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MicroRNA-134 activity in somatostatin interneurons regulates H-Ras localization by repressing the palmitoylation enzyme, DHHC9

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MicroRNA-134 (miR-134) serves as a widely accepted model for microRNA function in synaptic plasticity. In this model, synaptic activity stimulates miR-134 expression, which then regulates dendrite growth and spine formation. By using a ratiometric microRNA sensor, we found, unexpectedly, that miR-134 activity in cortical neurons was restricted to interneurons. Using an assay designed to trap microRNA-mRNA complexes, we determined that miR-134 interacted directly with the mRNA encoding the palmitoylation enzyme, DHHC9. This enzyme is known to palmitoylate H-Ras, a modification required for proper membrane trafficking. Treatment with bicuculline, a GABAA receptor antagonist, decreased DHHC9 expression in somatostatin-positive interneurons and membrane localization of an H-Ras reporter in a manner that depended on miR-134. Thus, although miR-134 has been proposed to affect all types of neurons, we showed that functionally active miR-134 is produced in only a selected population of neurons where it influences the expression of targets, such as DHHC9, that regulate membrane targeting of critical signaling molecules.

Considerable progress has been made in understanding the contributions of microRNAs to neuronal development, but microRNAs are also found in mature neurons, where their localization in dendrites, and possibly axons, has been proposed to contribute to synaptic plasticity (1–5). This is an appealing concept because local regulation of protein translation is essential for plasticity and microRNAs can regulate dendritic growth, spine formation, and growth cone guidance, properties generally believed to underlie plasticity. Activity-regulated microRNAs are particularly well suited to participate in these mechanisms. One of the best-studied activity-dependent microRNAs in brain is microRNA-134 (miR-134), which has been shown to regulate dendritic spine morphogenesis through effects on LIM domain kinase 1 (Limk1) (1) and dendrite growth through the translational repressor, pumilio 2 (Pum2) (5). Mutations in Limk1 have been linked to a human mental retardation syndrome (6), and Pum2-deficient mice showed behavioral abnormalities (7). Recent studies have demonstrated involvement of miR-134 in long-term potentiation (LTP) via regulation of sirtuin 1 (SIRT 1) and in epileptogenesis (8, 9). Like most studies involving brain microRNAs, these reports make the implicit assumption that miR-134 is expressed uniformly in excitatory neurons. It is well known, however, that brain neurons are highly heterogeneous, and in many regions, excitatory (largely glutamatergic) and inhibitory (largely GABAergic) interneurons form complex networks that balance opposing actions (10). Although many microRNAs are probably expressed in both excitatory and inhibitory neurons, some are likely to be restricted to one or the other subclass. Indeed, analysis of Argonaute (Ago)2 complexes in individual neuronal subtypes within the mouse brain identified a significant number of microRNAs that were enriched in GABAergic but not in glutamatergic neurons and vice versa, indicating that microRNA expression patterns are specific for neurons that share a common origin and neurotransmitter phenotype (11). Thus, characterizing the expression patterns of microRNAs within a heterogeneous population of neurons requires approaches that monitor microRNA function at the single-cell level.

In previous studies, we used a ratiometric microRNA sensor to monitor another activity-regulated microRNA, miR-132 (12). This allowed us to quantify miR-132 function in individual cells by comparing the ratios of GFP (fused to a sequence containing the microRNA binding site) to an internal control, DsRed, driven by a shared promoter. Using a similar sensor designed to monitor miR-134, we found that unlike miR-132, miR-134 was not active in the majority of cortical neurons. Rather, activity was detected in cells that also stained for neuropeptides somatostatin (SST) or calretinin. This indicated that miR-134 activity was largely limited to inhibitory GABAergic interneurons. The finding that functionally active miR-134 was produced in only a limited population of neurons must be considered in analysis of potential mRNA targets. Using the RNA-induced silencing complex (RISC)-trap method, designed to characterize microRNA-mRNA interactions biochemically (13), we identified the transcript encoding the palmitoylation enzyme, DHHC9, as a putative miR-134 target. Palmitoylation is a lipid modification critical for proper trafficking of membrane proteins (14). In most instances, the relationship between a particular palmitoylation enzyme and its targets is poorly defined, in that each enzyme has multiple targets and each target can be palmitoylated by multiple enzymes. This does not appear to

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1S.C. and X.A.C. contributed equally to this work.

