Neuronal calcium sensor-1 enhancement of InsP$_3$ receptor activity is inhibited by therapeutic levels of lithium

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Regulation and dysregulation of intracellular calcium (Ca$^{2+}$) signaling via the inositol 1,4,5-trisphosphate receptor (InsP$_3$R) has been linked to many cellular processes and pathological conditions. In the present study, addition of neuronal calcium sensor-1 (NCS-1), a high-affinity, low-capacity, calcium-binding protein, to purified InsP$_3$R type 1 (InsP$_3$R1) increased the channel activity in both a calcium-dependent and -independent manner. In intact cells, enhanced expression of NCS-1 resulted in increased intracellular calcium release upon stimulation of the phosphoinositol signaling pathway. To determine whether InsP$_3$R1/NCS-1 interaction could be functionally relevant in bipolar disorders, conditions in which NCS-1 is highly expressed, we tested the effect of lithium, a salt widely used for treatment of bipolar disorders. Lithium inhibited the enhancing effect of NCS-1 on InsP$_3$R1 function, suggesting that InsP$_3$R1/NCS-1 interaction is an essential component of the pathomechanism of bipolar disorder.

Introduction
Calcium is a ubiquitous intracellular signaling molecule that is required for initiating and regulating a wide range of neuronal functions, including neurotransmitter release, synaptic plasticity, neurite outgrowth, and neurodegeneration (1, 2). Considering the importance of calcium signals for cellular functions, it is not surprising that the inositol 1,4,5-trisphosphate receptor (InsP$_3$R) is also involved in pathological conditions that are related to disturbance in calcium homeostasis. Loss of InsP$_3$R type 1 (InsP$_3$R1) in mice is associated with ataxia and seizures (3), and loss of InsP$_3$R3 is found in humans with bile duct obstruction (4). In several neurodegenerative diseases, such as Alzheimer disease and Huntington disease, and in brain ischemia, InsP$_3$R1 appears to be selectively downregulated (5–7). Understanding the basis for the dysregulation of InsP$_3$R activity will be crucial for understanding the pathomechanism of these diseases.

A variety of signaling molecules have been shown to associate with InsP$_3$R and modulate its activity (8, 9); the most studied of these regulators is calcium, which influences channel activity both as an activator and an inhibitor, both directly and indirectly. A number of potential cofactors necessary for calcium-dependent modulation have been identified (8, 10, 11). Recently, neuronal calcium sensor-1 (NCS-1) has been shown to regulate intracellular calcium signaling (8, 12, 13). This protein contains EF hand motifs for rapid binding of calcium that results in major conformational changes in NCS-1 (14). Mutations in NCS-1 have been linked to pathological conditions, including human X-linked mental retardation (15). In chronic bowel disease, the expression level of NCS-1 in the enteric nervous system is drastically decreased (16), causing diminution in neurotransmitter secretion. In contrast, NCS-1 expression is increased in the prefrontal cortex of schizophrenic and bipolar patients (17). Unfortunately, little is known about the underlying pathomechanism of these human diseases although misregulation of calcium homeostasis has been proposed to play a role (18, 19).

To determine whether NCS-1 and InsP$_3$R1 functionally interact with each other and whether this interaction could be altered under neuropathological conditions, we examined the effect of NCS-1 on the single-channel properties of InsP$_3$R and on agonist-dependent intracellular release in cells. Since NCS-1 is upregulated in bipolar disorders and one of the first successful medications for this condition was lithium, which is still widely used, we tested the effect of lithium on the functional interactions between NCS-1 and InsP$_3$R1. Lithium has already been shown to interact with the phosphoinositol signaling pathway (20) by inhibiting phosphoinositol lipid turnover. The effects of lithium on NCS-1/InsP$_3$R1 interactions would complement the lipid effects and add a new pathway to be explored for therapeutic potential.

We found that addition of NCS-1 enhances the activity of the InsP$_3$R1, when monitored as the activity of single channels and as calcium transients in intact cells. This modulation is InsP$_3$- and calcium-dependent such that InsP$_3$-activated responses will be larger and more rapid due to NCS-1–enhanced activation of the InsP$_3$R1. We also found that lithium attenuates the NCS-1/InsP$_3$R1 association. This observation indicates that the signaling complex comprised of NCS-1 and the InsP$_3$R may be involved in the pathomechanism of bipolar disorders.

