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Current Understanding of the Role of Neuronal Calcium Sensor 1 in Neurological Disorders

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Abstract

Neuronal calcium sensor 1 (NCS-1) is a high-affinity calcium-binding protein and its ubiquitous expression in the nervous system implies a wide range of functions. To date, it has been implicated in regulation of calcium channels in both axonal growth cones and presynaptic terminals, pre- and postsynaptic plasticity mechanisms, learning and memory behaviors, dopaminergic signaling, and axonal regeneration. This review summarizes these functions and relates them to several diseases in which NCS-1 plays a role, such as schizophrenia and bipolar disorder, X-linked mental retardation and fragile X syndrome, and spinal cord injury. Many questions remain unanswered about the role of NCS-1 in these diseases, particularly as the genetic factors that control NCS-1 expression in both normal and diseased states are still poorly understood. The review further identifies the therapeutic potential of manipulating the interaction of NCS-1 with its many targets and suggests directions for future research on the role of NCS-1 in these disorders.

Keywords NCS-1 · Frequenin · Calcium-binding proteins · Schizophrenia · Bipolar disorder · X-linked mental retardation

Introduction

Neuronal calcium sensor 1 (NCS-1), a homolog of the *Drosophila* protein frequenin, is a high-affinity intracellular calcium-binding protein expressed in most neural tissues. It belongs to the family of intracellular neuronal calcium sensor (NCS) proteins, which are 185–205 amino acids long and contain 4 EF-hand calcium-binding sites (EF1-4) (Fig. 1) and have been described in detail elsewhere [1–3]. NCS-1 has both calcium- and magnesium-binding capabilities. While its EF1 domain is degenerated and cannot bind either ion [4, 5], it binds both calcium and magnesium at EF2 and EF3 [6]. Following myristoylation of the N-terminal domain of the protein, NCS-1 can also bind calcium, but not magnesium, at the EF4 domain [7]. Myristoylation is also important to the ability of NCS-1 to bind G protein receptor kinases (GRKs) [8, 9].

NCS1 mRNA and NCS-1 protein are expressed ubiquitously in chick [12], rat [13], and mouse neuronal tissues, with some expression in mouse astroglia [14] and rat astrocytes during development [15]. In the mouse brain, NCS-1 protein is expressed roughly equivalently in most regions, with highest levels of expression in the hippocampus and lowest in the caudate-putamen [14]. In rat, most brain areas express *NCS1* mRNA, with moderate expression in the caudate-putamen, nucleus accumbens, and hypothalamus, and no expression in layer I of the cortex [16]. NCS-1 is also found prominently in the spinal cord, with high immunoreactivity in rat dorsal root ganglia [17]. NCS-1 immunoreactivity is also found in the inner plexiform layer of the rat retina, and its developmental expression in the retina closely follows that of synaptophysin, suggesting a role in synaptogenesis [18]. Human genome-wide microarray data on the expression of NCS-1 DNA in the brain is depicted in Fig. 2.

At the subcellular level, NCS-1 immunoreactivity is most intense in axons, both myelinated and unmyelinated; dendrites; and cell bodies of neurons. Weak colocalization of NCS-1 and synaptophysin in the rat neuromuscular junction suggests it is present at the presynaptic nerve terminal [13]. Finally, NCS-1 immunoreactivity is also associated with microtubules [13] in neurons. It colocalizes with MAP-2 [14], suggesting an association with neuritic processes.

As NCS-1 is expressed in a variety of neuronal cell types throughout the nervous system, it is an attractive candidate for regulation of a variety of cellular processes. It binds a wide variety of targets, some of which it shares with calmodulin (CaM)

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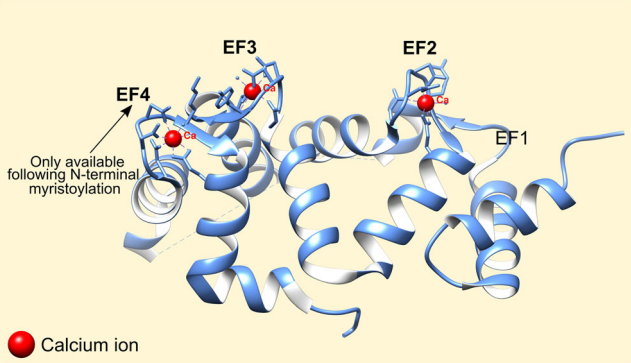

Fig. 1 Protein structure of NCS-1 (PDB 5AEQ) [10], with calcium ions in red and EF hand domains shown. Myristoylation of the N-terminal domain allows calcium binding to EF4. Structure was generated using UCSF Chimera [11]

[20, 21]. Furthermore, its implication in a variety of signaling pathways makes its dysfunction a potential basis for many different human diseases. This review will summarize the diverse known functions of NCS-1, particularly those relating to calcium channel regulation, synaptic plasticity, dopamine receptor regulation, and neurite outgrowth, which have been reviewed elsewhere [22–24]. A recent review has summarized these functions and has proposed an NCS-1-mediated mechanism of chemotherapy-induced peripheral neurodegeneration (CIPN) [25]; however, our review will relate the functions of NCS-1 to its involvement in other diseases, particularly as it pertains to neuropsychiatric disorders, neurodevelopmental disorders, and axonal injury.

NCS-1 Regulates Calcium Channels in Presynaptic Terminals

Well-characterized functions of NCS-1 are voltage-gated calcium channel (VGCC) regulation and neurite extension. These two functions are intertwined and to understand the potential roles of NCS-1 in disease, we first summarize the normal function of the protein.

Neurotransmitter release from presynaptic nerve terminals is initiated by entry of calcium through VGCCs. It is coordinated by a complex set of molecular determinants. Briefly, depolarization of the presynaptic terminal opens VGCCs, and calcium enters and activates assembly of the SNARE complex, leading to vesicle fusion to the presynaptic membrane and neurotransmitter release into the synaptic cleft [26]. The amount of calcium that enters the presynaptic nerve terminal is dependent on the type of VGCC expressed and protein modification of those VGCCs. For example, high-voltage activated (HVA) VGCCs containing $Ca_v1.3$, $Ca_v2.1$, and $Ca_v2.2$ α-subunits conduct L-type, P/Q-type, and N-type calcium currents, respectively. $Ca_v2.1$ and $Ca_v2.2$ VGCCs mediate neurotransmitter release in most central nervous system (CNS) synapses, while $Ca_v1.3$ is

