Neuronal calcium sensor-1 modulation of optimal calcium level for neurite outgrowth

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Neurite extension and branching are affected by activity-dependent modulation of intracellular Ca²⁺, such that an optimal window of [Ca²⁺]i is required for outgrowth. Our understanding of the molecular mechanisms regulating this optimal [Ca²⁺]i remains unclear. Taking advantage of the large growth cone size of cultured primary neurons from pond snail Lymnaea stagnalis combined with dsRNA knockdown, we show that neuronal calcium sensor-1 (NCS-1) regulates neurite extension and branching, and activity-dependent Ca²⁺ signals in growth cones. An NCS-1 C-terminal peptide enhances only neurite branching and moderately reduces the Ca²⁺ signal in growth cones compared with dsRNA knockdown. Our findings suggest that at least two separate structural domains in NCS-1 independently regulate Ca²⁺ influx and neurite outgrowth, with the C-terminus specifically affecting branching. We describe a model in which NCS-1 regulates cytosolic Ca²⁺ around the optimal window level to differentially control neurite extension and branching.

KEY WORDS: NCS-1, Neurite outgrowth, Activity-dependent calcium signals, fura-2 imaging, Lymnaea stagnalis

INTRODUCTION

Neurite extension and branching are affected by activity-dependent modulation of intracellular Ca²⁺ (Komuro and Rakic, 1996; Tang et al., 2003; Zheng, 2000), such that an optimal window of [Ca²⁺]i is required (see Gomez and Spitzer, 2000; Henley and Poo, 2004; Kater and Mills, 1991): lower levels stabilize growth cones and higher levels stall them, in both cases preventing extension. Cytosolic Ca²⁺ can come from many sources, including ionotropic receptors, Ca²⁺ channels, Ca²⁺-release channels and Ca²⁺ pumps (see Berridge et al., 2003). Our understanding of the molecular mechanisms regulating this optimal [Ca²⁺]i remains unclear.

Neuronal calcium sensor-1 (NCS-1) is a member of a superfamily of proteins that respond to local Ca²⁺ changes (Ames et al., 1997; Tanaka et al., 1995). It interacts with a variety of proteins that are involved in Ca²⁺ homeostasis (Burgoyne, 2004). In particular, NCS-1 modulation of L- (Rousset et al., 2003), non-L- (Weiss et al., 2000), N- (Rousset et al., 2003; Wang et al., 2001) and P/Q-type Ca²⁺ channels (Rousset et al., 2003; Tsujimoto et al., 2002; Weiss and Burgoyne, 2001) directly changes intracellular Ca²⁺ levels. It also affects intracellular Ca²⁺ concentration directly by modulating Ca²⁺-permeable transient receptor potential (TRP) channels (Hui et al., 2006) and InsP3 receptor activity (Schlecker et al., 2006), and indirectly by affecting K⁺ channel expression and function (Guo et al., 2002; Nakamura et al., 2001). It interacts with signaling-transduction pathways that are known to affect ion channel activity or expression, including dopamine receptors, G-protein dependent receptor kinase 2 (Kabbani et al., 2002) and cyclic nucleotide phosphodiesterase (Schlaud et al., 1996), and to regulate exocytosis via phosphatidylinositol-4 kinase β (Kapp-Barnea et al., 2003; Taverna et al., 2002; Zhao et al., 2001). Thus, NCS-1 is a likely candidate for dynamically regulating cytosolic Ca²⁺ levels.

There is compelling evidence that NCS-1 is involved in neurite development in a few systems. For instance, NCS-1 expression increases in grey matter and decreases in white matter during embryogenesis and early postnatal stages (Kawasaki et al., 2003). Overexpression of NCS-1 in NG108-15/rat myotube co-cultures (Chen et al., 2001), and of the Drosophila homologue Freq (Frq) in motor nerve terminals (Angaut-Petit et al., 1998), reduces the number of neurites. More recently, NCS-1 was shown to impede neurite extension without altering branching in NGF-treated PC12 cells by potentiating TRPC5-evoked Ca²⁺ currents (Hui et al., 2006). However, there has been no study to examine if and how NCS-1 is involved in regulation of the window level of Ca²⁺ permissive to neurite outgrowth.

In this study, we examined the affects of NCS-1 on intracellular Ca²⁺ and neurite outgrowth in cultured L. stagnalis snail PeA neurons. Using RNA interference, we found that NCS-1 knockdown enhanced neurite extension and branching, and reduced activity-dependent Ca²⁺ influx in growth cones. Using a C-terminal peptide of NCS-1, we show that the C-terminus modulates growth cone whole-cell voltage-dependent Ca²⁺ currents and enhances neurite branching. Our findings indicate that NCS-1 modulates neurite branching and extension by regulating Ca²⁺ influx through at least two mechanisms: one that specifically affects branching, and another that specifically affects extension.

MATERIALS AND METHODS

Animal and primary cell culture

Freshwater pond snails, L. stagnalis, were obtained from an inbred stock maintained at the Free University in Amsterdam, and were raised and maintained at the University of Toronto under a 12 hour light/dark cycle in aquaria containing 20°C water. All animals used in these studies were fed green leaf lettuce twice a week. Serotonergic Pedal A (PeA) neurons, which are involved in locomotion and are found in each pedal ganglion in clusters of approximately 30 cells (Spencer et al., 1995), were isolated as previously described (Feng et al., 1997; Syed et al., 1990). Briefly, 2-month-old snails having shell lengths of 15 to 20 mm were anesthetized for 10 minutes with 10% (v/v) Listerine. The central ring and the buccal ganglia were dissected from the snail in sterile snail saline, containing 51.3 mM NaCl, 1.7 mM KCl, 4.1 mM CaCl₂, 1.5 mM MgCl₂, adjusted to pH 7.9 using 1 M HEPES/NaOH (pH 8.05), and then incubated with 3 mg/ml trypsin (Type A, Sigma). The ganglia were separated into hemiganglia and immersed in trypsinized ganglia for 45 minutes. Neurons were isolated by gentle pipetting and mechanical dissection, followed by 3 mg/ml trypsin treatment for 5 minutes. Neurons were then incubated with 4 mg/ml trypsin for 5 minutes before washing and further culturing.