Results
Brain slices of adult rats were immunostained to show the colocalization of NCS-1 and InsP$_3$R1 in vivo. In the cerebellum, NCS-1...
was visible throughout the cell except the nucleus in Purkinje cells and stellate cells (Figure 1). InsP₃R1 was also found throughout the cell but with stronger staining in the cell body. Both proteins were detected in neurons of the hippocampus and deep layers of the prefrontal cortex (data not shown). Neurons in the upper layers of the cortex showed marked InsP₃R1 staining but no or little NCS-1 staining. These observations agree with previous reports about the cell type–specific expression of NCS-1 (21, 22). To show that there is a physical interaction between NCS-1 and InsP₃R1, both NCS-1 and InsP₃R1 were immunoprecipitated from cerebellar lysate. Both proteins were present in mouse cerebellar lysate (Figure 2). When calcium was present in the buffer, both proteins were present in the immunoprecipitate when using either anti–NCS-1 or anti-InsP₃R1 (Figure 2). When calcium levels were low due to the addition of calcium buffers, only the protein directly associated with the immunoprecipitating antibody was present (Figure 2). An additional band at 25 kDa was present in all antibody-treated lanes, including the control lane, which is neither NCS-1 nor InsP₃R. These results show that the interaction between NCS-1 and InsP₃R1 can be identified in native tissue and is calcium dependent.

To determine whether the interaction between NCS-1 and InsP₃R1 has a functional component, we added purified NCS-1 to InsP₃R1 after it had been incorporated into planar lipid bilayers and monitored InsP₃R-gated channel activity. Measurements were obtained using 300 nM free calcium, 0.5 mM ATP, and 2 μM InsP₃ on the cytoplasmic side of InsP₃R1. In the absence of NCS-1, the amplitude of the single-channel currents was 2 pA (Figure 3A), the open probability (Pₒ) was 4.0% ± 0.6% (n = 3; Figure 3, A and C), and the mean open time was 2.5 ms ± 0.3 ms (n = 3; Figure 3, A and B). Addition of NCS-1 (2 μg/ml) to the cytoplasmic side of the channel increased InsP₃R1 channel activity: the Pₒ was dramatically increased by a factor of 5 (21.0% ± 1.7%; n = 4; Figure 3, A and C), and the mean open time increased to 6.0 ms ± 0.6 ms (n = 4; Figure 3B). The amplitude of the single-channel currents remained unaltered (Figure 3A). The ability of NCS-1 to activate the channel was unchanged even when the NCS-1 concentration was varied from 0.45 μg/ml to 2.56 μg/ml. The enhancing role of NCS-1 on InsP₃R1 channel activity could, in principle, be attributed to the ability of NCS-1 to activate the channel in the absence of InsP₃. When NCS-1 was added to InsP₃R1 in the absence of InsP₃ over a range of calcium concentrations, no channel openings were observed (Figures 3A and 4C), showing that NCS-1 alone was unable to activate InsP₃R1.

When NCS-1 was tested on the activity of the ryanodine receptor type 2 (RyR2), there was no change in RyR2 channel activity at any calcium concentration tested (Figure 3D); the bell-shaped calcium-dependence curve of the RyR2 remained unaltered (data not shown). These results indicate that the NCS-1/InsP₃R1 interaction is functionally specific.

To test the requirement for calcium binding to NCS-1, we monitored channel activity over a range of calcium concentrations, and we used a mutated form of NCS-1 with a point mutation in EF hand 3 (E120Q). This mutation results in a protein with reduced calcium-binding ability but an unaltered calcium-dependent conformational change and unaltered ability to bind to protein partners (23). After addition of the E120Q mutant to InsP₃-activated channel at 300 nM free calcium, the Pₒ increased to 8.0% ± 1.7% (n = 3; Figures 3A and 4, A and C), but the mean open time was unchanged (2.2 ms ± 0.3 ms; n = 3; Figure 3B). When the free calcium concentration was raised serially from 0.01 μM to 1000 μM in the presence of 2 μg NCS-1, there was an increase in Pₒ (up to 41% ± 0.05% at 1000 μM calcium; n = 4) and mean open time (Figure 4, A–C). When the channel properties are plotted as a function of the free calcium concentration, 2 plateaus appear at pCa 6.5 and 4 (where pCa is the negative log of the free calcium concentration) that are comparable to the calcium-binding sites of the purified NCS-1 (24). In contrast, when E120Q was used, the Pₒ and mean open time remained constant as the calcium concentration was increased (Figure 4, A–C). Thus, NCS-1 acts on InsP₃R1 in both a calcium-independent and a calcium-dependent manner.