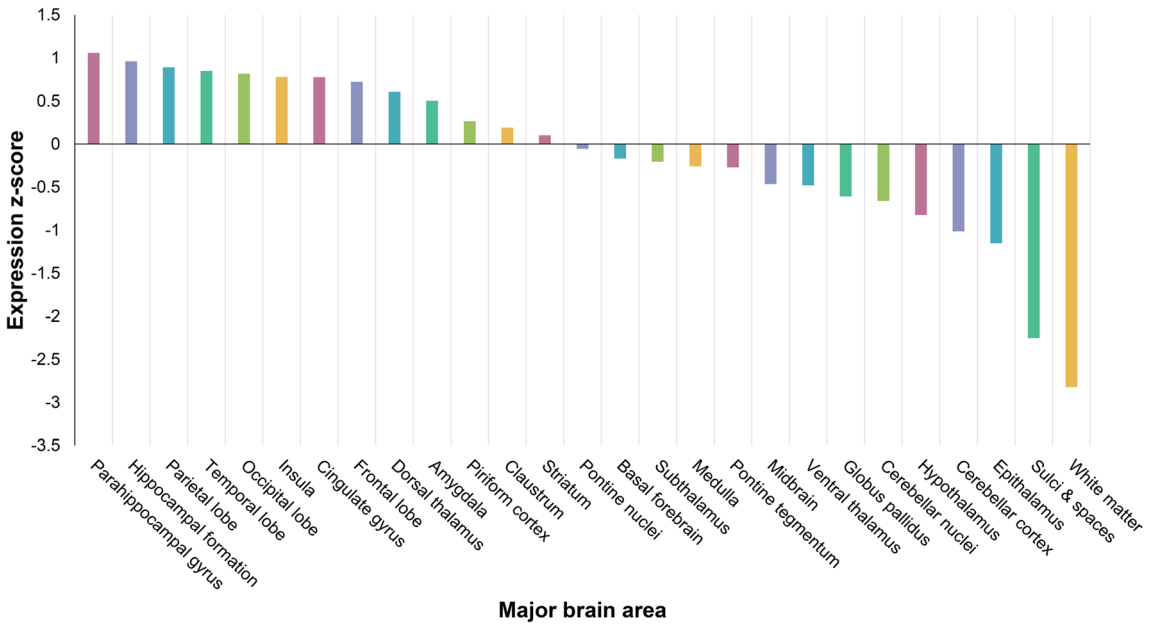

Fig. 2 Distribution and abundance of NCS-1 in adult human patients as determined by DNA microarray, presented as a Z-score compared to average expression. High expression of NCS-1 in the parahippocampal gyrus and hippocampal formation implies a role in memory acquisition and retrieval; however, the protein's high expression in many areas of the brain implies a wide range of functions. Microarray data was resourced from Allen Human Brain Atlas [19]. © 2010 Allen Institute for Brain Science. Allen Human Brain Atlas. Available from: human.brain-map.org

present in photoreceptor terminals [27]. Variability in residual presynaptic calcium contributes to diversity in the kinetics of neurotransmitter release, including mechanisms of short-term presynaptic plasticity [28]. Specifically, the mechanism of paired-pulse facilitation (PPF), where the amplitude of the second peak in a train of evoked EPSCs recorded from the postsynaptic cell is greater than the first, has been proposed to rely on buildup of residual calcium in the postsynaptic terminal. Putatively, increased presynaptic calcium availability after the second stimulation results in more vesicles fusing and therefore more neurotransmitter release, leading to a greater recorded second EPSC [29]. Facilitation of calcium currents via VGCC modification is therefore likely to enhance PPF.

VGCC regulation by NCS-1 was first demonstrated in *Xenopus* embryos [30]. Glial cell-derived neurotrophic factor (GDNF) application enhanced N-type calcium currents in the *Xenopus* neuromuscular junction, which was blocked by application of anti-frequenin/NCS-1 antisense oligos, suggesting frequenin/NCS-1 to be an effector protein of GDNF-mediated calcium channel regulation [30], likely due to GDNF-mediated activation of NCS-1. Contrastingly, in the *Xenopus* oocyte expression system, NCS-1 overexpression was shown to decrease L-type $Ca_v1.2$, P/Q-type $Ca_v2.1$, and N-type $Ca_v2.2$ currents calcium-dependently [31]. This effect was abolished by the NCS-1_{E120Q} mutation, which prevents calcium binding to the third EF-hand motif [31], suggesting that EF3 calcium-binding is necessary for NCS-1 downregulation of calcium currents. Frequenin also enhances evoked neurotransmitter release in *Drosophila* neuromuscular junction, where overexpression of frequenin and frequenin2 increased quantal content, despite decreasing the number of active zones per synaptic bouton [32], suggesting an effect of increased sensitivity to calcium influx in frequenin overexpressers. In rat NG108-15 cells cocultured with myocytes, NCS-1 overexpression also enhanced evoked release of acetylcholine while colocalizing partially with the exocytotic t-SNARE protein SNAP-25 [33]. This suggests that NCS-1 may modulate presynaptic release not only by modulating calcium currents, but potentially by interacting directly with the exocytotic machinery.

In mammalian neurons, NCS-1 has exhibited a similar effect on calcium currents, enhancing P/Q-type calcium currents activity-dependently in rat calyx of Held and enhancing PPF [34]. Likewise, NCS-1 modulates P/Q-type $Ca_v2.1$ channels to enhance PPF in presynaptic superior cervical ganglion neurons [35]. It does so by binding the IQ calmodulin-binding domain (CBD) of the $Ca_v2.1$ channel, as confirmed by yeast two-hybrid assay. The facilitation effect of NCS-1 requires this motif to be present on the $Ca_v2.1$ channel [35]. These effects are calcium dependent [36], suggesting that the effect of NCS-1 on calcium channels depends on an initial calcium influx. These experiments point to NCS-1 playing a modulatory role of calcium-controlled neurotransmitter release in neurons by directly binding the IQ CBD of calcium channels, where NCS-1 fine-tunes neurotransmitter release in response to small changes in intracellular calcium.

NCS-1 Regulates Voltage-Gated Calcium Channels in Neuronal Growth Cones

Along with its role in regulating presynaptic voltage-gated calcium channels, NCS-1 also plays a key role in regulating neuronal growth cone channels and regulating neurite extension. Growth cones are the dynamic protrusions of growing neuronal processes, and their accurate guidance by chemotropic attractive and repulsive cues is essential to proper brain circuit development [37]. In developing embryonic rat spinal cord, NCS-1 colocalizes with the growth cone protein GAP-43 and follows a developmental time course of expression with peaks in the late prenatal period [15], pointing to an association with neuronal growth cones in the developing spinal cord. This is supported by expression of NCS-1 in chick dorsal root ganglion neuronal growth cones, particularly near the polymerizing plus-ends of microtubules in the center of the growth cone, and in the peripheral leading edge of the lamellipodia [38] (Fig. 3).

