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Store-Operated Calcium Entry Regulates Transcription Factor Sp4 in Resting Neurons

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Abstract

Calcium (Ca^{2+}) signaling activated in response to membrane depolarization regulates neuronal maturation, connectivity and plasticity, but the role of store-operated Ca^{2+} entry (SOCE) in neurons is poorly understood. Here, we report that SOCE is regulated by membrane potential in cerebellar granule neurons (CGNs) in the opposite direction of voltage-gated Ca^{2+} channels; maximal activation of SOCE occurs in resting conditions where this Ca^{2+} influx promotes the degradation of transcription factor Sp4, a regulator of neuronal morphogenesis and function. We report that lowering $[\text{K}^+]_{\text{EXT}}$ caused depletion of intracellular Ca^{2+} stores and induced a Ca^{2+} influx with characteristics of SOCE. Under these conditions, we also observed the relocalization of the ER Ca^{2+} sensor STIM1. We found that compounds that block SOCE prevented the ubiquitination and degradation of Sp4 in low extracellular potassium ($[\text{K}^+]_{\text{EXT}}$). Further, we show that STIM1 knockdown blocked degradation of Sp4 while expression of a constitutively active STIM1 decreased Sp4 protein abundance under depolarization conditions. Our findings provide evidence for the dynamic regulation and downstream signaling effect of SOCE in neurons and suggest a new pathway for Ca^{2+} signaling to shape neuronal gene expression.

Introduction

Ca^{2+} signaling cascades induced by membrane depolarization regulate gene expression programs essential for the development, function, plasticity and survival of neurons (1, 2). Discrete Ca^{2+} influx resulting from glutamatergic receptors and the activation of voltage-dependent Ca^{2+} channels (VDCCs) influences transcription by controlling signaling pathways that guide the activity of transcription factors such as cAMP Response Element Binding protein (CREB) and Myocyte Enhancer Factor-2 (MEF2). In addition to these Ca^{2+} signals, neurons also express store-operated Ca^{2+} channels (SOCCs); although whether and how the activity of these channels contributes to the Ca^{2+} -dependent regulation of transcription factors in neurons is not known.

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The depletion of Ca^{2+} stored in the ER promotes the initiation of SOCE (also known as capacitative Ca^{2+} entry [CCE]). In non-excitabile cells, SOCE not only mediates refilling of Ca^{2+} stores but also supports Ca^{2+} signaling pathways important for the regulation of cellular processes such as exocytosis, enzyme function, cell proliferation, and gene expression (3–5). Studies have revealed that STIM1 and Orai channels (also termed CRAC modulators [CRACM]) are responsible for SOCE. STIM1 monitors Ca^{2+} concentrations in the ER via an EF-hand domain in its lumenally localized N-terminus. Association with Ca^{2+} prevents STIM1 oligomerization, whereas, when the ER Ca^{2+} pool is depleted, STIM1 forms multimers that ultimately translocate to ER-plasma membrane (PM) junctions where they recruit Orai channels to initiate SOCE (5). STIM and Orai homologs are expressed in many regions of the brain including the cerebellum, cortex, and hippocampus (6–8). Although it has been reported that SOCE can be triggered in neurons using store depletion agents like thapsigargin or cyclopiazonic acid (9–14), the regulation and signaling effects of this Ca^{2+} signal in neurons are poorly described.

Transcription factor Sp4 is predominantly found in neurons (15). Sp4 binds to GC-rich DNA sequences, which are recognized as important *cis*-acting elements for the appropriate expression of tissue-specific and housekeeping genes (16–18). Interestingly, Sp4 either activates or represses gene transcription in a context and promoter-specific manner (19, 20). The importance of this transcription factor for the maturation and function of the central nervous system is exemplified by, first, the observations that reduced expression of Sp4 interferes with normal dendrite patterning in cerebellar and hippocampal granule neurons (21, 22), and second, that *Sp4* hypomorphic mice exhibit memory and synaptic impairments (23, 24). Sp4 protein stability is regulated in response to changes in membrane potential such that under *resting* conditions Sp4 is rapidly degraded by the ubiquitin-proteasomal system (UPS) (25). In this study, we identify STIM1 and SOCCs as mediators of a Ca^{2+} influx that regulates Sp4 protein polyubiquitination and proteasomal degradation. These findings support the concept that neurons integrate input from both depolarization-induced Ca^{2+} signals and the graded activation of store-operated Ca^{2+} entry to regulate transcription factor activity.

Results

Dynamic regulation of Sp4 protein stability by membrane potential independently of glutamatergic neurotransmission, neuronal activity, and VDCCs

Altering PM potential by changing the $[\text{K}^+]_{\text{EXT}}$ is a well established method to investigate molecular signaling and regulated transcription factor activity in neurons (26–29). Recently, we have reported that decreasing the PM potential by lowering $[\text{K}^+]_{\text{EXT}}$ led to the rapid degradation of Sp4 by the UPS in CGNs (25). We confirmed this result (Fig. 1 A and B) and extended it by showing that raising $[\text{K}^+]_{\text{EXT}}$ from 25 to 65 mM KCl for 60 min led to a significant increase in Sp4 protein (Fig. 1C and D) in the absence of changes in *Sp4* mRNA expression (Fig. 1E). We therefore initiated studies to identify the membrane channels that regulate Sp4 stability in response to dynamic changes in PM potential.

We initially investigated whether membrane channels associated with neuronal activity signaled to promote Sp4 protein stability under depolarizing conditions. Consistent with a

recent report where we described signaling pathways controlling phosphorylation of Sp4 at S770 (30), inhibition of *N*-methyl-D-aspartate receptors (NMDARs) with MK-801 did not affect Sp4 protein stability (Fig. 2A and B). More unexpectedly, we also found that blocking voltage-gated Na⁺ channels with tetrodotoxin (Fig. 2C and D) did not promote the degradation of Sp4. Finally, although we previously reported that a 12 hr treatment with nimodipine reduced Sp4 protein (25), pharmacological inhibition of L-type VDCCs for a shorter time (2 hr) was not sufficient to decrease Sp4 abundance (Fig. 2E and F). The finding that inhibition of NMDARs, voltage-gated Na⁺ channels, or L-type VDCCs did not mimic the rapid effect of lowering [K⁺]_{EXT} suggested that a pathway distinct from well-characterized depolarization-induced Ca²⁺-dependent molecular cascades is involved in the control of Sp4 protein stability.