Mature PeA neurons were cultured in 24-well plates coated with poly-D-lysine (100 µg/ml) and laminin (20 µg/ml). Cells were plated at a density of 5000 cells per well in Neurobasal medium supplemented with 2% B27 supplement, 0.5 mM glutamine, 4.5 µg/ml gentamicin, 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µM ascorbic acid, and 100 nM dopamine. All culture media were prepared with Hanks balanced salt solution (HBSS) supplemented with 10% (v/v) fetal bovine serum. Culture medium was renewed every 3 days. After 3 days of plating, the medium was replaced with Neurobasal medium supplemented with 10% (v/v) fetal bovine serum and the neurons were maintained for at least 3 days before experimentation.

**Notes:**

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III. Sigma, ON, Canada) for 22 minutes. PeA neurons were isolated individually with gentle suction using a fire-polished Sigmaced (Sigma, Ontario, Canada) pipette (2 mm, WPI 1B200F), and subsequently transferred to a poly-L-lysine coated culture dish, where they were maintained in conditioned medium (CM) at room temperature for 24-36 hours. CM was prepared by incubating *L. stagnalis* brains in defined medium (DM) as previously described (Feng et al., 1997; Syed et al., 1990). The DM contained serum-free 50% (v/v) Liebowitz L-15 medium (GIBCO, Grand Island, NY), supplemented with 51.3 mM NaCl, 1.7 mM KCl, 4.1 mM CaCl₂, 1.5 mM MgCl₂, 10 mM glucose, 1.0 mM L-glutamine and 20 mg/ml gentamycin. The pH of this medium was adjusted to 7.9 with 1 M HEPES/NaOH (pH 8.05).

### Cloning

mRNA was extracted from three snail central ring ganglia using the Qiagen RNeasy Mini Kit (Qiagen, Ontario, Canada). We first cloned a partial fragment of *L. stagnalis* NCS-1 using first-strand cDNA using Transcriptor reverse transcriptase (Roche Applied Sciences, Quebec, Canada) primed with random 9-mers at 40°C for 25 minutes, then incubated at 70°C for 2 hours. PCR reactions (25 µl) contained cDNA, forward (5’-TACAARC-AGTCTTCTCCAATTTGGAGACC-3’) and reverse (5’-CCATCGTT-ATCCAGATCATACAGAYKRAANGCCCA-3’) degenerate primers, to generate an amplicon of ~300 bp. The PCR cycle consisted of a 10-minute activation step at 94°C, followed by 45 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 2 minutes at 70°C, with a final extension step at 72°C for 3 minutes. Utilizing the forward primer, DNA sequencing confirmed the identity of the PCR product. Cloning of the full-length NCS-1 gene was by 3’ and 5’ rapid amplification of cDNA ends (RACE) following the SuperSMART PCR cDNA Synthesis Kit (Clontech, Mountain View, CA) in a two-step PCR protocol with some changes. First-strand cDNA was synthesized as described in the kit using the included SMART CDS primer, followed by amplification with the included primer 2A. In the first PCR step, a gene-specific primer (Table 1) was designed based on the partial clone and run with primer-1 (5’-CGACGTGGAGTCATACCAGAAGGGAAC-3’), which overlaps with the 5’ end of primer 2A. In the second PCR step, a nested gene-specific primer (Table 1) was designed based on the partial clone and run with primer-2 (5’-TGGGACATCGTCCATCAACACCAGAGTC-3’), which overlaps with the 5’ end of primer 1. The 5’ end was cloned in a similar manner, except that a modified random 15-mer (5’-AAGCAGTGGATCACGAAGGAGTTCAACGCGGCTGATCGAG-3’) was used to prime first-strand synthesis in a reverse transcription at 25°C for 10 minutes prior to a 42°C incubation for 90 minutes. The PCR cycle used in RACE consisted of a 5 minute enzyme-activation step at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 3 minutes at 72°C, with a final extension step at 72°C for 5 minutes. RACE products were confirmed by sequencing. Table 1 lists the gene-specific primers used in RACE. A full-length clone of 704 bp was cloned, coding for a 191 amino acid protein (GenBank DQ99793).

### Synthesis of dominant-negative NCS-1 peptide

Peptides were synthesized at the Advanced Protein Technology Centre in Toronto, Canada. The C-terminal NCS-1 peptide (CTN) construct, which consisted of an N-terminal tyrosine ligated to the HIV TAT (YGRKKRRQRRR) protein transduction domain joined by a tetra-glycine spacer ([Gly]₄) at its C-terminus to the 34 amino acid sequence found at the C-terminus of NCS-1 (DKNADGKLTLQEFQEGSKADPSIVQAL-SLYDGLV), was cleaved from the preparatory column and acetylated. CTN shown), indicating that the TAT PTD domain did not alter the conformation of the rest of the peptide.

### dsRNA construct

Based on the *L. stagnalis* NCS-1 sequence we cloned, we designed a 27-mer NCS-1-specific dsRNA using SciTools RNAi Design software (IDT DNA); we purchased this dsRNA from IDT DNA (Kim et al., 2005). TrFiECTa control dsRNA was used as a control in our experiments (IDT DNA). The sequences for each dsRNA are as follows: LNCS dsRNA 5’GUUUUAUUCUGUCAGAGGGUUGA/5P-CAACGUCUUCUGAGGAAAUAGGdA, TrFiECTa control 5’UCAACAGAAGGAAAGGAAGGAAGGA/5P-CUCUCCUCUUCUUCUCCUCUGUdGdA, where 5P represents a 5’ phosphate, and dN represents a deoxynucleotide; the other bases in these 27-mers were ribonucleotides. For whole animal knockdown experiments, snails were pinned onto a dissection board, and injected in the head, above the central ganglia, with 2 µl of 20 µM NCS-1 dsRNA, control dsRNA or 2 µl water. Two days after the injections, animals were sacrificed and separated for western blot analyses into four groups of two for each treatment.

