Sigma-1 receptors regulate hippocampal dendritic spine formation via a free radical-sensitive mechanism involving Rac1·GTP pathway

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Sigma-1 receptors (Sig-1Rs) are endoplasmic reticulum (ER)-resident proteins known to be involved in learning and memory. Dendritic spines in hippocampal neurons play important roles in neuroplasticity and learning and memory. This study tested the hypothesis that Sig-1Rs might regulate dendritic spine formation in hippocampal neurons and examined potential mechanisms therein. In rat hippocampal primary neurons, the knockdown of Sig-1Rs by siRNAs causes a deficit in the formation of dendritic spines that is unrelated to ER Ca²⁺ signaling or apoptosis, but correlates with the mitochondrial permeability transition and cytochrome c release, followed by caspase-3 activation, Tiam1 cleavage, and a reduction in Rac1·GTP. Sig-1R-knockdown neurons contain higher levels of free radicals when compared to control neurons. The activation of superoxide dismutase or the application of the hydroxyl-free radical scavenger N-acetyl cysteine (NAC) to the Sig-1R-knockdown neurons rescues dendritic spines and mitochondria from the deficits caused by Sig-1R siRNA. Further, the caspase-3-resistant TIAM1 construct C1199DN, a stable guanine exchange factor able to constitutively activate Rac1 in the form of Rac1·GTP, also reverses the siRNA-induced dendritic spine deficits. In addition, constitutively active Rac1·GTP reverses this deficit. These results implicate Sig-1Rs as endogenous regulators of hippocampal dendritic spine formation and suggest a free radical-sensitive ER-mitochondrion-Rac1·GTP pathway in the regulation of dendritic spine formation in the hippocampus.

mitochondria | ROS | N-acetyl cysteine | learning and memory | caspase-3

Dendritic spines in the CNS are important for many functions. Dendritic atrophy in the neocortical region is related to aging-induced amnesia, and its reversal improves memory retention (1). Similarly, the loss of dendritic spine-related synapses is currently a strong pathologic correlate of cognitive decline, and synaptic dysfunction is evident long before synapses and neurons are lost (2). On the other hand, exposure to drugs of abuse including cocaine, nicotine, or morphine produces persistent changes, usually in the form of increased dendritic spines and arborizations, in cells in brain regions involved in incentive motivation and reward (3). These persistent changes are thought to represent the neuronal reorganization that contributes to some of the persistent sequelae associated with drug use, including the establishment of motivational conditioning and learning (3).

The morphology of dendritic spines and axons is determined by the dynamic cytoskeleton protein actin. Rho family small GTPases including Rho, Cdc42, and Rac1 regulate the dynamics of actin and are critical for neuronal polarization and morphogenesis (4–6). Rho proteins are regulated by guanine nucleotide exchange factors (GEFs). In early stages of neural morphogenesis, the activation of Cdc42 promotes the formation of filopodia, the long thin protrusions serving as primary precursors of axons and dendritic spines (7). However, Rac1 and its

specific GEF TIAM1 regulate the late stage of neural morphogenesis by targeting themselves to dendritic spines and controlling the formation of mature forms of spines (8–10). TIAM1-Rac1·GTP signaling is also important for dendrite branching. Thus, Tiam1-Rac1·GTP signaling is critical for the morphogenesis of dendritic spines especially in the later stages of neuronal maturation.

Originally mistaken as a subtype of opioid receptors (11), the Sigma-1 receptor (Sig-1R) (12–15) is now known to be a nonopioid endoplasmic reticulum (ER)-resident protein (16–19). Sig-1Rs are postulated to be involved in neuropsychiatric diseases including amnesia and addiction (13, 20, 21). By using CHO cells, we recently (17) identified the Sig-1R as a ligand-operated ER receptor chaperone that regulates Ca²⁺ signaling specifically by chaperoning type 3 IP₃ receptors. Importantly, Sig-1Rs regulate neuritogenesis in PC12 cells (22) and enhance cell differentiation in rat oligodendrocytes (23). Sig-1Rs also promote reconstitution of lipid rafts in plasma membranes (22). Taken together, these results suggest the possibility that Sig-1Rs might affect the morphogenesis of neurons in the CNS. However, this possibility has never been examined.