The Ca²⁺ channel inhibitor SKF96365 prevents Sp4 degradation by the UPS

We investigated a role for transient receptor potential canonical channels (TRPCs) or SOCCs using SKF96365 (SKF), a non-specific antagonist of TRPCs, SOCCs, and VDCCs. Based on prevailing models of Ca²⁺-dependent regulation of transcription factors, we predicted that addition of SKF under depolarizing conditions (25mM KCl) would be similar to the effect of lowering [K⁺]_{EXT}, leading to reduced Sp4 protein. Contrary to our expectations, however, we observed that addition of SKF not only increased Sp4 protein quantity in depolarizing conditions but also completely blocked Sp4 degradation in CGNs at rest (Fig. 3A and B). A similar result was obtained with 2-aminoethoxydiphenyl borate (2-APB) (Fig. 3C and D); a chemical unrelated to SKF that has been reported to antagonize some TRPCs as well as SOCCs at the concentration used (31, 32). SKF did not increase *Sp4* mRNA expression (Fig. 3E) but was very effective at preventing the polyubiquitination of Sp4 in hyperpolarized CGNs (Fig. 3F).

SKF has been shown to decrease phosphorylation of CREB at Ser133 (P-CREB) in CGNs (33). As a control, we confirmed that the addition of SKF decreased CREB phosphorylation. Interestingly, we found that SKF reduced phosphorylation of CREB only in the 25mM KCl condition (fig. S1A and B), while the residual P-CREB signal in the 5mM KCl condition was insensitive to SKF (P-CREB in the 5mM KCl condition was, however, eliminated by blocking NMDARs with MK-801; Fig. 2A as well as fig. S1C and D). Thus, while SKF blocks pathways that signal to both CREB and Sp4, an SKF-sensitive pathway in the 5mM KCl condition regulates only Sp4. We interpret this finding as an indication that Sp4 polyubiquitination is mediated by the *activation* of an SKF-sensitive pathway that is most active when CGNs are at rest and is distinct from the SKF-sensitive pathway supporting CREB Ser133 phosphorylation under depolarization conditions.

Proteasomal degradation of Sp4 in resting neurons requires extracellular Ca²⁺

Since SKF antagonizes PM channels that are permissive to Ca²⁺, we next examined whether Sp4 regulation by the UPS in low [K⁺]_{EXT} is in fact Ca²⁺-dependent. We found that chelating Ca²⁺ in the fresh culture medium using EGTA before adding it to the CGNs was sufficient to prevent Sp4 degradation in the 5mM KCl condition (Fig. 4A and B). Importantly, addition of EGTA to the fresh culture medium supplemented with 25mM KCl completely blocked depolarization-induced expression of c-Fos but had no effect on Sp4

protein (Fig. 4A). These results provide strong support for the idea that Sp4 protein degradation is promoted by a Ca^{2+} influx that operates in resting neurons and is distinct from depolarization-induced Ca^{2+} -dependent molecular cascades.

Lowering $[\text{K}^+]_{\text{EXT}}$ depletes caffeine-sensitive Ca^{2+} stores in CGNs

Although SKF and 2-APB antagonize both TRPCs and SOCCs, we favored a role for SOCCs in the hyperpolarization-induced regulation of Sp4 for two reasons: 1) application of 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), an agonist of several TRPCs, did not promote Sp4 degradation (fig. S2), and 2) most TRPCs are nonselective $\text{Na}^+/\text{Ca}^{2+}$ channels that cause membrane depolarization (34). Since SOCE is initiated by the depletion of Ca^{2+} stored in the ER, we reasoned that if SOCE regulates Sp4 protein stability then the quantity of Ca^{2+} in intracellular pools should rapidly decrease when CGNs are switched from depolarizing to resting culture conditions. To test this hypothesis, we used Ca^{2+} imaging and exploited the well-documented effect of caffeine to induce Ca^{2+} release from intracellular pools through the potentiation of ryanodine receptors (35, 36).

As a baseline, we applied caffeine to CGNs in 65 mM KCl and observed a sharp, pronounced Ca^{2+} transient (Fig. 5A). Treatment of CGNs in 25 mM KCl with caffeine also produced a Ca^{2+} transient (Fig. 5B) but the average amplitude of the Ca^{2+} release in the 25 mM condition was significantly smaller than the one observed in the 65 mM KCl imaging solution (Fig. 5C). Together, these results confirmed the presence of caffeine-sensitive Ca^{2+} stores in depolarized CGNs. In order to extend the observation that the amount of Ca^{2+} within the intracellular stores varied in response to $[\text{K}^+]_{\text{EXT}}$, we then repeated this experiment with CGNs placed in 5 mM KCl. In this case, we probed the effect of caffeine at two time-points, 7 and 60 min after changing to the 5 mM KCl imaging solution. Using this experimental strategy, we detected a caffeine-induced Ca^{2+} transient shortly after switching to the 5 mM KCl solution (Fig. 5D) but the effect of caffeine was lost when the cells were incubated in low KCl for 60 min before the addition of caffeine (Fig. 5E). This result demonstrates that caffeine-sensitive pools gradually deplete when switching CGNs from depolarized to resting culture conditions. Remarkably, we also found that if the resting CGNs that were unresponsive to caffeine were briefly (5 min) depolarized with 65 mM KCl solution before switching back to the 5 mM KCl solution, this was sufficient to make the cells responsive to caffeine again, indicating that the intracellular Ca^{2+} stores could be rapidly replenished (Fig. 5E). Together, these experiments corroborate similar observations of caffeine-induced Ca^{2+} release in dissociated neuronal cultures by other groups (37–39) and establish a direct correlation between Ca^{2+} stores and the degree of membrane depolarization such that in low $[\text{K}^+]_{\text{EXT}}$ intracellular Ca^{2+} pools are depleted.

Sustained SOCE in resting CGNs

Next, we investigated whether the rapid and persistent depletion of Ca^{2+} stores in resting CGNs was accompanied by the activation of a Ca^{2+} influx consistent with SOCE. To test this hypothesis, we removed extracellular Ca^{2+} during the pre-imaging period and added a cocktail of inhibitors to block any confounding Ca^{2+} influx from ionotropic glutamate receptors (NMDA and AMPA) and/or L-type VDCCs. We included this control because our previous analysis of CREB phosphorylation in resting CGNs showed persisting activity of

NMDARs under this condition (fig. S1C and D). When CGNs were treated in this way and imaged in conditions that correspond to resting PM potential (5_M KCl), we observed and measured a sustained Ca²⁺ influx shortly after adding back extracellular Ca²⁺ to the imaging solution (Fig. 6 and Movie S1). Most importantly, addition of SKF was sufficient to prevent this Ca²⁺ influx (Fig. 6 and Movie S2). Although the averaged increase in Fluo-4 signal intensity for this SKF-sensitive Ca²⁺ influx was small (F/F_0 = about +0.3), this result is consistent with previous measures of SOCE in other cell types as these channels are known to have very low but highly selective conductance for Ca²⁺ (40). Finally, taking these data together with our findings that SKF or the chelation of extracellular Ca²⁺ with EGTA was sufficient to prevent the hyperpolarization-induced Sp4 degradation, we propose that this Ca²⁺ influx is a key component in the cascade of events that leads to the regulation of Sp4 by the UPS.