### Western blot analysis

Protein extracts were prepared from snail ganglia and from rat brain (Sprague-Dawley, Charles River Lab, Wilmington, MA). Western blots were performed using NuPAGE Novex Bis-Tris Gel (Invitrogen, Ontario, Canada) following the manufacturer’s instructions. Specifically, extract samples containing 20 µg protein were separated by electrophoresis on a NuPAGE Novex Bis-Tris Gel in MES running buffer, and were then electrophoretically transferred from the gel to a 0.2 µm PVDF membrane (Invitrogen). Membranes were incubated with 5% blocking solution overnight at 4°C, followed by incubation with rabbit polyclonal anti-rat NCS-1 antibody (diluted 1:500; Biomol, Plymouth Meeting, PA) in 5% blocking solution for 2 hours at room temperature. After washing, membranes were incubated with horseradish peroxidase-conjugated rabbit secondary antibody (diluted 1:5000; Chemicon, Temecula, CA) in 3% blocking solution for 2 hours at room temperature. Antibody-labelled protein bands were visualized using an enhanced chemiluminescent reagent (Amersham Biosciences, Ontario, Canada), and analyzed by scanning densitometry with Kodak 1D image analysis software (Eastman Kodak, USA). Mouse anti-actin antibody (1:500; Chemicon, CA) was used as a control.

### Immunochemistry and confocal imaging

Cultured PeA cells were fixed with 1% paraformaldehyde in snail saline solution lacking NaCl overnight at 4°C. Cells were then washed three times with snail saline, and incubated with rabbit polyclonal anti-rat NCS-1 antibody (diluted 1:300) in snail saline solution containing 0.3% Triton X-100 and 0.5% BSA overnight at 4°C. Following three washes with snail saline, cells were incubated with FITC-conjugated anti-rabbit secondary antibody (diluted 1:600; Chemicon) for 2 hours at 20°C. After three washes with snail saline, and one wash with distilled water, the preparation was attached to a Cys residue that had been added to the N-terminus of CTN. The scrambled control peptide (sCTN) was constructed similarly to CTN except that the NCS-1 control peptide previously described (DIDGDQVYEEVFQDLSKLKGLKALPQ) was substituted for the C-terminal NCS-1 region (Tsujiimoto et al., 2002). These membrane-permeant CTN constructs allowed us to perform non-invasive studies on the effects of the NCS-1 C-terminus on neurite outgrowth. The N-terminal HIV-1 Tat protein transduction domain (PTD) conferred membrane permeability to the CTN (Vives et al., 1997). CD spectroscopy in the presence of trifluoroethanol indicated the presence of stable α-helices in the CTN at room temperature, similar to those seen in the NCS-1 C-terminus (not shown), indicating that the Tat PTD domain did not alter the conformation of the rest of the peptide.

| Table 1. Primers used to clone the full-length gene for *L. stagnalis* NCS-1 |
|--------------------------------|----------------------------------|
| RNA33 | 5’-CTN,-TCGAGCGGCCGCCCGGGCAGGTCGACGTGGACTATCCATG-3’ |
| 3’ | 5’-CGACGTGGAGTCATACCAGAAGGGAAC-3’ |

Sequences are listed 5’ to 3’.
mounted with permafluor (Fisher Scientific, Canada). Confocal images of NCS-1 reactivity were acquired using a TCS SL laser confocal microscope (Leica Confocal Software, version 2.5, build 1347. Leica Microsystems, Germany). z-series scan images of labelled cells were viewed under a 40× oil immersion lens using a He-Ne laser. The FITC-conjugated fluorophore was excited at 483 nm, and peak intensity emissions were determined at 543 nm. z-series scans of the cells were performed at a zoom level that afforded the largest possible view of cells and their neurites. Each plane was averaged three times using a step size between each plane of approximately 0.5 μm. Background fluorescence was determined using three regions not encompassing cells throughout the entire z-stack. Confocal data were analysed using LCS Lite Leica Confocal Software. In the double-labelled experiments, in addition to the rat-NCS-1 antibody, mouse β-actin antibody (diluted 1:500; Chemicon) was also incubated with the cells followed by Alexa-568-conjugated goat anti-mouse antibody (diluted 1:800; Molecular Probes) in the same manner as the rat NCS-1 antibody.

Neurite growth
PeA cells were cultured in CM at 20°C for 24-36 hours. Cells were then treated without or with 5 mM NCS-1 dsRNA, 5 mM control dsRNA, 50 μM CTN or 50 μM scrambled CTN. Neurite growth pattern images were collected at the time of treatment, and at several later timepoints, through 22 hours, using an Olympus inverted microscope (CK X41) with an Olympus C5050 digital camera. Images were analysed using ImageTool 3.1 software. We considered branches to be outgrowths when segments were longer than 10 μm. Using the 40× objective, neurite lengths were measured from the edge of the somata to the visible tip of the growth cone. Neurite extension rates were defined as the difference between the current and previous lengths divided by the time elapsed between the two measurements.

Intracellular recording and ratiometric fura-2 Ca2+ imaging
Intracellular Ca2+ ([Ca2+]i) was measured as described previously using a fura-2 ratiometric Ca2+ imaging system (Feng et al., 2002). Cells were incubated for 30 minutes with 4 μM Fura-2 AM (Molecular Probes), and then washed with snail saline three times before imaging. The experiments were carried out in the dark to prevent photobleaching of the dye. Fura-2 was alternately excited at 340 and 380 nm by illumination generated by a 100 W Hg/Xe-arc lamp that had passed through 340 and 380 nm excitation filters; this process was controlled by Image Pro 5 software (PTI). The fluorescence signal was reflected via a 430 nm dichroic mirror, passed through a 510 nm emission filter and detected and digitized by an intensified charged-coupled device (ICCD) camera (Roper Scientific) in Image Pro 5 at 1-10 Hz. The fluorescence intensity (Poenie-Tsien) ratios of images acquired at 340 and 380 nm were calculated using Image Pro 5. Cells were stimulated by action potentials generated by 1 second depolarizing current pulses (Axopatch 700B controlled by Clampex, Axon Instruments) from a sharp electrode containing a saturated solution of KCl or K2SO4. The number of action potentials used was controlled by changing the amplitude of the current pulses. The fluorescent signal evoked by the action potentials was recorded with a time-lapse protocol. The peak fluorescent signal was measured and compared under various conditions. The bath solution was composed of three times using a step size between each plane of approximately 0.5 μm. Background fluorescence was determined using three regions not encompassing cells throughout the entire z-stack. Confocal data were analysed using LCS Lite Leica Confocal Software. In the double-labelled experiments, in addition to the rat-NCS-1 antibody, mouse β-actin antibody (diluted 1:500; Chemicon) was also incubated with the cells followed by Alexa-568-conjugated goat anti-mouse antibody (diluted 1:800; Molecular Probes) in the same manner as the rat NCS-1 antibody.