We tested if the ER Sig-1R might regulate neuronal morphology in this study, specifically dendritic spine formation in rat primary hippocampal neurons. We also examined potential underlying mechanisms of such effect, if present. We report here that Sig-1Rs regulate dendritic spine formation via a potential ER-mitochondrion-Rac1·GTP pathway, apparently due to this receptor's ability to regulate the redox state of neurons.

Results

Sig-1Rs Regulate the Morphogenesis of Hippocampal Primary Neurons. We examined the roles of Sig-1Rs in the morphogenesis of hippocampal primary neurons in vitro by silencing Sig-1R expression using siRNAs. Small hairpin RNAs, constructed in the pSI-REN vector, were transfected into primary neurons by using the liposome-delivering system in this initial portion of the study. Rat hippocampal primary neurons were thus transfected with siRNAs (17, 23) together with pEGFP-N1 vectors on DIV 7. Morphologies were observed on DIV 14 or 21. Control neurons receiving transfection of control siRNAs (siCon-tf) on DIV 14 showed a

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Fig. 1. Inhibition of dendritic morphogenesis in Sig-1R-knockdown hippocampal neurons. (A–F) Effects of siSig-1R-tf on dendrite formation. Neurons were transfected with siRNAs on DIV 7 and were stained on 14 or 22 DIV with anti-MAP-2B antibodies. The number of established primary dendrites and branches were counted. Primary dendrites are defined as neurites originating from the neuronal soma and are at least longer than two times the diameter of the cell body. (Scale bar, 100 μ m.) *** P < 0.001; n = 3, five to 10 neurons were quantified in each individual experiment. (G–J) Effect of siSig-1R-tf on spine formation. Neurons were transfected on DIV 7 with siCon (G and I) or siSig-1R (H and J) vectors in combination with EGFP. Neurons were stained with rhodamine phalloidin for F-actin. Note elongated filopodia in siSig-1R-tf neurons. (K and L) Quantitative assessments were made of the effects of siSig-1Rs on protrusion length, dendrite branching, and filopodium formation. The control neurons at DIV 22 had protrusions mostly < 2 μ m in length and mushroom shaped, which is characteristic of dendritic spines, whereas the siRNA neurons failed to retract protrusions (i.e., exhibited elongated filopodia). Protrusions equals dendritic spines plus filopodia. (Scale bar, 50 μ m.) *** P = 0.0002; n = 2, at least 20 neurons were captured in each experiment, and two to three dendrites were randomly selected from each neuron for counting of protrusions. (M) Synaptophysin immunostaining. (N) Effects of siSig-1Rs on synaptic activity. FM4-64 (red)-labeled neurons were depolarized with KCl for measurement of synaptic activity. (Scale bars, M 20 μ m and N 5 μ m.)

typical morphology of a stage V neuron, having several long dendrites with tapering and branching characteristics (Fig. 1 A–F). The Sig-1R-siRNA-transfected (siSig-1R-tf) neurons, however, showed a reduction of extension and branching of dendrites. Long filopodia-like structures protruding from soma were often observed in siSig-1R-tf neurons. Similar effects were seen on DIV 21 in siSig-1R-tf neurons. Sig-1Rs also affected the formation and maturation of dendritic spines. Results are shown in Fig. 1 G–L. On DIV 16, control neurons formed filopodia-like long protrusions on dendrites, with majority of them (>60%) showing F-actin-enriched head structures at the tip. On DIV 22, control neurons formed clusters of stubby mushroom-shaped spine heads and possessed no filopodia, indicating the maturation of spines. In contrast, siSig-1R-tf neurons formed long and thin protrusions, and only few possessed spine heads at the tip. Further, F-actins were seen diffusing over the whole cytoplasm in the filopodia of siSig-1R-tf neurons, whereas F-actins discretely accumulated at the tip of filopodia in control neurons (Fig. 1 G–J). For siSig-1R-tf neurons,

the longer the culture time, the more pronounced the abnormal phenotype was seen, such as increased numbers of elongated filopodia-like protrusions lacking head structures. In general, silencing of Sig-1R expression increased the length of protrusions by 59% on DIV 16 and 159% on DIV 22, respectively. These results suggest a dysregulation of stage-dependent signalings for actin compartmentalization/polymerization in Sig-1R-deficient neurons.