Changes in [K⁺]_{EXT} rapidly modify STIM1 distribution in dissociated CGNs

Depletion of Ca²⁺ from the ER in non-excitable cells promotes STIM1 oligomerization—a required step for the association of STIM1 with SOCCs at ER-PM junctions and the initiation of SOCE (5). We therefore examined whether modifying the [K⁺]_{EXT} was sufficient to change the distribution of STIM1 in CGNs. To test this, we expressed full-length YFP-tagged STIM1 and monitored the YFP fluorescent signal using live-cell imaging. Because highly overexpressed STIM1 can promote the non-physiological and constitutive oligomerization of STIM1 (41), we took care to use the lowest detectable level of YFP-STIM1 for these experiments. As predicted, transfected CGNs displayed diffuse YFP-STIM1 fluorescence when the cells were depolarized with 65_M KCl solution (Fig. 7A, left panel). However, when the KCl concentration was lowered from 65 to 5_M, we observed a striking redistribution of YFP-STIM1 fluorescence into puncta around the cell body as well as along the axon and fine processes within 30 min (Fig. 7A, right panel). Quantification of the number of YFP-STIM1 puncta along neurites and around cell bodies of live CGNs revealed a significant difference between resting membrane potential (5_M KCl) and the conditions of moderate (25_M) and strong (65_M) depolarization (Fig. 7B). These findings suggest the formation of STIM1 multimers and their relocalization to ER-PM junctions in resting conditions (5, 41). Notably, within 5 min of returning the cells from 5_M to 65_M KCl solution, the STIM1 localization pattern was restored to being diffuse (fig. S3). This rapid relocalization of STIM1 is consistent with our finding that caffeine-sensitive Ca²⁺ stores are refilled within the same timeframe (Fig. 5E). Finally, we observed that CGNs express endogenous STIM1 while the related STIM2 was almost undetectable (fig. S4A) and, in contrast to YFP-STIM1, YFP-STIM2 did not form puncta when [K⁺]_{EXT} was lowered from 65 to 5_M (fig. S4B and C).

In order to confirm that the relocalization of STIM1 in response to changes in [K⁺]_{EXT} was mediated by changes in luminal Ca²⁺, we repeated this experiment with mutant YFP-STIM1^{D76A}. Amino acid substitution D76A prevents Ca²⁺ binding to the luminal EF-hand domain of STIM1, which leads to the constitutive activation of SOCE (42). In contrast to wild-type STIM1, YFP-STIM1^{D76A} was localized in puncta regardless of the [K⁺]_{EXT} (Fig. 7C). This result suggests that association of Ca²⁺ with the luminal EF-hand domain of STIM1 limits the oligomerization process in CGNs. Finally, because activation of SOCE

ultimately involves the recruitment of Orai channels by STIM1 multimers (5), we also examined whether the distribution of overexpressed Orai channels would parallel that of YFP-STIM1 under resting conditions. As expected, we found that either His-tagged Orai1 (Fig. 7D–F) or His-tagged Orai2 (fig. S5) formed clusters that were closely juxtaposed to YFP-STIM1 puncta. This close association of YFP-STIM1 multimers and His-tagged-Orai channels in CGNs is comparable to what has been observed in non-excitable cells (43, 44).

STIM1 expression and function affects Sp4 protein

Since the SOCC inhibitor SKF blocked ubiquitination and degradation of Sp4 under culture conditions that correlated with depletion of Ca^{2+} stores and formation of STIM1 puncta, we further investigated a direct connection between STIM1 and the regulation of Sp4 protein. Using short hairpin RNA (shRNA), we knocked down endogenous STIM1 and assessed whether depletion of STIM1 in CGNs prevented the degradation of co-transfected FLAG-Sp4 in low $[\text{K}^+]_{\text{EXT}}$ conditions. Efficacy and specificity of the STIM1 shRNA was validated in Neuro2A cells (Fig. 8A). CGNs were co-transfected with plasmids encoding GFP, FLAG-Sp4 and either the STIM1 shRNA or a control shRNA. We then quantified FLAG-Sp4 immunofluorescence in transfected CGNs treated for 60 min with fresh culture medium supplemented with 65 or 5mM KCl. Cells transfected with the control shRNA displayed significantly less FLAG immunofluorescence in the 5mM KCl condition than the 65mM KCl condition, indicating that, similar to endogenous Sp4, the abundance of FLAG-tagged Sp4 was regulated by $[\text{K}^+]_{\text{EXT}}$. CGNs transfected with the STIM1 shRNA had significantly more FLAG immunofluorescence than control cells in 5mM KCl (Fig. 8B). These loss-of-function results provide evidence that STIM1 is required for the regulation of Sp4 by the PM potential. To complement this knockdown experiment, we co-expressed FLAG-tagged Sp4 with either WT YFP-STIM1 or the constitutively active STIM1^{D76A} mutant. We observed that, despite being exposed to a strong depolarizing condition (65mM KCl), neurons transfected with STIM1^{D76A} were less likely to display immunopositive nuclei with FLAG-Sp4 (Fig. 8C–E). Further, those neurons that did stain in depolarized CGNs mimics the effect of low $[\text{K}^+]_{\text{EXT}}$ to reduce Sp4 protein expression by engaging SOCE, leading to a Ca^{2+} influx that activates the downstream signaling pathway responsible for the regulation of Sp4 by the UPS.

Discussion

The data presented here reveal a surprising connection between low $[\text{K}^+]_{\text{EXT}}$ condition that favors resting PM potential, the depletion of intracellular Ca^{2+} stores, and the induction of STIM1-mediated SOCE in dissociated CGNs (fig. S7). We further show that this Ca^{2+} influx regulates transcription factor Sp4 by promoting its polyubiquitination and proteasomal degradation in a manner that is distinct and separate from well known depolarization-induced Ca^{2+} pathways. Together, our findings reveal exciting new details about how Ca^{2+} signaling operates in neurons and add an unsuspected dimension to our understanding of Ca^{2+} -mediated transcription in these cells.

