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Modulation of Ca_v2.1 Channels by Neuronal Calcium Sensor-1 Induces Short-term Synaptic Facilitation

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Abstract

Facilitation and inactivation of P/Q-type Ca²⁺ currents mediated by Ca²⁺/calmodulin binding to Ca_v2.1 channels contribute to facilitation and rapid depression of synaptic transmission, respectively. Other calcium sensor proteins displace calmodulin from its binding site and differentially modulate P/Q-type Ca²⁺ currents, resulting in diverse patterns of short-term synaptic plasticity. Neuronal calcium sensor-1 (NCS-1, frequenin) has been shown to enhance synaptic facilitation, but the underlying mechanism is unclear. We report here that NCS-1 directly interacts with IQ-like motif and calmodulin-binding domain in the C-terminal domain of Ca_v2.1 channel. NCS-1 reduces Ca²⁺-dependent inactivation of P/Q-type Ca²⁺ current through interaction with the IQ-like motif and calmodulin-binding domain without affecting peak current or activation kinetics. Expression of NCS-1 in presynaptic superior cervical ganglion neurons has no effect on synaptic transmission, eliminating effects of this calcium sensor protein on endogenous N-type Ca²⁺ currents and the endogenous neurotransmitter release machinery. However, in superior cervical ganglion neurons expressing wild-type Ca_v2.1 channels, co-expression of NCS-1 induces facilitation of synaptic transmission in response to paired pulses and trains of depolarizing stimuli, and this effect is lost in Ca_v2.1 channels with mutations in the IQ-like motif and calmodulin-binding domain. These results reveal that NCS-1 directly modulates Ca_v2.1 channels to induce short-term synaptic facilitation and further demonstrate that CaS proteins are crucial in fine-tuning short-term synaptic plasticity.

Keywords

short-term plasticity; neuronal calcium sensor-1; Ca_v2.1

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Introduction

Short-term plasticity on the time scale from milliseconds to seconds causes rapid, bidirectional changes in synaptic strength including facilitation, augmentation, post-tetanic potentiation, and depression (Zucker and Regehr, 2002). Integration of different patterns of short-term plasticity allows synapses to perform filtering and computational functions and, therefore, short-term plasticity is crucial for neural coding and information processing (Abbott and Regehr, 2004). Synaptic facilitation is caused by residual Ca^{2+} that builds up in the presynaptic terminal during repetitive action potentials and acts on Ca^{2+} sensors that are distinct from those responsible for fast exocytosis (Blatow et al., 2003; Catterall and Few, 2008; Zucker and Regehr, 2002). However, the effector mechanisms that respond to residual Ca^{2+} and enhance neurotransmitter release remain elusive.

P/Q-type Ca^{2+} currents conducted by $\text{Ca}_v2.1$ channels initiate neurotransmission at most fast conventional synapses (Dunlap et al., 1995; Jun et al., 1999; Llinas et al., 1992; Westenbroek et al., 1995). Facilitation and inactivation of P/Q-type Ca^{2+} currents are mediated by Ca^{2+} /calmodulin (CaM) binding to the IQ-like motif and calmodulin-binding domain (CBD) in the C-terminal domain of the $\alpha_12.1$ subunit (Catterall and Few, 2008; DeMaria et al., 2001; Lee et al., 2000; Lee et al., 1999; Lee et al., 2003). The steep dependence of neurotransmission on presynaptic Ca^{2+} entry predicts that this modulation should have a profound impact on short-term plasticity (Dodge and Rahamimoff, 1967). In agreement with this expectation, regulation of $\text{Ca}_v2.1$ channels by CaM induces facilitation followed by rapid depression of synaptic transmission in transfected superior cervical ganglion (SCG) neurons (Mochida et al., 2008).

CaM is the founding member of a large family of Ca^{2+} sensor (CaS) proteins that are differentially expressed in the central nervous system and retina (Burgoyne et al., 2004; Burgoyne and Weiss, 2001; Haeseleer et al., 2002). Interaction of Ca^{2+} -binding protein 1 (CaBP1) with CBD induces fast inactivation of $\text{Ca}_v2.1$ channels, which results in rapid synaptic depression in transfected SCG neurons (Few et al., 2011; Leal et al., 2012; Lee et al., 2002; Mochida et al., 2008). In contrast, visinin-like protein 2 (VILIP-2) binding to $\text{Ca}_v2.1$ slows the rate of inactivation and enhances short-term synaptic facilitation (Lautermilch et al., 2005; Leal et al., 2012; Nanou et al., 2012).