Sig-1R-Knockdown Inhibits Synapse Formation. Active synapses express specific proteins and receptors for proper function. We next asked if the knockdown of Sig-1Rs might affect the formation of synapses. In control neurons, staining for NR-1, GluR2/3, and PSD-95 showed puncta of strong immunoreactivities on dendritic spines. However, no such puncta were observed in the long filopodia-like protrusions in siSig-1R-tf neurons; these proteins remained in the dendritic shafts (Fig. S1 in *SI Appendix*). Axon terminals in siSig-1R-tf neurons also exhibited significantly less immunoreactivities for synaptophysin (Fig. 1M). Synaptic activities assessed by the depolarization-induced uptake of

Fig. 2. Sig-1R-knockdown causes TIAM1 GEF cleavage and the inactivation of Rac. (A and B) Immunocytochemistry for Rac and Tiam1 in AAV-siCon-td or AAV-siSig-1R-td neurons at DIV 22. (Scale bar, 5 μ m.) (C) TIAM1 cleavage detected by Western blots. Note the decrease of intact TIAM1 (200 kDa) with concomitant increases of cleaved TIAM1 (120, 85, and 74 kDa) in AAV-siSig-1R-td neurons. (D–G) Effect of AAV-siSig-1R on lipid raft distributions of total Rac (D–G) or Rac-GTP (F and G). * $P < 0.05$; $n = 3$. (H and I) Lipid raft distributions of TIAM1 and its proteolytic products.

FM4–64 showed that the siSig-1R-tf-induced protrusions did not form functional synapses (Fig. 1N). These findings suggest that the filopodia-like long protrusions caused by siSig-1Rs failed to form functional synapses.

Sig-1R-Knockdown Inhibits Activation of Rac1 Rho GTPase. Rac1 regulates dendritic growth and spine maturation but not filopodium formation (8–10). Since the knockdown of Sig-1Rs stunt dendritic growth and synapse formation but not the formation of filopodia, we hypothesized that the Sig-1R might regulate Rac1 signaling in neurons. Both Rac1 and TIAM1 clustered in dendritic shafts and spines of control neurons (Fig. 2A and B). However, intensities of Rac1 and TIAM1 in filopodium-like protrusions on the dendrites of siSig-1R-tf neurons were markedly decreased (Fig. 2A and B). The expression levels of these proteins were examined by Western blotting, a technique that requires more protein to be analyzed. Since gene transfer using liposomes yields only a few percentage of successful transfection in primary neurons, we used an AAV-based gene delivery system that yields >70% efficacy in gene expression. Details of vector constructions are described in *SI Methods* and Fig. S2 in *SI Appendix*. Western blots showed that the knockdown of Sig-1Rs (i.e., AAV-siSig-1R transduced or abbreviated as AAV-siSig-1R-td) did not affect the total level of Rac1 protein in hippocampal neurons. However, the knockdown caused a down-regulation of full-length TIAM1, accompanied by increases in its proteolytic products (Fig. 2C).

Rac1 is compartmentalized in lipid rafts of postsynaptic membranes (24). We found (Fig. 2D) that Rac 1 existed in both raft (fractions 3, 4, and 5) and nonraft fractions (fractions 6 and higher). The knockdown of Sig-1Rs did not apparently affect the distribution pattern of Rac1 in raft or nonraft fractions (Fig. 2E). However, when active forms of GTP-binding Rac1 (i.e., Rac1-GTP) were

measured by a pull-down assay, the Rac1-GTP in the raft fractions (fractions 4 and 5) was reduced in AAV-siSig-1R-td neurons (Fig. 2F and G). The Sig-1R-knockdown also caused a decrease of intact TIAM1 in the raft fractions. The cleaved TIAMs, which were increased in AAV-siSig-1R-td neurons, were present only in nonraft fractions (Fig. 2H and I). These data suggest that: (i) The active full-length TIAM1 preferentially exists at the raft where TIAM1 serves to activate Rac1 and (ii) the knockdown of Sig-1Rs decreases the amount of full-length TIAM1 at rafts, leading to a reduction of Rac1 activation. How the knockdown of Sig-1Rs might reduce the full-length TIAM1 is examined in the next section.