Neuronal SOCE

SOCE is the major pathway by which extracellular Ca^{2+} enters non-excitable cells (3). Excitable cells like neurons coexpress a wide range of Ca^{2+} -permeable channels, including ionotropic glutamatergic receptors and voltage-gated channels, and the contribution of store-operated Ca^{2+} influx to the net pattern of ionic conductance is not well described for these cells. Although a function for this specific mode of Ca^{2+} influx in the nervous system has been an intriguing question (45), and the key components of SOCE are expressed in the brain (6–8), efforts to confirm operation of neuronal SOCE remain limited (35). Studies that have reported evidence suggesting that SOCE can occur in the nervous system have generally relied on the use of pharmacological agents like thapsigargin to deplete internal Ca^{2+} stores (9–14). In contrast, here, we observed depletion of caffeine-sensitive Ca^{2+} stores, induction of an SKF-sensitive Ca^{2+} influx comparable in intensity to SOCE, and rapid and reversible formation of STIM1 puncta in CGNs by simply decreasing $[\text{K}^+]_{\text{EXT}}$ to levels that bring the PM potential to rest. We believe these data support the novel concept that activation of this Ca^{2+} signal in dissociated neuronal cultures may be inversely related to the degree of membrane depolarization. Consistent with this scenario, Usachev and Thayer have reported that a Ca^{2+} influx induced by store depletion and insensitive to antagonists of VDCCs was facilitated by hyperpolarizing the membrane of rat dorsal root ganglion cells (46). In addition, hyperpolarization-induced STIM/Orai-mediated SOCE has been described in human myoblasts, where it is required for the Ca^{2+} -dependent differentiation of these excitable cells into multinucleated myotubes (47, 48). Finally, there is evidence that the ER in primary neuronal cultures and even organotypic slice cultures are depleted or only partially filled under resting conditions (35, 49), a characteristic that we confirmed for dissociated CGNs in this study. Taken together, these reports support the view that the hyperpolarization-induced SOCE that we describe here is not a phenomenon specific to dissociated CGNs but rather a fundamental characteristic that is shared by many groups of excitable cells, including possibly most types of neurons.

STIM1, STIM2 and neuronal SOCE

Studies in non-excitable cells support both overlapping and unique functions for the STIM1 and STIM2 homologs. Previous studies in neurons have suggested a primary role for STIM2 (71), and, in apparent contrast, a primary role for STIM1 to control SOCE in cortical neurons (72). Multiple factors may explain the discrepancies between these studies, including major differences in experimental approach as well as the possible impact of non-SOCE Ca^{2+} signals. Further, our study indicates that changes in membrane depolarization can rapidly alter the concentration of intracellular Ca^{2+} pools and influence the activity of SOCE in dissociated neurons; thus, the level of PM depolarization may need to be taken into account in studies of neuronal SOCE. Interestingly, in our study we observed that the balance in expression between endogenous STIM1 and STIM2 proteins in primary CGNs appeared to favor STIM1 (fig. S4A) while both STIMs may be more equally expressed in Neuro2A cells (Fig. 8A). Furthermore, we failed to see the formation of YFP-STIM2 puncta under conditions that consistently resulted in clear, distinct YFP-STIM1 puncta in CGNs (fig. S4B and C). These data suggest that the relative expression and function of STIM1 and STIM2 may vary by neuronal cell type and, in cerebellar granular neurons, STIM1, and not STIM2, contributes to SOCE in response to changes in membrane potential. Above all, our

results highlight the complexity of Ca^{2+} dynamics in neuronal cells and show the advantage that identifying downstream molecular targets of SOCE in neurons, like Sp4, may provide in future investigation of this signal as a complimentary measure to Ca^{2+} imaging.

SOCE-dependent regulation of transcription factor activity

Our study reveals for the first time that Sp4 is a downstream effector of SOCE. This represents an important new connection between the activity of SOCE and the regulation of transcription. STIM1-mediated SOCE in non-excitabile cells is known to influence signaling pathways that control transcription factor activity. In T lymphocytes, for instance, SOCE promotes the expression of Nuclear Factor of Activated T cells (NFAT) target genes via the Ca^{2+} -dependent phosphatase calcineurin, which acts to increase nuclear accumulation of this transcription factor (50). More recently, muscarinic receptor activity in SH-SY5H cells has been shown to induce SOCE and then enhance transcription factor NF- κ B function (51). Finally, interaction of STIM1 with SOCCs has also been found to promote cAMP accumulation and PKA activation (52), raising the possibility that the formation of this molecular complex may also influence the activity of PKA-regulated TFs, such as CREB, CREM, and ICER (53). Together, these studies offer candidate signaling pathways that could participate in the SOCE-dependent regulation of Sp4 and open up the possibility that other transcription factors, in addition to Sp4, are regulated by SOCE in resting neurons.

Ca^{2+} -mediated transcription in neurons

A major finding of our study is that the abundance of Sp4 protein ranges across a continuum where the lowest expression of this transcription factor correlates with the maximal activation of an SKF-sensitive Ca^{2+} influx under conditions that favor resting PM potential. This introduces a new variable to consider when thinking about how Ca^{2+} signaling participates in the control of gene expression in neurons. Over the years, considerable efforts have been focused on understanding how Ca^{2+} influx resulting from glutamatergic neurotransmission, neuronal activity, and membrane depolarization influence activity of transcription factors like CREB and MEF2 (1, 2, 54). Although the spatiotemporal characteristics of different Ca^{2+} signals has been recognized to play a key role in engaging one molecular cascade over another (55, 56), the majority of these studies have presented the regulation of Ca^{2+} -dependent transcription factors as occurring in a temporally constrained and largely ON/OFF manner in response to PM depolarization. Our results challenge this view of Ca^{2+} -mediated transcription in neuronal cells by showing that amount of transcription factor Sp4 is distributed over a continuum that we believe reflects the graded activation of SOCE by the membrane potential. Based on this observation, we propose that this represents an *analog* control by neuronal SOCE over transcription. In sum, we believe that the demonstration that Ca^{2+} signaling not only controls depolarization-induced transcription factors but also modifies transcription factor expression under resting membrane conditions through SOCE represents a major new insight about how Ca^{2+} signaling in neurons regulates specific programs of gene expression that enable these cells to respond and adapt to changes in their extracellular environment.