Neuronal calcium sensor-1 (NCS-1) is the classical example of facilitation of synaptic activity and secretion by CaS proteins (Pongs et al., 1993; Sippy et al., 2003; Tsujimoto et al., 2002; Weiss et al., 2010). Frequentin, the *Drosophila* homologue of NCS-1, increases synaptic facilitation (Pongs et al., 1993) and modulates Ca^{2+} entry through functional interaction with the protein encoded by *cacophony*, the only gene encoding a $\text{Ca}_v2 \alpha 1$ subunit in *Drosophila* (Dason et al., 2009). In rat hippocampal cell cultures and at the Calyx of Held synapse, NCS-1 induces Ca^{2+} -dependent synaptic facilitation (Sippy et al., 2003; Tsujimoto et al., 2002). In contrast, overexpression of a dominant-negative NCS-1 enhances activity of non-L-type Ca^{2+} channels in neuroendocrine cells, suggesting that NCS-1 negatively regulates these channels (Weiss et al., 2000; Weiss and Burgoyne, 2001). Direct binding of NCS-1 to the C-terminal domain of $\text{Ca}_v2.1$ has been shown in biochemical

experiments (Lian et al., 2014). However, the effects of NCS-1 interaction with Ca^{2+} channels in these preparations differ, and the mechanism of NCS-1 regulation of Ca^{2+} currents and modulation of synaptic plasticity remains uncertain. Here we report that NCS-1 reduces inactivation of $\text{Ca}_v2.1$ channels through interaction with the IQ-like motif and CBD in the C-terminal domain and thereby enhances short-term synaptic facilitation in SCG neurons. These results reveal the molecular mechanism of NCS-1-induced facilitation and demonstrate that CaS proteins are key players in the diversity of short-term synaptic plasticity.

Experimental methods

Binding assays

Binding assays were performed as previously described (Magupalli et al., 2013; Nanou et al., 2012). NCS-1-MBP or MBP alone was immobilized on amylose beads (New England Biolabs, Beverly, MA) that were extensively washed with PBS buffer. The MBP proteins were incubated with 4 μg of CBD-GST, IQ-GST or GST alone proteins for 2 hrs at 4 °C. The binding buffer contained (in mM): 200 NaCl, 1 MgCl_2 , 20 Tris-HCl and 0.1% Triton X-100. Ca^{2+} and EGTA were added as described. After extensive washing, bound proteins were eluted at 97 °C with sample buffer (2 $\times$) and separated on a NuPAGE gel (Invitrogen). Immunoblotting was performed with monoclonal anti-GST (Sigma) or anti-MBP (New England Biolabs) antibodies. Bound protein ligands were quantified by immunoblotting of the gel with a secondary antibody against GST or MBP covalently tagged with horseradish peroxidase, and measurement of horseradish peroxidase luminescence in the linear range of the luminometer. Analysis of the blots was done using the National Institutes of Health ImageJ program, and relative binding was normalized to control GST or MBP.

Transfection and voltage clamp recording of tsA-201 cells

Whole-cell voltage clamp recording were acquired from transfected tsA-201 cells (Nanou et al., 2012). Human embryonic kidney tsA-201 cells were grown in DMEM/Ham's F12 with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 100 units/ml penicillin and streptomycin (Invitrogen) to ~80% confluence at 37 °C and 10% CO_2 . NCS-1 was subcloned into an eGFP-expressing vector (pEGFP-N1) such that the resulting chimeric protein consisted of NCS-1 followed by eGFP in a single polypeptide. Cells were transfected with cDNA encoding Ca^{2+} channel subunits $\alpha_12.1$ (2 μg) or $\alpha_12.1_{\text{IMAA}}/\text{CBD}$ (2 μg), β_{2a} (1.5 μg), $\alpha_2\delta$ (1 μg) and NCS-1-eGFP (0.005 μg) using TransIT-LT1 (Mirus Bio LLC, Madison, WI). Finally, 24h after transfection, the cells were used for electrophysiological recordings. Whole cell voltage clamp recordings were acquired from transfected tsA-201 cells at room temperature. The recordings were done in an extracellular solution containing (in mM) 10 CaCl_2 or 10 BaCl_2 , 150 Tris, 1 MgCl_2 (305 mosM) and with an intracellular solution consisting of (in mM) 120 N-methyl-D-glucamine, 60 Hepes, 1 MgCl_2 , 2 Mg-ATP , and 0.5EGTA (295 mosM). The pH of both solutions was adjusted to pH 7.3 with methanesulfonic acid. Recordings were made with a HEKA EPC 10 patch clamp amplifier using PULSE software (HEKA Elektronik, Lambrecht, Germany) and filtered at 3 kHz. Leak and capacitive transients were subtracted using a P/-4 protocol. Voltage protocols were adjusted to compensate for the more negative voltage dependence of

activation in extracellular Ba^{2+} solution. Data analysis was performed using IGOR (Wave-Metrics, Lake Oswego, OR). Activation curves were fit to determine values for the voltage of half-activation ($V_{1/2}$) and the slope (k) using the following Boltzmann equation:

$y = (y_{\max} - y_{\min}) / (1 + \exp((V_{1/2} - V)/k)) + y_{\min}$. All average data represent the mean $\pm$ SEM. Statistical significance was determined using Student's unpaired t test.

Expression of $\text{Ca}_v2.1$ in cultured SCG neurons

SCG neurons were cultured for 6 weeks as described (Leal et al., 2012; Mochida et al., 2008; Mochida et al., 1996) to allow synapse formation. cDNA encoding $\alpha_12.1$ (2.5 μg) or $\alpha_12.1_{\text{IMAA/CBD}}$ (2.5 μg), NCS-1-eGFP (0.5 μg) or eGFP were microinjected into the nuclei of SCG neurons through glass micropipettes along with fast green dye as described (Leal et al., 2012; Mochida et al., 2008; Mochida et al., 1996).

