Autism spectrum disorder susceptibility gene **TAOK2** affects basal dendrite formation in the neocortex

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How neurons develop their morphology is an important question in neurobiology. Here we describe a new pathway that specifically affects the formation of basal dendrites and axonal projections in cortical pyramidal neurons. We report that thousand-and-one-amino acid 2 kinase (TAOK2), also known as TAO2, is essential for dendrite morphogenesis. TAOK2 downregulation impairs basal dendrite formation in vivo without affecting apical dendrites. Moreover, TAOK2 interacts with Neuruphin 1 (Nrp1), a receptor protein that binds the secreted guidance cue Semaphorin 3A (Sema3A). TAOK2 overexpression restores dendrite formation in cultured cortical neurons from *Nrp1*−/− mice, which express Nrp1 receptors incapable of binding Sema3A. TAOK2 overexpression also ameliorates the basal dendrite impairment resulting from Nrp1 downregulation in vivo. Finally, Sema3A and TAOK2 modulate the formation of basal dendrites through the activation of the c-Jun N-terminal kinase (JNK). These results delineate a pathway whereby Sema3A and Nrp1 transduce signals through TAOK2 and JNK to regulate basal dendrite development in cortical neurons.

Pyramidal neurons are abundant in brain regions associated with complex cognitive functions, including the cortex, hippocampus and amygdala. An understanding of the distinct physiology and morphology of these neurons is key to the elucidation of the mechanisms underlying sophisticated cognitive functions in normal and disease conditions. Several lines of evidence suggest that aberrant dendritic arborization may contribute to neurodevelopmental and psychiatric disorders with delayed onset, such as autism spectrum disorders.

In general, pyramidal neurons have a dendritic tree that is divided into two domains: the apical dendrite, which extends toward the pial surface, and basal dendrites, which emerge from the base of the cell body. The majority of synapses received by neocortical pyramidal neurons form on the basal dendrites. However, little is known about the molecular pathways that control the formation of basal dendrites.

TAOK1 and TAOK2 serine/threonine protein kinases are known to activate mitogen-activated protein (MAP) kinase (MAPK) pathways (JNK, p38 or extracellular signal-regulated kinase), leading to the modulation of gene transcription. In humans, the gene encoding TAOK2, a member of the MAP kinase kinase kinase (MAPKKK) family, is located on chromosome 16p11.2, a region that has recently been shown to carry substantial susceptibility to autism spectrum disorders and schizophrenia. TAOK2 mRNA is also a direct target of the fragile X protein, FMRP, whose loss or dysfunction leads to an autistic phenotype. TAOK2 selectively activates mitogen-activated protein kinases (MEKs) and serves as a regulator of p38 MAPK. In addition, TAOK2 modulates the actin cytoskeleton in non-neuronal cells through the activation of JNK. TAOK2 is subjected to alternative splicing to produce the TAOK2α (140 kDa) and TAOK2β (120 kDa) isoforms, of which only TAOK2α stimulates the JNK pathway.

Here we demonstrate that TAOK2 downregulation selectively impairs the formation of basal dendrites and axonal elongation. We found that TAOK2 interacted with Nrp1, the receptor of the secreted guidance cue Sema3A that controls basal dendrite arborization. Sema3A induced TAOK2 phosphorylation, thereby activating TAOK2. In conditions in which Nrp1 was either not expressed or was not capable of binding Sema3A, basal dendrite formation deficits could be restored by TAOK2 overexpression. TAOK2 downregulation also led to JNK inactivation that manifested as a decrease of JNK phosphorylation in cultured cortical neurons.