To determine whether the interaction between NCS-1 and InsP₃R1 was functional in vivo, we monitored agonist-induced calcium release in nerve growth factor–differentiated (NGF-differentiated) PC12 cells. Both proteins were detected in these cells (Figure 5A). PC12 cells that stably overexpressed NCS-1 were

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**Figure 1**

Immunolocalization of NCS-1 and InsP₃R1 in neurons in vivo. Slices from cerebellum of adult rat brain were immunostained for NCS-1 (far left) and InsP₃R (second from left). Both proteins were found throughout the cerebellum with an overlapping distribution (second from right) but were concentrated in Purkinje cells (see detail of single cell in far right panel).

**Figure 2**

Coimmunoprecipitation of NCS-1 and InsP₃R1 in rat cerebellum. Lanes show mouse cerebellar lysate (lane 1); beads treated with cerebellar lysate but without antibody (lane 2); immunoprecipitate (IP) with anti–NCS-1 with calcium buffered (lane 3); immunoprecipitate with anti–NCS-1 with 50 μM free calcium present (lane 4); immunoprecipitate with anti-InsP₃R with calcium buffered (lane 5); and immunoprecipitate with anti-InsP₃R with 50 μM free calcium present (lane 6). Top and bottom panels show immunoblots for InsP₃R1 and NCS-1, respectively.
stimulated with 50 μM ATP for 1 minute, and the changes in cytosolic calcium levels were monitored using Fluo-4, a calcium-sensitive fluorescence dye. PC12 cells stably expressing the empty vector were used as controls. Cells containing NCS-1 showed a larger response to ATP stimulation than control cells (Figure 5B: all values presented as maximum ratio of fluorescence intensity over baseline [F/F₀], 1.5 ± 0.06 for control cells, n = 37; 3.8 ± 0.5 for NCS-1 cells, n = 22). Removal of ATP from the chamber reduced the fluorescence to baseline levels. These results show that the functional effects of NCS-1 on InpR1 observed using purified proteins are maintained in intact cells.

NCS-1 may play a role in the pathophysiology of several neuropsychiatric disorders (17). Because lithium has been used for the treatment of bipolar disorders for more than 50 years, we tested its effects on the ability of NCS-1 to enhance the activity of InpR1. InpR1 channel activity increased at least 2-fold after addition of NCS-1 (Figure 3A). Addition of lithium increased the activity of NCS-1 on InpR1 by about 20-30% (Figure 3B). This inhibitory effect of lithium on NCS-1 was strongly dependent on the concentration of lithium (Figure 3C).

Discussion

Our experiments show that NCS-1 modulates calcium signaling by enhancing InpR1-mediated activity of InpR1 and thereby amplifying the calcium signal. This modulation is calcium dependent such that elevated cytosolic calcium concentrations will increase even more rapidly due to enhanced NCS-1 activation of InpR1. Furthermore, we found that lithium attenuates the NCS-1/InpR1 association, suggesting that the interaction is associated with the pathomechanism of bipolar disorders.

Calcium is a major intracellular messenger that is involved in the regulation of many cellular functions, some of which appear to be opposing functions, such as apoptosis and cell proliferation (2). One way that a single molecule such as calcium could be used to orchestrate such diverse signals and regulate so completely dissimilar cellular processes is by modulating the spatial and temporal pattern of the calcium signals. For that purpose, the existence of a large "calcium-signaling toolkit" (9) has been...
suggested, where the selected tools would be unique for a given cell type. We suggest that NCS-1 is one of the proteins that modulate calcium signaling.

NCS-1, a member of the family of calcium-binding proteins, is predominantly expressed in neurons and neuroendocrine cells (27, 28). It is involved in many diverse neuronal signaling pathways from modulation of neurotransmitter release (29) to attenuation of dopamine receptor desensitization (30). In cultured hippocampal neurons, synapses with elevated levels of NCS-1 show paired-pulse facilitation whereas synapses with little NCS-1 respond to repetitive stimulation with depression. This phenomenon can be explained by elevated calcium levels at synapses overexpressing NCS-1. Based upon the results presented here, the molecular basis for the observed switch is likely to be the NCS-1–induced enhancement of InsP\(_3\)R1 activity. Other phenomena may also be regulated by NCS-1 levels. For example, Caenorhabditis elegans overexpressing NCS-1 shows improved memory whereas NCS-1 knockout animals display impaired memory function. In humans, the highest levels of NCS-1 are found in brain regions such as the hippocampus and areas that are associated with memory and sensory processing (31).