Synaptic transmission between SCG neurons

Conventional intracellular recordings were made from two neighboring neurons using microelectrodes filled with 1M KAc (40–60 M Ω). EPSPs were recorded from a non-injected neuron, whereas action potentials were generated in the injected presynaptic neuron expressing $\text{Ca}_v2.1$ channels and NCS-1 proteins by passing 1–2 nA of current for 5 ms. All endogenous synaptic transmission was blocked by bath application of 3 μM ω -conotoxin GVIA in a modified Krebs's solution consisting of (in mM) 136 NaCl, 5.9 KCl, 2 CaCl_2 , 1.2 MgCl_2 , 11 glucose and 3 Na-Hepes (pH 7.4). Each protocol was repeated 10 times. EPSP amplitudes were averaged and normalized to the first EPSP of each trial and plotted against action potential number. Mean EPSP trains (Fig. 6B) were fit with biexponential functions $C + A\exp(-t/\tau_1) + (1 - A - C)\exp(-t/\tau_2)$. The absolute values of τ_1 describing facilitation were poorly defined by the data and were fixed at reasonable values for fitting. Mean EPSP trains (Figure 5 and Fig. 7) were fit with single exponential functions $C + A\exp(-t/\tau)$. Best-fit (solid lines) and 95% confidence estimates for these fits (dotted lines) are provided in the figures.

Results

Interaction between $\text{Ca}_v2.1$ and NCS-1

Although NCS-1 shares structural and amino acid sequence similarity with other CaS proteins, it was not known prior to this work whether it binds directly to $\text{Ca}_v2.1$ channels. Therefore, as a first step toward characterizing its potential functional effects on $\text{Ca}_v2.1$ channels, we used in vitro binding methods to measure its interaction with the CaS protein regulatory site on $\text{Ca}_v2.1$. To test direct binding of NCS-1 to the IQ-like motif and the CBD in the CaS protein regulatory site, we used MBP-tagged NCS-1 immobilized on amylose beads and GST-tagged IQ-like domain and CBD. In control experiments, GST alone did not bind to either NCS-1-MBP or MBP (Fig. 1A, lanes 1–3). CBD-GST bound specifically to immobilized NCS-1-MBP in the presence of 500 μM Ca^{2+} (Fig. 1A, lane 4). Binding of CBD-GST to NCS-1-MBD is Ca^{2+} -dependent, as binding was lost in the presence of EGTA (Fig. 1A, lanes 4 and 5). In contrast to CBD-GST, we observed specific binding of the IQ-GST to NCS-1-MBP (Fig. 1A, lanes 6 and 7), but addition of EGTA had no effect (Fig. 1A, lane 8) indicating that binding of IQ-GST to NCS-1-MBP is Ca^{2+} -independent. These

results demonstrate differential Ca^{2+} -dependent interactions of NCS-1 with the two components of the CaS protein regulatory site.

Previous studies have shown that mutation of the IQ-like motif by substitution of two Ala residues for the initial Ile and Met to give the mutant IM-AA reduces binding affinity for CaS proteins (DeMaria et al., 2001; Lee et al., 2003). We used a GST-tagged IM-AA mutant IQ domain to test the effect of this mutation on binding of NCS-1. We observed that alanine substitutions in the IQ-like domain caused a significant decrease in binding (Fig. 1B. Ca^{2+} : IQ + NCS-1, 0.57 ± 0.06 vs. IMAA + NCS-1, 0.24 ± 0.06 ; EGTA: IQ + NCS-1, 0.56 ± 0.06 vs. IMAA + NCS-1, 0.29 ± 0.08 ; $*p < 0.05$). The remaining binding activity was Ca^{2+} independent as for the wild-type IQ-like motif. These results support the conclusion that NCS-1 binds directly to the CaS protein regulatory site including the IQ-like motif.

Modulation of $\text{Ca}_v2.1$ channels by NCS-1

We carried out whole-cell voltage clamp studies of tsA-201 cells transfected with $\text{Ca}_v2.1$ alone or together with NCS-1 during 1-s test pulses. Co-expression of NCS-1 did not alter the peak current amplitude, IV-relationship (Fig. 2A, $p = 0.89$) or voltage-dependence of half-maximal activation (Fig. 2B, $V_{1/2}$: 8.28 ± 0.62 mV vs. 8.68 ± 1.01 mV, $p = 0.73$). The 0 to 63% rise time of the $\text{Ca}_v2.1$ current was calculated to compare the rate of activation (Fig. 2C). No significant change of the rate of activation was observed (Fig. 2C, 1.62 ± 0.25 ms vs. 1.51 ± 0.11 ms, $p = 0.78$).

