Regulation of CREB signaling through L-type Ca\textsuperscript{2+} channels by Nipsnap-2

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A recent study identified Nipsnap1 as an auxiliary protein inhibiting TRPV6 ion channel activity. Based upon this finding, we investigated the role of Nipsnap1, and the closely related Nipsnap2, in Ca\textsuperscript{2+} channel regulation. Here, we find that overexpression of Nipsnap2 caused a 45% increase in currents though L-type Ca\textsuperscript{2+} channels in a neuronal cell line, while siRNA knockdown of Nipsnap2 greatly reduced L-type currents. The increased influx through L-type Ca\textsuperscript{2+} channels due to Nipsnap2 overexpression led to increased phosphorylation of the transcription factor cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) along with enhanced expression of several transcription factors and CREB target genes. These experiments highlight a novel role of Nipsnap2 in transcriptional regulation via L-type Ca\textsuperscript{2+} channels.

Introduction

The Nipsnap family of proteins was identified concurrently in 1998 by Seroussi and colleagues during genomic sequencing of human chromosome 22,1 and by Wang and colleagues during a screen to identify sequences upregulated in glioblastoma cancer where epidermal growth factor receptor was amplified.2 Because the C. elegans Nipsnap gene is present in an operon harboring proteins with homology to 4-nitrophenylphosphate (NIP-) and synaptosomal associated protein 25 (-SNAP) domains, it was therefore named Nipsnap. To date, four members of the Nipsnap family (i.e., Nipsnaps1–4) have been identified.1,3,4 While expression of all four Nipsnaps has been reported within the brain,1,3 the function(s) subserved by this family of proteins has remained elusive. Two studies reported possible links between Nipsnaps and epilepsy3 and cognitive deficits in phenylketonuria, a genetic metabolic disorder,6 although these roles have not been substantiated. The first of these phenomenological studies showed increased Nipsnap1 expression in kainite-treated mice, whereas the second reported a reduction in Nipsnap1 gene in microarray analysis in a mouse model of phenylketonuria. Beyond reporting changes in expression, neither study provided any definitive evidence of a role for Nipsnaps. A more recent study described the mitochondrial localization of Nipsnap1 in neurons and a potential role in mitochondrial processes through interaction with enzymatic complexes.7 This finding is consistent with a prior report describing Nipsnap1–4 as mitochondrial proteins and demonstrating that Nipsnap3 and 4 bind inhibitors of apoptosis proteins.8

A putative role for Nipsnaps in vesicular transport has been inferred from their presence in the same cluster of genes as SNAP-25, which is a soluble N-ethylmaleimide-sensitive attachment factor (SNARE) protein involved in synaptic vesicle fusion and docking.9 In 2004, a role in vesicular transport was confirmed in a study by Lee and Colleagues4 who identified target for Salmonella secreted protein C (TassC or Nipsnap4) in yeast and demonstrated its presence within a biochemical complex with the Salmonella enterica virulence factor SpiC, which has been linked to interference of intracellular vesicular trafficking via inhibition of the phagosome-lysosome maturation.10 This study found that suppression of Nipsnap4 in Salmonella devoid of SpiC allowed the Salmonella to survive within vacuoles in the infected host macrophages.

Interestingly, despite publication of only a handful of studies on Nipsnap proteins and their potential functions since their discovery, there is recent evidence linking Nipsnaps to ion channel regulation. In bioinformatic studies aimed at discovering proteins or protein complexes that mediate the Ca\textsuperscript{2+}-selective transient receptor potential vanilloid channels 5 and 6 (TRPV5/6)-dependent regulation of Ca\textsuperscript{2+} within epithelial cells,
Bindels and coworkers found Nipsnap1 as a TRPV5/6-associated protein.11 These authors reported a biochemical complex between Nipsnap1 and TRPV5/6, and mapped Nipsnap1 binding motifs within TRPV6. Additionally, they reported Nipsnap1 to be a negative regulator of TRPV6-mediated Ca2+ influx as both acute (i.e., Nipsnap1 in the patch pipette) and long-term (i.e., Nipsnap1 overexpression) treatments caused an inhibition of Ca2+ currents. The authors concluded that Nipsnap1 was a novel auxiliary protein inhibiting TRPV5/6 channel activity.

