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 ($InsP_3R$) 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 $InsP_3R$ type 1 ($InsP_3R1$) 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 phosphoinositide signaling pathway. To determine whether $InsP_3R1$/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 $InsP_3R1$ function, suggesting that $InsP_3R1$/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 ($InsP_3R$) is also involved in pathological conditions that are related to disturbance in calcium homeostasis. Loss of $InsP_3R$ type 1 ($InsP_3R1$) in mice is associated with ataxia and seizures (3), and loss of $InsP_3R3$ is found in humans with bile duct obstruction (4). In several neurodegenerative diseases, such as Alzheimer disease and Huntington disease, and in brain ischemia, $InsP_3R1$ appears to be selectively downregulated (5–7). Understanding the basis for the dysregulation of $InsP_3R$ activity will be crucial for understanding the pathomechanism of these diseases.

A variety of signaling molecules have been shown to associate with $InsP_3R$ 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 $InsP_3R1$ 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 $InsP_3R$ 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 $InsP_3R1$. 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/$InsP_3R1$ 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 $InsP_3R1$, 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 $InsP_3R1$. We also found that lithium attenuates the NCS-1/$InsP_3R1$ association. This observation indicates that the signaling complex comprised of NCS-1 and the $InsP_3R$ 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 $InsP_3R1$ in vivo. In the cerebellum, NCS-1

Nonstandard abbreviations used: CaBP1, calcium-binding protein 1; $InsP_3R$, inositol 1,4,5-trisphosphate receptor; $InsP_3R1$, inositol 1,4,5-trisphosphate receptor type 1; NCS-1, neuronal calcium sensor-1; NGF, nerve growth factor; P_o, open probability; RyR2, ryanodine receptor type 2.

Conflict of interest: The authors have declared that no conflict of interest exists.

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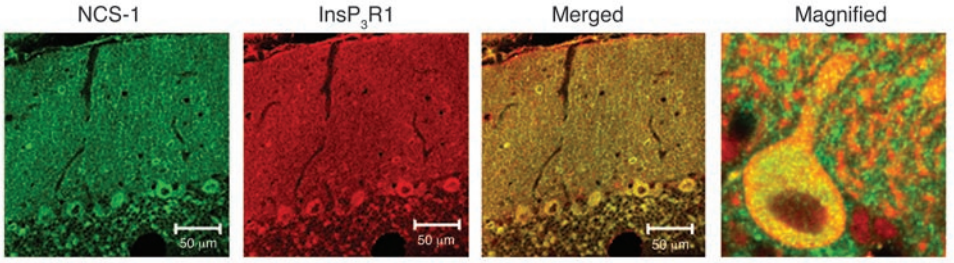

Figure 1
Immunolocalization of NCS-1 and $InsP_3R1$ in neurons in vivo. Slices from cerebellum of adult rat brain were immunostained for NCS-1 (far left) and $InsP_3R$ (second from left). Both proteins were found throughout the cerebellum with an overlapping distribution (second from right) but were concentrated in Purkinje cells (see detail of single cell in far right panel).

was visible throughout the cell except the nucleus in Purkinje cells and stellate cells (Figure 1). $InsP_3R1$ 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 $InsP_3R1$ 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 $InsP_3R1$, both NCS-1 and $InsP_3R1$ 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-$InsP_3R1$ (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 $InsP_3R$. These results show that the interaction between NCS-1 and $InsP_3R1$ can be identified in native tissue and is calcium dependent.

To determine whether the interaction between NCS-1 and $InsP_3R1$ has a functional component, we added purified NCS-1 to $InsP_3R1$ after it had been incorporated into planar lipid bilayers and monitored $InsP_3$-gated channel activity. Measurements were obtained using 300 nM free calcium, 0.5 mM ATP, and 2 μM $InsP_3$ on the cytoplasmic side of $InsP_3R1$. In the absence of NCS-1, the amplitude of the single-channel currents was 2 pA (Figure 3A), the open probability (P_o) 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 $InsP_3R1$ channel activity: the P_o 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 $InsP_3R1$ channel activity could, in principle, be attributed to the ability of NCS-1 to activate the channel in the absence of $InsP_3$. When NCS-1 was added to $InsP_3R1$ in the absence of $InsP_3$ over a range of calcium concentrations, no channel openings were observed (Figures 3A and 4C), showing that NCS-1 alone was unable to activate $InsP_3R1$.