**RESULTS**

**Expression profile of TAOK2 in neurons and cerebral cortex**

To examine the subcellular expression profile of TAOK2, we analyzed TAOK2 immunoreactivity in mouse cortical neurons dissociated at embryonic day (E) 17 and cultured 2 days in vitro (DIV). TAOK2 preferentially localized to growth cones (Fig. 1a,b). The growth cone is a region where actin, but not microtubules, accumulates (Fig. 1b).
Figure 1 Distribution of TAOK2 and activated TAOK2 in cultured neurons and the developing cerebral cortex. (a) TAOK2 localizes to the growth cones (arrowheads) of isolated cortical neurons. (b) TAOK2 (red) localizes with actin (green) in growth cones. (c) Activated TAOK2 (pTAOK2; green) localizes to the neurite shaft of isolated cortical neurons. (d) TAOK2 and pTAOK2 are preferentially present in the intermediate zone (IZ) and cortical plate (CP) of the developing cortex. (e) Western blotting. TAOK2α expression is constant during early cortical embryonic development but increases considerably at perinatal (E19, P0) and adult time points. The TAOK2β isoform is absent before E19. Focal adhesion kinase (FAK) is used as a loading control. Scale bars, 10 μm (a,c), 5 μm (b) and 200 μm (d); Hoechst stains nuclei.

and where the actin cytoskeleton is the most dynamic. In contrast, TAOK2 activated by phosphorylation on Ser181 (pTAOK2) localized to the neurite shaft, where microtubules also accumulate (Fig. 1c). This pattern of TAOK2 expression suggests that TAOK2 may act as a coordinator of actin and microtubule dynamics.

In the mouse brain, TAOK2 and pTAOK2 are preferentially expressed in the intermediate zone and the cortical plate of the developing cortex (E18), and their expression in the ventricular zone is low (Fig. 1d). Western blot analysis using whole-cell extracts from the cortices of mice at different embryonic and postnatal ages demonstrated that the long isoform of TAOK2 (TAOK2α; 140 kDa) was expressed throughout early cortical embryonic development and increased in perinatal (E19 and postnatal day 0) and adult mice. In contrast, the short isoform of TAOK2 (TAOK2β; 120 kDa) was only observed perinatally and in the adult (Fig. 1e). In addition, in DIV2 E17 cortical neurons, we detected expression of TAOK2α but not TAOK2β (data not shown). These results suggest that TAOK2α is likely to be the TAOK2 isoform most important for neuronal differentiation. We therefore focused our subsequent studies on TAOK2α.

TAOK2 affects differentiation in cultured cortical neurons

The remodeling of the actin-based cytoskeleton is an important regulatory step in axon and dendrite formation. As it has been shown that TAOK2 modulates the organization of the actin cytoskeleton in non-neuronal cells, we found TAOK2 expression to be concentrated in actin-rich structures, we asked whether TAOK2 loss of function and gain of function affects neuronal differentiation. For this, we designed three specific short hairpin RNAs (shRNAs) targeting different coding sequences of Taok2 to acutely knock down the expression of TAOK2. We confirmed the specificity of our shRNA constructs with respect to their ability to downregulate endogenous neuronal TAOK2 in cortical neurons at E17 from embryos that had been transfected by in utero electroporation at E15 with constructs expressing Taok2 shRNA or control shRNA and expressing membrane-bound GFP (F-GFP). Neurons were cultured for 48 h before being processed for immunocytochemistry using antibodies to neuronal TAOK2 in cortical neurons at E17 from embryos that had been transfected by in utero electroporation at E15 with constructs expressing Taok2 shRNA or control shRNA and expressing membrane-bound GFP (F-GFP). Neurons were cultured for 48 h before being processed for immunocytochemistry using antibodies to

compared with those in control-transfected neurons (Fig. 2a,c). In addition, TAOK2 downregulation decreased the number of neurites per neuron (Fig. 2a,d) and the number of secondary branches per cell (Fig. 2a,e). TAOK2 autophosphorylation is known to activate TAOK2 (refs. 10,13); accordingly, the overexpression of TAOK2 increased phosphorylated, active TAOK2 (Supplementary Fig. 1g,h). TAOK2 overexpression in cultured cortical neurons increased the number of primary, but not secondary, neurites compared with those in control neurons (Fig. 2a,d).

In addition, we analyzed whether TAOK2 downregulation or overexpression affects polarization in cultured neurons. We found that TAOK2 downregulation impaired axon formation (Supplementary Fig. 2a). Of note, TAOK2 overexpression did not affect the number of polarized (axon-bearing) neurons (Supplementary Fig. 2a), but it did increase the proportion of neurons that elaborated multiple axons (Supplementary Fig. 2b,c). To determine whether these phenotypes resulted specifically from the downregulation of TAOK2, we coexpressed Taok2 shRNA 1 with a shRNA-resistant version of TAOK2 cDNA (human TAOK2 cDNA, TAOK2), which rescued the Taok2 shRNA 1 phenotypes. This molecular replacement experiment further demonstrated the specificity of Taok2 shRNA 1 treatment (Fig. 2a–e and Supplementary Fig. 2a). Together, these data demonstrate that TAOK2 is critical for the morphological differentiation of cultured cortical neurons.