Inspired by this latter report, the aim of the present study was to investigate if Nipsnap1 or the related Nipsnap2 homolog are part of the functional interactome of L-type Ca2+ channels. We found that Nipsnap2, but not Nipsnap1, was a positive regulator of L-type Ca2+ channels although it did not appear to interact biochemically with Ca1.2. In addition to increasing L-type currents, overexpression of Nipsnap2 led to enhanced activation of the transcriptional factor CREB along with increased expression of several related transcription factors, suggesting a putative link between Nipsnap2 and transcription.

Results

The function of the Nipsnap family of proteins is largely unknown. The observed modulation of TRPV6 by Nipsnap1 suggests that they may work as ion channel regulators. Nipsnap1 and 2 share similar patterns of expression in the brain, although Nipsnap1 is heavily expressed in liver while Nipsnap2 appears to more abundant in skeletal muscle and heart.3,12 Based upon the expression profiles of Nipsnap1 and 2, and their presence in a screen for voltage-gated Ca2+ channels (data not shown), we choose to study the role of these proteins in regulation of L-type voltage-gated Ca2+ channels as these channels are expressed in brain, heart and skeletal muscle.13 Modulation of L-type voltage-gated Ca2+ channels by Nipsnaps was studied in a catecholaminergic A differentiated (CAD) neuronal cell line.14 CAD cells were selected for these studies as they express only L-type Ca2+ channels although it did not appear to interact biochemically with Ca1.2. The function of the Nipsnap family of proteins is largely unknown. The observed modulation of TRPV6 by Nipsnap1 suggests that they may work as ion channel regulators. Nipsnap1 and 2 share similar patterns of expression in the brain, although Nipsnap1 is heavily expressed in liver while Nipsnap2 appears to more abundant in skeletal muscle and heart.3,12 Based upon the expression profiles of Nipsnap1 and 2, and their presence in a screen for voltage-gated Ca2+ channels (data not shown), we choose to study the role of these proteins in regulation of L-type voltage-gated Ca2+ channels as these channels are expressed in brain, heart and skeletal muscle.13 Modulation of L-type voltage-gated Ca2+ channels by Nipsnaps was studied in a catecholaminergic A differentiated (CAD) neuronal cell line.14 CAD cells were selected for these studies as they express only L-type Ca2+ channels as well as for their ease of genetic manipulation.14 Inward Ba2+ currents which typically inactivated almost completely were recorded from these cells (Fig. 1A). Consistent with previous results in reference 14, the threshold for activation of these currents was around -50 mV and the maximum value occurred between -20 to -10 mV (Fig. 1B). Forty-eight hours after transfection, the Ba2+ current density was significantly higher in Nipsnap2 + EGFP-transfected CAD cells than in EGFP- or Nipsnap1 + EGFP-transfected CAD cells (Fig. 1C). On average, the Ba2+ current density was increased by about 45%, from -6.23 ± 0.24 pA/pF (n = 7) in EGFP expressing CAD cells to -8.95 ± 0.97 pA/pF (n = 8) (at -20 mV, p < 0.001, One-way ANOVA) in Nipsnap2 + EGFP expressing CAD cells compared with -6.67 ± 0.77 pA/pF (n = 9) in Nipsnap1 + EGFP expressing CAD cells (p > 0.05 vs. the other transfected conditions). Importantly, Nipsnap2 did not augment a non-L-type current in these cells as Nifedipine (20 μM), a well-known dihydropyridine inhibitor of L-type Ca2+ channels15 almost completely blocked (>98%) the upregulated Ca2+ currents (Fig. 1D and E). Both Nipsnap1 and Nipsnap2 expressed in CAD cells as 3xFlag fusion proteins were detected by an anti-flag antibody after transfection, confirming expression (Fig. 1F).