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/$InsP_3R1$ 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 $InsP_3$-activated channel at 300 nM free calcium, the P_o 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 P_o (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 P_o and mean open time remained constant as the calcium concentration was increased (Figure 4, A–C). Thus, NCS-1 acts on $InsP_3R1$ in both a calcium-independent and a calcium-dependent manner.

To determine whether the interaction between NCS-1 and $InsP_3R1$ 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

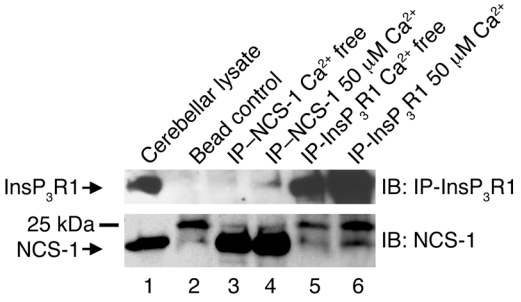

Figure 2
Coimmunoprecipitation of NCS-1 and $InsP_3R1$ in rat cerebellum. Lanes show mouse cerebellar lysate (lane 1); beads treated with cerebellar lysate but without antibody (lane 2); immunoprecipitate (IP) with anti–NCS-1 with calcium buffered (lane 3); immunoprecipitate with anti–NCS-1 with 50 μM free calcium present (lane 4); immunoprecipitate with anti-$InsP_3R$ with calcium buffered (lane 5); and immunoprecipitate with anti-$InsP_3R$ with 50 μM free calcium present (lane 6). Top and bottom panels show immunoblots for InsP3R1 and NCS-1, respectively.