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Most brain regions comprise numerous neural cell types that are distinct in function and molecular characteristics. We examined microRNA-134 (miR-134) activity in cortical cultures using a microRNA sensor and discovered that miR-134 was induced in response to neuronal activity in somatostatin- and calretinin-expressing interneurons but not in pyramidal neurons. We identified the palmitoylation enzyme DHHC9 as a direct target of miR-134 and showed that it contributed to the regulation of H-Ras in somatostatin interneurons. H-Ras is a signaling molecule crucial for neuronal development and function. This study uncovered an activity-dependent miR-134 regulatory pathway in cortical interneurons that can potentially affect membrane localization of multiple signaling molecules.

Significance

Author contributions: S.C., X.A.C., and R.H.G. designed research; S.C., X.A.C., and S.W.E. performed research; S.C., X.A.C., and S.W.E. analyzed data; and S.C., X.A.C., and R.H.G. wrote the paper.

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be the case for H-Ras, which appears to be palmitoylated pre-dominantly by DHHC9 (15). This modification is required for directing H-Ras to the neuronal membrane, where it mediates morphological changes and may participate in LTP and memory formation (16). Our studies indicate that in SST⁺ neurons, DHHC9 is down-regulated by neuronal activity through a pathway that involves miR-134. The consequent reduction of DHHC9 expression disrupted localization of H-Ras from plasma membrane microdomains. Thus, although previous studies have highlighted miR-134 as an activity-dependent microRNA localized in the synaptodendritic compartment, we show that functionally active miR-134 is produced in only a selected population of neurons where it influences the expression of targets, such as DHHC9, which influence the membrane targeting of critical signaling molecules such as H-Ras.

Results

Activity-Dependent Induction of miR-134 in SST-Expressing Interneurons.