We also examined voltage-dependent properties of activation and inactivation of Ba2+ currents in these cells. The steady-state activation of Ca2+ channels from EGFP, Nipsnap1 + EGFP or Nipsnap2 + EGFP overexpressing CAD cells was well described by the Boltzmann relation (see Materials and Methods). For EGFP-expressing cells, V1/2 was -32.9 ± 0.6 mV (n = 8), which was significantly different from that of Nipsnap1 + EGFP transfected CAD cells (-36.7 ± 0.7 mV, n = 8; p < 0.05, Student’s t-test) but not from those expressing Nipsnap2 + EGFP (-35.2 ± 1.3, n = 8; p = 0.13; Student’s t-test). The slope factors for the Boltzmann fits (mV/e-fold change in conductance) were not different between the three transfected conditions: 6.6 ± 0.4 mV (n = 8) for EGFP transfected CAD cells, 6.3 ± 0.67 (n = 8) for Nipsnap1 + EGFP transfected CAD cells and 6.6 ± 1.1 (n = 8) for Nipsnap2 + EGFP transfected CAD cells (p > 0.05, One-way ANOVA).

The voltage-dependent properties of inactivation were determined by applying 5 sec conditioning pre-pulses that ranged successively from -120 to +30 mV in 0 mV voltage steps, followed by a 50 ms second step depolarization to 0 mV. The 0 mV test voltage was chosen as it is appropriately saturating on the Gm vs. Vm relation and at this test potential, the current amplitude is ~20% smaller than the peak current amplitude allowing for the best possible voltage clamp with minimal series resistance error. The normalized test pulse voltage-peak current amplitude relations was plotted against its corresponding holding potential and fitted with the Boltzmann equation. The voltage dependence of inactivation was similar for currents recorded from EGFP, Nipsnap1 + EGFP or Nipsnap2 + EGFP overexpressing CAD cells, as shown by the conductance vs. voltage relations. For EGFP-expressing CAD cells, V1/2 was -48.7 ± 1.1 mV (n = 7), which was not significantly different from the V1/2 values of -50.5 ± 0.7 (n = 7) or -48.7 ± 1.0 (n = 7) in Nipsnap1 + EGFP or Nipsnap2 + EGFP overexpressing CAD cells, respectively (p > 0.05, Student’s t-test). The slope factors were also similar: 4.9 ± 0.8 mV (n = 7) for EGFP transfected CAD cells; 4.5 ± 0.4 (n = 7) for Nipsnap1 + EGFP transfected CAD cells; and 4.9 ± 0.7 (n = 7) for Nipsnap2 + EGFP transfected CAD cells (p > 0.05). These results indicate that Nipsnap2 increases currents through L-type Ca2+ channels.

To further confirm that Nipsnap2 manipulation affected L-type Ca2+ channels, we next asked if knockdown of Nipsnap2 using short interfering RNA (siRNA) would decrease Ba2+ current density. Nipsnap2 siRNA (100 nM) caused a ~50–60% reduction in Nipsnap2 protein as assessed by immunoblotting and immunocytochemistry (Fig. 2A and B). Consistent with the knockdown, we observed that whole-cell recordings of CAD cells transfected with Nipsnap2 siRNA for two days in culture demonstrated reduced Ba2+ currents to -2.4 ± 0.3 pA/pF (n = 8); an ~73% reduction compared with Nipsnap2 overexpressing CAD cells (Fig. 2C and D). Voltage-gated activation or inactivation properties were not affected.

Channels

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Calcium channels, CaV1.1–4. Of these, only CaV1.2 and CaV1.3 are expressed in appreciable amounts within the brain. CaV1.2 is the predominant isoform expressed within CAD cells, with a ~260-fold greater expression compared with CaV1.3, as determined by quantitative RT-PCR (data not shown). So, since both CaV1.2 and CaV1.3 are present in CAD cells, we tested whether Nipsnap2 led to increased expression of either of these L-type calcium channels.