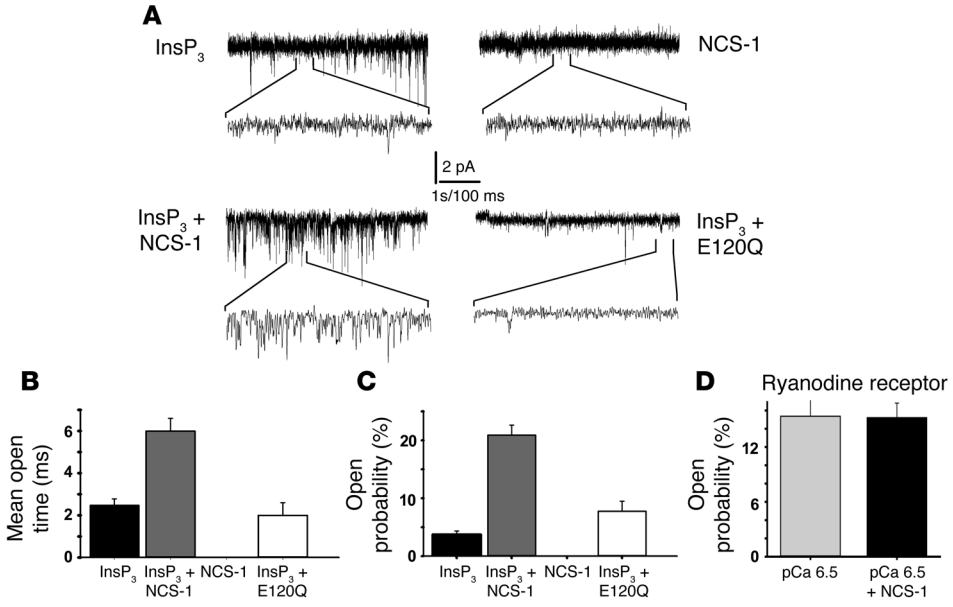

Figure 3

Effect of NCS-1 and the E120Q mutant of NCS-1 on the activity of $InsP_3R1$. (**A**) Single-channel recordings of $InsP_3R1$ where downward deflections are channel openings. The top pair of traces on the left were recorded after addition of 300 nM free calcium and 2 μM $InsP_3$, and the bottom left pair of traces show activity after addition of 2 μg/ml NCS-1 in the presence of 2 μM $InsP_3$. In each case, the uppermost trace represents 10 seconds of continuous recording, and the bottom trace shows a 10-fold expansion of the time scale. On the right, the top pair of traces were recorded in the presence of 2 μg/ml NCS-1 in the absence of $InsP_3$, and the bottom pair of traces were recorded after addition of 2 μg/ml E120Q, a mutant of NCS-1, in the presence of 2 μM $InsP_3$. Averaged values from at least 3 experiments for mean open time (**B**) and for P_o (**C**) of $InsP_3R1$ corresponding to the traces shown in **A**. Note that NCS-1 alone did not activate $InsP_3R1$. (**D**) Addition of NCS-1 (2 μg/ml) had no effect on the activity of the RyR2 activated by 300 μM calcium.

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/F_0], 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 $InsP_3R1$ 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 $InsP_3R1$. $InsP_3R1$ channel activity increased at least 2-fold after addition of NCS-1 (Figure 3A). Addition of lithium offset the enhancing effects of NCS-1 on $InsP_3R1$ channel activity to near baseline levels (5% ± 2.1%, n = 3; Figure 6B). This inhibitory effect of lithium on the NCS-1 action was strongly dependent on the lithium concentration, with an IC_{50} of 350 μM (Figure 6C). In the absence of NCS-1, addition of lithium had no effect on $InsP_3$-gated channel activity (Figure 6C). Similarly, lithium significantly decreased the amplitude of the calcium-induced fluorescence change in cells overexpressing NCS-1 (3.8 ± 0.56 before lithium incubation, 1.9 ± 0.1 after cells were incubated in 10 mM lithium for 30 minutes before stimulation; Figure 5, C and D; n = 18). Lithium had no effect on the intracellular calcium transients in control cells (1.5 ± 0.06 before lithium incubation, 1.4 ± 0.06 after lithium incubation; n = 22). To test the possibility that pretreatment with lithium would alter the calcium concentration in the intracellular stores, the stores were depleted of intracellular calcium by treatment with 1 μM thapsigargin for 2 minutes. The maximum amplitude of measured fluorescence change was 1.4 ± 0.03 (n = 31) in control cells and 1.7 ± 0.06 (n = 59) in cells pretreated with 10 mM lithium for 30 minutes. With the slight elevation of calcium in the stores after lithium treatment, the calcium signal after ATP stimulation should have increased rather than decrease as observed (Figure 5).

Lithium did not alter the ability of NCS-1 to immunoprecipitate $InsP_3R1$ (Figure 6A). To determine whether lithium binds to NCS-1, we monitored the change in Trp fluorescence of NCS-1. Lithium decreased the fluorescence emission of NCS-1 to the same degree as calcium (25, 26) irrespective of the presence or absence of physiological levels of calcium (350 nM; data not shown). Addition of calcium in the presence of therapeutic levels of lithium (1 mM) produced no further decrease in fluorescence emission. These data are consistent with a model where lithium has fixed the NCS-1 in a calcium-independent state that then attenuates activity- and NCS-1–dependent changes in $InsP_3R1$. Further experiments are required to identify the binding sites and the affinity of NCS-1 for lithium in the presence and absence of calcium.

Discussion

Our experiments show that NCS-1 modulates calcium signaling by enhancing $InsP_3$-mediated activity of $InsP_3R1$ 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 $InsP_3R1$. Furthermore, we found that lithium attenuates the NCS-1/$InsP_3R$ 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