This expression pattern depends on the presence of intracellular calcium, as depleting ER stores disrupted the organization of NCS-1 expression. The same study showed colocalization of NCS-1 with the inositol 1,4,5-triphosphate receptor ($InsP_3R1$), a promoter of neurite extension (Fig. 4), in the growth cone. Application of an $InsP_3R1$ blocker disrupted the NCS-1 distribution pattern and resulted in neurite growth arrest and neurite retraction, an effect that was similarly achieved by micro chromophore-assisted laser inactivation (CALI) of NCS-1 [38]. Therefore, NCS-1 is a clear regulator of neurite extension, potentially via regulation of $InsP_3R1$. The role of NCS-1 in neurite extension is supported by overexpression studies, where overexpression of NCS-1 in rat NG108-15 cells cocultured with myocytes resulted in neurite outgrowth suppression while increasing rate of synapse formation [33]. NCS-1 demonstrates a similar inhibitory effect on neurite extension in PC12 cells, where a dominant-negative NCS-1 peptide exhibited an enhancing effect on neurite outgrowth [40]. It seems to signal by binding TRPC5, a growth cone transient receptor potential calcium channel which inhibits neurite extension (Fig. 4). Furthermore, the dominant-negative peptide inhibited TRPC5 only at specific calcium concentrations [40], suggesting that NCS-1 serves to activate TRPC5 within a narrow range of intracellular calcium concentrations. As such, NCS-1 may serve as a sensor for the optimal intracellular calcium concentration required for mammalian

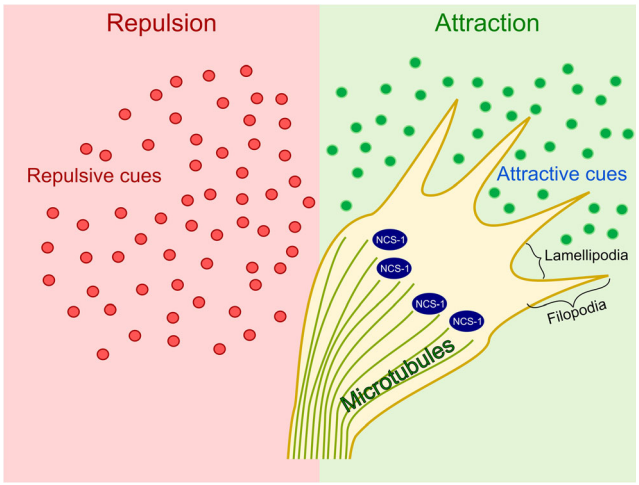

Fig. 3 The neuronal growth cone responds to both repulsive (red) and attractive (green) tropic cues secreted in the extracellular milieu. Changes in microtubule dynamics cause extension of the lamellipodial leading edge and smaller filopodia away from repulsive cues, towards attractive cues [39]. NCS-1 is associated with the center of the growth cone, near the polymerizing plus-ends of the microtubules [38]

neurite outgrowth, as its effect on regulation of TRPC5 channels is calcium concentration dependent.

Our lab has further demonstrated that NCS-1 negatively regulates neurite extension in invertebrates, particularly in *Lymnaea stagnalis*. The protein is expressed in somata, growth cones, and branch points of *Lymnaea* primary cultured neurons, and knockdown of NCS-1 results in abnormal branching and increased growth rate of neurites [44]. This effect involves changes in calcium dynamics in growth cones, as calcium imaging revealed a lower calcium influx per action potential in NCS-1-knockdown neurons, an effect that was mimicked by injection of NCS-1 C-terminal dominant-negative peptide. Dominant-negative peptide injection also increased branching complexity in these neurons [44]. These data suggest that the C-terminal of NCS-1 is required for the protein's inhibitory effect on neurite branching and outgrowth, and that the function of NCS-1 includes modulation of calcium influx in neuronal cells, likely via growth cone calcium channel modulation. This was confirmed by measuring the effect of NCS-1 C-terminal dominant-negative peptide on calcium currents in *Lymnaea* neuronal growth cones, where application of the dominant-negative peptide decreased growth cone current density [45]. These results identify NCS-1 as a crucial enhancer of calcium currents in *Lymnaea* growth cones and suggest that this function of NCS-1 inhibits neurite branching and growth.

NCS-1 in Learning and Memory

Influence of NCS-1 on Short-Term Plasticity The function of NCS-1 as a regulator of calcium channels implicates it as a modulator of presynaptic plasticity mechanisms, such as PPF/paired-pulse depression (PPD), as these mechanisms involve calcium dynamics. NCS-1 regulates PPF in cervical ganglion neurons, as discussed previously [35]. In cultured hippocampal neurons, NCS-1 transfection resulted in consistent PPF at synapses, whereas control synapses demonstrated robust PPD [46]. NCS-1 allowed PPF to occur at smaller interstimulus intervals (ISI) than in control synapses, where PPD dominated at those ISIs. This effect of NCS-1 requires calcium binding,

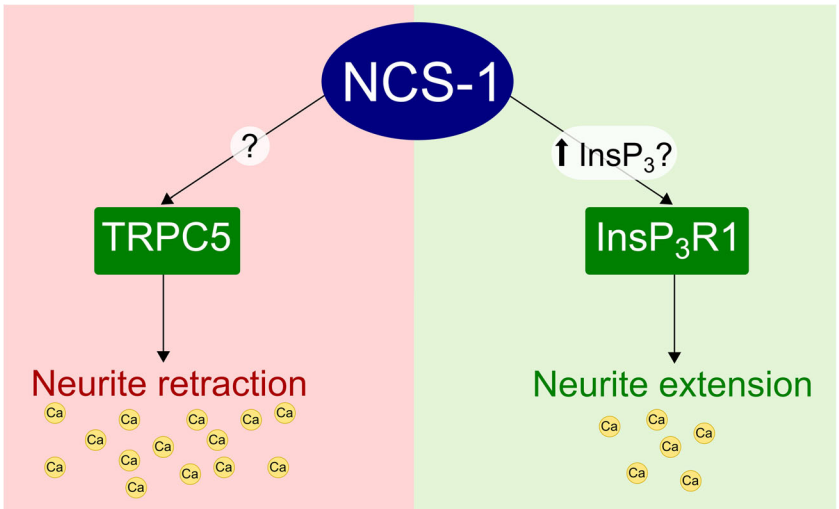

Fig. 4 Effects of NCS-1 on neurite extension and retraction via $InsP_3R1$ and TRPC5 modulation. NCS-1 interaction with $InsP_3R1$, potentially through elevation of $InsP_3$ or direct modulation, results in release of calcium from intracellular stores and moderate elevation in the cytosolic calcium concentration [41]. NCS-1 also interacts with TRPC channels through an unknown mechanism, allowing extracellular calcium to enter the growth cone and resulting in a large elevation in the cytosolic calcium concentration [42]. Optimal moderate calcium levels lead to neurite extension, while high calcium levels lead to neurite retraction [43]

as mutating the calcium-binding domain of NCS-1 significantly reduced its activity. However, overexpression of NCS-1 did not modify conductance of calcium channels, nor basal NT release probability [46]. Interestingly, PPF was blocked in Schaffer collateral-CA1 cultures transfected with a NCS-1 C-terminal regulatory region mutant [47]. Taken together, these experiments identify the C-terminal regulatory region of NCS-1 as a potent calcium-dependent enhancer of presynaptic plasticity in a mechanism separate from its established role in regulating voltage-gated calcium currents.