**Figure 1.** Nipsnap2 increases L-type Ca\(^{2+}\) channel current density in CAD cells. (A) Voltage protocol used to evoke Ba\(^{2+}\) currents (top). Representative family of Ba\(^{2+}\) current responses in CAD cells transfected with EGFP (topmost traces), Nipsnap1 + EGFP (middle traces) and Nipsnap2 + EGFP (bottommost traces) elicited by voltage steps from -70 to +70 mV from a holding potential of -80 mV. (B) Summary of current-voltage (I–V) relationships for the various transfected conditions (n = 7–9 cells per condition). (C) Peak current density (pA/pF) measured at -10 mV was similar between EGFP and Nipsnap1 (p > 0.05; Student’s t-test, n = 7–9 each). In contrast, Nipsnap2 expression caused a significant increase in current density vs. vector expressing CAD cells (*p < 0.05; Student’s t-test). Unless otherwise indicated, values are mean ± SEM and some error bars are smaller than the symbols. (D) Representative current response, at -20 mV, from a Nipsnap2-overexpressing CAD cell before and after application of 20 \(\mu\)M Nifedipine. (E) Summary of percent inhibition by Nifedipine in the various transfected conditions. The peak currents were not significant (n.s.) from each other (p > 0.05, Student's t-test). (F) Representative immunoblot (n = 4) of equal amounts of transfected CAD cells lysates demonstrating successful expression of Nipsnap proteins. An anti-flag antibody was used to detect the expressed proteins. Values are mean ± S.E.M. and some error bars are smaller than the symbols.

were not different between Nipsnap2 and scramble siRNA conditions. Collectively, these results confirm that Nipsnap2 has a specific effect on L-type current density. Because Nipsnap1 did not affect L-type current density in CAD cells, it was not tested any further in this study.

It is possible that the increase in L-type current density observed under these conditions is due to increased expression of Ca\(^{2+}\) channels. There are four distinct isoforms of L-type calcium channels, Ca\(_{1.1–4}\). Of these, only Ca\(_{1.2}\) and Ca\(_{1.3}\) are expressed in appreciable amounts within the brain.\(^{16}\) Ca\(_{1.2}\) is the predominant isoform expressed within CAD cells, with a ~260-fold greater expression compared with Ca\(_{1.3}\), as determined by quantitative RT-PCR (data not shown). So, since both Ca\(_{1.2}\) and Ca\(_{1.3}\) are present in CAD cells, we tested whether Nipsnap2 led to increased expression of either of these L-type calcium channels. Relative mRNA levels of Ca\(_{1.2}\)
A previous study found that Nipsnap1 was able to modulate TRPV6 through a biochemical interaction with the receptor. Based upon this observation and our finding that CaV1.2 mRNA was upregulated by Nipsnap2 (Fig. 3A), we asked if Nipsnap2 could bind to CaV1.2. CaV1.2 was chosen as the qPCR data suggested that Nipsnap2 was able to increase mRNA expression.

were significantly increased by ~4.8-fold in Nipsnap2 transfected CAD cells compared with control transfected CAD cells (Fig. 3A). Relative expression of the CaV1.3 isoform was not altered by Nipsnap2 overexpression. As expected, CAD cells transfected with Nipsnap2 showed an increase in Nipsnap2 mRNA levels compared with control transfection.

A previous study found that Nipsnap1 was able to modulate TRPV6 through a biochemical interaction with the receptor.17 Based upon this observation and our finding that CaV1.2 mRNA was upregulated by Nipsnap2 (Fig. 3A), we asked if Nipsnap2 could bind to CaV1.2. CaV1.2 was chosen as the qPCR data suggested that Nipsnap2 was able to increase mRNA expression.
The biochemical interaction between Nipsnap1/2 and Ca$_{\text{\textit{v}}}$,1.2 was tested in CAD cells overexpressing Nipsnap1- or Nipsnap2–3xFlag. CAD cells were transfected using polyethylenimine (PEI) and lysates were made 48 h later. Immunoprecipitations (IPs) were then performed on the freshly prepared lysates using an anti-Flag antibody. Nipsnap1 and Nipsnap2–3xFlag showed enrichment in the IPs although no immunoreactive Ca$_{\text{\textit{v}}}$,1.2 was observed in these fractions (Fig. 3B). These findings suggest that although Nipsnap2 is able to enhance L-type currents this does not appear to be via a direct biochemical action on Ca$_{\text{\textit{v}}}$,1.2. In addition, although expression of Nipsnap2 increased mRNA expression of Ca$_{\text{\textit{v}}}$,1.2 there was not a correlative change in Ca$_{\text{\textit{v}}}$,1.2 protein levels (data not shown).