Voltage-clamp recording
Whole-cell (ruptured) patch-clamp recordings were performed on growth cone regions using an Axopatch 700A amplifier connected to an analog-to-digital interface Digidata 1322 that was linked to a personal computer running pClamp9 (Axon Instruments, Foster City, CA). Patch pipettes (Sutter borosilicate glass, BF 150-86-15; Novato, CA) were pulled using a Sutter P-87 microelectrode puller, and subsequently fire polished using a Narishige microforge (Narishige), as described previously (Hui et al., 2005). Pipettes (4-10 MO) were filled with solution, containing 29 mM CsCl, 2.3 mM CaCl2, 2 mM MgATP, 0.1 mM GTP-Tris, 11 mM EGTA and 10 mM HEPES, adjusted to pH 7.4 with CsOH. Currents were recorded during perfusion with a Ca2+ solution containing 10 mM CaCl2, 45.7 mM TEA-Cl, 1 mM MgCl2, 10 mM HEPES and 2 mM 4-AP, adjusted to pH 7.9 with TEA-OH. Currents were elicited by stepping from a holding potential of −100 mV to the test potential using Clampex (Axon Instruments).

Growth cones were transected with a glass pipette and included adjacent neurite. srCTN, or CTN, was added to the cell-culture dish, and sometimes the recording pipette solution. Data were filtered at 1 kHz (~3 dB) using a 4-pole Bessel filter, and digitized at a sampling frequency of 2 kHz. Data were analysed using Clampfit (Axon Instruments). All curve fittings were carried out using Origin 7 (Microcal Origin, MA). Current-voltage relations were fit to the modified Boltzmann equation: \[ \frac{1}{1 + e^{-\frac{(V - V_h)}{S}}} \] and branch points (1.91±0.3), Differences were considered significant if P<0.05.

**RESULTS**
Expression of NCS-1 protein in L. stagnalis neurons
NCS-1 is believed to be highly conserved across species (Burgoyne, 2007). Using degenerate primers corresponding to sequences in a conserved region between the second and third EF-hand of NCS-1 (see Materials and methods), we amplified a ~300 bp DNA segment from L. stagnalis brain. This was followed by 5' and 3' RACE to yield a gene encoding for a 191 amino acid protein (GenBank Accession no. DQ099793). The sequence of this L. stagnalis protein is most similar to that of the Aplysia Californica aplycinin protein (Dyer et al., 1996) (95% identity) and very similar to the sequences of other known NCS-1 orthologues (Fig. 1A). To further confirm that the L. stagnalis NCS-1 protein is structurally conserved with those of its mammalian counterpart (also known as Freq), we used a rabbit polyclonal anti-rat NCS-1 antibody in western blots of snail brain protein preparations and in immunocytostaining of isolated cells (see below). In the western blots, a strong single band was detected migrating at a rate similar to that of rat NCS-1 (17-28 kDa, Fig. 1B). These results confirm that NCS-1 is highly conserved between species.

If NCS-1 is involved in neurite outgrowth, it should be expressed in growth cones. We examined the expression pattern of NCS-1 in L. stagnalis pedal A (PeA) neurons by immunocytochemistry and confocal fluorescence imaging. PeA neurons cultured for 3 days in CM showed NCS-1 immunoreactivity in neurites and soma (Fig. 1C). In contrast to previous studies (Bourne et al., 2001; O'Callaghan et al., 2002), immunoreactivity was diffusely distributed throughout the cytosol rather than being most intense in the membranes. This pattern is similar to that seen in Aplysia (Dyer et al., 1996) and may indicate that the N-terminal myristoylation domain, which mediates membrane localization for other NCS proteins (Ames et al., 1995), is concealed at resting levels of Ca2+. Relative to primary neurites (Fig. 1D, m=5), NCS-1 expression levels were significantly higher in soma (2.95±0.39, P<0.05), growth cones (1.89±0.1, P<0.05) and branch points (1.91±0.3, P<0.05). Secondary neurites showed slightly higher levels (1.27±0.3) but not significantly different from those observed in primary neurites. The higher levels of NCS-1 observed in growth cones and branching points reaffirm the notion that the protein is involved in neurite outgrowth.
NCS-1 knockdown affects both neurite elongation and branching

We next examined how NCS-1 affects neurite outgrowth by using a 27-mer NCS-1-specific dsRNA molecule to knockdown the gene. Although the 27-mer design has been well established in mammalian systems (Kim et al., 2005), its usefulness in invertebrates is unclear. We evaluated the effectiveness of this dsRNA in gene silencing by injecting 2 μl of either the NCS-1 dsRNA (20 μM), or a control dsRNA (20 μM), into the snail central ganglia and examined the protein level from brain protein extracts 2 days later. The NCS-1 dsRNA treatment, but not control dsRNA treatment, significantly reduced the expression of NCS-1 protein by 35.7±5.1% of the mock group (P<0.05, n=4) without affecting that of β-actin (Fig. 2A,B). No significant difference was found in NCS-1 expression between the control and mock groups.