Role of NCS-1 in Long-Term Plasticity Interestingly, NCS-1 is also involved in some forms of synaptic plasticity independent of its modulation of calcium channels. Induction of metabotropic glutamate receptor (mGluR)–mediated long-term potentiation (LTP) in the hippocampus correlates with upregulation of NCS-1 [48]. mGluR-mediated long-term depression (LTD) in the Perirhinal cortex also involves NCS-1, where blocking the function of NCS-1 using both dominant-negative peptide and RNA interference (RNAi) approaches blocked the induction of mGluR- but not NMDAR-LTD [49]. Further research is required to identify the mechanism by which NCS-1 induces mGluR-LTD in this brain area, and its physiological relevance. *Ncs-1* mRNA has been shown to be significantly upregulated in the dentate gyrus following long-term potentiation (LTP) induction, an effect that was prevented by applying an N-methyl-D-aspartate receptor (NMDAR) antagonist and subsequent inhibitor of NMDAR-dependent LTP [50]. This suggests that NCS-1 may play a role as a downstream effector protein in the expression of LTP.

Impact of NCS-1 on Learning and Memory Behavior The role of NCS-1 in regulating synaptic plasticity in the hippocampus implicates it as an important physiological correlate for learning and memory, as learning and memory have been classically shown to be blocked by inhibitors of NMDAR-mediated LTP in the hippocampus [51–54]. Therefore, the hypothesis has persisted that LTP in the hippocampus is at least partially responsible for the physiological basis of learning and memory [55, 56]. *C. elegans* worms demonstrate isothermal tracking (IT), a learning behavior where worms will travel radially along an isotherm around a heat source to remain within a particular temperature range (15–26 °C) [57]. When food is exhausted, the animal will search for a new isotherm within the preferred range and acquire the association with that temperature. *NCS-1* knockout worms show a defective IT phenotype, with germ-line rescue rescuing the IT behavior. Furthermore, a loss-of-function mutation in NCS-1, rendering it unable to bind calcium, also resulted in the defective IT phenotype, suggesting that calcium binding is required for this learning behavior. Finally, overexpression of NCS-1 resulted in better IT performance than in control worms [57]. Therefore, NCS-1 plays a crucial role in regulating a learning behavior in *C. elegans*.

Behavioral studies performed in mice have also demonstrated the role of NCS-1 in regulating spatial learning. Overexpression of NCS-1 in the dentate gyrus (DG) results in an enhancement of rapid acquisition of spatial memory, as measured by increased performance in the Morris water maze and displaced object recognition test, and is accompanied by a subsequence increase in LTP [58]. Interestingly, this NCS-1-mediated enhancement of LTP and spatial learning requires NCS-1 interaction with D2-like dopamine receptors (D2Rs) [58], as application of a dominant-negative peptide designed to block the NCS-1/D2R interaction blocked the enhancement effect. This suggests an involvement of dopaminergic signaling in mediating the enhancement effect of NCS-1 on spatial learning. This interaction of NCS-1 with D2Rs is unlikely to be used normally by the circuitry and instead may have a modulatory effect specifically on LTP and spatial learning, as both NCS-1 overexpression and application of the DNIP to control mice had no effect on basal synaptic transmission in the DG [58]. Other roles of the NCS-1/D2R interaction will be discussed later in this review.

Additionally, NCS-1 knockout mice demonstrate deficient spatial learning, also measured by decreased performance in the Morris water maze [59]. NCS-1 knockout is accompanied by decreased brain-derived neurotrophic factor (BDNF) expression, decreased dopamine expression, and decreased calcium/calmodulin-dependent protein kinase II-α (CaMKII-α) phosphorylation in the hippocampus, basal ganglia, and cortex. Additionally, high-voltage electric potential (HELP) stimulation, which has been previously shown to enhance BDNF expression and spatial learning in mice [60], was shown to result in an increase in NCS-1 in the hippocampus [59]. These studies suggest that NCS-1 plays a crucial role in regulating spatial learning, and that it may be regulated by the mechanism which upregulates BDNF, dopamine, and phosphorylated CaMKII-α in spatial learning. NCS-1 knockout mice also demonstrated impaired performance on the spontaneous novel object recognition test [61], suggesting impairment of non-spatial long-term memory.

Another interesting behavioral consequence of NCS-1 expression manipulation in mice may involve modulation of dopaminergic circuits. Along with the spatial and non-spatial memory deficits mentioned above, NCS-1 overexpression has also been shown to increase exploratory behavior of a safe environment [58], suggesting a reduction in anxiety in NCS-1-overexpressing mice. Subsequently, NCS-1 knockout mice demonstrated reduced novelty-induced exploratory behavior as measured by the open field task [61], suggesting increased anxiety. This behavior was rescued by administration of diazepam, an anxiolytic. Furthermore, knockout mice demonstrated increased depressive-like behavior, as assessed by the forced swim and tail suspension tests, which was interestingly not rescued by administration of imipramine, a tricyclic antidepressant [61].

As both anxiety [62, 63] and depression [64, 65] are postulated to have a dopaminergic component to their pathophysiology, the described anxiety- and depressive-like behaviors in NCS-1 knockout mice may be due to dysregulated NCS-1 modulation of D2Rs. However, the expression of these behaviors may also be linked to abnormal dopaminergic signaling in the mesolimbic and mesocortical dopamine systems, which are associated with motivation and reward [66]. In fact, NCS-1 knockout mice chose less effortful food options, even when the more effortful food was a preferred food, compared to wild-type controls [67]. This was accompanied by a decrease in dopamine release in the nucleus accumbens core (NAc), implicating NCS-1 as a modulator of presynaptic dopamine release. However, the number of D2Rs remained constant between NCS-1 knockout and WT mice [67], suggesting that NCS-1 regulates the G protein signaling cascade mediated by activation of D2Rs in NAc cells, rather than the number of D2Rs. These results suggest a motivation deficit in NCS-1 knockout mice, potentially due to decreased dopamine release and dysregulation of D2Rs in the mesolimbic and mesocortical dopamine motivation circuits.

