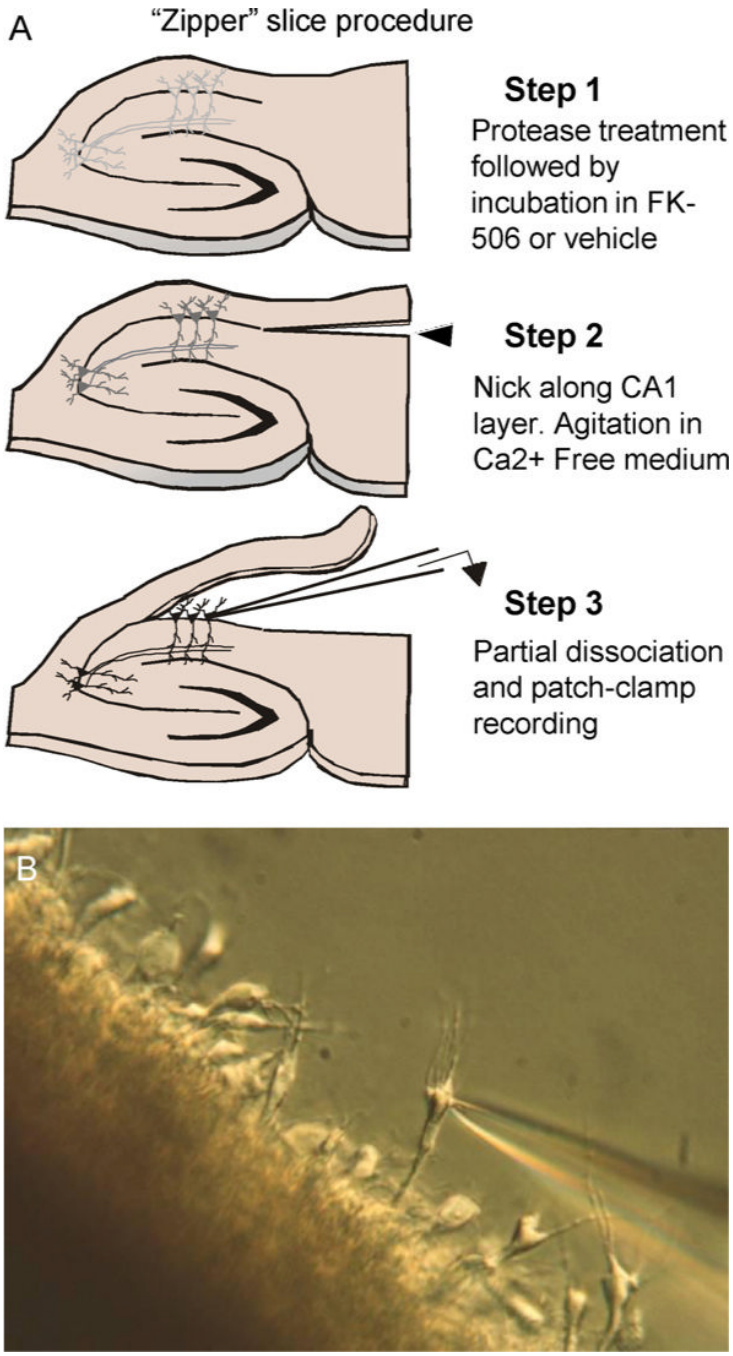

Fig. 1.

The hippocampal "zipper" slice protocol. (A) Steps involved in the partial dissociation or "unzipping" procedure. Step 1: slices are first incubated briefly in ACSF containing proteases to initiate the breakdown of extracellular matrix constituents. Afterwards, slices are exposed to FK-506 or vehicle. Step 2: prior to recording, slices are "nicked" along the CA1 cell body layer with a scalpel blade to start the unzipping process, and then gently agitated in Ca^{2+}-free ACSF until the slice dissociates or unzips along the CA1 cell layer. Step 3: slices then are transferred to a perfusion style recording chamber, and cleanly exposed CA1 pyramidal neurons are isolated with glass microelectrodes and VSCC activity is elicited and recorded in on-cell patch mode. (B) Photomicrograph of cleanly exposed CA1 pyramidal neurons

following the unzipping procedure. A glass microelectrode (coming from bottom right corner) is sealed on to one of these neurons for the recording of VSCC activity.