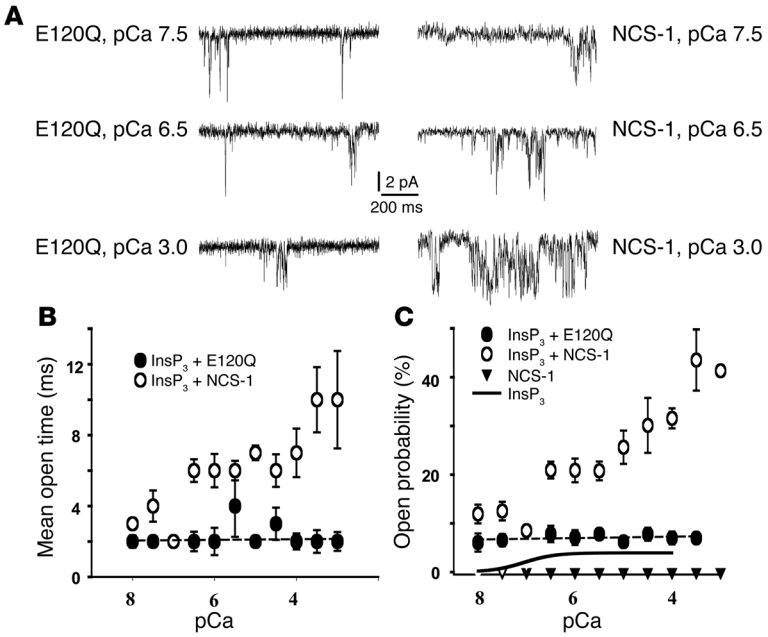

Figure 4
Calcium dependence of the effect of NCS-1 and its E120Q mutant on $InsP_3R1$. Single-channel recordings of $InsP_3R1$ at the calcium concentrations indicated in the figure in the presence of E120Q and $InsP_3$ (**A**, left traces) or NCS-1 and 2 μM $InsP_3$ (**A**, right traces). Averaged values from at least 3 experiments for the mean open time (**B**) and the P_o (**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 $InsP_3R1$ 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).

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 $InsP_3R1$ 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 $InsP_3R$ (8, 13, 25, 32). An initial report suggested that calcium-binding protein 1 (CaBP1) could activate $InsP_3R$, even in the absence of $InsP_3$ (32), but subsequent reports have shown that CaBP1 is an effective inhibitor of calcium release through $InsP_3R$ and that $InsP_3$ 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 NSC-1 enhanced the respon-

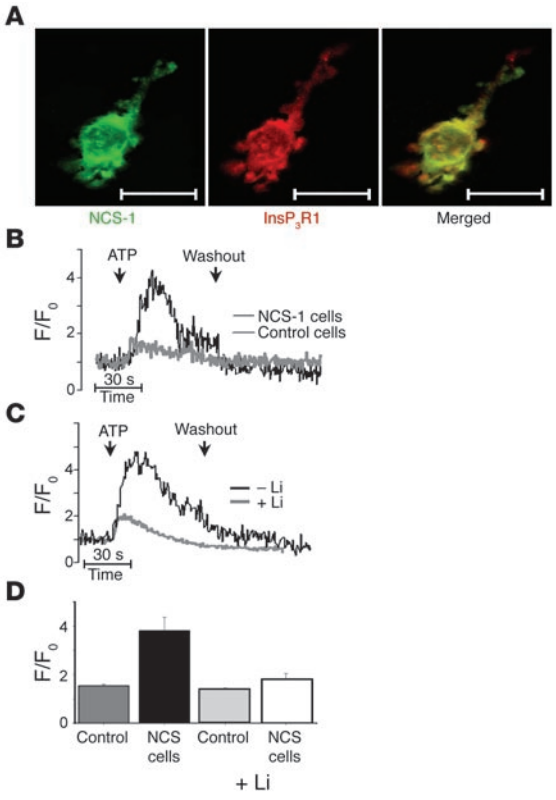

Figure 5
PC12 cells overexpressing NCS-1 show increased intracellular calcium release after stimulation by extracellular ATP. (**A**) Wild-type PC12 cells contain both NCS-1 (green) and $InsP_3R1$ (red). Scale bar: 50 μm. (**B**) Representative traces showing calcium release evoked by the addition of 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.