As the behavioral tests mentioned earlier involved mostly effortful tasks (e.g., Morris water maze, forced swim, open field test), impaired performance may be attributed to decreased motivation instead of real anxious or depressive behaviors. Importantly, a lack of motivation may lead to decreased performance in the Morris water maze, which, as discussed, has been attributed to impaired spatial learning in NCS-1 knockout mice [58]. NCS-1 knockout mice demonstrated significantly decreased swim speed compared to WT controls, which suggests a motivational deficit [58]. However, since NCS-1 knockout mice also display impaired performance on the spontaneous novel object recognition test, which is not as effortful as the aforementioned tests [68], the decreased performance demonstrated by NCS-1 knockout mice in the Morris water maze may well be due to a real deficit in spatial memory, rather than reflective of a lack of motivation. Regardless, it may be of interest to examine the effect of NCS-1 knockout on less effortful memory tasks and evaluate physiological correlates of memory, such as those that have already been examined in spatial learning (CaMKII-α phosphorylation, BDNF) [59].

As demonstrated, NCS-1 has a broad range of molecular functions, and therefore an essential role in overall brain function. NCS-1 regulates growth cone calcium dynamics, neurite extension, and synaptogenesis, influencing formation of neural circuits. Furthermore, NCS-1 modulates synaptic plasticity, which may underlie the learning and memory behaviors for which NCS-1 is essential. NCS-1 is an extremely important protein for normal brain functioning, and its dysregulation would result in devastating consequences for all neuronal processes which it regulates.

Potential Implication of NCS-1 in Dopamine Dysregulation in Schizophrenia and Bipolar Disorder

Its regulation of dopaminergic signaling makes NCS-1 a protein of interest in the study of the mechanisms of neuropsychiatric disorders that involve dysregulation of dopamine receptors. The dopamine hypotheses of schizophrenia and bipolar disorder (BPD) have been reviewed extensively elsewhere [64, 69–75]. Briefly, as reviewed by Howes and Kapur [69], the current dopaminergic hypothesis of schizophrenia includes hallmarks such as increased dopamine accumulation and release from striatal dopaminergic neurons and elevated striatal D2R and dopamine D3-like receptor (D3R) density. Most antipsychotics block striatal D2Rs to normalize dopaminergic signaling. However, the authors note that though many genes involved with dopaminergic signaling and various environmental factors have been identified as risk factors for schizophrenia, the causes of dopamine dysfunction in schizophrenia are complex and poorly understood, particularly in terms of the interactions of identified risk factors. Furthermore, schizophrenia is a complex diagnosis, with different patients exhibiting different clinical features and courses [69].

The dopamine hypothesis of BPD encounters some of the same problems. Cousins et al.'s [75] 2009 review acknowledges that much of the current insight into the pathophysiology of BPD comes from the mechanisms of action of the drugs that treat it, and therefore only implicates dopamine by inference. As BPD is characterized by both manic and depressive states, a simple dopamine model of BPD postulates that mania is caused by excess dopamine activity while depression is caused by decreased dopamine activity. However, the authors caution that no consistent pattern of abnormality in dopamine signaling has been identified in BPD patients [75].

A recent review has identified multiple studies that support a hypothesis for abnormal phasic striatal dopamine signaling, resulting in dysfunctional reward processing, in both schizophrenia and bipolar disorder [74]. However, molecular mechanisms of this dysfunction are still poorly understood, and it is therefore of interest to continue to study proteins that can modify dopaminergic systems, as they pertain to neuropsychiatric diseases.

NCS-1 has been implicated in a variety of behaviors involving dopaminergic circuits, such as anxiety- and depressive-like behavior [61] and motivation behavior [67], as discussed previously. Furthermore, as discussed, NCS-1 interacts with D2Rs in learning and memory behavior, making it a protein of interest in diseases involving dopamine dysregulation and motivation systems. This interaction is supported by the colocalization of D2Rs with NCS-1 postsynaptically in primate prefrontal cortex [76]. NCS-1 was demonstrated by both co-immunoprecipitation and fluorescence anisotropy

experiments to bind D2Rs directly, with the D2R peptide likely binding the 60 first residues of two NCS-1 monomers cooperatively [77]. This interaction was confirmed by the crystal structure of the calcium/NCS-1/D2R complex, where NCS-1 bound two D2Rs in its hydrophobic ligand-binding site [10]. The effect of this interaction on the activity of D2Rs is unclear, though overexpression of NCS-1 has been shown to decrease D2R phosphorylation [9], and a recent study has implicated the NCS-1/D2R interaction as necessary to sensitization of presynaptic D2Rs (Fig. 5a) [78], which will be discussed in greater detail later in this review.

Along with its demonstrated role in binding D2Rs, NCS-1 has also been shown to modulate G protein–coupled receptor kinase 2 (GRK2)–mediated desensitization of activated D2Rs in the rat dopamine system (Fig. 5b) [9]. NCS-1 decreased the number of internalized D2/3Rs when GRK2 was overexpressed, and decreased D2R phosphorylation in the absence of GRK2 overexpression. Furthermore, while NCS-1 binding D2Rs was not calcium dependent, NCS-1 was shown to bind GRK2 calcium-dependently and demonstrated enhanced binding with elevated cAMP. This effect was demonstrated in HEK293 cells, while overlapping immunoreactivity for D2R, NCS-1, and GRK2 was seen in striatal cell bodies [9]. Another study has reported elevated levels of NCS-1 in the dorsolateral prefrontal cortex of schizophrenic and BPD patients [79]. Therefore, this effect of GRK2-mediated NCS-1 desensitization of D2Rs may occur in schizophrenia and BPD as NCS-1 is expressed in striatum, though further research is required to determine if NCS-1 levels may be abnormal in schizophrenic/BPD striatum and whether blocking D2R desensitization by NCS-1 may alleviate some symptoms of schizophrenia/BPD.

Finally, a common drug for the treatment of BPD, lithium [80], has been shown to disrupt the interaction between NCS-1 and $InsP_3R1$ in PC12 cells [81]. Though this interaction has been suggested to mediate the regulatory effect of NCS-1 on neurite extension [38], it is also a general mechanism of calcium release from intracellular stores (Fig. 5c). This suggests that the NCS-1/$InsP_3R1$ interaction may be related to the physiological cause of BPD and may point to a role of altered calcium homeostasis in the brain in BPD. Subsequent research should focus on the role of $InsP_3R1$ in BPD, its potential interaction with D2Rs as dopamine signaling is altered in the disorder, and whether NCS-1/$InsP_3R1$/D2Rs might form a complex involved in BPD. Also, the NCS-1/$InsP_3R1$ interaction should be considered as to whether it may be a source of some of the unwanted side effects of lithium, which may occur due to changes in cytosolic calcium concentration because of disruption of the $InsP_3R1$/NCS-1 interaction, modulation of calcium release from the endoplasmic reticulum, and dysregulation of calcium signaling within the cell.