Previous studies in hippocampal and cortical cultures showed elevated levels of the miR-134 precursor transcript (pre–miR-134) following either treatment with brain-derived neurotrophin factor (BDNF) or KCl-induced membrane depolarization (1, 5). Using cultures of rat hippocampal neurons (DIV6), we compared the BDNF-stimulated induction of miR-134 with that of miR-132, another activity-dependent microRNA involved in the development of dendrites and dendritic spines (17, 18). Using quantitative PCR (qPCR) and Taqman microRNA assays to monitor levels of precursor and mature microRNAs, respectively, we detected relatively small increases in pre-miR-134 and mature miR-134 following BDNF treatment over a time course of 6 h (Fig. 1A). Levels of pre–miR-134 peaked 2 h after BDNF treatment (1.6 ± 0.5-fold above basal levels) and returned to baseline by 6 h. Correspondingly, mature miR-134 levels were not significantly elevated above basal level after 6 h. In contrast, pre–miR-132 levels increased by 18-fold over the same time period. Similar results were obtained after treatment with 16 mM KCl. We hypothesized that induction of miR-134 may be restricted to specific cell types and therefore may not be apparent in assays using heterogeneous populations of cells. To assess the induction of mature miR-134 in individual cells, we generated a ratiometric sensor specific for miR-134, following a strategy that we had used previously for measurement of miR-132 (Fig. 1B). This approach monitors miR-134 activity through its regulation of a GFP transcript containing three reiterated miR-134 microRNA recognition elements (MREs) in its 3′UTR. Comparing GFP levels to that of an internally expressed DsRed control lacking these MREs provides a ratiometric measurement for microRNA activity. To test this system, we first assessed the efficacy of our miR-134 sensor in SH-SYSY neuroblastoma cells using flow cytometry to analyze the GFP/DsRed ratio in individual cells. These studies revealed a dose-dependent response of the miR-134 sensor to exogenous miR-134 (Fig. 1C). A control sensor lacking MREs was completely unresponsive to added miR-134. The effect of exogenous miR-134 was also apparent at the sensor transcript level (Fig. 1D). To monitor miR-134 induction in individual cortical neurons, we analyzed activity of the miR-134 sensor in primary cultures characterized using specific molecular markers. In particular, we examined cells that stained for the neuropeptide, somatostatin, which represents a well-characterized subtype of inhibitory interneuron. Cortical cultures were transfected with either the miR-134 sensor or control at DIV7, stimulated at DIV8 with bicuculline (a GABA receptor antagonist that blocks GABA receptors and thereby is predicted to increase neural excitability), and examined at DIV9 using relative intensity measurements (Fiji software). The population of SST-expressing interneurons was identified by immunostaining (Fig. 1E). The GFP/DsRed ratio for the control sensor in these cells had a mean value of 1.09; the ratio for the miR-134 sensor was 0.62, indicating a robust increase in miR-134 activity (Fig. 1F). We next analyzed sensor activity in neighboring large excitatory pyramidal neurons. In these cells, the difference between the control and miR-134 sensors was not significantly different (P = 0.385; Fig. 1F). In contrast, miR-132 activity measured using a specific sensor was readily apparent in these pyramidal neurons (Fig. S1). These results indicated that miR-134 is induced in SST⁺ neurons but not in pyramidal neurons, likely explaining the modest increase of miR-134 in preparations of heterogeneous cortical cultures where SST-expressing cells constitute a relatively small minority. To determine whether miR-134 levels were, in fact, induced after stimulation, we repeated the assays in the presence or absence of bicuculline. With the control sensor, bicuculline treatment did not significantly alter the GFP/DsRed ratio (Fig. 1G). However, the miR-134 sensor in SST⁺ neurons responded robustly (Fig. 1G), demonstrating that miR-134 induction in SST⁺ neurons is activity-dependent. To determine whether miR-134 activity was induced in other subtypes of interneurons, we examined calretinin-positive interneurons. SST- and
calretinin-positive neurons have been identified as the main interneuronal population postnatally (P0-P14) in rat neocortex, whereas other major interneuronal subtypes such as parvalbumin (PV)- and vasointestinal peptide-positive neurons emerge later (19). The sensor assays in cortical culture showed that similar to SST+ neurons, calretinin neurons expressed miR-134 upon bicuculline treatment, and miR-134 activity was absent in untreated cells (Fig. 1G). These data suggested that miR-134 is induced in at least two major subtypes of early developing neocortical interneurons.

Palmitoylation Enzyme DHHC9 Is a Target of miR-134. We identified DHHC9 as a putative miR-134 target by using the RISC-trap assay that we had developed previously to capture miRNA-mRNA interactions (13). This assay, performed in HEK293 cells, uses an epitope-tagged dominant negative form of the RISC component, GW182, to stabilize microRNA-mRNA complexes. The endogenous DHHC9 transcript enriched by 4.5-fold in the presence of miR-134, compared with another neuronal microRNA, miR-124 (Fig. 2F). To confirm that DHHC9 was a miR-134 target, we fused the DHHC9 3' UTR to a luciferase cDNA and assayed luciferase activity in HEK293 cells and primary hippocampal cultures. Luciferase activity was significantly down-regulated by exogenous miR-134 in both types of cells (Fig. 2B). Within the DHHC9 3' UTR, we identified three putative miR-134 MREs with perfect seed matches (Fig. 2C, designated M1, M2, and M3). Mutation of M1 and M3 individually partially stabilized a FLAG-DHHC9-3'UTR reporter; mutation of both sites rendered the reporter completely refractory to miR-134 regulation, compared with a scrambled microRNA and with the DHHC9 reporter lacking its 3' UTR (Fig. 2D).