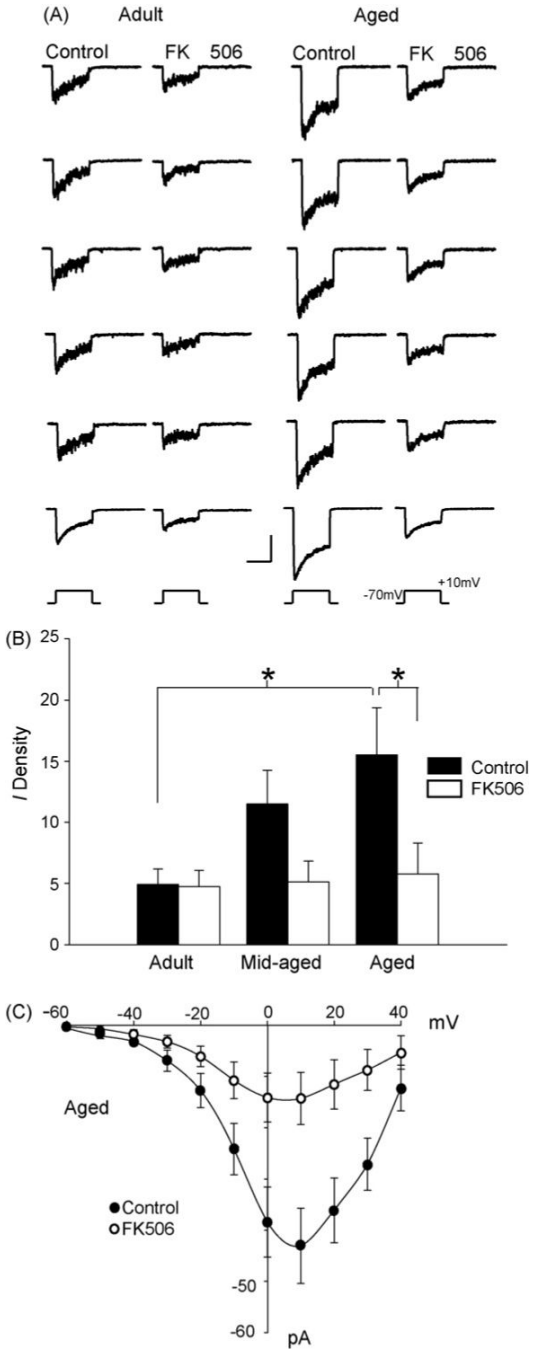

Fig. 2.

The potent CN inhibitor FK-506 blocks the aging-related increase in VSCC activity. (A) Representative VSCC current traces, and the average ensemble current (lowest trace) generated from 45-step depolarizations in multichannel cell-attached patches from adult and aged rat slices treated with and without the CN inhibitor, FK-506 (5 μM). Maximal VSCC activity was elicited by stepping the patch membrane from −70 to +10 mV for 150 ms. Scale bars: 10 pA/100 ms. (B) Mean±S.E.M. current densities in each age group, with and without FK-506. Aging is associated with increased VSCC activity and this increase is prevented by FK-506. (C) *I*/*V* relationships in the aged group were not affected by FK-506, even though maximal current was reduced across most voltage steps. *$p < 0.05$.