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TAOK2 affects basal dendrite formation and axon elongation

We then sought to determine the effect of TAOK2 on the post-migratory differentiation of cortical neurons in vivo. We electroporated E15 mouse embryos in utero with Taok2 shRNA, control shRNA or rat Taok2 cDNA (rTaok2) plasmids together with an F-GFP construct. Mice were killed at P7 and the dendritic morphology of neurons evaluated. Both the knockdown and overexpression of TAOK2 disrupted cortical neuronal differentiation in vivo. In layer II–III of the in utero electroporated brains, the Taok2 RNA–transfected neurons had significantly fewer primary dendrites as compared to controls (Fig. 3a,b). TAOK2 overexpression, by contrast, increased the number of primary dendrites (Fig. 3a,b). Sholl analysis of dendritic arbors from shRNA-transfected neurons revealed that TAOK2 downregulation produced less complex basal dendritic arbors than control shRNA transfection. Taok2 shRNA–transfected neurons had significantly fewer dendritic processes with intersections between 15 μm and 55 μm from the cell soma (Fig. 3c). In contrast, TAOK2 overexpression increased the complexity of the dendritic arbor compared to that in control-transfected neurons (Fig. 3c). Notably, however, Sholl analysis demonstrated that TAOK2 downregulation or TAOK2 overexpression did not affect the apical dendrites of transfected neurons (Fig. 3d and Supplementary Fig. 3a).

To determine whether the basal dendrite phenotype was exclusive to upper-layer neurons or was a more general feature of pyramidal neurons in the different cortical layers, we knocked down TAOK2 expression in neurons of a deeper layer (layer V) by in utero electroporating E15 embryos with Taok2 shRNA or control shRNA plasmids together with an F-GFP construct, then evaluated the dendritic morphology of transfected neurons in layer V at P4. TAOK2 downregulation using either shRNA 1 or 2 also impaired the basal dendrite complexity of layer V pyramidal neurons (Supplementary Fig. 3b,c), without affecting apical dendrite complexity (Supplementary Fig. 3d). These findings demonstrate that TAOK2 loss of function or gain of function impairs pyramidal neuron basal dendrite formation in the developing neocortex.

We also examined the contribution of TAOK2 to axon elongation during brain development. We performed sequential in utero transfection as previously reported33. mCherry fluorescent protein–expressing and control shRNA constructs were introduced into E15 mouse cortex, followed by the introduction of Venus-expressing and Taok2 shRNA the constructs into the contralateral hemisphere. Callosal axons from transfected neurons crossing into the midline were examined at P7. TAOK2 downregulation impaired axon elongation, and axons were absent in the midline (Fig. 3e,f). By contrast, TAOK2 overexpression caused some transfected axons to deviate from the axonal tract (Supplementary Fig. 3e). These results strongly support action of TAOK2 in axon elongation in the mouse in vivo, as was reported in Drosophila19.
TAOK2 interacts with Neuropilin 1 to modulate differentiation

Previous studies have shown that the Sema3A–Nrp1/PlexinA4 signaling cascade controls basal dendritic arborization. Nrp1Sema mice also develop axonal projection defects in the corpus callosum and the hippocampus. These defects vary from mild phenotypes, in which some axons deviate from the axonal tract, to more severe ones, in which callosal axons defasciculate and do not cross the midline. We hypothesized that TAOK2 may interact with this pathway to modulate neuronal differentiation. To test this idea, we performed coimmunoprecipitation experiments to probe for an interaction between TAOK2 and Nrp1. We coexpressed TAOK2 and Nrp1-mCherry in HEK-293T cells, and we found that TAOK2 interacted with Nrp1-mCherry (Fig. 4a). To investigate whether this complex also forms in vivo, cortices from P0 mice were homogenized and subjected to coimmunoprecipitation using antibodies to Nrp1 and TAOK2. Nrp1 associated with TAOK2 in lysates from P0 mouse cortices (Fig. 4b).