We established that DHHC9 was expressed in SST+ interneurons (Fig. S2). To determine whether DHHC9 protein was regulated by endogenous miR-134, we generated a ratiometric reporter similar to the microRNA sensors described above but incorporating the DHHC9 3' UTR (Fig. 2E). This reporter was expressed in cortical cultures at DIV7, and cells were treated with bicuculline or control for 24 h and examined in both SST+ interneurons and pyramidal neurons on DIV11 using ratiometric analyses of fluorescence intensity. In SST+ cells, bicuculline significantly down-regulated GFP expression (Fig. 2E). In contrast, pyramidal neurons exhibited no difference in the mean GFP/DsRed ratio following bicuculline treatment (Fig. 2E).

H-Ras Palmitoylation Is Critical for Its Subcellular Localization. We next asked whether the regulation of DHHC9 in SST+ neurons by miR-134 affected membrane trafficking of the DHHC9 palmitoylation target, H-Ras. Most studies examining H-Ras localization have used reporter constructs, such as GFP fused to the H-Ras coding sequence. To minimize confounding effects from ectopic H-Ras expression (20), we generated a GFP fusion that contained only the carboxyl-terminal H-Ras trafficking domain. This domain (designated HRaCS24) has been shown to be sufficient to localize H-Ras to cell membranes and includes two cysteine residues (C181 and C184) that, when palmitoylated, promote H-Ras trafficking (21–24). We first examined the subcellular localization of wild-type GFP-HRaCS24 in SST+ interneurons, compared with a mutant isoform that could not be palmitoylated (C181S, C184S). Confocal microscopy revealed a distinct punctate pattern of wild-type GFP-HRaCS24 throughout the soma and processes of SST+ neurons (Fig. 3B). The distinct microdomain localization of GFP-HRaCS24 is indistinguishable from the expression pattern of the GFP-tagged full-length H-Ras (GFP-Hras) (Fig. S3). In contrast, the palmitoylation-defective mutant (C181S, C184S) exhibited a diffuse distribution of green fluorescence throughout the cell body and processes, consistent with the nonspecific targeting of mutated GFP-HRaCS24 due to the absence of palmitoylation (24). To quantify the difference in GFP-HRaCS24 localization, we used line scans measurements of GFP intensity, captured across 30 μm of continuous dendritic length for each SST+ neuron (Fig. 3C). The high-intensity peaks (max) represented the localization of GFP-HRaCS24 in puncta, and the lower-intensity values represented diffused GFP (min). These measurements showed a significantly different profile of GFP-HRaCS24...
Fig. 3. Palmitoylation is critical for localizing H-Ras in SST⁺ neurons. (A) The C-terminal membrane-targeting domain of H-Ras contains two palmitoylation sites (designated Pal; C181 and C184) and a farnesylation motif (designated Far; CAAX). GFP fusions to the wild type and the palmitoylation mutant (C181S, C184S) are illustrated. (B) Subcellular localization of GFP-HRasC24 in SST⁺ neurons. Dendrites of SST⁺ neurons expressing either the wild-type or the palmitoylation defective H-Ras membrane-targeting domain are shown; localization of the wild-type H-Ras to microdomains was diminished by mutating the two palmitoylation sites. (C) Quantitative analysis using linescan measurement of GFP intensity in 30 μm of continuous dendritic length depicted in (B). Each peak denotes microdomain localization; relative distance reflects the span of the segment analyzed in μm. (D) Quantification of linescan data was performed by measuring differences (Δ intensity) between the maximum (max) and minimum (min) intensity of each peak. The Δ intensity plotted represented the average of the five highest peaks in each dendrite. The wild type and palmitoylation mutant had a significantly different Δ intensity. *** P < 0.0001 (n = 10 neurons). Student t test, mean ± SE. (Scale bars, 5 μm.)