Other members of the calcium-binding protein family have functional effects on InsP₃R (8, 13, 25, 32). An initial report suggested that calcium-binding protein 1 (CaBP1) could activate InsP₃R, even in the absence of InsP₃ (32), but subsequent reports have shown that CaBP1 is an effective inhibitor of calcium release through InsP₃R and that InsP₃ is required for this effect (8, 25). Comparisons of the properties of CaBP1 and NCS-1 showed that the calcium-binding affinity was similar, but when cells were maximally stimulated to release intracellular calcium (25), expression of CaBP1 inhibited the response whereas expression of NCS-1 enhanced the response.

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**Figure 4**

Calcium dependence of the effect of NCS-1 and its E120Q mutant on InsP₃R1. Single-channel recordings of InsP₃R1 at the calcium concentrations indicated in the figure in the presence of E120Q and InsP₃ (A, left traces) or NCS-1 and 2 μM InsP₃ (A, right traces). Averaged values from at least 3 experiments for the mean open time (B) and the Pₒ (C) are shown for the effect of the addition of either NCS-1 (open circles) or the mutant form E120Q (filled circles). There is a calcium-independent activation as shown at low calcium concentrations after addition of E120Q and a calcium-dependent activation shown after addition of NCS-1. The purified InsP₃R1 used in this study displays no inhibition at calcium levels over the negative log of the free calcium concentration (pCa) 6.5 (black line; data taken from ref. 40).

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**Figure 5**

PC12 cells overexpressing NCS-1 show increased intracellular calcium release after stimulation by extracellular ATP. (A) Wild-type PC12 cells contain both NCS-1 (green) and InsP₃R1 (red). Scale bar: 50 μm. (B) Representative traces showing calcium release evoked by the addition of 50 μM ATP for 1 minute followed by a washout (dark trace: PC12 cells overexpressing NCS-1; light trace: PC12 cells transfected with empty vector). (C) Representative traces comparing the intracellular calcium release after preincubation with lithium (30 minutes, 10 mM lithium; light trace) with NCS-1 cells that were not treated with lithium (dark trace). Note that lithium did not alter the fluorescence signal but did decrease the NCS-1–dependent increase in calcium release to control levels. (D) Averaged values from at least 3 experiments.
siveness of the cells (8, 25). This diversity of effect allows the numerous members of the calcium-binding protein family to be specific yet potent regulators of InsP$_3$R function.

The interactions between NCS-1 and InsP$_3$R are not only of importance for physiological functions in neurons, but accumulating evidence suggests an association with pathological conditions. Recently, NCS-1 levels have been shown to be increased in the prefrontal cortex of schizophrenic and bipolar patients (17). Both conditions are also associated with disturbances in calcium homeostasis and alterations in the phosphoinositide signaling pathway (19, 33). Platelets of affected people are often used as models for neurons because they have similar signaling features and it is possible to obtain samples from affected individuals. In platelets from unmedicated patients, the increase in intracellular calcium after thrombin stimulation is significantly higher than that measured in healthy controls (33). Our observations can explain these phenomena with the enhancing effect of NCS-1 on InsP$_3$R1 activity, which affects intracellular calcium release but not resting calcium levels.

It now appears that NCS-1 plays a multifaceted role in the pathophysiology of neuropsychiatric disorders. Our results show that lithium, a commonly used therapeutic reagent for bipolar disease, antagonizes NCS-1–mediated InsP$_3$R1 enhancement by altering the calcium-dependent properties of NCS-1. That therapeutic levels of lithium could inhibit this property of NCS-1 even in the presence of physiological levels of calcium shows the importance of the activity of this family of proteins and their interactions with target proteins. Our experiments using the calcium-binding deficient NCS-1 (E120Q mutant) lend further support for the importance of the regulation of the calcium-binding proteins on the activity of InsP$_3$R1. We conclude that the positive enhancement of InsP$_3$R1 action by NCS-1 is a widely used mechanism to increase calcium signals and is important in many physiological processes. The role of NCS-1 in pathological conditions suggests that it is a promising target for treatment of neuropsychiatric disorders.

Methods

Immunoprecipitation and immunocytochemistry. Microsomes were made from mouse cerebella as previously described (34). Cerebellar lysates were made by homogenization of mouse cerebella in RIPA buffer (Santa Cruz Biotechnology Inc.), followed by 2 spins at 16,000 g for 5 minutes at 4°C. For immunoprecipitation, either the cerebellar microsomes or lysates were incubated with antibody; the 2 antibodies used were anti–NCS-1 (Santa Cruz Biotechnology Inc.) and anti-InsP$_3$R type I (35). The immunoprecipitates were resolved by SDS-PAGE and transferred to a PVDF membrane, and immunoreactive bands were visualized using standard methods. Frozen mouse cerebella were purchased from Pel-Freeze Biologicals.