Knockdown of Sig-1Rs Activates Caspase-3 in an ER-Stress and Apoptosis-Independent Manner. TIAM1, by activating Rac1, is known to maintain the structural configuration of dendritic spines (8–10). TIAM1 in the motor neuron is inactivated by caspase-dependent proteolysis when cells undergo stress (25). To determine whether increased cleavage of TIAM1, as seen above in AAV-siSig-1R-td neurons, is associated with caspase activation, active forms of several caspase members were analyzed by Western blots. As shown in Fig. S4 in *SI Appendix*, in the absence of ER stressors like thapsigargin, AAV-siSig-1R-td neurons showed a reduction of procaspase-3 with a concomitant increase in active caspase-3, seen as the cleaved form, when compared to the control (Fig. S4A, lane 2 vs. lane 1). There was no effect on the level of ER stress-responding proteins including caspase-12, GRP94, calnexin, ATF4, and BiP (Fig. S4B and C, lane 2 vs. lane 1 in each panel). These results suggest that Sig-1R-knockdown did not elicit ER stress and that the knockdown of Sig-1Rs might have caused an increase in caspase-3 in an ER stress-independent manner. We thus decided to use thapsigargin, a well-known ER stressor that causes an increase in many of the ER stress-responding proteins detailed above. Inasmuch as the knockdown of Sig-1Rs did not cause ER stress, we postulated that the caspase-3 increase seen in AAV-siSig-1R-td neurons should continue to be present if neurons are challenged with thapsigargin, whereas the thapsigargin-elicited ER stress-responding proteins should not be affected, or more specifically enhanced, by the Sig-1R-knockdown. Indeed, we found that the caspase-3 activation continued to be seen in thapsigargin-treated Sig-1R-knockdown neurons (Fig. S4A), whereas no further enhancement of the ER stress-responding proteins was seen in thapsigargin-treated Sig-1R-knockdown neurons (Fig. S4C). Thus, the activation of caspase-3 induced by the Sig-1R-knockdown may not involve ER stress but instead may involve other, as yet unidentified, pathway(s).

To ascertain whether the knockdown of Sig-1Rs would cause apoptosis, Hoechst 33342 staining was used to detect chromatin condensation in control and transfected or transduced neurons. Staurosporine-treated (STS; 1 μ M for 24 h) neurons served as positive controls for apoptosis. In both AAV-siCon-td and AAV-siSig-1R-td neurons, only a very small fraction of cells underwent apoptosis. No difference in the percentage of apoptotic cells was seen in AAV-siCon-td and AAV-siSig-1R-td neurons (Fig. S4D). Therefore, Sig-1R-knockdown in hippocampal primary neurons did not induce apoptosis.

Sig-1R-Knockdown Does Not Affect Ca^{2+} Dynamics in Hippocampal Neurons but Nevertheless Causes Mitochondrial Dysfunction. Consistent with other reports (26, 27), we found that type-1 IP_3 receptors, and not type-3 receptors, were the predominant IP_3 receptors in hippocampal neurons. As Sig-1R chaperones are known to regulate only type-3 IP_3 receptors but not the type-1 receptors (17), we found that the basal or activated level of mitochondrial or cytosolic Ca^{2+} transient/concentration in hippocampal neurons was not affected by Sig-1R-knockdown (Fig. S3A–D in *SI Appendix*). We found, however, that Sig-1R-knockdown caused a disruption of mitochondrial membrane potential (28) as well as an enhanced release of cytochrome *c* from mitochondria (Fig. S3E–L in *SI Appendix*). Thus, Sig-1R-knockdown caused the activation of caspase-3 by increasing