Fig. 4. miR-134 and DHHC9 regulate H-Ras trafficking in SST⁺ neurons. (A and B) H-Ras localization changes after exogenous miR-134 expression. (A) Linescan measurement of GFP intensity in control- or miR-134-expressing neurons. (B) Quantification of linescan data by measuring Δ intensity (*** P = 0.0004, n = 15 neurons). (C) DHHC9 shRNA blocked expression of epitope-tagged DHHC9 in HEK293 cells. sh-control represents a nonspecific shRNA. (D) Constructs of shDHHC9 and sh-control in plasmids expressing tdTomato are shown. (E and F) DHHC9 regulates H-Ras localization in SST⁺ neurons. Linescan measurement of GFP intensity in dendrites expressing GFP-HRasC24 along with sh-control or shDHHC9. (F) DHHC9 knockdown significantly reduced Δ intensity (** P = 0.0024, n = 11 neurons). GFP intensity was measured from 30 μm of continuous dendritic length (A, B, E, and F). Student t test, mean ± SEM.

miR-134 Regulation of DHHC9 Regulates Trafficking of H-Ras in SST⁺ Neurons. To determine whether miR-134 affected GFP-HRasC24 localization, we examined the effects of scrambled control or miR-134 mimics in dissociated cortical cultures stained immunohistochemically for SST expression. The distinct punctate pattern of GFP-HRasC24 was retained in the presence of control microRNA, whereas expression of miR-134 caused a diffused pattern of GFP fluorescence in the SST⁺ cells (Fig. 4A and B). This effect was somewhat less pronounced than with the C181S, C184S palmitoylation mutant, so we asked whether DHHC9 depletion resulted in a similar mislocalization. To examine the contribution of DHHC9 to GFP-HRasC24 localization in SST⁺ cells, we generated a plasmid that encodes a short-hairpin RNA to DHHC9 (shDHHC9) downstream of a tdTomato fluorescence marker. In HEK293 cells, shDHHC9 was capable of depleting coexpressed Flag-DHHC9 protein levels (Fig. 4C and D) and endogenous levels of DHHC9 in Neuro2A cells (Fig. S4). We identified shDHHC9-expressing cells by presence of the tdTomato fluorescence and analyzed GFP-HRasC24 localization in the population that was also positive for SST. Introduction of shDHHC9 increased levels of diffused GFP fluorescence compared with control (Fig. 4E and F). Moreover, the effect of DHHC9 depletion on GFP-HRasC24 was comparable to that seen after expression of miR-134. Together, our data suggest that miR-134 regulates DHHC9-dependent palmitoylation and is important for H-Ras localization in SST⁺ interneurons.

Bicuculline Treatment Changes H-Ras Localization in an miR-134-Dependent Manner in SST⁺ Neurons. We next showed that bicuculline, like exogenous miR-134, altered localization of GFP-HRasC24 and GFP-HRas full-length protein in SST⁺ neurons (Fig. 5A and B and Fig. S3). To determine whether the bicuculline effect depended upon miR-134, we examined GFP-HRasC24 localization in cells cotransfected with a miR-134 locked nucleic acid (LNA) inhibitor. The inhibitor LNA-miR-134, but not a scrambled control, completely blocked the bicuculline effect (Fig. 5C–F). These data suggest that miR-134 regulates DHHC9-dependent palmitoylation and is important for H-Ras localization in SST⁺ neurons.
**Discussion**