During 1-s test depolarizations, co-expression of NCS-1 with $\text{Ca}_v2.1$ slowed the rate of inactivation significantly (Fig. 3A). The residual Ca^{2+} current of NCS-1-expressing cells at the end of a 1-s test pulse was significantly larger than that of cells transfected with $\text{Ca}_v2.1$ alone (Fig. 3B, 0.28 ± 0.03 vs. 0.40 ± 0.04 , $*p < 0.05$). To analyze NCS-1 regulation in response to more physiological stimuli, Ca^{2+} currents were recorded during trains of repetitive 5 ms depolarizations to 20 mV at 100 Hz. In $\text{Ca}_v2.1$ -transfected cells, the Ca^{2+} current showed facilitation followed by progressive inactivation, similar to previous studies (Fig. 3C) (Lautermilch et al., 2005; Lee et al., 2000; Nanou et al., 2012). Co-expression of NCS-1 resulted in enhanced facilitation and slowed inactivation of Ca^{2+} current (Fig. 3C).

Enhanced facilitation and rapid inactivation of $\text{Ca}_v2.1$ channels are Ca^{2+} -dependent (Lee et al., 1999). We used Ba^{2+} substitution for Ca^{2+} to determine whether the effects of NCS-1 were also Ca^{2+} -dependent. Substitution of Ca^{2+} with Ba^{2+} shifted the voltage-dependence activation to more negative membrane potential, but there was no difference in $V_{1/2}$ between cells transfected with $\text{Ca}_v2.1$ alone or with NCS-1 (Fig. 2D, $V_{1/2}$: 2.86 ± 1.28 mV vs. 2.55 ± 1.55 mV; $p = 0.88$). After replacing Ca^{2+} with Ba^{2+} as the charge carrier, the rate of inactivation was similar between cells transfected with $\text{Ca}_v2.1$ alone or with NCS-1 (Fig. 3D–F), indicating that NCS-1 specifically modulates Ca^{2+} -dependent inactivation. The Ca^{2+} -dependent component of inactivation is caused by association of endogenous CaM with C-terminal domain of $\text{Ca}_v2.1$ (DeMaria et al., 2001; Lee et al., 1999; Lee et al., 2003). CaM was shown to be endogenously expressed in tsA-201 cells and to bind to the CaS protein regulatory site (Lee et al., 1999). Therefore, our results suggest that NCS-1 binds to the CaS protein regulatory site, displaces CaM, and thereby regulates $\text{Ca}_v2.1$ channels by reducing Ca^{2+} -dependent inactivation.

Function of the IQ-like motif and CBD in modulation of Cav2.1 channels by NCS-1

To test whether facilitation is mediated by interaction between NCS-1 and Cav2.1, we studied the Cav2.1_{IMAA/ CBD} mutant, which is known to abolish the interaction between the Cav2.1 channel and CaS proteins (DeMaria et al., 2001; Lautermilch et al., 2005; Lee et al., 1999; Lee et al., 2003; Nanou et al., 2012). Cav2.1_{IMAA/ CBD} was transfected alone or together with NCS-1. Co-expression of NCS-1 with Cav2.1_{IMAA/ CBD} had no effect on Ca²⁺-dependent inactivation during a single depolarization or repetitive depolarizations (Fig. 4A–C) and had no effect on the peak current amplitude ($p = 0.86$) or voltage-dependence of activation (Fig. 4D $V_{1/2}$: 8.92 ± 1.3 mV vs. 8.41 ± 1.15 mV; $p = 0.78$). Overall, these results indicate that association of NCS-1 with the bipartite CaS regulatory site in the C-terminal domain of Cav2.1 is required for NCS-1 modulation of P/Q-type Ca²⁺ currents.

NCS-1 and endogenous neurotransmission in superior cervical ganglion neurons

Cultured SCG neurons transfected with wild-type or mutant calcium channels are an advantageous experimental system for studies of the role of calcium channel regulation in synaptic plasticity (Leal et al., 2012; Magupalli et al., 2013; Mochida et al., 2008; Mochida et al., 2003). Endogenous neurotransmission in cultured SCG neurons is mediated by N-type Ca²⁺ currents, which can be blocked by ω -conotoxin GVIA (Mochida et al., 2008; Mochida et al., 2003). In response to a train of depolarizing stimuli, endogenous synapses between SCG neurons show rapid depression but no facilitation (Fig. 5). In order to assess the effects of NCS-1 on endogenous neurotransmission in SCG neurons, we transfected NCS-1 by injection of cDNA into the presynaptic neuron (Materials and methods). We found no significant effects of presynaptic expression of NCS-1 on either short-term facilitation or rapid depression of endogenous neurotransmission mediated by N-type Ca²⁺ currents (Fig. 5). These negative results show that the endogenous N-type calcium channels are not modulated by NCS-1, and they also eliminate effects of NCS-1 on the endogenous exocytosis machinery as important contributors to facilitation in SCG neurons. Therefore, SCG neurons are an appropriate null background for studies of the effects NCS-1 on short-term synaptic plasticity of synaptic transmission mediated by transfected Cav2.1 channels.