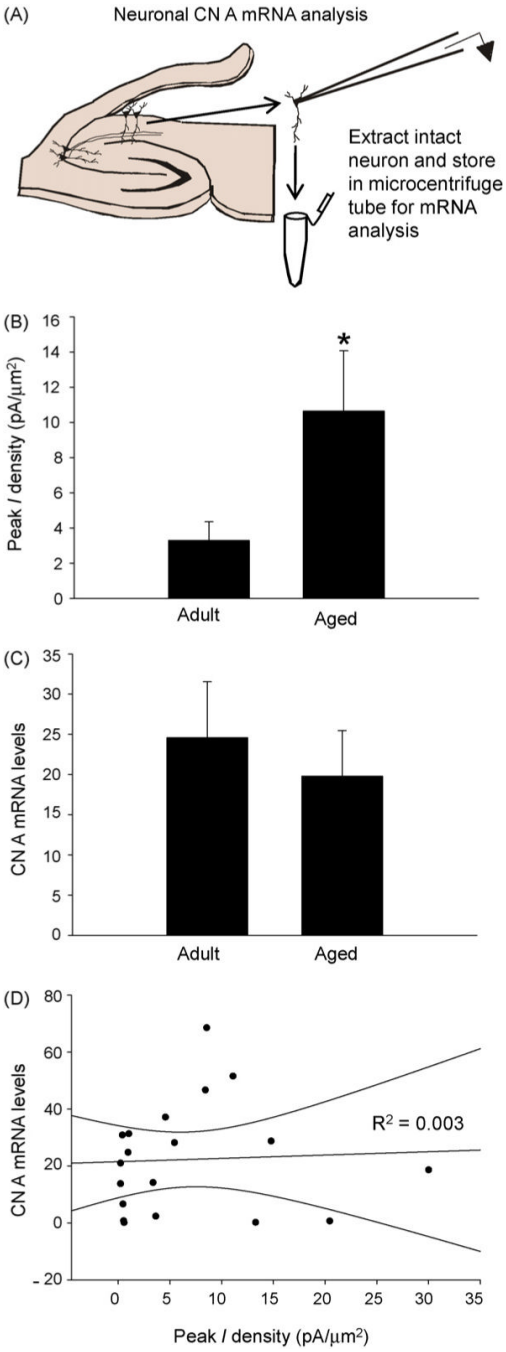

Fig. 3.

Neuronal VSCC activity and CN mRNA levels are not co-regulated. (A) Cartoon illustration of the single cell collection procedure. After VSCC recordings, individual neurons are extracted from the slice using the recording microelectrode. Neurons are harvested in a separate collection pipette, with membranes and dendritic processes largely intact, and then stored in a pre-chilled microcentrifuge tube containing pre-amplification buffer (see Section 2) until real-time RT-PCR analysis. (B) Similar to the global data set shown in Fig. 2, VSCC activity in the subset of collected cells (10 young-adult cells, 9 aged) was also greater in the aged group. (C) Unlike VSCC activity, CN A mRNA levels in collected neurons were not increased in the aged

group. (D) Within-cell correlational analysis across all cells that were recorded and collected showed no significant correlation between VSCC activity levels and CN A mRNA content.

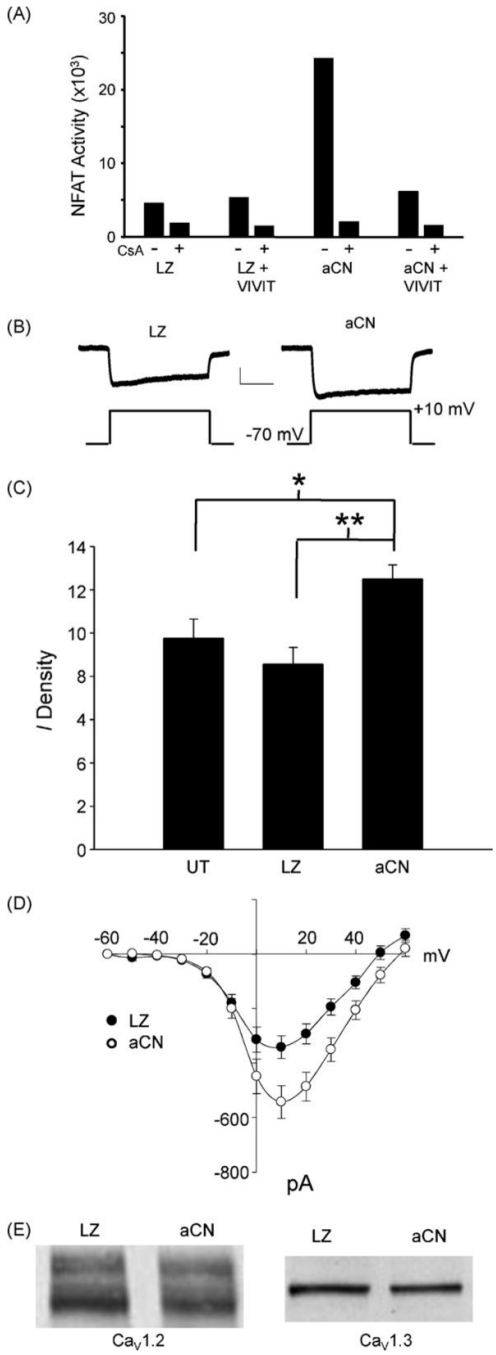

Fig. 4.