As we observed an enhancement of L-type currents with Nipsnap2 overexpression we next sought to determine if downstream signaling of L-type Ca$^{2+}$ channels was also affected. Previous reports have shown signaling to the nucleus by an L-type Ca$^{2+}$ channel complex. This signaling involves phosphorylation, at Ser 133, of the transcription factor CREB which drives expression of genes involved in synaptic function. To investigate if increased L-type current density observed in Nipsnap2 overexpressing CAD cells can signal to CREB, we measured the activation of CREB. First, we tested the idea that Nipsnap2-mediated increase in Ca$^{2+}$ influx via L-type Ca$^{2+}$ influx increases CREB activation. CAD cells expressing dsRed (vector) or Nipsnap2 were depolarized for 40 min by 75 mM KCl; a 40 min depolarizing stimulation has been previously shown to induce sustained CREB phosphorylation in neurons. Nipsnap2 caused a marked increase in the ratio of phosphorylated CREB/CREB (pCREB/CREB) (Fig. 4A). Next, to examine if this increase was dependent upon L-type channels, Nipsnap2-overexpressing CAD cells were depolarized for 40 min in the presence or absence of two concentrations of Nifedipine. Consistent with these findings, a 40 min stimulation with 75 mM KCl caused a greater than 2-fold increase in the ratio of phosphorylated CREB/CREB (pCREB/CREB) over unstimulated control CAD cells (Fig. 4B and C). In the presence of 2 or 10 μM Nifedipine, KCl-stimulated CREB Ser$^{133}$ phosphorylation was reduced by 30 and 75%, respectively, compared with untreated cells (p < 0.05 vs. vehicle-treated control, Student’s t-test; Fig. 4C). CREB phosphorylation was not affected by incubation of Nipsnap-2 overexpressing CAD cells with ω-conotoxin GVI A (1 μM) or ω-agatoxin IVA (1 μM), inhibitors of N- and P/Q-type Ca$^{2+}$ channels, respectively (Fig. 4B and C). These results show Nipsnap2 increased CREB phosphorylation by increasing influx of Ca$^{2+}$ via L-type Ca$^{2+}$ channels in CAD cells.

Overexpression of Nipsnap2 leads to increased CREB phosphorylation, possibly due to increased calcium influx through L-type Ca$^{2+}$ channels. There is evidence that CREB has the ability to autoregulate its expression via the cAMP-response-element within its promoter. Thus, we investigated expression levels of CREB following overexpression of Nipsnap2. Compared with vector control, CREB mRNA levels were increased in CAD cells overexpressing Nipsnap2 (Fig. 4E). We then asked whether the expression of other transcription factors involved in CREB-target gene expression were also altered. Activating transcription factor 2 (ATF2) is a basic leucine zipper domain (bZip) transcription factor activated through calcium signaling which binds to the CRE sequences to regulate transcription. Another CREB-like transcription factor is transducer of regulated CREB activity 1 (TORC1). Calcium influx through voltage-gated Ca$^{2+}$ channels triggers dephosphorylation and nuclear translocation of TORC1 where it aids in the activation of CREB target genes. Relative mRNA levels of ATF2 and TORC1 were also increased in Nipsnap2 overexpressing cells (Fig. 4E). As a negative control, we also examined the expression levels of a CREB-unrelated transcription factor, Signal transducer and activator of transcription 1 (STAT1) is a transcription factor activated by interferon signaling. Relative mRNA levels of STAT1 were not affected by Nipsnap2 overexpression (Fig. 4E). We also wanted to determine if the increased expression of transcription factors involved in CREB signaling resulted in increased CREB-target gene expression. Salt inducible kinase 1 (SIK1) is a CREB-target gene which is activated in response to prolonged CREB/TORC signaling. Induction of SIK1 expression initiates a negative feedback mechanism through phosphorylation of TORC1

Figure 3. Nipsnap2 increases Ca$_{\text{\textit{v}}}$,1.2 mRNA expression. (A) Quantification of mRNA transcripts from CAD cells transfected with vector (control) or Nipsnap2. Total mRNA was isolated 48 h following transfection and all values are normalized to max for each gene. Significance was determined with a Student’s t-test (*p < 0.05; n = 6 for each gene for each condition). (B) CAD cells were transfected with Nipsnap1- or Nipsnap2–3xFlag fusion proteins before lysates were generated and immunoprecipitated using an anti-Flag antibody. Western blots displaying expression and subsequent immunoprecipitations (IPs) of 3xFlag fusion proteins along with Ca$_{\text{\textit{v}}}$,1.2 from transfected CAD cell lysates (n = 3).
mRNA levels were increased in Nipsnap 2 overexpressing cells. 