NCS-1 modulation of short-term synaptic plasticity mediated by Cav2.1 channels

We transfected Cav2.1 channels without or with NCS-1 in SCG neurons by injection of cDNA in the presynaptic neuron to assure that the expressed proteins have no postsynaptic effects, and we blocked endogenous N-type Ca²⁺ currents with ω -conotoxin GVIA (see Materials and methods). Since neural information is encoded in bursts of synaptic activity *in vivo*, we stimulated the synapses at various frequencies to elicit short-term synaptic plasticity (Fig. 6). Repetitive stimulation at 10 Hz produced short-term depression of EPSPs, which was similar in control synapses and NCS-1 synapses (Fig. 5B, EPSP due to stimulus 3; $p = 0.48$). We observed little or no facilitation followed by rapid depression of synaptic transmission for stimuli at 20 Hz in control synapses (Fig. 6A, B), but synapses expressing NCS-1 showed enhanced facilitation and slower depression at 20 Hz (Fig. 6A, B). More rapid stimulation at 30 Hz induced greater facilitation in control synapses and larger increases in facilitation and in the rate of rapid depression in the presence of NCS-1 (Fig. 6B). Peak synaptic facilitation measured from the EPSPs in response to stimulus 3 was

significantly enhanced at 20 Hz and 30 Hz in NCS-1-expressing synapses (CON: 0.78 ± 0.12 vs NCS-1: 1.45 ± 0.11 , $*p < 0.01$ for 20 Hz; 0.95 ± 0.10 vs 1.63 ± 0.17 , $*p < 0.01$ for 30 Hz). These results show that expression of NCS-1 induced enhanced synaptic facilitation during trains of evoked action potentials.

Function of the IQ-like motif and CBD in short-term plasticity

To test whether direct binding of NCS-1 to the CaS protein regulatory site on Cav2.1 is required for modulation of short-term plasticity by NCS-1, we transfected SCG neurons with Cav2.1_{IMAA/ CBD} alone or with NCS-1. Co-expression of NCS-1 had no effect on Cav2.1_{IMAA/ CBD}-driven synaptic transmission in response to trains of stimulation at 10, 20, and 30 Hz (Fig. 7). Thus, NCS-1 expression did not alter either facilitation or depression in response to trains of stimulation in Cav2.1_{IMAA/ CBD}-expressing synapses, indicating that its effects on short-term synaptic plasticity require binding to the CaS regulatory site on Cav2.1 channels.

Modulation of paired-pulse facilitation by NCS-1

Facilitation and rapid depression of synaptic transmission can also be measured in paired-pulse stimulation experiments. To determine whether presynaptic expression of NCS-1 could affect this rapid form of short-term synaptic plasticity, we evoked EPSPs by paired stimuli with different interstimulus intervals as shown in Fig. 8A. Paired pulse ratios (PPRs) for synaptic transmission mediated by Cav2.1 were approximately 1.0 (Fig. 8A, B, CON: PPR_{30ms}, 1.06 ± 0.11 , CON: PPR_{50ms}, 1.09 ± 0.09) at the interstimulus intervals we examined using 2 mM [Ca]_i, with no indication of paired-pulse facilitation or depression. In contrast, NCS-1-expressing synapses exhibited significant paired-pulse facilitation (PPF) at interstimulus intervals between 30 ms and 50 ms (Fig. 8A–C; NCS-1: PPR_{30ms}, 1.58 ± 0.12 , NCS-1: PPR_{50ms}, 1.47 ± 0.13 , $*p < 0.05$). Paired-pulse facilitation induced by NCS-1 was not caused by a change of initial neurotransmitter release probability since there was no difference between amplitudes of the first EPSP between control synapses and NCS-1-expressing synapses (Fig. 8D, left panel, $p = 0.56$). Moreover, the shapes of EPSPs were identical for control synapses and NCS-1-expressing synapses (Fig. 8A), ruling out changes in the kinetics of the postsynaptic component as the basis of the facilitation. These data show that NCS-1 induces paired-pulse facilitation by changing release probability in an activity-dependent manner.

In contrast to the results with wild-type Cav2.1 channels, transfection of NCS-1 with Cav2.1_{IMAA/ CBD} did not induce facilitation at any interstimulus interval (Fig. 8B). As for wild-type Cav2.1, expression of Cav2.1_{IMAA/ CBD} alone or with NCS-1 did not alter the basal neurotransmission compared with synapses expressing Cav2.1 (Fig. 8D, right panel, $p = 0.63$). These data demonstrate that mutation of the IQ-like motif and deletion of the CBD eliminate the modulation by NCS-1 and therefore suggest that direct binding of NCS-1 to Cav2.1 via the IQ-like motif and CBD is required for NCS-1 modulation of short-term plasticity.

Discussion

Our results show that NCS-1 can modulate P/Q-type Ca^{2+} currents and short-term synaptic plasticity through direct interaction with the CaS protein regulatory site in the C-terminal domain of presynaptic $\text{Ca}_v2.1$ channels. These results provide strong evidence that expression of NCS-1 can override ubiquitous CaM-dependent regulation of $\text{Ca}_v2.1$ channels and allow neurons to change their patterns of short-term synaptic facilitation and depression. Evidently, differential expression of NCS-1 in neurons can contribute to the diversity of presynaptic short-term synaptic plasticity and the resulting broad range of neuronal integration and computation through its modulation of Ca^{2+} -dependent inactivation of presynaptic $\text{Ca}_v2.1$ channels.