We studied the effects of the NCS-1 dsRNA on individually isolated L. stagnalis PeA neurons by adding it to the culture medium, thereby avoiding potential cytotoxic or secondary effects of more invasive methods. The expression level of NCS-1 was determined to ensure that the strategy was effective. In cells treated with 5 nM dsRNA for 24 hours, the level of NCS-1 was substantially reduced in all regions relative to β-actin (Fig. 2C,D). As with the in vivo ganglial knockdown results, the relative expression level of NCS-1 to β-actin in the different regions was significantly decreased by ~30% after the NCS-1 dsRNA treatment (soma 0.84±0.06, n=15; neurite 0.94±0.13, n=6; branching point 0.97±0.08, n=8; and growth cone 1.10±0.16, n=5; P<0.05) compared with the control dsRNA (soma 1.48±0.08, n=10; neurite 1.51±0.12, n=6; branching point 1.44±0.05, n=4; and growth cone 1.60±0.12, n=6). Because the knockdown was only partial, we anticipate that any functional differences we saw in dsRNA and control treatments reflect only a ~30% reduction in NCS-1.

Having established the effectiveness of the dsRNA treatment, we examined the effects of NCS-1 knockdown on neurite outgrowth. The neurons were grown for 24 hours after isolation in order to allow for recovery and some neurite growth to which we could compare. The cells were subsequently treated with 5 nM dsRNA (NCS-1, control or mock) or water (mock) and allowed to grow for another 24 hours. The relatively short treatment period was appropriate in order to minimize variations arising from changing conditioned media, which was necessary due to evaporation. Even with only 24 hour treatment with the NCS-1 dsRNA, there was significant reduction in NCS-1 expression (Fig. 2) and change in the neuron morphology (Fig. 3A).

NCS-1 knockdown altered both neurite branching and extension in the cultured neurons. Many cells did not sprout neurites during the first 24 hours before dsRNA-treatment, and were not examined further. Of the remaining cells, the number of newly formed neurite branches after NCS-1 dsRNA treatment (3.9±0.6, n=57 cells) was significantly larger than the control dsRNA (1.0±0.3, n=43 cells) and the mock control (1.5±0.4, n=24 cells) treatments (Fig. 3B). We measured the length of the neurites before and after the treatments, and found that 75 to 80% of the neurites advanced and the remaining neurites either retracted or remained unchanged during the period of treatment (24 hours). The fraction of neurites which advanced was not affected by the treatments. As the NCS-1 dsRNA treatment altered the degree of branch of the neurons and extension...
of new branches requires advancement of neurites, we opted to measure extension rates and retraction rates of individual neurites. During this period, the average rate of growth of individual neurites was 6.44±0.49 µm/hour (n=57) in the NCS-1 dsRNA-treated neurons, 3.47±0.49 µm/hour (n=43) in the control dsRNA-treated neurons and 3.77±0.67 µm/hour in the mock group (Fig. 3C). This increase was significantly greater in the NCS-1 dsRNA-treated neurons compared with that in the control dsRNA-treated neurons and mock group (P<0.05). The mean retraction rates varied from 0.76 to 1.16 µm/hour (mock control 1.17±0.46; control RNA 0.76±0.11; NCS-1 RNA 0.95±0.16); the difference among the conditions, however, was not statistically significant (P>0.05). Our findings indicate that NCS-1 is involved in both neurite branching and extension processes in PeA neurons, and that a reduction of ~30% NCS-1 is sufficient to influence these processes.

**NCS-1 knockdown reduces growth cone Ca²⁺ signals**

Ca²⁺ homeostasis affects neurite outgrowth (Henley and Poo, 2004; Kater and Mills, 1991), so we examined whether NCS-1 modulates activity-dependent Ca²⁺ influx using ratiometric fura-2 calcium imaging. Activity was induced by somatic stimulation of 1 second current pulses via an intracellular sharp electrode. As expected, higher frequencies of action potentials (APs) resulted in higher peaks in [Ca²⁺], as shown by higher ratiometric fluorescent intensities (Fig. 4). The maximal fluorescent signal always coincided with the last spike of each action potential train, so we recorded and compared with the maximal signals under different conditions. The responsiveness of the NCS-1 dsRNA-treated cells to APs was dramatically weaker than that of the mock or the control dsRNA-treated cells (Fig. 4A). We quantified the activity-dependency of the Ca²⁺ signal by plotting the maximal fura-2 ratio (F_max/F_A0) against the number of action potentials used to evoke the response (APs per second). As shown in Fig. 4B, the change in peak ratio intensity (evoked level–basal level) was linearly correlated to the number of action potentials at least up to 7 Hz. Summarized in Fig. 4C, the responsiveness of the NCS-1 dsRNA-treated cells had a significantly lower degree of Ca²⁺ influx per action potential (0.010±0.004 AU/AP; n=43) than the control dsRNA (0.027±0.003 AU/AP; n=3) and mock treated cells (0.037±0.006, n=4). The measured basal Ca²⁺ levels, however, were similar between the treatments (AU; control 0.89±0.07; control dsRNA 0.86±0.04; NCS-1 dsRNA 0.99±0.06; P>0.05). These results support the hypothesis that NCS-1 affects activity-dependent Ca²⁺ influx in growth cones.

**The NCS-1 C-terminal peptide reduces growth cone Ca²⁺ current activities**

The C-terminus of NCS-1 has been shown to regulate activity-dependent currents from presynaptic voltage-dependent Ca²⁺ channels (Tsujimoto et al., 2002). Assuming that NCS-1 modulates growth cone Ca²⁺ channels similarly, its effects on neurite outgrowth could be directly due to this modulation. To test this hypothesis, we
used a dominant-negative peptide to compete with C-terminal NCS-1 interactions (CTN) and examined its effects on three parameters: (1) voltage-dependent Ca\textsuperscript{2+} currents; (2) activity-dependent Ca\textsuperscript{2+} influx; and (3) neurite outgrowth properties.