Therefore, despite the complexities of the disorders, there are clear potential functional links between NCS-1, D2R regulation, and schizophrenia and BPD. Further elucidation of these links may lead to attractive therapeutic targets, particularly in disruption of NCS-1/D2R interactions, as well as regulation of NCS-1/$InsP_3R1$ interactions.

Role of NCS-1 in Selective Vulnerability of Substantia Nigral Dopaminergic Neurons in Parkinson's Disease

NCS-1 has also been implicated in D2R regulation in Parkinson's disease (PD). Though D2Rs are typically found postsynaptically in striatal neurons in the basal ganglia [82], substantia nigra (SN) dopaminergic neurons, which selectively degenerate in PD and have intrinsic pacemaker activity [83], also express somatodendritic dopamine D2 autoreceptors (D2ARs). Activation of D2ARs inhibits pacemaker activity by opening potassium channels, leading to hyperpolarization [84]. Normally, in response to increased dopamine release, adult SN D2ARs do not desensitize (i.e., they are sensitized) in response to a saturating dose of dopamine, and a saturating dose of dopamine will effectively inactivate the neuron. However, in mouse models of PD, SN D2ARs rapidly desensitize and are able to restore firing activity even while a saturating dose of dopamine is applied [85].

A recent study has identified a potential mechanism involving NCS-1 for this desensitization of D2ARs in PD [78]. In the study, juvenile SN dopaminergic neurons displayed a similar desensitization response to PD SN D2ARs. However, this desensitization could be reversed, and an adult-like phenotype could be induced by application of L-DOPA or cocaine for induction of a transient high dopamine state. Reversal of the desensitization phenotype in juvenile SN dopaminergic neurons required internal calcium provided by $Ca_v1.3$ L-type calcium channel activity and calcium-dependent interaction of NCS-1 with D2ARs (Fig. 5a). As desensitized D2ARs are seen in SN dopaminergic neurons in PD, changes in NCS-1-mediated regulation of desensitization of D2ARs may be a mechanism by which SN dopaminergic neurons are selectively vulnerable in PD. However, *Ncs-1* and *D2AR* mRNA transcripts were shown to be elevated in brain extracts from humans with PD in this study, suggesting that the interaction between NCS-1 and D2ARs is not as simple as decreased NCS-1 resulting in desensitized PD SN dopaminergic neurons. Further research is required to determine if targeting NCS-1, or $Ca_v1.3$ L-type calcium channels, in order to induce desensitization in conjunction with L-DOPA administration may have promising therapeutic value [78]. This mechanism may also be of interest to schizophrenia pathophysiology, as D2Rs have been

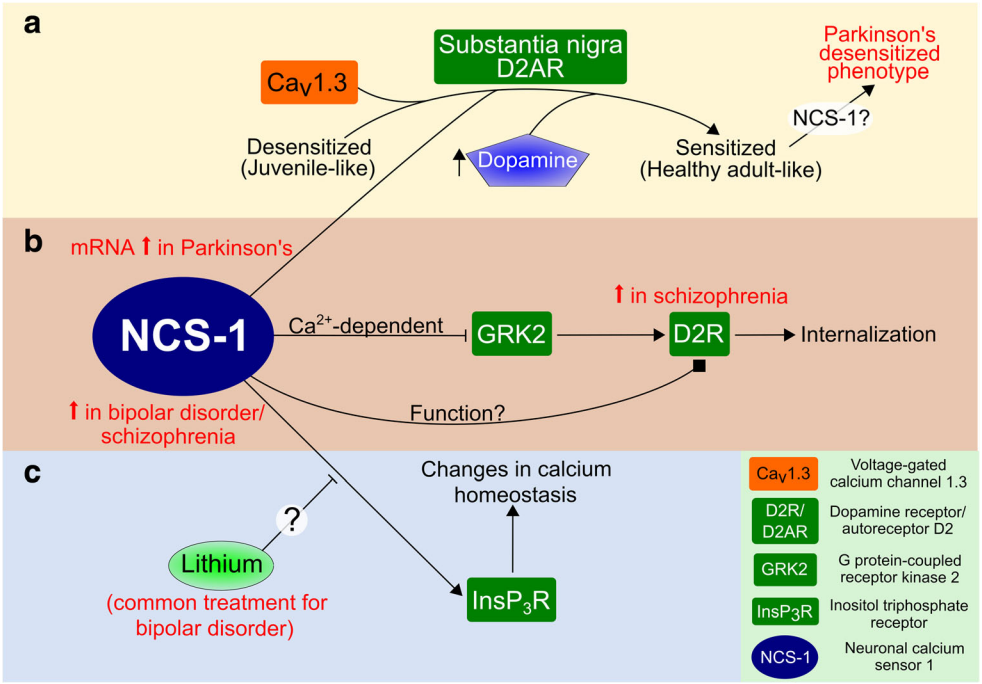

Fig. 5 NCS-1 involvement in disorders involving dopaminergic circuit dysfunction. (a) NCS-1 may act as a developmental switch to sensitize substantia nigra (SN) D2-like autoreceptors (D2ARs) in adulthood, L-type voltage-gated calcium channel ($Ca_v1.3$)-dependently. The mechanism responsible for switching the adult healthy SN D2AR phenotype from sensitized to desensitized in Parkinson's disease (PD) is not known, but may involve NCS-1, mRNA transcripts of which are upregulated in PD. (b) Upregulated NCS-1 in schizophrenia and bipolar disorder (BPD) may block G protein–coupled receptor kinase 2 (GRK2)–mediated dopamine D2-like receptor (D2R) internalization, resulting in increased number of D2Rs and heightened sensitivity to dopamine. (c) The interaction between NCS-1 and inositol triphosphate receptor ($InsP_3R$), leading to changes in calcium homeostasis, is disrupted by lithium, a common treatment for BPD, and therefore may implicate changes in calcium homeostasis as a mechanism for BPD

shown to be upregulated in the SN of schizophrenic patients [86], and reward prediction circuits involving the SN have behaviorally been shown to potentially be disrupted in schizophrenic patients [87]. Therefore, it may be important to examine the potential role of SN D2ARs and their interactions with NCS-1 as it relates to the pathophysiology and treatment of schizophrenia.

Disruption of Synaptogenesis in X-Linked Mental Retardation May Be Caused by NCS-1 Dysregulation by IL1RAPL1

Interleukin 1 receptor accessory protein-like 1 (IL1RAPL1) is an important interactor of NCS-1 [88]. IL1RAPL1 is thought to regulate excitatory synaptogenesis. It induces formation of the presynaptic terminal by transsynaptically binding immunoglobulin (Ig)-like domains in protein tyrosine phosphatase δ (PTPδ) [89, 90] while inducing formation of the postsynaptic terminal by interacting with both Rho GTPase activating protein 2 (RhoGAP2) [91] and MCF.2 cell line–derived transforming sequence-like (Mcf2l), activating RhoA-ROCK signaling via its intracellular C-terminal domain [92]. Furthermore, IL1RAPL1 contains a PDZ-binding domain in its C-terminus, and seems to contribute to synaptogenesis by activating c-Jun terminal kinase (JNK) [93], phosphorylating postsynaptic density protein 95 (PSD-95), and clustering it in the postsynaptic density (PSD) [94].