Direct binding between NCS-1 and $\text{Ca}_v2.1$ channels

We found that NCS-1 interacts with the IQ-like domain of $\text{Ca}_v2.1$ channels (residues 1848–1964) in a Ca^{2+} -independent manner and binds to the downstream CBD (residues 1959–2035) in a Ca^{2+} -dependent manner. A recent paper published after submission of this work for review also showed that NCS-1 bound directly to the C-terminal domain of $\text{Ca}_v2.1$ channels; however, it bound to a peptide containing residues 1898–2035 in a Ca^{2+} -dependent manner and did not bind to the CBD (Lian et al., 2014). These two studies are similar in that both show specific, calcium-dependent interactions of NCS-1 with peptides derived from the C-terminal regulatory domain of $\text{Ca}_v2.1$ channels. However, the details of the binding reaction and its Ca^{2+} -dependent regulation differ between the two sets of results. Differences in the peptide fragments studied and in the ionic conditions used to study the binding interactions may account for these differences in results. Our model for biphasic regulation of $\text{Ca}_v2.1$ channel function by sequential interactions with the N- and C-terminal lobes of CaS proteins envisions reciprocal Ca^{2+} -dependent conformational changes in the CaS proteins and their interaction site(s) that drive channel regulation (Catterall and Few, 2008; Lee et al., 2003); therefore, it is not surprising that different peptides derived from the interacting sites would have differences in Ca^{2+} -dependent binding properties. Our results presented here connect these binding interactions directly to calcium channel regulation and synaptic plasticity.

NCS-1 binding reduces Ca^{2+} -dependent inactivation of $\text{Ca}_v2.1$ channels

In transfected tsA-201 cells, we found that Ca^{2+} -dependent inactivation of $\text{Ca}_v2.1$ channels is specifically reduced by NCS-1. No effect was observed on Ba^{2+} currents, confirming that the effects of this CaS protein require Ca^{2+} entry. Previous studies had not resolved the cellular or molecular mechanism through which NCS-1 may regulate the function of $\text{Ca}_v2.1$ channels. When expressed in dissociated hippocampal neurons, NCS-1 did not alter the non-L-type Ca^{2+} current, which likely included N-type, P/Q-type, and possibly R-type Ca^{2+} currents (Sippy et al., 2003). Specific effects of NCS-1 on P/Q-type Ca^{2+} current may have been obscured by these other Ca^{2+} currents that are not responsive to NCS-1. Micro-injected NCS-1 protein enhanced facilitation of P/Q-type Ca^{2+} current at the Calyx of Held synapse via acceleration of voltage-dependent activation (Tsujimoto et al., 2002). This effect could have been mediated indirectly through an intermediary protein, and therefore may be distinct from the modulation of P/Q-type Ca^{2+} current via direct interaction between NCS-1 and the

CaS protein regulatory site on the Cav2.1 channel that we describe here. Alternatively, effects on P/Q-type Ca^{2+} currents in the Calyx of Held could arise through differential modulation of Cav2.1 channels by Cav β subunits or other proteins in that synapse.

NCS-1 interaction with Cav2.1 channels induces synaptic facilitation

NCS-1 has been reported to modulate both basal neurotransmission (Olafsson et al., 1995; Wang et al., 2001) and short-term synaptic plasticity (Pongs et al., 1993; Sippy et al., 2003; Tsujimoto et al., 2002) via a variety of molecular mechanisms. Synaptic facilitation depends on elevation of residual Ca^{2+} during repetitive stimulation (Zucker and Regehr, 2002). One model proposes that facilitation is due to elevation of residual Ca^{2+} following saturation of local calcium buffers (Zucker and Regehr, 2002). If NCS-1 acts as such a calcium buffer, over-expression of NCS-1 should result in decreased facilitation, which is opposite to our findings. Moreover, in our experiments, it is not likely that NCS-1 enhances facilitation by acting as a fast, saturable buffer (Zucker, 2001), since neither basal neurotransmission nor neurotransmission initiated by N-type Ca^{2+} currents is affected by NCS-1 expression.

Another model for short-term facilitation is modulation of synaptic vesicle function. Could NCS-1 instead modulate exocytosis machinery? To address this question, we recorded endogenous synaptic transmission driven by Cav2.2 channels in SCG neurons and found that basal neurotransmission was not affected by co-expression of NCS-1 and short-term facilitation remained unchanged. These results indicate that expression of NCS-1 specifically modulates short-term plasticity mediated by Cav2.1 channels and leaves the endogenous exocytosis machinery unaffected. Evidently, NCS-1 primarily acts as a calcium sensor for modulation of Cav2.1 channels and short-term plasticity in the presynaptic nerve terminals of SCG neurons.