Anti–NCS-1 or anti-InsP$_3$R type I antibodies (from the same sources as above) were used to probe fixed rat brain slices or PC12 cells. PC12 cells were differentiated with 100 ng/ml NGF (New England Biolabs Inc.) for 5 days prior to fixation. Tissue samples were prepared as previously described (36).

Purification of the proteins and measurement of NCS-1 properties. InsP$_3$R were purified and reconstituted as described previously (37). Similarly, NCS-1 was produced and purified as described previously (38). Lithium binding
to NCS-1 was monitored by fluorescence spectroscopy. NCS-1 protein was suspended in 50 mM Tris and 100 mM KCl, pH 7.2, and was titrated with the desired concentrations of lithium and incubated for 5 minutes; emission spectra were recorded at the excitation of 280 and 295 nm.

Single-channel measurements. Planar lipid bilayers were formed by painting a solution of phosphatidylethanolamine/phosphatidylserine (3:1; 30 mg/ml in decane) across a 200-μm hole in the side of a polystyrene cup (Warner Instruments) separating 2 chambers with a volume of 1 ml each. Then the purified InsP₃R₁ were incorporated into the bilayer (37). After addition of InsP₃, InsP₃R₁ was activated, and single-channel activity was recorded. NCS-1 was then added to the cytoplasmic side of the channel. For the NCS-1 concentration-response curve, channel activity was recorded after adding NCS-1 to a final concentration of 0.45 μg/ml, 0.9 μg/ml, 1.34 μg/ml, 1.92 μg/ml, and 2.56 μg/ml. The free calcium concentration was kept constant at 300 nM, and InsP₃ concentration was 2 μM. For the calcium concentration-response curve, channel activity was recorded after the free calcium concentration was incrementally increased (10 nM, 30 nM, 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 30 μM, 100 μM, 300 μM, and 1000 μM) at a fixed NCS-1 concentration of 2 μg and a constant InsP₃ concentration of 2 μM. These experiments were repeated using the E120Q mutant of NCS-1 to determine the effect of calcium on NCS-1. To examine the ability of NCS-1 to activate InsP₃R₁, channel activity was first observed in the presence of InsP₃. Then, after InsP₃ was removed, NCS-1 alone was added, and the level of channel activity was recorded.

To examine the effect of lithium on the ability of NCS-1 to alter channel activity, lithium was added to the cytoplasmic side incrementally (0.5 mM, 1 mM, 1.5 mM, 2.5 mM, 4 mM, and 5 mM) in the presence of fixed concentrations of InsP₃ (2 μM), calcium (300 μM), and NCS-1 (1.92 μg/ml). The effect of lithium was also tested in the absence of NCS-1.

Cell culture and generation of NCS-1 and E120Q overexpressing PC12 cells. PC12 cells were grown as described previously (39), and stable transfectants of PC12 cells were obtained as reported (13). Cells were detached using 0.25% trypsin/1 mM EDTA (Invitrogen Corp.) and plated on cover slips (Fisher Scientific International) in a dilution of 30,000 cells/ml. The plated cells were differentiated with 100 ng/ml NGF (New England Biolabs Inc.) for 5 days.

Calcium imaging. Confocal microscopy was used to measure intracellular calcium in PC12 cells (35). Differentiated PC12 cells were loaded with Fluo-4 AM (Invitrogen Corp.) and then mounted onto the stage of a Zeiss Axiovert 135 inverted microscope. The cells were perfused continuously with artificial cerebrospinal fluid (124 mM NaCl, 10 mM glucose, 25 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM KCl, 2 mM CaCl₂, and 2 mM MgCl₂; pH 7.35) using a 4-chambered superfusion reservoir that allowed rapid changes of perfusion solutions. Cells were stimulated with 50 μM ATP for 1 minute. Increases in calcium were expressed as the ratio of fluorescence intensity to baseline (F/F₀). Background fluorescence was automatically subtracted from all measurements. There was no change in size, shape, or location of cells during the experiments.

To determine the effect of lithium on ATP-evoked calcium release, cells were preincubated in growth medium containing 10 mM lithium for 15 minutes. The cells were then loaded with Fluo-4 and monitored as described above, except that the dye solution was supplemented with 10 mM lithium.

Statistics. All averaged values shown are mean ± SEM. Each value for bilayer experiments represents an average of at least 3 experiments; for calcium imaging, at least 10 experiments were averaged.

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