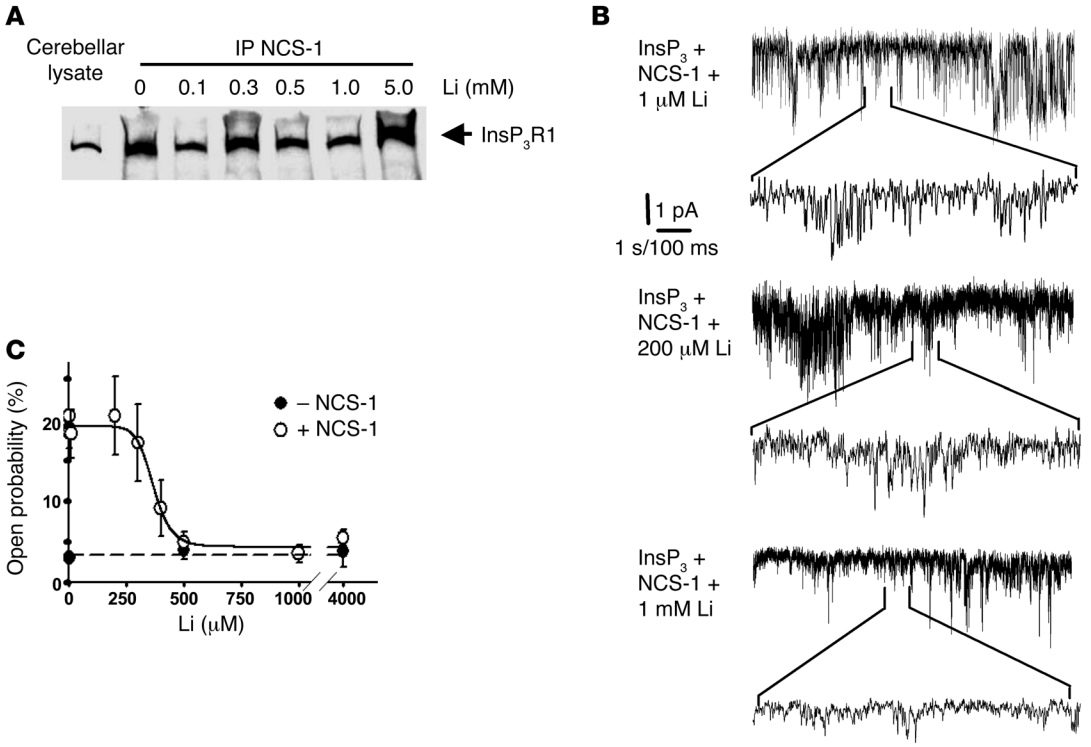

Figure 6
Lithium reduces the NCS-1–mediated increase in $InsP_3R1$ activity. (**A**) Addition of lithium (concentrations as indicated) did not alter the ability of NCS-1 to immunoprecipitate $InsP_3R1$. (**B**) Single-channel recordings of $InsP_3R1$. Traces show the activity in the presence of 300 nM free calcium, 2 μM $InsP_3$, and 2 μg/ml NCS-1 and were recorded after addition of 1 μM, 200 μM, and 1 mM lithium (in the top, middle, and bottom panels, respectively). (**C**) Averaged values from at least 3 experiments for P_o. The concentration dependence for lithium on the NCS-1–mediated enhancement of $InsP_3R$ activity in the presence (open circles) and absence (filled circles) of NCS-1. The IC_{50} for lithium was approximately 350 μM.

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 $InsP_3R$ function.

The interactions between NCS-1 and $InsP_3R$ 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 $InsP_3R1$ 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 $InsP_3R1$ 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 $InsP_3R1$. We conclude that the positive enhancement of $InsP_3R1$ 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-$InsP_3R$ 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-$InsP_3R$ 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. $InsP_3R$ 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 $InsP_3R1$ were incorporated into the bilayer (37). After addition of $InsP_3$, $InsP_3R1$ 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 $InsP_3$ 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 $InsP_3$ 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 $InsP_3R1$ in the absence of $InsP_3$, channel activity was first observed in the presence of $InsP_3$. Then, after $InsP_3$ 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 $InsP_3$ (2 μM), calcium (300 nM), 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 transfected PC12 cells were obtained as reported (13). Cells were detached using 0.25% trypsin/1 mM EGTA (Invitrogen Corp.) and plated on glass 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 $NaHCO_3$, 1.25 mM NaH_2PO_4, 2.5 mM KCl, 2 mM $CaCl_2$, and 2 mM $MgCl_2$; 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/F_0). 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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