Relevance of SOCE and Sp4 to neurodegenerative and neuropsychiatric disorders

Deficiencies in STIM1 and Orai channels that cause aberrant SOCE contribute to the pathophysiology of cardiovascular, pulmonary, and autoimmune disorders (57, 58). For a long time, it has been recognized that dysregulation of Ca^{2+} homeostasis is likely playing a central role in the etiology of neurodegenerative disorders (59, 60) and other recent evidence suggests that this may be also a feature that is common to several major psychiatric disorders (61). Although previous studies have examined SOCE in neurons under pathological conditions linked to Alzheimer's disease (62, 63), Huntington's disease (64), or nerve damage of sensory neurons (65), the link between this specific Ca^{2+} signal and brain disorders remains elusive because it is not clear under what physiological conditions neuronal SOCE functions. Our study offers a new way of thinking about neuronal SOCE that may provide insights into dysregulation of Ca^{2+} homeostasis associated with certain brain disorders. As a possible example of this, genetic variations in *Sp4* have been associated with schizophrenia and bipolar disorder (24, 66, 67) and we have reported that Sp4 protein but not mRNA levels in the cerebellum and prefrontal cortex were reduced in postmortem bipolar disorder subjects and inversely correlated with negative symptoms in schizophrenia (25, 68). Our current results suggest the possibility that dysregulation of upstream signaling potentially controlled by STIM1-mediated SOCE may play a role in the aberrant expression of Sp4 and contribute to the etiology of these affective disorders.

Materials and Methods

Cell culture and transfection

Primary granule neurons were obtained from dissociated cerebella of 6-day old Long Evans rat pups as described (69). All animal work was approved by Tufts University Institutional Animal Care and Use Committee and carried out in accordance with institutional guidelines. The isolated cells were resuspended in Basal Medium Eagle (BME) supplemented with 10% FetalClone II (Hyclone, Logan, UT), 20mM KCl (25mM final concentration), 2mM glutamine, penicillin (50 units/ml), and streptomycin (50µg/ml). Cells were seeded in 12- or 6-well dishes coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO) at a density of 1.0×10^6 or 3.0×10^6 cells/well, respectively. Cytosine arabinofuranoside (AraC, 10µM) was added to the culture medium 18–24 hrs after plating. Unless indicated otherwise, cultures were maintained for 6–10 days prior to experimental treatments and cell harvesting. For experiments involving pharmacological inhibitors, the cells were typically pre-treated for 60 min with the compound before incubating them with fresh culture medium supplemented with the same pharmacological agent.

Dissociated CGNs were transfected at DIV2 by Ca^{2+} phosphate precipitation. For each transfection, culture medium was replaced with warm DMEM and then DNA- Ca^{2+} phosphate precipitate was added to the cells for 20 min. After this time period, cells were washed twice with warm DMEM and fresh culture medium supplemented with AraC was added to each well. For STIM1 knockdown experiments, cells were analyzed at DIV5. Finally, for experiments comparing the impact of STIM1 WT and constitutively active STIM^{D76A} overexpression on FLAG-Sp4 protein levels, cells were analyzed at DIV4.

Neuro2A cells were cultured in DMEM [supplemented with 10% FetalClone II (Hyclone, Logan, UT), penicillin (50 units/ml), and streptomycin (50 µg/ml)] and transfected overnight using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Transfected cells were selected with puromycin (4 µg/ml) for 48 hr.

Antibodies and pharmacological compounds

The anti-Sp4 (sc-645), anti-CREB (sc-186), anti-ubiquitin (sc-8017), anti-ERK2 (sc-1647) as well as the horseradish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phospho-CREB^{Ser133} (#9198), anti-phospho-ERK1/2^{Thr202/Tyr204} (#4370), anti-c-Fos (#2250), anti-STIM1 (#4916), and anti-STIM2 (#4917) antibodies were from Cell Signaling Technologies (Beverly, MA). Anti-GAPDH (MAB374) and anti-Sp1 (#07-645) antibodies were from Millipore Corporation (Billerica, MA). The anti-6X His (ab18184) was purchased from Abcam (Cambridge, MA), the anti-GFP (A10262) was from Invitrogen (Grand Island, NY) and the M2 mouse anti-Flag antibody (F1804) was from Sigma-Aldrich (St. Louis, MO).

MK-801, nimodipine, CNQX, EGTA, SKF96365, OAG, and caffeine were from Sigma-Aldrich. MG132, ALLN, and 2-APB were from EMD Biosciences (La Jolla, CA, USA). ω -Conotoxin GVIA and ω -Agatoxin IVA were from Peptide Institute Inc (Osaka, Japan). Tetrodotoxin (TTX) and DL-2-Amino-5-phosphonopentanoic acid (APV) were from Tocris Bioscience (Bristol, UK).

Plasmids

The full-length 3X FLAG-tagged human Sp4 was described before (21). The following plasmids were purchased from Addgene: the pEX-CMV-SP-YFP-STIM1 (Plasmid 18857) and pEX-CMV-SP-YFP-STIM1^{D76A} (Plasmid 18859) were initially described in (42), the pEX-CMV-SP-YFP-STIM2 (Plasmid 18862) was first presented in (70), while the pcDNA 3.1-Orai1-Myc-His (Plasmid 21638) and the pcDNA 3.1-Orai2-Myc-His (Plasmid 16369) were described in (7).

The pLKO.1-puro Empty Plasmid DNA Control Vector (SHC001) and the pLKO.1-puro STIM1 (TRCN0000175008) short hairpin RNAs (shRNAs) were purchased from Sigma-Aldrich. The sequence targeted by the STIM1 shRNA corresponded to mouse STIM1 nucleotides 1668 to 1688 (gcagtactacaacatcaagaa; NCBI Reference Sequence: NM_009287), which has 100% homology with the rat sequence (NCBI Reference Sequence: NM_001108496).

Western blotting

For Western blot analyses, cells were rapidly collected in ice-cold RIPA buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 0.5% Igepal-630, 0.5% deoxicollic acid, 0.1% SDS, 1 mM EDTA) supplemented with a cocktail of protease inhibitors (Complete Protease Inhibitor without EDTA, Roche Applied Science, Indianapolis, IN) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail A, Santa Cruz Biotechnology). One volume of 2X Laemmli buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 0.15% Bromophenol Blue, 20% glycerol, 200 mM β -mercaptoethanol) was added and the extracts were boiled for 5 min. Samples were

adjusted to an equal concentration after protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA).

Lysates were separated using SDS-PAGE and transferred to a nitrocellulose membrane. After transfer, the membrane was blocked in TBST (Tris-buffered saline and 0.1% Tween 20) supplemented with 5% milk and probed with the indicated primary antibody at 4°C overnight. After washing with TBST, the membrane was incubated with the appropriate secondary antibody and visualized using ECL reagents according to manufacturer guidelines (Pierce, Thermo Fisher Scientific, Rockford, IL).