We designed the CTN peptide to satisfy two criteria. First, the peptide should replicate the original study on C-terminal modulation of presynaptic Ca\textsuperscript{2+} channels (Tsujimoto et al., 2002). Second, it should be easily introduced into many cells. With these in mind, the peptide was made from the last 34 amino acids of the NCS-1 C-terminus, as in the previous study, linked at its N-terminus by a tetraglycine spacer to the HIV-TAT protein transduction domain sequence (see Fig. S1A in the supplementary material), which allows it to readily translocate across cell membranes (Vives et al., 1997). A scrambled control peptide (srCTN) was similarly synthesized (see Materials and methods). Using a BODIPY-tagged CTN, we found that 5 minute incubation with \textit{L. stagnalis} PeA neurons was enough to allow for the fluorescent signal to invade the cytosol (see Fig. S1B in the supplementary material), showing that a short exposure was sufficient for CTN to access the intracellular space of the neurons. This is consistent with previous studies.

**Fig. 3. NCS-1 knockdown enhances neurite outgrowth in \textit{L. stagnalis}**. (A) Phase-contrast images of a single PeA neuron before and 24 hours after addition of water (right), 5 nM control (middle) or NCS-1-specific (left) dsRNA. Cells were isolated and cultured in CM for 24 hours before treatment. (B,C) Neurite outgrowth was examined for the degree of branching (B) and the rate of extension (C). Both the number of new neurites (the degree of branching) and the rate of neurite extension were significantly higher after the NCS-1-specific dsRNA treatment compared with controls. The number of new neurites: mock control 1.50±0.39; control dsRNA (ctrl dsRNA) 1.04±0.39; and NCS-1 dsRNA 3.93±0.65. The rate of the extension (μm/hour): mock control 3.77±0.67; control dsRNA 3.47±0.49; NCS-1 dsRNA 6.44±0.49. The data are presented as means±s.e.m. and obtained from 24 control, 43 control dsRNA and 57 NCS-1-specific dsRNA-treated cells. Asterisk indicates significant difference (P<0.05) from control condition.

**Fig. 4. NCS-1 knockdown affects the action potential-induced fura-2 Ca\textsuperscript{2+} signal in growth cones in an activity-dependent manner.** (A) Representative epifluorescent images of Ca\textsuperscript{2+} signals in \textit{L. stagnalis} neurites treated with the control dsRNA or NCS-1 dsRNA. The Ca\textsuperscript{2+} ratiometric signals excited at 340 and 380 nm (F\textsubscript{340}/F\textsubscript{380}) were obtained at rest (basal) or elicited by two, four or six depolarization-induced action potentials. Action potentials were evoked by 1 second depolarization using an intracellular sharp electrode impaled into the soma. (B) Representative examples showing that the Ca\textsuperscript{2+} signal (F\textsubscript{340}/F\textsubscript{380}) was as a linear function of the number of action potentials. The Ca\textsuperscript{2+} signal from a single growth cone region was plotted against the number of action potentials used to evoke the response. The line represents a linear best fit of the data, which shows no apparent saturation of the signal within the range of action potentials used. The slopes of the fits are 0.037±0.004 (control), 0.031±0.003 (control dsRNA) or 0.011±0.001 (NCS-1 dsRNA). The slope was used to quantify the dynamics of Ca\textsuperscript{2+} change resulting from action potential stimulation. (C) Summary of the slope of the fit described in B from three to five cells with treatments as indicated. The mean slope dependencies are 0.037±0.006 (control, n=4), 0.027±0.003 (control dsRNA, n=3), and 0.010±0.004 (NCS-1 dsRNA, n=5). The data are presented as means±s.e.m. Asterisk indicates significant difference (P<0.05) from the controls. AP, action potentials.
reports of rapid uptake of TAT and other protein-transduction-domain-tagged peptides (Dunican and Doherty, 2001; Green et al., 2003). The distribution of the labelled CTN appeared to be Gaussian along the z-axis for somata, neurites and growth cones (see Fig. S1C in the supplementary material) and only excluded from the nucleus, similar to a previous study (Voero-Askani et al., 2000) that showed that TAT-linked peptides have relatively uniform access to all compartments of the cell without any strong specificity for any particular organelle or membrane.

Having shown that CTN sufficiently invades the cell, we examined its effects on voltage-dependent Ca\textsuperscript{2+} currents in growth cones. Currents were recorded for either transected growth cones that were untreated or treated for at least 5 minutes with either 50 μM CTN or srCTN. Representative records of Ca\textsuperscript{2+} currents from a transected growth cone (Fig. 5, insert) and the I-V plots of the current under different treatments are shown in Fig. 5A, and Fig. 5B, respectively. The activity peaked rapidly upon depolarization and decayed slowly to a sustained level over the course of the 200 millisecond step, which is typical of currents from high voltage-decoupled Ca\textsuperscript{2+} channels. As the size of the growth cones varied

The NCS-1 C-terminal peptide decreases activity-dependent Ca\textsuperscript{2+} dynamics in growth cones

We have shown that NCS-1 knockdown reduces intracellular Ca\textsuperscript{2+} influx and a peptide of its C-terminus reduces voltage-dependent Ca\textsuperscript{2+} currents. Consequently, we asked whether the RNA interference effects were mimicked by the C-terminal peptide. We examined the acute effects of 50 μM CTN (or srCTN) on activity-dependent Ca\textsuperscript{2+} signals in growth cones of PeA cells. After the peptide was added to the bath, 5 minutes were allotted before recording to ensure that the peptide was in the cells. In some cases, the activity-dependent Ca\textsuperscript{2+} signalling was recorded before peptide treatment to allow for paired assessment of the changes, avoiding any cell-specific differences. Fig. 6A shows representative Ca\textsuperscript{2+} signals in the neurites of a sprouted PeA cell in response to one, three or six action potentials before and after CTN treatment. As with the dsRNA treatments, the changes in fluorescence intensity ratio (F\textsubscript{340/380}) in growth cones exhibited a positive linear relationship with the number of action potentials for up to 7 Hz (Fig. 6B). After the CTN-treatment (0.019±0.002 AU/AP) this correlation was reduced compared with control (0.037±0.006 AU/AP; n=4) was significantly reduced by CTN (0.021±0.003 AU/AP; n=8; P<0.05), but not by srCTN (0.042±0.004 AU/AP, n=3), indicating that NCS-1 affects activity-dependent Ca\textsuperscript{2+} influx in these regions. The basal Ca\textsuperscript{2+} level under resting conditions was not significantly affected by the peptide (control 0.89±0.07 AU; CTN 0.98±0.06 AU; srCTN 0.88±0.09 AU). Our findings indicate that the C-terminus of NCS-1 is at least partly responsible for regulating activity-dependent Ca\textsuperscript{2+}-dynamics in growth cones.