TAOK2 is activated by phosphorylation on Ser181, which resides in the activation loop of the kinase. To determine whether Sema3A/Nrp1 activates TAOK2, we first examined whether active TAOK2 colocalizes with Nrp1. In the mouse E19 cerebral wall, we found that pTAOK2 and Nrp1 colocalized preferentially in the intermediate zone and lower cortical plate, where axons elongate and deeper layer neurons begin to form dendrites (Fig. 4c,d).

Next, we examined whether the Nrp1 ligand Sema3A modulates TAOK2 phosphorylation in cultured primary neurons. Cortical neurons dissociated from E17 mouse embryos and cultured for 48 h were treated with Sema3A (2 µg ml⁻¹) for 30 min, 1 h, 2 h, or 6 h. pTAOK2 immunoreactivity after Sema3A treatment increased significantly between 1 and 6 h (Fig. 4e). Western blot analysis of lysates from neurons after Sema3A treatment revealed that endogenous TAOK2 phosphorylation was also induced after Sema3A treatment (Fig. 4g). Normalized to control: control, 1 ± 0.03-fold; Sema3A, 1.32 ± 0.14-fold increase versus control; n = 3 experiments per duplicate; P = 0.0401 by t test). Finally, we quantified pTAOK2 in cortical extracts from mice homozygous for a knock-in mutation that renders
Figure 4 TAOK2 interacts with Nrp1 to modulate TAOK2 phosphorylation.
(a) Immunoprecipitation (IP) with anti-mCherry demonstrating the interaction of Nrp1 and TAOK2. HEK-293T cells were transfected with Nrp1-mCherry and Myc- TAOK2. Input, 5% of the total protein used for immunoprecipitation. IB, immunoblot. (b) TAOK2 immunoprecipitates with Nrp1 in the developing cortex. Input, 5% of the total protein used for immunoprecipitation; IgG, immunoglobulin G. (c) Nrp1 and pTAOK2 are preferentially expressed in the intermediate zone (IZ) and cortical plate (CP) of the developing cortex. (d) Magnification of white box in c; Nrp1 and pTAOK2 co-localize in the developing cortex. (e-g) Sema3A treatment (2 μg ml−1) increases pTAOK2 immunoreactivity in cultured cortical neurons. (e) Control neuron (top) and a neuron treated with Sema3A for 2 h (bottom). (f) Quantification of pTAOK2 immunoreactivity from the longest neurite of control and Sema3A-treated cultured cortical neurons (control, n = 77 cells, three cultures; Sema3A 30 min, n = 25 cells, two cultures; Sema3A 1 h, n = 32 cells, three cultures; Sema3A 2 h, n = 99 cells, three cultures; Sema3A 6 h, n = 25 cells, three cultures; P < 0.0001 by one-way ANOVA, post hoc Dunnett test **P < 0.01 and NS, not significant). (g) Immunoblot of cultured cortical neuron lysates showing that the 2-h Sema3A treatment increases pTAOK2 immunoreactivity. (h) Immunoblot of cortical lysates from Nrp1Sema−/− heterozygous (+/−) and Nrp1Sema− homozygous (−/−) P7 mice littersmates showing less pTAOK2 immunoreactivity in the −/− mouse than in the +/− littersmate. FAK and tubulin are used as loading controls. Scale bars, 500 μm (c) and 10 μm (d,e).

The endogenous Nrp1 receptor incapable of binding Sema3A (Nrp1Sema−). In agreement with our in vitro experiments, we found that pTAOK2 amounts were lower in the cortical neurons of homozygous Nrp1Sema−/− mice than in those of heterozygous (+/−) littersmates (Fig. 4h; normalized to +/−; for +/−, 1 ± 0.10, n = 9 brains; for −/−, 0.56 ± 0.09, n = 4 brains; P = 0.0310 by t-test). These results suggest that Sema3A signaling activates TAOK2 kinase activity.