The following procedure was used to quantify Western blots. First, equal quantity of protein lysate was analyzed by SDS-PAGE for each biological replicate. Second, the exposure time of the film to the ECL chemiluminescence was the same for each biological replicate. Third, all the exposed films were scanned on an Epson Perfection V500 Photo Scanner in grayscale at a resolution of 300 dpi. Fourth, the look-up table (LUT) of the scanned tiff images was inverted and the intensity of each band was individually estimated using the selection tool and the histogram function in Adobe Photoshop CS 8.0 software. Finally, the intensity of each band was divided by the intensity of its respective loading control (GAPDH) to provide the normalized value used for statistical analysis.

Detection of polyubiquitinated Sp4

To assess polyubiquitination of Sp4 in treated DIV10 CGNs, Sp4 was immunopurified from the nuclear fraction using rabbit agarose-conjugated polyclonal anti-Sp4 antibody (Santa Cruz Biotechnology). Immunopurified material was separated using SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. In order to enhance the immunodetection of ubiquitin conjugated to Sp4, the nitrocellulose membrane was placed between two layers of Whatman filter paper, submerged in distilled water, and autoclaved for 30 minutes. After autoclaving, the membrane was blocked in blocking solution (TBST supplemented with 5% milk) for 30 min and finally incubated overnight with an anti-ubiquitin antibody (Santa Cruz Biotechnology, clone P4D1, 1:200) in blocking solution. Subsequent steps were carried out according to the Western blot procedure described above.

Real time RT-PCR

After experimental treatment, total RNA was isolated from CGNs cells using the TRIzol method (Invitrogen). The concentration of total RNA was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and first strand cDNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad). Real time PCR reactions were performed using gene specific primers and monitored by quantification of SYBR Green I fluorescence using a Bio-Rad CFX96 Real-Time Detection System. Expression was normalized against *gapdh* expression. The relative quantification from three biological replicates was performed using the comparative cycle threshold (CT) method.

Primers for real time RT-PCR:

Sp4-F 5'-AGCGATCAGAAGAAGGAGGAG-3'

Sp4-R 5'-GTTGCTTGATTTTCACCAGGA-3'

GAPDH-F 5'-ATGACCACAGTCCATGCCATC-3'

GAPDH-R 5'-CCAGTGGATGCAGGGATGATGTTC-3'

Calcium measurements

For Ca^{2+} imaging experiments, CGNs were seeded on poly-D-lysine-coated 35mm glass bottom multiwell culture plates (MatTek Corp. Ashland, MA). The cells were loaded with the fluorescent Ca^{2+} indicator Fluo-4 by incubation in 25mM KCl imaging solution (10mM HEPES [pH 7.3], 120mM NaCl, 1mM MgCl_2 , 2mM CaCl_2 , 10mM glucose) containing 2 μM of the acetoxymethyl ester of the dye (Fluo-4 AM; Invitrogen, Molecular Probes, Eugen, OR, USA) for 45 min at room temperature. After loading, cells were washed three times with HEPES imaging solution supplemented with either 65, 25, or 5mM KCl according to the experiment and incubated for 30–60 min at room temperature to allow de-esterification of the dye. Adjusting the NaCl concentration was used to control for the osmotic balance between the imaging solutions with different KCl concentration. For imaging solution where CaCl_2 was omitted, the concentration of MgCl_2 was increased accordingly to maintain osmotic balance. Data collection, image processing, and analyses were carried out with SlideBook (Intelligent Imaging Innovations, Denver, CO), ImageJ (NIH), and Microsoft Excel. Fluo-4 fluorescent signal in all experiments was quantified at the level of the cell body.

Live-cell imaging and YFP-STIM1 puncta quantification

Dissociated CGNs cultured on poly-D-lysine-coated 35mm glass bottom multiwell culture plates (MatTek Corp) were transfected at DIV2 with 0.25 μg of DNA plasmid using the Ca^{2+} phosphate technique. Two days after transfection, the cells were washed three times with HEPES imaging solution supplemented with 65mM KCl and incubated 10 min before beginning the imaging session to stabilize the cells to the stage temperature (37°C). Adjusting the NaCl concentration between the different imaging solutions (65, 25, and 5mM KCl) was used to control the osmotic balance.

Quantification of YFP-STIM1 puncta was performed according to the following procedure. First, high-resolution digital images of live primary CGNs expressing YFP-STIM1 and incubated for 60 min in equiosmotic 65, 25, or 5 mM KCl imaging solution were collected using a 63X oil objective. Second, the length of visible neurites for each capture was measured and recorded. Third, background signal was removed by image segmentation using the clustering-based thresholding tool in ImageJ (NIH). The same background/foreground cutoff threshold was used for images of all three KCl conditions. Finally, distinct foreground objects greater than 4 pixels along neurites and cell bodies were manually counted as puncta and presented as the average number of YFP-STIM1 puncta per 20 μm of neurite length or per cell body for each experimental condition.

Immunocytochemistry

Indirect immunofluorescence detection of antigens was carried out using CGNs cultured on poly-D-lysine-coated coverslips in 12-well plates. After experimental treatment, CGNs cells were washed twice with phosphate-buffered saline (PBS) and fixed for 30 minutes at room

temperature with 4% paraformaldehyde (PFA) in PBS. After fixation, cells were washed twice with PBS, permeabilized with PBST (PBS and 0.25% Triton X-100) for 20 min, blocked in blocking solution (5% normal serum in PBS) for another 30 min, and finally incubated overnight at 4°C with the first primary antibody in blocking solution. Next day, coverslips were extensively washed with PBS and incubated for two hours at room temperature in the appropriate fluorophore-conjugated secondary antibody solution (Alexa 488- or Alexa 594-conjugated secondary antibody [Molecular Probes, Invitrogen] in blocking solution). After washes with PBS, the coverslips were incubated again overnight in primary antibody solution for the second antigen and the procedure for conjugation of the fluorophore-conjugated secondary antibody was repeated as above. Finally, cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and coverslips were mounted on glass slides with Prolong Antifade reagent (Invitrogen, Molecular Probes).

CGNs cultured on coverslips were imaged with a Spot RT2 color digital camera mounted on a Nikon E800 microscope. Image preparation, assembly and analysis were performed with ImageJ and Photoshop CS. Change in contrast and evenness of the illumination was applied equally to all images presented in the study. The following procedure was used for pixel intensity measurements. First, original raw tiff files of transfected CGNs were opened in Photoshop CS and pixel intensity corresponding to nuclear FLAG-Sp4 immunofluorescence was measured from 30 pixel spots. Second, for each measure of nuclear immunofluorescence pixel intensity, a measure of background pixel intensity from the same image channel was acquired and subsequently subtracted from the nuclear immunofluorescence pixel intensity value. Finally, mean pixel intensity of FLAG-Sp4 was calculated by averaging values from three independent biological replicates.