Knocking out IL1RAPL1 has been shown to decrease spine and synapse density [95] and impair theta-burst stimulation LTP (TBS-LTP) in the hippocampus [94], and dysregulation of the normal excitatory-inhibitory balance during cerebellar development [96]. Knockout also has behavioral effects, where IL1RAPL1-KO mice display impairments in various types of memory [95, 97]. Meanwhile, overexpression of IL1RAPL1 in zebrafish olfactory sensory neurons stimulated synaptic vesicle accumulation in putative presynaptic terminals and stimulated presynaptic differentiation [98] and increased the number of excitatory synapses N-terminal-dependently in a neuron-fibroblast co-culture [94]. Recently, IL1RAPL1 has also demonstrated a role in circuit formation, as knockout increases dendritic branching in CA1 and CA2 hippocampal neurons, while overexpression decreases branching [97]. This effect of IL1RAPL1 on dendritic arborization occurs before synaptogenesis, but also relies on PTPδ signaling [97].

Evidently, IL1RAPL1 is a critical protein for synaptogenesis and dendritic branching in early neuronal development, and this critical role is emphasized by the consequences of IL1RAPL1 mutations. Human genetics approaches have identified various IL1RAPL1 mutations as associated with non-specific X-linked mental retardation (XMR) [99–104]. XMR is a complex disorder characterized by sub-average general intellectual functioning and decreased cognitive abilities, and is extremely genetically heterogeneous [105]. Many IL1RAP1 mutations associated with XMR cause dysfunction or deletion of the extracellular N-terminal Ig-like domain of IL1RAPL1. Still other mutations associated with XMR lack the C-terminal domain of IL1RAPL1 [99]. Both types of mutations suggest a role for dysregulation of signaling associated with synaptogenesis in the pathophysiology of the disease.

IL1RAPL1 interacts with NCS-1, with the intracellular C-terminal domains of both proteins participating in the interaction [88]. NCS-1 and IL1RAPL1 appear to colocalize at the plasma membrane and have opposing effects on growth hormone (GH) release from PC12 cells in response to adenosine triphosphate (ATP) application [88]. While NCS-1 overexpression increases GH exocytosis, IL1RAPL1 overexpression inhibits release [88] upstream of NCS-1, seemingly by inhibiting NCS-1 activation of N-type voltage-gated calcium currents [106]. Interestingly, IL1RAPL1-overexpressing PC12 cells exposed to NGF, which normally will extend neurites, showed significantly decreased neurite lengths compared to non-transfected cells [106]. This effect is NCS-1 dependent, as knockdown of NCS-1 in IL1RAPL1-transfected PC12 cells restored normal neurite elongation [106]. These results suggest a functional link between disrupted synaptogenesis in XMR associated with IL1RAPL1 C-terminal mutations (Fig. 6a) and the demonstrated role of NCS-1 in regulating both neurite extension and voltage-gated calcium channels. However, as IL1RAPL1 has a variety of targets with a demonstrated role in regulating synaptogenesis, future research should examine if NCS-1 is dysregulated in XMR, with an emphasis on searching for NCS-1 mutations associated with XMR. NCS-1 may therefore be determined as a potential target for gene therapy approaches in treating XMR.

Involvement of NCS-1 in Fragile X

Another neurodevelopmental disorder in which NCS-1 may be involved is fragile X syndrome (FXS). Ric8a is a guanine exchange factor which binds *Drosophila* frequenin-2 (Frq2), as well as human NCS-1, preferentially in the absence of calcium [107]. Interestingly, in situ hybridization of these proteins showed them both to be localized to synaptic active zones, and both Ric8a knockdown and Frq2 overexpression resulted in a decrease in the number of presynaptic active zones. This suggests an opposing regulatory role of Frq2 and Ric8a on synaptogenesis. Frq2 was determined to be upstream of Ric8a in this mechanism, as Ric8a overexpression did not yield an effect when Frq2 was overexpressed. Manipulating levels of Ric8a and Frq2 also yielded similar and independent effects on NT release probability [107]. This study suggests Frq2 as a regulator of both synapse number and NT release probability, with both Ric8a-dependent and Ric8a-independent mechanisms. FXS is one of the most common forms of heritable mental retardation [108], and a *Drosophila* model of the syndrome has been shown to be partially rescued by administration of FD44, a blocker of NCS-1/Ric8a interaction [109]. The fragile X flies treated

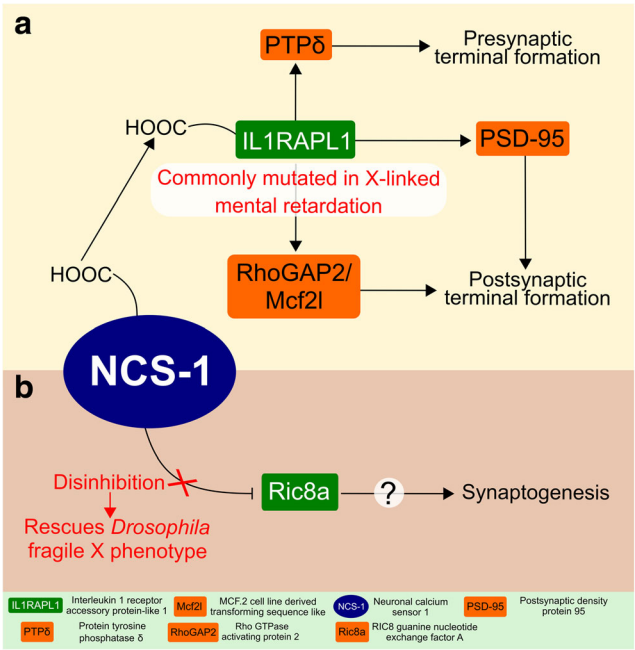

Fig. 6 NCS-1 regulation of synaptogenesis may play a role in neurodevelopmental disorders. (a) NCS-1 interacts via its C-terminal domain with the C-terminus of interleukin 1 receptor accessory protein–like 1 (IL1RAPL1), which is commonly mutated in X-linked mental retardation (XMR). The normal function of IL1RAPL1 is to induce formation of the presynaptic terminal via interaction with protein tyrosine phosphatase δ (PTPδ) and formation of the presynaptic terminal via activation of Rho GTPase activating protein 2 (RhoGAP2) and MCF.2 cell line–derived transforming sequence-like (Mcf2l) and promotion of postsynaptic density protein 95 (PSD-95) clustering. Mutation of the C-terminus of IL1RAPL1 may disrupt normal regulation by NCS-1 and lead to disrupted synaptogenesis in XMR. (b) A recent study has shown that disruption of the interaction of NCS-1 with Ric8a, which is a negative regulator of synaptogenesis, rescues a *Drosophila* fragile X phenotype, implying a role of increased synaptogenesis in the pathology of fragile X. Tight regulation of NCS-1 therefore may be required for the right amount of synaptogenesis to occur in the developing brain, and disruption of synaptogenesis, either by reduction or by increase, may result in neurodevelopmental disorders