C-terminus of NCS-1 affects neurite branching

We have shown that, similar to NCS-1 RNA knockdown, the NCS-1 C-terminus interactions affected activity-dependent Ca\textsuperscript{2+} influx and voltage-dependent Ca\textsuperscript{2+} currents (Figs 5 and 6). We next examined whether the NCS-1 C-terminus interactions are
responsible for the effects of NCS-1 knockdown on neurite outgrowth. As in the dsRNA experiments, CTN was added to PeA neurons 24 hours after plating the cells, and the change in outgrowth was examined 24 hours later. Again, ~80% of neurites advanced and the remaining neurites either retracted or remained unchanged during the period of treatment (24 hours). From cells that started with similar degrees of growth (Fig. 7A, 0 hours), CTN-treated cells showed more elaborate branching 24 hours after treatment than did the controls. On average, there were significantly more new branches in CTN-treated neurons (4.57±0.81, n=44) compared with mock (1.50±0.39, n=24) and srCTN-treated neurons (0.83±0.25, n=21) (Fig. 7B, P<0.05). Treatment of srCTN had no significant effect compared to mock. The degree of neurite branching in CTN-treated cells was similar to that in NCS-1 dsRNA-treated cells (Fig. 3B), supporting the idea that the C-terminus of NCS-1 may be involved in neurite branching.

In contrast to branching, CTN did not affect neurite extension to the same degree as NCS-1 knockdown. The extension rate of CTN-treated neurites (3.43±0.38 μm/hour) was similar to that in the mock control (3.77±1.67 μm/hour) and srCTN-treatment (3.79±1.02 μm/hour). Similarly, no significant difference was observed in the retraction rates from the three groups (mock control 1.17±0.46; srCTN 1.8±0.48; CTN 0.93±0.22; P>0.05). However, dsRNA-treated neurons (6.44±0.49 μm/hour) grew twice as fast as untreated neurons (3.77±0.67 μm/hour). These findings suggest that the involvement of NCS-1 in neurite extension does not require C-terminal interactions that are competed with by CTN.
NCS-1 regulates neurite branching

DISCUSSION

Neurite outgrowth depends on the level of intracellular Ca\(^{2+}\) (see Gomez and Spitzer, 2000; Henley and Poo, 2004; Kater and Mills, 1991). Too little Ca\(^{2+}\) and the neurites do not grow; too much Ca\(^{2+}\) and the growth stalls. An optimal window level of Ca\(^{2+}\) may be necessary for neurite outgrowth (Kater and Mills, 1991). Many sources of Ca\(^{2+}\) have been well studied and their regulation well established (Berridge et al., 2003), but to our knowledge there have been no studies on how the window level of Ca\(^{2+}\) is achieved and what molecules are involved. Here we report that NCS-1 regulates neurite outgrowth by modulating the local Ca\(^{2+}\) concentration to at least two different stimulated levels: the higher level affects neurite branching only, and the lower level affects both branching and extension. Our findings suggest that there are at least two distinct regions on NCS-1 that function independently to regulate the level of cytosolic Ca\(^{2+}\) levels, thereby modulating neurite outgrowth.

Ca\(^{2+}\) is an important regulator of neurite extension and growth cone pathfinding (Gomez and Spitzer, 2000). Intracellular Ca\(^{2+}\) transients can modulate the rate of neuronal migration in cerebellar microexplant cultures (Komuro and Rakic, 1996) and cortical cultures (Tang et al., 2003), and can also induce growth cone turning in cultured Xenopus neurons (Zheng, 2000). Neurite branching is also an activity-dependent event (see Dent et al., 2003). Inhibiting neuronal activity or blocking Ca\(^{2+}\)-conducting channels such as the AMPA (Uesaka et al., 2005) or NMDA (Ruthazer et al., 2003) receptors suppresses branching; and repetitive Ca\(^{2+}\) transients induced by netrin-1 facilitated release of intracellular Ca\(^{2+}\) stores promotes branching (Tang and Kalil, 2005). In parallel with these findings, we show that NCS-1 affects both neurite outgrowth and activity-dependent Ca\(^{2+}\) influx at the growth cones.

One target of NCS-1 was recently found by others to be a canonical TRP channel, TRPC5 (Hui et al., 2006). When overexpressing the dominant-negative NCS-1 mutant E120Q in PC12 cells, the Ca\(^{2+}\) influx through TRPC5 was reduced and the rate of neurite extension was enhanced, but branching was unchanged. There are some similarities between these findings to ours. First, we found that dsRNA knockdown of NCS-1 expression in \textit{L. stagnalis} neurons increased the rate of neurite extension. Second, in both NCS-1 knockdown and CTN treatment, the activity-dependent Ca\(^{2+}\) influx was suppressed compared with untreated cells. In contrast to the NCS-1 E120Q mutant study, we found that NCS-1 is also involved in neurite branching via its C-terminus. We believe that this discrepancy lies in the use of different experimental tools. The mutation in E120Q abolishes the third NCS-1 EF-hand motif (EF-3), which is required for Ca\(^{2+}\)-dependent conformational changes, leaving other EF-hands and functional domains intact (Weiss et al., 2000). NCS-1 knockdown encompasses these effects, as it reduces the expression level of the protein; but CTN competes with the C-terminus interactions and may not affect EF-3 interactions. Thus, NCS-1 has two distinct effects on neurite outgrowth properties that are mediated by different structural domains of NCS-1: one involving EF-3 and another involving the C-terminus.