Statistics

All graphical representations are presented as mean $\pm$ standard error of mean (SEM) unless specified otherwise. The statistical methods are described in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Sp4 protein abundance is regulated by membrane potential and the ubiquitin-proteasome system

(A) Sp4 polyubiquitination was determined in lysates from CGNs cultured in the presence or absence of the proteasome inhibitor MG132 and either 25mM KCl (normal culture condition) or switched to 5mM for 30 min. Lysates were immunoprecipitated with anti-Sp4 and Western blotted with anti-Ub or anti-Sp4 as indicated. (B) CGNs treated with the 26S proteasome inhibitor MG132 (10μM) or the calpain inhibitor ALLN (20μM) and Sp4 levels determined by Western blot. Sp1 was used as a negative control. (C) Western analysis of Sp4 from CGNs treated for 60 min with fresh culture medium supplemented with the indicated concentrations of KCl. The band specific to Sp4 (marked by arrowhead) migrates just above the 100 kD marker. (D) Graph shows mean ($n = 6$) Sp4/GAPDH ratio ($\pm$ SEM) for cells treated as in (C). One-way ANOVA revealed a significant difference between KCl groups [$F_{2,15} = 17.69$, $p < 0.0001$]. Tukey's HSD post hoc test, $*p < 0.05$; $****p < 0.0001$. (E) *Sp4* mRNA expression in CGNs treated for 60 min with three different concentrations of $[K^+]_{EXT}$. Changing $[K^+]_{EXT}$ did not affect Sp4 mRNA expression in CGNs. Bars represent mean fold-change measured by RT-qPCR (error bars indicate range from three biological replicates).

Figure 2. NMDARs, neuronal activity, and L-type VDCC are not required for regulation of Sp4 by membrane potential

(A) Western blot of Sp4 protein expression in CGNs treated with DMSO (vehicle) and two concentrations of MK-801 in 5 or 25mM KCl. c-Fos and PCREB^{S133} were used as positive controls. (B) Graph shows mean Sp4/GAPDH ratio ($\pm$ SEM) from five biological replicates. A two-way ANOVA reveals a significant effect of KCl on Sp4 protein expression, $F_{1,24} = 50.23$, $p < 0.0001$. The effect of MK-801 is not significant, $F_{2,24} = 0.82$, $p > 0.05$. There is no significant interaction between KCl and MK-801, $F_{2,24} = 0.11$, $p > 0.05$. Post hoc comparisons between two means were conducted using the Bonferroni correction, *** $p < 0.001$, **** $p < 0.0001$. (C) Western blot of Sp4 protein expression in depolarized CGNs treated with TTX. Cells were treated with two different concentrations of TTX (1 and 10µM) for two periods of time (2 and 18 hr) before cell lysis. The band specific to Sp4 migrates just above the 100 kD marker. c-Fos was used as a positive control. (D) Graph shows mean Sp4/GAPDH ratio ($\pm$ SEM) from three biological replicates. A two-way ANOVA shows no significant effect for the concentration factor [$F_{1,8} = 0.12$, $p > 0.05$], the time factor [$F_{1,8} = 0.90$, $p > 0.05$], or the interaction between the two factors [$F_{1,8} = 2.82$, $p > 0.05$]. (E) Western blot of Sp4 protein expression for control and CGNs treated with nimodipine for 2 hours. P-ERK was used as positive control. (F) Graph shows mean Sp4/GAPDH ratio ($\pm$ SEM) from five biological replicates. A two-way ANOVA reveals a significant effect of KCl on Sp4 protein expression, $F_{1,24} = 50.99$, $p < 0.0001$. The effect of nimodipine was not significant [$F_{2,24} = 0.64$, $p > 0.05$] nor was the interaction between KCl and nimodipine [$F_{2,24} = 0.08$, $p > 0.05$]. Post hoc comparisons between two means were conducted using the Bonferroni correction, *** $p < 0.001$, **** $p < 0.0001$.

Figure 3. Pharmacological inhibition of SOCCs prevents hyperpolarization-induced degradation of Sp4

(A) Western blot analysis of Sp4 in CGNs treated with the indicated concentrations of SKF or vehicle. Cells were pre-treated for 60 min with SKF or vehicle before changing to fresh culture medium with the indicated KCl concentration (supplemented with SKF or vehicle) for an additional 60 min. (B) Graph shows mean ($n = 4$) Sp4/GAPDH ratio ($\pm$ SEM) for cells treated as in (A). A two-way ANOVA shows a significant effect of KCl, $F_{1,18} = 31.96$, $p < 0.0001$. The effect of SKF is also significant, $F_{2,18} = 17.75$, $p < 0.0001$. Addition of SKF increased Sp4 protein abundance and eliminated the statistical difference between 25 and 5 μM KCl. The interaction effect between KCl and SKF was not significant, $F_{2,18} = 2.81$, $p = 0.087$. Post hoc comparisons between two means were conducted using the Bonferroni correction, *** $p < 0.001$, **** $p < 0.0001$. (C) Western analysis of Sp4 protein expression in CGNs treated with 2-APB or vehicle control. Cells were pre-treated with 2-APB or vehicle for 60 min before incubating the cells with fresh culture medium (supplemented with 2-APB or vehicle) for the same period of time. The band specific to Sp4 migrates just above the 100 kD marker. Sp1 protein expression was used as negative control. (D) Graph shows mean Sp4/GAPDH ratio ($\pm$ SEM) from four biological replicates. A two-way ANOVA reveals a significant effect of KCl, $F_{1,12} = 24.23$, $p < 0.0005$. The effect of 2-APB was also significant, $F_{1,12} = 21.49$, $p < 0.001$. Addition of 2-APB increased Sp4 protein abundance and eliminated the difference between 25 and 5 μM KCl. The interaction effect between KCl and 2-APB was not significant, $F_{1,12} = 2.66$, $p > 0.05$. Post hoc comparisons between two means were conducted using the Bonferroni correction, *** $p < 0.001$. (E) Sp4 mRNA expression in control and SKF-treated CGNs assessed by RT-qPCR. Bar represents mean fold-change (error bar indicates range from three independent replicates). (F) Sp4 was

immunoprecipitated from lysates of cells treated with MG132 or MG132+SKF as indicated. Immunoprecipitates were analyzed by Western blot with antiubiquitin to reveal amount of Sp4 polyubiquitination.