Our results show that specific expression of NCS-1 in presynaptic neurons can functionally compete with endogenous CaM for regulation of Cav2.1 channels and synaptic transmission. Endogenous CaS proteins are highly expressed in central neurons, so it is likely that similar regulation of synaptic plasticity and neurotransmission takes place. The interaction between NCS-1 and Cav2.1 channels enhances presynaptic Ca^{2+} entry by reducing inactivation of P/Q-type Ca^{2+} current, leading to facilitation of synaptic transmission as predicted by the supralinear dependence of neurotransmission on presynaptic Ca^{2+} entry (Dodge and Rahamimoff, 1967). The fact that modulation of Ca^{2+} current and short-term plasticity is largely eliminated when mutant Cav2.1^{IMAA/ CBD} channels are expressed demonstrates that direct binding of NCS-1 is required and eliminates effects on other possible presynaptic targets. Our results do not exclude additional mechanisms of NCS-1 regulation of synaptic plasticity in other physiological settings, but regulation of Cav2.1 channels by direct binding to their CaS protein regulatory site is responsible for its effects on short-term synaptic plasticity in transfected SCG neurons.

NCS-1 and diversity of short-term synaptic plasticity

NCS-1, like VILIP-2, CaBP1, and other CaS proteins, has a similar structure to their common ancestor, CaM, with two pairs of EF-hand Ca^{2+} -binding domains separated by a central alpha helix (Burgoyne and Weiss, 2001). NCS-1 is able to bind three Ca^{2+} ions in the

submicromolar range with affinities considerably higher than those of CaM (Mikhaylova et al., 2009). The higher affinity of NCS-1 for Ca^{2+} likely contributes to its ability to override the regulatory effects of ubiquitously expressed CaM. The highest level of expression of NCS-1 was found in the hippocampus, cerebellum, and retina in rat brain (Braunewell and Gundelfinger, 1999; Paterlini et al., 2000). Therefore, cell-type specific modulation of short-term plasticity by NCS-1 should add to the diversity of the short-term synaptic plasticity in these neurons. Our previous results with CaBP1 and VILIP-2 indicate that these CaS proteins can also override the ubiquitous effects of CaM and modulate $\text{Ca}_v2.1$ channels and short-term synaptic plasticity in a bi-directional manner, fine-tuning synaptic plasticity by enhancing facilitation or accelerating depression (Leal et al., 2012). Overall, our results suggest that modulation of presynaptic Ca^{2+} entry due to regulation of facilitation and/or inactivation of P/Q-type Ca^{2+} current by NCS-1 and other CaS proteins may be a crucial determinant of short-term synaptic plasticity and the encoding properties of neurons through activity-dependent modulation of presynaptic function.

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Highlights

1. NCS-1 directly interacts with the IQ-like motif and CBD domain of Cav2.1 channels.
2. NCS-1 reduces Ca^{2+} -dependent inactivation of P/Q-type Ca^{2+} current.
3. Presynaptic expression of NCS-1 induces short-term synaptic facilitation.
4. Presynaptic effects of NCS-1 are lost when the IQ-like motif and CBD are mutated.

Figure 1.

Binding of IQ domain and calmodulin binding domain (CBD) with NCS-1. (A) Binding of GST-tagged IQ domain and CBD domain with MBP-tagged NCS-1 immobilized on amylose resin beads (Bait) in the presence of 200 μM Ca^{2+} , 500 μM Ca^{2+} , or 10 mM EGTA detected by immunoblot with anti-GST antibodies. 1) NCS-1-MBP+GST; 2) MBP+GST; 3) MBP+GST; 4) NCS-MBP+CBD-GST, 500 μM Ca^{2+} ; 5) NCS-MBP+CBD-GST, 10 mM EGTA; 6) MBP+IQ-GST; 7) NCS-MBP+IQ-GST; 8) NCS-MBP+IQ-GST; (B) Binding of GST-tagged IQ domain and IM-AA domain with MBP-tagged NCS-1 immobilized on amylose resin beads (Bait) in the presence of 200 μM Ca^{2+} or 10 mM EGTA detected by immunoblot with anti-GST antibodies. Relative binding to NCS-1 was calculated by determining the ratio between IQ-GST or IMAA-GST and NCS-1-MBP. Bound ligands were quantified by direct photon counting of luminescence from the antibodies bound to epitope tags in the linear range of the luminometer. * $p < 0.05$, one-way ANOVA, *post-hoc* Tukey test.

Figure 2.

NCS-1 expression does not affect IV-relationship or voltage-dependent activation. (A) Mean IV curve in tsA-201 cells transfected with Ca_v2.1 alone (black, n = 6) or co-transfected with NCS-1 (red, n = 6). The current was activated by depolarizing steps from -40 mV to 60 mV for 10 ms. (B) Voltage dependence of activation of Ca_v2.1 channels alone (black; n = 13), or with NCS-1 (red; n = 11) with Ca²⁺ as permeant ion. (C) The 0 to 63% rise time of Ca_v2.1 current was calculated in tsA cells transfected with Ca_v2.1 alone (black, n = 6) or co-transfected with NCS-1 (red, n = 6). (D) Voltage dependence of activation of Ca_v2.1 channels alone (black; n = 12), or with NCS-1 (red; n = 7) with Ba²⁺ as permeant ion.