Active CN stimulates whole-cell VSCC activity in hippocampal neurons. (A) Representative gene reporter experiment showing that NFAT-dependent luciferase expression is markedly greater in AdaCN-DsRed2-treated cultures, relative to Ad-LacZ-GFP-treated cultures. Induction of luciferase expression is completely suppressed by the CN inhibitor, cyclosporine (CsA, 5 μM) and also by the NFAT inhibitor VIVIT, indicating the specific involvement of CN. (B) Representative whole-cell current records from individual neurons during a voltage step from −70 to +10 mV. Cells were infected with Ad-LacZ-GFP (LZ) or with Ad-aCN-DsRed2 (aCN). Scale bars: 200 pA/50 ms. (C) Mean±S.E.M. whole-cell maximal current densities in untreated (UT) and adenoviral-treated groups. Current density was increased in

the aCN group relative to UT and LZ groups, which did not differ from each other. (D) *I*/*V* relationships recorded in Ad-LacZ-GFP and Ad-aCN-DsRed2 cultures. aCN increased current amplitudes, but did not alter the voltage dependence of VSCCs. (E) Representative Western blots for $Ca_V1.2$ and $Ca_V1.3$ subunits in LZ and aCN-treated cultures. L-VSCC protein levels were not affected by CN activity. *$p < 0.05$; **$p < 0.01$.

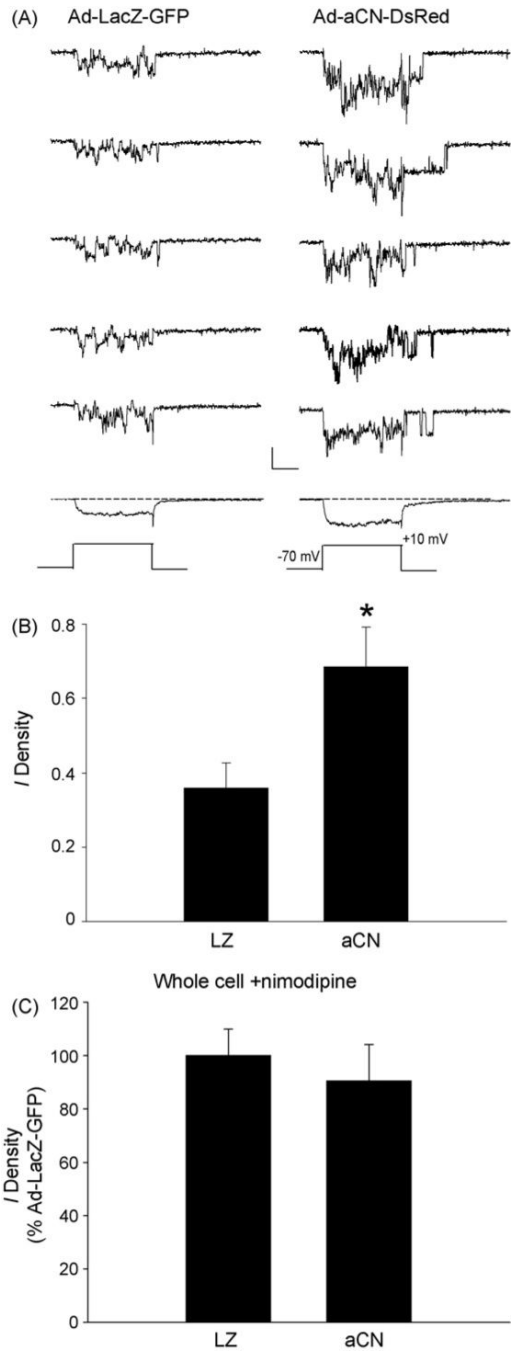

Fig. 5.

Effects of active CN are selective for L-VSCCs. (A) Representative VSCC current traces, and the average ensemble current (lowest trace) generated from 45 step depolarizations in multichannel cell-attached patches from Ad-LacZ-GFP and Ad-aCN-DsRed2-treated cells. Patch pipettes included 0.5 μM Bay K 8644 to elicit predominantly L-VSCC activity. (B) Mean ± S.E.M. current densities in adenoviral treatment groups, showing that CN activation is associated with a significant increase in L-VSCC activity. (C) Mean ± S.E.M. whole-cell maximal current densities in Ad-LacZ-GFP and Ad-aCN-DsRed2-treated cultures pre-incubated in 10 μM nimodipine to remove the influence of L-VSCCs from the whole-cell

current record. Without the availability of L-VSCCs, aCN no longer affects VSCC current density. *$p < 0.01$.