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Figure 4. Extracellular Ca^{2+} is required for hyperpolarization-induced Sp4 degradation
 (A) Western analysis of Sp4 after incubation of CGNs for 60 min in control or EGTA-treated culture medium. Sp1 and c-Fos were used as negative and positive controls, respectively. (B) Graph shows mean ($n = 6$) Sp4/GAPDH ratio ($\pm$ SEM) of cells treated as in (A). A two-way ANOVA indicates a main significant effect of KCl [$F_{1,20} = 11.12$, $p < 0.005$] and EGTA [$F_{1,20} = 8.06$, $p < 0.05$]. The interaction effect between the two factors is significant, $F_{1,20} = 8.23$, $p < 0.01$. Graphical representation shows that EGTA affects Sp4 protein abundance only when the extracellular concentration of KCl is 5mM. Post hoc comparisons between two means were conducted using the Bonferroni correction, $**p < 0.01$; $***p < 0.001$.

Figure 5. Depletion of caffeine-sensitive intracellular Ca^{2+} stores in resting CGNs
 (A and B) CGNs loaded with Fluo-4 and treated with 65 mM KCl (A) or 25 mM KCl (B) imaging solution were stimulated with caffeine (50 mM) for the period indicated (gray box) to release Ca^{2+} from intracellular pools. In both A and B experiments, cells were switched to 5 mM KCl solution to establish $F/F_0 = 1$. (C) The amount of Ca^{2+} released by the application of caffeine was quantified as the difference between F/F_0 [CAF Peak] and F/F_0 [Baseline] (as indicated in B). Data from each biological replicate is presented and the horizontal bars represent the mean ($\pm$ SEM) for each condition. $*p < 0.05$, two-tailed t -test. (D) CGNs loaded with Fluo-4 as in (A) were pre-incubated in 65 mM KCl for 5 min and then switched to equiosmotic 5 mM KCl imaging solution and stimulated with caffeine 7 min later. (E) The experiment was repeated exactly as in (D) but the cells were incubated in the 5 mM KCl solution 60 min before caffeine stimulation. In both D and E experiments, the cells were re-depolarized with 65 mM KCl solution after the initial caffeine stimulation to demonstrate cellular responsiveness and to show the rapid replenishment of intracellular pools (E). All results are expressed as F/F_0 and were replicated in at least 3 biological replicates. Traces are the average of 72 (A), 60 (B), 68 (D), and 66 (E) individual cells from one biological replicate. The gray outlines represent $\pm$ SEM for each measured time-points.

Figure 6. Induction of a Ca^{2+} influx consistent with SOCE in resting CGNs

CGNs (DIV4) loaded with Fluo-4 as in Figure 5 were then pre-incubated for 60 min in 5_MM KCl imaging solution without Ca^{2+} and supplemented with a cocktail of inhibitors (APV, 100 μM ; CNQX, 4 μM ; nimodipine, 20 μM) $\pm$ SKF (30 μM). After beginning the imaging session, the pre-incubation solution was replaced by fresh equiosmotic 5_MM KCl imaging solution with the indicated inhibitors and 2_MM Ca^{2+} to reveal SOCE in resting CGNs. Video of Ca^{2+} influx under these conditions is included as Supplementary Material (Movies S1 and S2). Traces are the average of 104 (Control) and 94 (SKF96365) individual cells from three independent biological replicates. The gray outlines represent $\pm$ SEM for each measured time-points.

Figure 7. Changes in membrane potential induce dynamic relocation of STIM1 in primary CGN neurons

(A) YFP-STIM1 fluorescence from a transfected CGN was imaged in real-time first when the cell was in 65mM KCl imaging solution (left panel) and then 30 min later after changing to an equiosmotic 5mM KCl imaging solution (right panel; a representative cell is shown). Arrowheads in each panel correspond to the same region. The lower panels are high magnification of a 20µm neurite stretch from the corresponding upper panels. (B) Quantification of number of YFP-STIM1 puncta along neurites (left panel) and cell bodies (right panel) of live CGNs when the cells were incubated in imaging solution with the indicated concentration of extracellular KCl. One-way ANOVA revealed a significant difference between KCl groups for the quantification of puncta along neurites [$F_{2,108} = 84.32$, $p < 0.0001$] and cell bodies [$F_{2,84} = 29.46$, $p < 0.0001$]. Tukey's HSD post hoc test, $***p < 0.0001$. Note that we applied a stringent threshold to isolate the YFP-STIM1 puncta from the background. This manipulation likely prevented puncta with weaker fluorescence intensity from being counted, particularly in neurites. (C) Real time imaging of a CGN expressing the mutant YFP-STIM1^{D76A} treated first with 65mM (left panel) and then 5mM (right panel) KCl imaging solution. Arrowheads in the corresponding upper and lower panels indicate the same position. (D to F) CGN co-transfected with YFP-STIM1 and Orai-His were imaged with an anti-GFP antibody (D) and an anti-His antibody (E). The merged captures are presented in (F). Arrowheads indicate the exact same position in each panel. Scale bar = 20µm.

Figure 8. STIM1 regulates Sp4 protein

(A) Lysates from Neuro2A cells transfected with STIM1 shRNA or control shRNA were analysed by Western blot with the indicated antibodies. Here, cells were co-transfected with a plasmid expressing GFP to control for transfection efficiency. (B) Quantification of FLAG immunofluorescence in dissociated CGNs co-transfected with FLAG-Sp4 and shRNA vector and treated for 60 min with either 65mM KCl ($n = 131$) or 5mM KCl ($n = 141$) culture medium. FLAG immunofluorescence was also quantified in CGNs transfected with FLAG-Sp4 and STIM1 shRNA and treated for 60 min with 5mM KCl ($n = 83$) culture medium. One-way ANOVA revealed a significant difference between groups, [$F_{2,352} = 39.02$, $p < 0.0001$]. Tukey's HSD post hoc test, $****p < 0.0001$. (C and D) Representative immunostaining of a FLAG-Sp4 co-transfected with YFP-STIM1 WT (C) or YFP-STIM1^{D76A} mutant (D) in CGNs. Arrowheads indicate the same position in each panel. Scale bar = 20μM. (E) Contingency table summarizing the number of FLAG immunopositive and immunonegative nuclei counted in dissociated CGN cultures co-transfected with FLAG-Sp4 and human WT STIM1 or constitutively active STIM1^{D76A}. (F) Quantification of FLAG immunofluorescence in the immunopositive FLAG-Sp4 nuclei co-transfected with either WT STIM1 ($n = 57$) or constitutively active STIM1^{D76A} ($n = 30$). $**p < 0.01$, two-tailed t -test.