Consistent with this negative feedback, relative SIK1, which, combined with nuclear export, leads to reduced CREB signaling. 

Collectively, these results support Nipsnap2 as a regulator of transcription, and more specifically of CREB signaling and transcription factor expression.

**Figure 4.** Nipsnap2 mediated increase in L-type Ca\(^{2+}\) channels results in CREB activation. (A) CAD cells transfected with Nipsnap2 (+) or vector (-) were stimulated with a depolarizing concentration of KCl (75 mM) for 40 min, then lysed and analyzed by western blotting using anti-phosphoCREB or anti-CREB antibodies. Nipsnap2 overexpression increased pCREB/CREB levels compared with vector control. (B) CAD cells expressing Nipsnap2 were either unstimulated (U) or treated with 75mM KCl for 40 min in the presence of vehicle (0.01% MPL, C) or inhibitors of L-type (Nifedipine; Nif, 2 and 10 \(\mu\)M), N-type (\(\omega\)-conotoxin GVIA; Ctx, 1 \(\mu\)M) or P/Q-type (\(\omega\)-agatoxin GIVA; Aga, 1 \(\mu\)M) Ca\(^{2+}\) channels and subjected to western blot analysis as in (A). (C) Summary of ratio of phosphorylated CREB to total CREB (pCREB/CREB) from Nipsnap2 transfected CAD cells. (D) The graph shows the average \pm SEM ratios of pCREB:CREB from the various conditions. Asterisks (*) represent statistically significant differences between unstimulated and vehicle, 2 \(\mu\)M Nif and 10 \(\mu\)M Nif using an ANOVA with Dunnett’s post-hoc test (p < 0.05). The ampersand (&) indicates statistically significant difference between unstimulated and Nif (both concentrations) (p < 0.05; Student’s t-test). The numbers in parentheses represent number of independent samples from at least two separate transfections. (E) Total mRNA was isolated from CAD cells transfected with Nipsnap2 or vector control. Nipsnap2 overexpression led to increased expression of multiple transcription factors responsible for the induction of CREB-target genes, as well as the CREB-target gene SIK1. Expression levels of an unrelated transcription factor were not affected. Significance was determined with a Student’s t-test (\(t^p < 0.05\); n = 6 for each gene for each condition).

**Discussion**

This study identifies Nipsnap2 as a positive regulator of L-type Ca\(^{2+}\) channels. Notably, the Nipsnap2-mediated increase in Ca\(^{2+}\) influx via the L-type Ca\(^{2+}\) channel coupled to activation of the transcription factor CREB. Nipsnap2, however, was not able to immunoprecipitate Ca\(_{\alpha1.2}\) from CAD cell lysates suggesting that Nipsnap2 is not a biochemical partner of the Ca\(^{2+}\) channel. Together, these findings posit a potential role for Nipsnap2 in transcriptional activation via recruitment of CREB activity. Despite the close homology between Nipsnap1 and 2, there was only a slight shift in activation due to Nipsnap1 and no alteration in L-type current density.

The first question asked in this study was whether Nipsnaps could modulate L-type Ca\(^{2+}\) channels. We found that L-type Ca\(^{2+}\) channel density was significantly increased by Nipsnap2, but not Nipsnap1, overexpression in CAD cells suggesting either a change in insertion of channels in the membrane or a change in the open probability of the channel. Further studies into the modulation of L-type Ca\(^{2+}\) channels found that knockdown of Nipsnap2 significantly reduced L-type currents. Based upon these findings it appears that Nipsnap2 expression positively correlates with changes in L-type currents. Furthermore we observed an increase in Ca\(_{\alpha1.2}\) mRNA without a subsequent change in protein expression. Regulation of L-type currents by Nipsnap2 also appears to occur indirectly as Ca\(_{\alpha1.2}\) and Nipsnap2 were not present in the same biochemical complex. These findings suggest that Nipsnap2 works to increase L-type currents through a signaling mediated increase in surface expression or single channel activity of L-type Ca\(^{2+}\) channels.