Fig. 3. Free radicals in Sig-1R-knockdown neurons. (A) Generation of superoxide anions by GSH and sodium selenite. Sodium selenite was mixed with GSH at 0.2 mg/mL. $O_2^{\bullet -}$ were monitored by lucigenin-based chemiluminescence (lucigenin at 50 μ M). (B) Effect of hippocampal neuronal lysates on $O_2^{\bullet -}$ generated by GSH/selenite. Neurons were transduced with AAV-siCons or AAV-siSig-1Rs for 10 days before assay. (C) The AUC, calculated from assays in panel B (between the 10th and 25th intervals), seen with increasing lysates (0, 0.03, 0.1, 0.3, 1, and 3 μ g protein). (D) Effect of AAV-siSig-1R-td on the NADPH-induced generation of $O_2^{\bullet -}$ in living hippocampal neurons. NADPH at 45 μ M was applied to neurons in the presence of 50 μ M lucigenin at the beginning of the fifth interval (AUC from 10th to 40th interval). (E) Inhibition of AAV-siSig-1R-td-induced mitochondrial permeability transition by free radical scavengers NAC and Tempol. The puncta of JC-1 positive mitochondria in dendrites were measured. *** $P < 0.001$; $n = 5$, each group in triplicates. (F) Effects of free radical scavengers on dendritic spine formation in AAV-siSig-1R-td neurons. Neurons were transduced with AAV-siSig-1Rs on DIV 13 when 50 μ M Tempol, NAC, or NAL were also added. Protrusion lengths were measured on day 5 posttransduction. Frequencies of various protrusion lengths are shown; $n = 3$ independent experiments. Representative morphologies are shown at the Top. (Scale bar, 5 μ m.)

mitochondrial cytochrome *c* release. However, this action of Sig-1Rs in this regard did not involve mitochondrial Ca^{2+} .

Reduced Radical Scavenging Activity in Sig-1R-Knockdown Neurons.

In addition to Ca^{2+} overload in mitochondria, free radical-related oxidative stress is also known to cause mitochondrial membrane disruption, which leads to mitochondrial permeability transition (MPT) (29–31). Thus, we next examined if free radicals are involved in the siSig-1R-induced MPT. First, we examined if the Sig-1R-knockdown affected the free radical scavenging activity of neurons. An *in vitro* test, employing the selenite-catalyzed superoxide generation system (32), was used. Selenite at 0.06 mM effectively induces superoxide anion generation upon the addition of glutathione (GSH; 0.2 mg/mL) (Fig. 3A). The superoxides ($\text{O}_2^{\cdot -}$) thus generated were monitored by lucigenin-based chemiluminescence. Fig. 3B shows that lysates from AAV-siSig-1R-td neurons were less effective in scavenging $\text{O}_2^{\cdot -}$ when compared to control lysates. The scavenging activity was lysate protein-dose-dependent. Analyses of the area-under-the-curve (AUC) indicate an $\text{EC}_{50} = 0.0144 \mu\text{g}$ protein for AAV-siCon-td controls and $0.3791 \mu\text{g}$ protein for AAV-siSig-1R-td neurons (Fig. 3C). We next examined if similar results might be seen if assays were done in the living system. Accordingly, NADPH was applied to living neurons to generate $\text{O}_2^{\cdot -}$. NADPH induced a rapid generation of $\text{O}_2^{\cdot -}$ in both groups, but higher levels of detectable $\text{O}_2^{\cdot -}$ were seen in AAV-siSig-1R-td neurons (Fig. 3D). These results with the NADPH assay suggest that either AAV-siSig-1R-td neurons are less effective in scavenging $\text{O}_2^{\cdot -}$ or more $\text{O}_2^{\cdot -}$ are generated in those neurons.