with FD44 demonstrated improved associative learning and normal synapse numbers [109]. This suggests that the NCS-1/Ric8a interaction may be an important target for fragile X treatment, and that dysregulation of synapse number and probability of release may underlie the pathophysiology of the disease (Fig. 6b). The next steps may include testing FD44's effects in mammalian models of fragile X syndrome and determining the upstream regulators and downstream targets of the interaction.

NCS-1 May Be a Therapeutic Target for Treatment of Axonal Injury

Under normal circumstances, neurons in the mammalian CNS are unable to recover from axonal injury, leading to neuronal degeneration. Axonal injury is a widely studied field and the mechanisms of CNS regeneration failure have been reviewed extensively [110–114], and summarizing them is beyond the scope of this review. However, it is widely accepted that CNS axons, unlike PNS axons, are unable to regenerate following injury or transection due to prohibitive factors in the extracellular milieu, such as regeneration-inhibitory molecules secreted by glial cells and myelin, physical barriers to regeneration in the form of the glial scar, and proinflammatory factors which promote Wallerian degeneration and apoptosis. These factors make recovery from spinal cord injury (SCI) very difficult and therapeutic options are limited [114]. Therefore, it is of great importance to identify mechanisms by which regeneration can be induced in CNS axons, in hopes of developing novel therapies for SCI and other CNS axonal lesions.

Recent research has identified NCS-1 as an exciting new candidate in treating SCI. Following unilateral vagal axotomy, NCS-1 expression has been shown to be significantly increased in neurons of the dorsal motor nucleus of the vagus nerve (DMV) [115]. Administration of colchicine to disrupt tubulin polymerization and axonal transport increased NCS-1 expression as well, suggesting that NCS-1 is upregulated in response to disruption of microtubules. NCS-1 overexpression rendered hydrogen peroxide–treated PC12 cells more resistant to cellular damage and rescued cells from death in B27 withdrawal. Calcium binding by NCS-1 is required for this protective effect, as expressing NCS-1_{E120Q} eliminated any protective effect of the protein. Expressing the NCS-1_{E120Q} mutant resulted in significant neuronal death on the injured side following unilateral vagal axotomy [115]. Though the vagus nerve is a part of the peripheral nervous system, which generally demonstrates more regenerative capacity, more recently, NCS-1 overexpression in primary cultured cortical neurons was shown to cause sprouting of neurites [116]. This effect was accompanied by an increase in phospho-Akt in NCS-1-overexpressing neurons. Furthermore, NCS-1 transfection improved recovery in rats following pyramidotomy, as measured by mobility tests, and was associated with an increase in the number of collaterals sprouting across the midline into the denervated corticospinal tract, compared to controls. Also, corticospinal neurons overexpressing NCS-1 did not exhibit typical cell soma shrinkage following the lesion [116]. Taken together, these results suggest that NCS-1 upregulation may contribute to recovery from axonal injury and promote survival of transected neurons and may be an attractive therapeutic target for treatment of SCI and other common axotomies (Fig. 7).

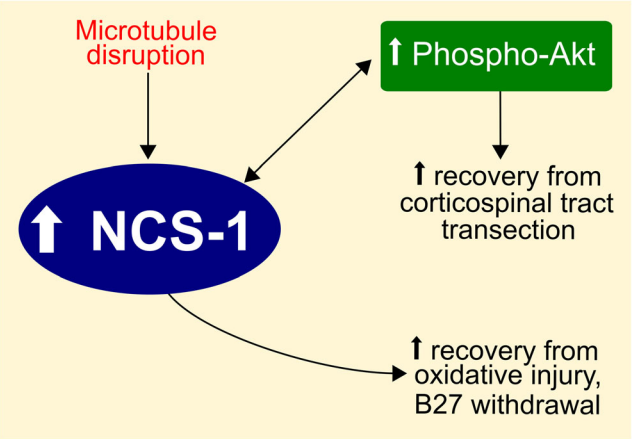

Fig. 7 NCS-1 is upregulated following microtubule disruption and promotes functional recovery from corticospinal tract transection, recovery from oxidative injury, and recovery from B27 withdrawal [115]. Recovery from corticospinal tract transection occurs in tandem with a phosphorylated-Akt (phospho-Akt) increase, though it is not known if phospho-Akt upregulates NCS-1 or vice versa [116]

Conclusion

The ubiquitous expression of NCS-1 in neuronal tissues and variety of protein targets implicates it as an important protein in a variety of neuronal processes. However, there are almost certainly many more signaling pathways which include NCS-1 as an effector protein. Future directions of study may include potential interactions of NCS-1 with the presynaptic exocytotic machinery (including SNAP-25) and elucidating mechanisms of calcium channel–independent NCS-1 enhancement of PPF. Many questions also remain concerning the seemingly independent regulation of microtubules from growth cone calcium channels by NCS-1 in the process of neurite extension. More in vivo studies are required to determine effects of NCS-1 knockdown at different developmental timepoints, and its effects on circuit refinement. In the context of synaptic plasticity, it may be of interest to examine whether NCS-1 is implicated in mGluR-LTD in other brain areas that demonstrate it, such as the ventral tegmental area [117] and the hippocampus [118]. A final suggested future direction of basic study is to examine the

involvement of the NCS-1/D2R complex as it pertains to spatial learning, and to attempt to control for deficits in motivation or depressive behaviors by testing spatial and other types of learning using less effortful behavioral tests.

Future research may wish to examine whether the NCS-1/$InsP_3R1$ interaction in BPD is related to GRK2-mediated D2R internalization, and to further elucidate these mechanisms. Also, the mechanisms of D2 autoreceptor (D2AR) regulation by NCS-1 in substantia nigra dopaminergic neurons in schizophrenia should be investigated. Rescue of fragile X syndrome with disruption of the NCS-1/Ric8a interaction may be a valuable area of study but requires further testing in mammalian fragile X models. Finally, selective upregulation of NCS-1 may be a promising therapy for spinal cord injury or CNS axotomy, but the interaction of NCS-1 with inflammatory pathways and causes of its upregulation must be further studied.

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