Since L-type channels are believed to link membrane depolarization to regulation of gene expression, we explored the possibility that Nipsnap2 regulation of L-type channels may couple to an increase in transcriptional activation. A large body of work has established that membrane depolarization results in transduction of Ca\(^{2+}\) signals into the nucleus to initiate transcriptional activation that is dependent on L-type Ca\(^{2+}\) channels. Activation of the transcription factor CREB by phosphorylation of CREB at Ser 133 has been used an index of the initiation of transcription. Using this assay, our results show that Nipsnap2 overexpression itself can increase the levels of phosphorylated CREB (Fig. 4A), suggesting increased transcription. Importantly, our data showed that Nipsnap2-mediated increase in CREB phosphorylation was blocked upon inhibition of the L-type Ca\(^{2+}\) channel with Nifedipine. This suggests that the Nipsnap2-mediated increase in L-type currents translated into enhanced Ca\(^{2+}\) signaling as observed by amplified CREB phosphorylation. The role of Nipsnap2 in transcription was further supported by the finding that several CREB related transcription factors and gene targets were upregulated in cells transfected with Nipsnap2. Ca\(_{\alpha1.2}\) and Ca\(_{\alpha1.3}\)
have been reported to be differentially involved in the regulation of phospho-CREB levels. Activation of CaV_1.3 is important in CREB signaling when the depolarizing stimulus is low (e.g., 20 mM KCl), whereas CaV_1.2 becomes more influential as the level of depolarizing stimulus is increased (e.g., 45–90 mM KCl). Since our depolarization stimulus was 75 mM KCl, presumably an increase in Ca^{2+} influx via CaV_1.2 channels accounts for Nipsnap2-mediated increase in CREB activation. Interestingly, the 5' region of the gene encoding CaV_1.2 contains a CRE-sequence, and increased CREB signaling can lead to upregulation of CaV_1.2. As we observed an increase in CREB expression this may explain the increase in CaV_1.2 mRNA expression, although it is currently unclear why protein levels were unaffected. Given that little is known about the function of Nipsnap2, the mechanism of action on L-type currents is elusive and will require additional work. One possible explanation may be that as a putative transcriptional regulator Nipsnap2 is able to enhance L-type currents by affecting activity of other transcription factors such as NKF2.5, Mef2c, AP-1, CRE and hormones that bind in the promoter region of CaV_1.2. For instance, CaV_1.2 mRNA is upregulated at the transcriptional level by testosterone binding to hormone binding sites in its promoter or angiotensin II increasing CaV_1.2 promoter activity.

Another possibility is that Nipsnap2 modulates signaling proteins that directly regulate CaV_1.2 through post-translational modification or direct binding. However, very little is known about the function of Nipsnap2 beyond the current study and additional investigations are required.

In conclusion, this study showed that L-type currents positively correlate with Nipsnap2 expression which results in increased transcriptional activation. The modulation of L-type Ca^{2+} channels does not apparently occur through a direct biochemical interaction as Nipsnap2 and CaV_1.2 were not present in the same biochemical complex. The involvement of Nipsnap2 in regulation of L-type Ca^{2+} channels is entirely consistent with its expression in tissues with high expression levels of L-type Ca^{2+} channels such as skeletal muscle and heart, suggesting the testable hypothesis that Nipsnap2 may be involved in gene transcription in these tissues. Studies to identify the role of Nipsnap2 in downstream events following CREB activation, namely CRE activation and transcription are currently in progress.