Free Radical Scavengers Block Mitochondrial Dysfunction and Rescue Dendritic Spine Formation in Sig-1R-Knockdown Neurons.

a causal relation between redox state and the alteration of mitochondria

drial function in AAV-siSig-1R-td neurons, we next examined if free radical scavengers might block the MPT in those neurons. N-acetyl cysteine (NAC) or 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (Tempol), scavengers of hydroxyl or superoxide dismutase activator, respectively, successfully blocked the MPT caused by the knockdown of Sig-1Rs (Fig. 3E). The nitric oxide synthase inhibitor nitro-L-arginine (NAL) was, however, ineffective. Thus, siSig-1R-induced disruption of mitochondrial membrane potential is, at least, in part due to the neurons' deficiency in scavenging free radicals.

Free radical scavengers were also used to examine if they might block the aberrant spine development in AAV-siSig-1R-td neurons. Indeed, NAC and Tempol successfully rescued spine formation in Sig-1R-knockdown neurons (Fig. 3*F*). Specifically, NAC- or Tempol-treated AAV-siSig-1R-td neurons were able to retract filopodia and form dendritic spines. NAL was again less effective in this regard (Fig. 3*F*).

Caspase-3-Resistant TIAM1 Construct Rescues the Spine Formation in Sig-1R-Knockdown Neurons. Our hypothesis suggests that the final

pathway of Sig-1R-knockdown effects on dendritic spine formation is related to the inactivation of Rac1, which in turn is hinged upon TIAM1 cleavage by caspase-3. To provide evidence for the involvement of Sig-1Rs in this pathway, we examined if the deficit of spine formation caused by Sig-1R-knockdown might be rescued by a caspase-3-resistant TIAM1 construct. We therefore transfected siSig-1R-tf neurons with either an N-terminal-truncated TIAM1 construct called C1199, which is more stable when compared to full-length TIAM1 (10), or a construct derived from C1199, called C1199DN, that is fully resistant to caspase-3 (25). Initially, we used Neuro-2a cells, in lieu of primary hippocampal neurons, to characterize and confirm the properties of C1199 and C1199DN, because of the need for the abundant samples available from Neuro-2a cells for such an extensive characterization. We specifi-

Fig. 4. Caspase-3-resistant TIAM1 construct C1199DN and its effects on Rac-GTP formation and dendritic spine maturation in Sig-1R-knockdown neurons. Effect of constitutively active Rac on dendritic spine formation in Sig-1R-knockdown neurons. (A) Characterizing the GEF activity between the full-length TIAM1, a more stable N terminus-truncated TIAM1 construct C1199, and a caspase-3-resistant TIAM1 construct C1199DN using Neuro-2a cells. Neuro-2a cells were transfected with various TIAM1 constructs: Full-length (FL) TIAM1, C1199, and C1199DN. All three TIAM1s equally increased the active form of Rac. (B) Resistance of C1199DN to proteolytic cleavage caused by siSig-1R-tf in Neuro-2a cells. The asterisk (*) indicates the nonspecific band. (C–E) In primary hippocampal neurons, overexpression of C1199 and C1199DN rescued aberrant dendritic spine morphologies caused by siSig-1R-tf. Note the greater potency of C1199DN in rescuing the morphologies. *** $P < 0.001$ (F) The effect of constitutively active Rac1 (Q61L) on dendritic spine formation in siSig-1R neurons. DIV 18 neurons were cotransfected with siRNA-Sig1R vectors together with constitutively active Rac1 or an empty vector. The morphologies were obtained at DIV 22; $n = 3$ experiments. At least 5–10 neurons were counted in each experiment. Scale bar, 5 μm .

cally examined the susceptibility of C1199 and C1199DN to siSig-1R-induced proteolysis. In Neuro-2a cells, both C1199 and C1199DN were able to increase the level of Rac-GTP (Fig. 4A). Both full-length TIAM1 and C1199 underwent slight cleavage in siCon-tf Neuro-2a cells (Fig. 4B, lanes 3 and 5). In siSig-1R-tf Neuro-2 cells, the cleaved form of full-length TIAM1 or C1199 increased, when compared to that seen in respective control cells (Fig. 4B, lanes 4 and 6 vs. lanes 3 and 5, respectively). However, the caspase-3-resistant construct C1199DN was resistant to cleavage both in control cells and in Sig-1R-null cells (Fig. 4B, lanes 7 and 8). These results indicated that C1199 and C1199DN would be useful tools to test our hypothesis.