Figure 3.

Modulation of $\text{Ca}_v2.1$ channels by NCS-1. (A) Mean normalized Ca^{2+} currents in tsA-201 cells transfected with $\text{Ca}_v2.1$ alone (black, $n = 16$) or co-transfected with NCS-1 (red, $n = 9$). $*p < 0.05$, unpaired t test. (B) Mean ratio of Ca^{2+} residual current amplitude measured at the end of the 1 s pulse (I_{res}) over peak current amplitude (I_{peak}) from tsA-201 cells transfected with $\text{Ca}_v2.1$ alone (black) or cotransfected with NCS-1 (red). (C) Mean normalized Ca^{2+} current during 100 Hz train of 5 ms pulses to +20 mV for $\text{Ca}_v2.1$ alone (black, $n = 15$) or co-transfected with NCS-1 (red, $n = 11$). (D) Mean normalized Ba^{2+} currents in cells transfected with $\text{Ca}_v2.1$ alone (black, $n = 14$) or co-transfected with NCS-1 (red, $n = 7$). (E) Mean ratio of Ba^{2+} residual current amplitude measured at the end of the 1 s pulse (I_{res}) over peak current amplitude (I_{peak}) from tsA-201 cells transfected with $\text{Ca}_v2.1$ alone (black) or cotransfected with NCS-1 (red). (F) Mean normalized Ba^{2+} current during 100 Hz train for $\text{Ca}_v2.1$ alone (black, $n = 17$) or co-transfected with NCS-1 (red, $n = 7$).

Figure 4.

Functions of IQ domain and CBD domain in modulation of Ca_v2.1 channel by NCS-1. (A) Mean normalized Ca²⁺ currents in cells transfected with Ca_v2.1_{IMAA/ CBD} (black, n = 11) or co-transfected with NCS-1 (red, n = 9). (B) Mean ratio of Ca²⁺ residual current amplitude measured at the end of the 1 s pulse (I_{res}) over peak current amplitude (I_{peak}) from tsA-201 cells transfected with Ca_v2.1_{IMAA/ CBD} alone (black) or cotransfected with NCS-1 (red). (C) Mean normalized Ca²⁺ current during 100 Hz train for Ca_v2.1_{IMAA/ CBD} alone (black, n = 11) or with NCS-1 (red, n = 7). (D) Voltage dependence of activation of Ca_v2.1_{IMAA/ CBD} channels alone (black; n = 6) or with NCS-1 (red; n = 6).

Figure 5.

NCS-1 modulation of synaptic transmission mediated by endogenous N-type Ca^{2+} current. Mean normalized EPSP amplitude at 30 Hz. EPSPs were normalized to the first EPSP of each train and plotted against action potential number for endogenous $\text{Ca}_v2.2$ alone (black) and with NCS-1 (red) ($n = 6$).

Figure 6.

Modulation of short-term plasticity during bursts of neuronal activity by NCS-1. (A) Representative EPSPs evoked by repetitive action potentials at 20 Hz for 1 s in the presynaptic neurons expressing $\text{Ca}_v2.1$ alone (black) or co-transfected with NCS-1 (red). Data from 10 sweeps repeated every 30 s at each frequency were averaged. (B) Mean normalized EPSP amplitude at 10, 20 and 30 Hz frequency. EPSPs were normalized to the first EPSP of each train and plotted against action potential number for $\text{Ca}_v2.1$ alone (black) and with NCS-1 (red). Points represent mean $\pm$ SEM. $n = 6-10$

Figure 7. NCS-1 modulation of synaptic transmission mediated by $\text{Ca}_v2.1_{\text{IMAA/ CBD}}$. Mean normalized EPSP amplitude at 10, 20 Hz and 30 Hz for neurons injected with $\text{Ca}_v2.1_{\text{IMAA/ CBD}}$ alone (black) and with NCS-1 (red), $n = 6-10$.

Figure 8.

Modulation of paired-pulse plasticity by NCS-1. (A) Representative averages of 10 EPSPs evoked by paired-action potentials with 30-ms or 50-ms interstimulus intervals in presynaptic neurons expressing $\text{Ca}_v2.1$ alone (black) and in neurons co-injected with NCS-1 (red), recorded in the presence of ω -conotoxin GVIA (3 μM). (B) Paired-pulse ratio (PPR) plotted against interstimulus interval. $*p < 0.05$, two-way ANOVA, *post-hoc* Bonferroni test. (C) PPR for 30- and 50-ISI for WT (black), WT+NCS-1 (red) neurons. $*p < 0.05$. (D) Averaged initial EPSP amplitude (mV) for WT (black, left) and WT+NCS-1 (red, left) and $\text{Ca}_v2.1^{\text{IMAA}/\text{CBD}}$ (black, right) and $\text{Ca}_v2.1^{\text{IMAA}/\text{CBD}}+\text{NCS-1}$ (red, right).