Several studies have suggested that microRNAs contribute to the establishment and maintenance of synaptic plasticity in developing and mature neurons (1, 3, 25, 26). MicroRNAs regulated by neuronal activity are particularly suited for this role. The first example of an activity-regulated microRNA in mammalian brain was miR-132, which regulates expression of p250GAP, a GTPase-activating protein involved in remodeling the actin cytoskeleton within dendritic spines (17, 18). miR-134 has also been proposed to regulate dendritic spine morphology and synaptic plasticity (1, 8). miR-134 provides a compelling model for these functions because unlike miR-132, it has been localized to the synaptodendritic compartment. Of note, miR-132 has been shown to increase the size and density of dendritic spines (27, 28), whereas miR-134 appears to have the opposite effect (1, 9). Because of their seemingly antagonistic actions, we set out to determine whether miR-134 and miR-132 were, in fact, expressed concurrently. Earlier studies indicated that miR-132 was expressed generally throughout the brain, including large pyramidal neurons (17, 18, 27, 29). Because the preponderance of neurons in the cortex are excitatory, we hypothesized that miR-134, at a minimum, would be expressed in this neuronal subtype. Using a ratiometric sensor approach, we confirmed that miR-134 was induced by bicuculline but found that the induction was restricted and included SST+ interneurons.

In previous studies, the participation of miR-134 in regulating dendritic spines was determined by examining a complex mixture of neurons from hippocampus and cortex (1, 8, 9). Unlike pyramidal neurons, mature interneurons often lack spines, and when present, they are few in number with an irregular distribution (30, 31). Consequently, regulation of spine morphology may be less important in interneurons than it is in excitatory, glutamatergic neurons. It remains possible, however, that miR-134 could be involved in the degeneration of dendritic spines in early stages of interneuron development. Indeed, some of the SST+ interneurons did have a few dendritic filopodia, possibly reflecting their immaturity (Fig. S5). Nonetheless, our findings do not support the idea that miR-134 contributes directly to dendritic spine morphology in pyramidal neurons because our sensor does not detect miR-134 activity in this population. Conceivably, the spine phenotype observed in excitatory neurons could result from the miR-134 activity in SST+ interneurons because these neurons innervate the distal dendrites of principal neurons, where they are believed to contribute to the integration of synaptic inputs and synaptic plasticity (32). In this study, we tested SST- and calretinin-positive neurons, which are found at relatively early postnatal days, but our experimental model did not allow us to test the interneuron subtypes that emerge in more mature brain. It is possible that miR-134 is expressed in those other interneuron subtypes as well. The analysis of Ago2 complexes from mouse cortex showed that microRNA expression profiles are largely maintained among PV-, SST-, and glutamate decarboxylase GAD2-expressing interneurons but are strikingly different from glutamatergic neurons (11).

The idea that an activity-regulated microRNA could control expression of a protein palmitoyltransferase has two implications. First, it suggests that a microRNA could regulate a battery of synaptic proteins concurrently by controlling their delivery to synaptic membranes. This mode of regulation would not require the individual mRNAs to share a common microRNA recognition site because a single protein palmitoyltransferase could modify multiple protein targets. Second, this process, because it is indirect, would not require the microRNA and synaptic target to be colocalized in the same subcellular compartment. This could allow microRNAs to regulate proteins at the growth cone, for example, a region generally not thought to be a prominent site of mRNA translation. Thus, modulation of protein palmitoyltransferases by activity-regulated microRNAs could provide an avenue for influencing synaptic function that could be particularly relevant for mature neurons. This mode of regulation is not entirely unprecedented—miR-138 was shown to regulate expression of APT1, a depalmitoylation enzyme, in quiescent neurons. This mode of regulation would not require the individual mRNAs to share a common microRNA recognition site because a single protein palmitoyltransferase could modify multiple protein targets. Second, this process, because it is indirect, would not require the microRNA and synaptic target to be colocalized in the same subcellular compartment. This could allow microRNAs to regulate proteins at the growth cone, for example, a region generally not thought to be a prominent site of mRNA translation. Thus, modulation of protein palmitoyltransferases by activity-regulated microRNAs could provide an avenue for influencing synaptic function that could be particularly relevant for mature neurons. This mode of regulation is not entirely unprecedented—miR-138 was shown to regulate expression of APT1, a depalmitoylation enzyme, in quiescent neurons, activating Gαi3 in the synapse and causing spine shrinkage (3, 26). In contrast to miR-134, however, miR-138 expression is repressed by neuronal activity (3, 26). We used H-Ras localization to monitor DHHC9 activity in SST+ neurons. Both shRNA-mediated reduction of DHHC9 and exogenous expression of miR-134 delocalized H-Ras from microdomains. We propose that the effect of miR-134 on H-Ras localization in SST+ neurons occurs through DHHC9 down-regulation. Exactly how changes in H-Ras localization contribute to interneuron function is unknown. In pyramidal neurons, H-Ras activity is increased in glutamate-stimulated dendritic spines and axonal growth cones (20, 33). Additionally, H-Ras contributes to presynaptic neurotransmitter release via the ERK/Synapsin I pathway (34). Conceivably, effects
of the miR-134/DHHC9/H-Ras pathway on axonal growth and other presynaptic mechanisms in SST⁺ interneurons could influence dendritic spine formation in principal cells. Finally, previous studies of miR-134 have largely addressed activities within the synaptodendritic compartment (1,5,8,9). We observed miR-134 activity in the soma, suggesting additional functions. Consistent with our findings, miR-134 was not enriched in the synaptosomal fraction of cortical neurons, compared with a total homogenate (35). Although some actions of miR-134 might be localized to the soma, downstream effects could nonetheless involve synaptic regions through the effects of DHHC9 on SST⁺ and other protein targets.