Hippocampal primary neurons were subsequently transfected with a C1199 or C1199DN plasmid along with siSig-1Rs. To best monitor the effects of those constructs on the spine formation, plasmids were transfected into neurons specifically on DIV 14, a time when filopodia begin to form spines in primary cultures. Cytotoxicity caused by this late transfection was minimized by the

following modifications in the protocol: (i) Neurons seeded at higher density (1×10^5 cells/well); (ii) pretreatment with kineuretic acid and MgCl_2 1 h before transfection; and (iii) neurobasal medium used as a diluent of the DNA-lipofectamine complexes. This modified protocol yielded no cytotoxicity. Our results showed that the overexpression of C1199 in siSig-1R-tf neurons slightly improved the maturation of dendritic spines, but filopodium-like protrusions still dominated the phenotype (Fig. 4C). This result is consistent with another report showing that branched filopodia and lamellapodia were occasionally observed in neurons overexpressing C1199 (10). The overexpression of caspase-3-resistant C1199DN in siSig-1R-tf neurons, however, successfully rescued dendritic spine formation in those neurons notably by decreasing the number of long filopodia while concomitantly increasing mature spines with mushroom-shaped head structures (Fig. 4 C–E).

Constitutively Active Rac Rescues the Spine Formation in Sig-1R-

Knockdown Neurons. To provide additional evidence for our hypothesis on the ER-mitochondrion- Rac1·GTP pathway, we examined if constitutively active Rac1 (i.e., Q61L) might rescue the deficit of dendritic spines caused by the Sig-1R-knockdown. Our results indicate that constitutively active Rac successfully rescued the formation of dendritic spines in siSig-1R-tf neurons. The siSig-1R-tf neurons cotransfected with Q61L on DIV 18 were able to form dendritic spines on DIV 22 (Fig. 4F).

Discussion

In this study we have demonstrated that a potential ER-mitochondrion-Rac-GTP pathway plays an important role in dendritic spine and synapse formation in hippocampal neurons and that the redox state of neurons is an integral and critical element in this pathway. Because Sig-1Rs are implicated in memory and cognition (33, 34), our findings also provide a mechanistic insight into the action of Sig-1Rs in neuroplasticity and cognition.

ER and mitochondria have been reported to participate in dendritic spine formation (35–39). Our results provide additional insights on how these two intracellular organelles may participate in dendritic spine formation in a unique fashion. Contrary to the notion that ER does so by regulating Ca^{2+} efflux via ryanodine or IP_3 receptors on the ER membrane (35–37), our results indicate that the ER might regulate dendritic spine formation in hippocampal neurons via a free radical-related mechanism that might not involve Ca^{2+} signaling. Mitochondria supply energy for cells and are important in neural development (38, 39). Our results indicate that mitochondria might participate in dendritic formation via a TIAM1-Rac1-GTP signaling pathway.

Our results also indicate that caspase-3 plays an important role in the development of neurons. Although caspase-3 is recognized as a protease that executes death signals, growing evidence suggests that caspase-3 may also be important in synaptic plasticity and growth cone motility (40–43). We have thus added one more important physiological role to the action of caspase-3 and shown that it regulates dendritic spine formation in hippocampal neurons. Although Sig-1R-knockdown increased caspase-3 activity in primary neurons in this study, the knockdown did not cause apoptosis. As such, the increase in caspase-3 activity caused by Sig-1R-knockdown might represent an early stage of dendritic spine dysfunction that is before apoptosis and might still be rescued by some therapeutic intervention including the use of free radical scavengers (see below).

One of the most important outcomes from this study is the demonstration that an ER protein can affect the activation of Rac1 and associated neuroplasticity by the level of free radicals in neurons. We do not know at present how Sig-1Rs modulate free radicals or reactive oxygen species (ROS) in the neuron. Mitochondria are the main source of free radicals/ROS levels in the cell, but the ER can also generate free radicals (44). The exact mechanism whereby free radicals are controlled by the ER Sig-1Rs warrants further investigation. Gene microarray and proteomic studies are being planned to provide some clues in this

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