Materials and Methods

Cell culture, transfection, antibodies and reagents. CAD cells were grown at 37°C and in 5% CO_2 in Ham's F12/DMEM medium + GlutaMax™ (GIBCO, cat #10565018), supplemented with 8% fetal bovine serum and 1% penicillin/streptomycin (100% stocks, 10,000 U/ml penicillin G sodium and 10,000 μg/ml streptomycin sulfate). Cells were passaged every 6–7 d at a 1:25 dilution. CAD cells were transfected with plasmid DNA encoding pRES-Nipsnap1–3xFlag-DsRed or pRES-Nipsnap1–3xFlag-DsRed and enhanced green-fluorescent protein (EGFP) or EGFP alone (control) using Lipofectamine 2000 (Invitrogen, cat #11668019), transfection with 100 nM of control or Nipsnap siRNA (Santa Cruz Biotechnology, cat #SC-149979) along with EGFP was also performed using Lipofectamine 2000. EGFP was used for assessment of transfection efficiency as well as to isolate cells for whole cell patch electrophysiology. For co-immunoprecipitations experiments CAD cells were transfected using PEI. DNA was mixed with PEI (Sigma, cat #40872–7) in OptimEM (Invitrogen) and vortexed briefly before incubating at RT for 5 min. The optimal DNA:PEI was determined experimentally for each stock of PEI and -60–70% transfection efficiency was routinely observed. The DNA:PEI mixture was then added drop wise to -70% confluent CAD cells and experiments were completed 2–3 d following transfection. Antibodies directed against CaV_1.2 (cat #L5746) were purchased from UC Davis/NIH NeuroMab Facility; Anti-Flag M2 antibody (cat #F1804) was acquired from Sigma. Antibodies recognizing β-tubulin (cat #G7121) were purchased from Promega. Antibodies against CREB (cat #9104) and Phospho-CREB (Ser 133, cat #9198) were obtained from Cell Signaling Technology. Horseradish peroxidase-conjugated goat anti-rabbit/mouse secondary were purchased from Jackson Immunoresearch or Sigma. Alexa-conjugated goat anti-rabbit/mouse secondary antibodies were from Invitrogen. All reagents were from Sigma unless otherwise indicated.

Isolation and cloning of Nipsnap1 and 2 cDNAs. Total RNA was isolated from neonatal day 2 rat brains (Omega Biotech). A cDNA library of mRNA sequences was then generated by annealing OligodT primers and performing RT-PCR using Superscript reverse transcriptase (Invitrogen). Nipsnap1 and Nipsnap2 DNAs were amplified from this cDNA library using gene-specific primers. The correctly amplified products were cloned into and pRES-3x-Flag-DsRed. This construct yielded a single vector that expressed both 3x-Flag fused Nipsnaps along with DsRed or GFP. All clones were sequence verified (Cogenics) before use in transfection experiments.

Electrophysiology. After 5 d in culture, cells were subjected to standard whole cell patch-clamp technique using an EPC-10 amplifier (HEKA Electronics) as described previously in reference 37 and 38. Current signals were filtered at 2 kHz, digitized at 5.71 kHz and analyzed with pClamp software (Molecular Devices). Data were leak subtracted on line by a standard P/4 protocol. Membrane capacitance (C_m) was determined as described previously in reference 34, and used to normalize currents. All experiments were performed at room temperature (~22°C).

Data analysis. The data are given as mean ± SEM. Statistical differences between two means were determined by Student’s t-tests (p < 0.05). Steady-state inactivation curves were fitted with a Boltzmann function: \( I_{h0} = I_{max}/(1 + \exp[(V - V_{1/2})/k]) \), where the current amplitude \( I_{h0} \) has decreased to half-amplitude at \( V_{1/2} \) with an \( e \)-fold change over \( k \) mV. The cell surface area was estimated from the whole-cell capacitance measurements assuming that \( C_m = 1 \mu F/cm^2 \).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Two 60 mm² plates of transfected CAD cells (~80% confluence) were lysed and homogenized and quantitative RT-PCR was performed as previously described in reference 39. Resulting mRNA levels were compared with L27,
a ribosomal housekeeping gene. For direct comparison of relative expression levels, normalized to max for target gene.

**Immunoprecipitations.** Immunoprecipitations were performed on CAD cell lysates transfected with Nipsnap1- or Nipsnap2–3xFlag constructs using PEI. Two days following transfection cell lysates were prepared and then incubated with 2 μg of anti-Flag M2 antibody for 2 h at 4°C with gentle agitation. Protein-G-agarose was then added and incubated for an additional 2 h. Samples were subsequently washed three times with lysis buffer before being analyzed by immunoblotting.

**Immunoblotting.** To monitor CREB phosphorylation by immunoblotting, CAD cells were stimulated with physiological saline solution with 75 mM KCl for 40 min at 37°C. Blockers were added 30 min prior to the stimulation and were kept throughout the stimulation period. Cell lysates were prepared, normalized to total protein amount and analyzed by immunoblotting as described in references 27 and 30.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

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