Materials and Methods

Primary cortical and hippocampal neurons were prepared from E20 Sprague–Dawley rats (Charles River) and maintained in Neurobasal medium supplemented with B-27 and GlutaMax (Life Technologies). Where indicated, neurons were treated with 20 μM bicuculline (Focis Bioscience) or 16 mM KCl. All assays and experiments were approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University.

Immunocytochemistry and Confocal Microscopy. Primary cortical neurons grown on poly-L-lysine–coated coverslips in 12-well plates were fixed at DIV 8–12 in 4% (wt/vol) paraformaldehyde/PBS and permeabilized and blocked in blocking buffer containing 2% (vol/vol) horse serum, 1% BSA, and 0.5% Triton X-100. Primary antibodies, goat anti-somatostatin (1:100; Santa Cruz, CA), and mouse anti-calretinin (1:1,000; Swant), and secondary antibodies, Alexa Fluor-647 Donkey anti-goat IgG and Alexa Fluor-647 goat anti-mouse IgG (Life Technologies), were used in blocking buffer for immunodetection of SST⁺ and calretinin-expressing neurons. Confocal microscopy was performed using Olympus FV1000 (Advanced Light Microscopy Core facility, Oregon Health and Science University).

Image Analysis. For measurement of GFP and DsRed intensity from microRNA sensors, Z-stack images of neurons were taken with 40× objectives and compressed into a single image. In each neuron, two to three dendrites were analyzed for intensity of fluorescence using Fiji software. Statistical analysis was performed using paired t test with two-tailed P value by GraphPad Prism software. For H-Ras localization, Z-stack images of neurons were taken with 60× objectives with twofold magnification. Images were compressed into a single image for analysis by LineScan tool using Metamorph software. In SST⁺ neuron, primary dendrite projecting at least 30 μm from the soma was chosen to measure the intensity of GFP. Using peaks detected by linescan, Δ intensity was calculated by subtracting the basal level intensity (min) from the maximum intensity (max) of each peak. Five highest peaks were chosen to calculate the average Δ intensity of a dendrite, and 10–15 neurons were chosen for analysis of one sample group. Statistical analysis was performed using paired t test with two-tailed P value by GraphPad Prism software.

Detailed information on cell culture and transfection, molecular method including DNA constructs and mutagenesis, qRT-PCR, Western blot analysis, and RISC-trap assay is provided in SI Materials and Methods.

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