NCS-1 contains four EF-hand Ca\(^{2+}\)-binding motifs and can coordinate three Ca\(^{2+}\) ions when saturated (Fig. 8C,D) (Ames et al., 2000). The first N-terminal EF-hand (EF-1) is non-functional (Cox et al., 1994); the second EF-hand (EF-2) binds Ca\(^{2+}\) with a relatively low affinity (Kd: 10 \(\mu\)M) compared with the third (EF-3) and fourth (EF-4) EF-hands (Kd: 400 nM) (Ames et al., 2000). Mutation of EF-3 (E120Q) upregulates non-L-type Ca\(^{2+}\) channels in chromaffin cells (Weiss et al., 2000), and, as mentioned above, suppresses TRPC5 channels in PC12 cells (Hui et al., 2006). These opposing effects of...
E120Q may arise from interactions on other regions of NCS-1. For example, the C-terminus encompassing part of EF-4 is involved in activity-dependent facilitation of the presynaptic P/Q type Ca\(^{2+}\) channels in the Calyx of Held (Tsujimoto et al., 2002). Although Ca\(^{2+}\) binding to the EF-hands in NCS-1 may be highly cooperative (Ames et al., 2000), these studies suggest that the different regions of NCS-1 can function independently of each other.

The binding affinities of the EF-hands in NCS-1 appear to be compatible with the optimal window level of Ca\(^{2+}\) that permits neurite outgrowth (Kater and Mills, 1991). In both mammalian (Connor, 1986) and invertebrate cultured neurons (Cohan et al., 1987), intracellular Ca\(^{2+}\) concentrations of 40-80 nM produce stable growth cones; 100-300 nM Ca\(^{2+}\) concentrations induce neurite outgrowth; and higher levels retard growth (Kater et al., 1989).

Domains EF-3 and EF-4 in NCS-1 have Ca\(^{2+}\)-binding affinities around 400 nM, just above the concentration threshold that is necessary for optimal neurite outgrowth; and EF-2 has a binding affinity around 10 \(\mu\)M, which lies in the range of outgrowth inhibition (Ames et al., 2000). Consequently, NCS-1 appears to have suitable structural features to respond to levels of intracellular Ca\(^{2+}\) around the optimal window necessary for neurite outgrowth.

Based on our findings, we envision a four-stage mechanism where NCS-1 is involved in activity-dependent neurite outgrowth (Fig. 8). First, at basal levels of Ca\(^{2+}\), NCS-1 may or may not be involved in limited outgrowth. Second, an optimal range of neuronal activity evokes Ca\(^{2+}\) influx (200–300 nM) to promote both neurite extension and branching. Higher activity, leading to higher Ca\(^{2+}\) influx, may inhibit both processes through an NCS-1-dependent mechanism, as described in stages three and four. Third, increasing Ca\(^{2+}\) allows it to bind to one or more of the high-affinity sites on NCS-1, consequently inhibiting elongation while still promoting branching. The elevation in Ca\(^{2+}\) in this stage may be partly due to NCS-1 facilitation of TRPC5 channels (Hui et al., 2006). Fourth, raising Ca\(^{2+}\) to higher levels allows it to bind to the low-affinity site on NCS-1 and also inhibit branching. The Ca\(^{2+}\) source at this stage may involve NCS-1 facilitation of growth cone voltage-dependent Ca\(^{2+}\) channels, leading to a positive-feedback mechanism to limit outgrowth until the stimulus is relaxed (i.e. Ca\(^{2+}\) levels fall to, and below that, of stage three). In this manner, branching and subsequent extension could be controlled by an NCS-1-dependent mechanism involving dynamic changes in the intracellular Ca\(^{2+}\) level. This simple model emphasizes that different windows of Ca\(^{2+}\) are permissive for neurite extension and branching, with NCS-1 modulating both processes in a [Ca\(^{2+}\)]-dependent manner.

The individual stages of our model of NCS-1-dependent outgrowth can be attributed to different regions of the protein. The third EF-hand (EF-3) was previously shown to be selectively involved in neurite extension (Hui et al., 2006). Disrupting this Ca\(^{2+}\)-binding site, as in the E120Q mutant, prevented NCS-1 inhibition of neurite extension in stage three of the model without affecting stage four: branching remained unaffected. In the current study, we show that the C-terminus is selectively involved in neurite branching. Competing with NCS-1 C-terminal interactions via the CTN peptide alleviated inhibition of branching in stage four without affecting stage three: neurite extension remained impaired. We show that NCS-1 is responsible for both effects, as knockdown by RNA interference affected both stages three and four, consequently preventing suppression of both branching and extension. As with other functional properties of this protein (Burgoyne et al., 2004), the different domains in NCS-1 appear to act independently in its modulation of neurite outgrowth, with the third EF-hand responsible for extension and the C-terminus responsible for branching.

The mechanism underlying neurite branching remains unclear but is thought to be an activity-dependent event (Tang et al., 2003; Zheng, 2000) (see also Dent et al., 2003). Our study suggests that NCS-1 is involved perhaps by fine-tuning local Ca\(^{2+}\) transients. It is the spatial localization of Ca\(^{2+}\) transients that determines the pathway of axonal development. In particular, Ca\(^{2+}\) transients in the growth cone stimulate axon extension (Gu and Spitzer, 1995) and Ca\(^{2+}\) transients in the filopodia induce growth cone turning (Gomez et al., 2001). The downstream actions of Ca\(^{2+}\) in neurons include control of growth cone traction (Conklin et al., 2005), polymerization of cytoskeletal structural elements (Lautermilch and Spitzer, 2000) and membrane insertion at growth cones (Lockerbie et al., 1991; Wood et al., 1992), which have direct effects on neurite outgrowth behaviour. By creating local increases in [Ca\(^{2+}\)], at growth cones, the neurite can be steered (Henley and Poo, 2004). Consequently, the ability of NCS-1 to modulate Ca\(^{2+}\) dynamics at developing growth cones endows it the ability to regulate neurite outgrowth.

In conclusion, we showed that by modulating different levels of cytosolic Ca\(^{2+}\), NCS-1 can influence neurite outgrowth properties. NCS-1 not only facilitates the functioning of the fully mature synapse, but it is also crucial during the conception of the synapse by modulation of Ca\(^{2+}\) dynamics, and neurite extension and branching.

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Supplementary material
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References
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