TAOK2 and Sema3A modulate JNK activity in cortical neurons
We next sought to elucidate the downstream effectors by which the interaction of TAOK2 with the Sema3A-Nrp1 signaling pathway modulates neuronal differentiation in cortical pyramidal neurons. The JNKs have been shown to be important for many aspects of neuronal differentiation25. TAOK2ε, unlike TAOK2β, stimulates the JNK pathway in cell lines13. TAOK2 overexpression indirectly activates endogenous JNK1 in HEK-293T cells through the preferential phosphorylation of MEK3 and MEK6 (ref. 5). In addition, TAOK2 has been shown to modulate the dual phosphorylation of JNK1 at Thr183 and Tyr185 (refs. 5,27). Sema3A has also been shown to activate the JNK1-c-Jun signaling pathway in cultured dorsal root ganglion neurons28. Phosphorylated JNK1 and 2 can modulate neurite initiation, axon formation and dendritic architecture in cultured neurons29–33 and is required for the maintenance of neuronal microtubules in axons and dendrites in vivo34. The deactivation of JNK1 by BDNF treatment destabilizes microtubules and induces axonal branching35.

On the basis of these findings, we hypothesized that JNK1 may mediate the effect of TAOK2 and Sema3A on neuronal differentiation.

To determine whether TAOK2 modulates JNK1 in cortical neurons, we introduced Taok2 shRNA 1 construct or control plasmids together with an F-GFP construct into E15 mouse embryos via in utero electroporation and isolated cortical neurons at E17. These neurons were cultured for 48 h before being subjected to immunocytochemistry using antibodies to phosphorylated JNK1 (pJNK; pJNK; Thr183 and Tyr185), which represents the active form of JNK1. TAOK2 downregulation decreased pJNK1 immunoreactivity in the longest neurite of shRNA-transfected neurons, compared to that of control-transfected neurons (Fig. 5a,b). Additionally, in Ht22 cells transfected with Taok2 shRNA 1, endogenous pJNK1 (top panel, lower band, 46 kDa) was significantly reduced (Fig. 5c; normalized to control; control, 1 ± 0.17; Taok2 shRNA 1, 0.76 ± 0.09, n = 4 experiments per triplicate; P = 0.0233 by t-test).

As TAOK2 interacts with Nrp1, we asked whether treatment with the Nrp1 ligand Sema3A modulates JNK1 phosphorylation in cultured primary neurons. Since JNK1 phosphorylation is enriched in developed axons31, we used cultured neurons during the initiation of polarization to avoid endogenous JNK1 phosphorylation, which could mask that induced by our Sema3A treatment. Cortical neurons dissociated from E17 mouse embryos and cultured for 48 h were
treated with Sema3A (2 µg ml⁻¹) for 30 min, 2 h, or 6 h. The levels of pJNK1 were measured using immunocytochemistry and western blot analysis. pJNK1 immunoreactivity was significantly increased at 2 h and 6 h following Sema3A treatment in the longest neurite analyzed (the putative axon, 40 µm < neurite length < 150 µm) compared to that in non-treated control cells (Fig. 5d,e). Western blot analysis of lysates harvested from neurons following 6 h of Sema3A treatment revealed that endogenous JNK1 phosphorylation (pJNK, top panel, lower band, 46 kDa) was also induced following Sema3A treatment (Fig. 5f; normalized to control: control = 1 ± 0.06, Sema3A = 1.59 ± 0.19; n = 3 experiments per duplicate; P = 0.0014 by t test). We also examined the levels of pJNK1 in cortical extracts from Nrp1Sema−/− littermate mice. In agreement with our in vitro experiments, we found that pJNK1 levels were reduced in the cortical neurons of homozygous Nrp1Sema−/− mice compared with those in heterozygous littermates (Fig. 5g; normalized to +/−; for +/−, 1 ± 0.11, n = 9 brains; for −/−, 0.54 ± 0.13, n = 4 brains; P = 0.0382 by t test).

Sema3A and TAOK2 may regulate JNK1 by means of a common mechanism; alternatively, they may regulate JNK1 by means of distinct pathways. To differentiate between these possibilities, we evaluated JNK1 activation after Sema3A treatment in the absence or presence of TAOK2. Primary neurons were infected with recombinant lentivirus carrying control or Taok2 shRNA 1 soon after plating. Three days later, we quantified pJNK1 after the addition of exogenous Sema3A (2 µg ml⁻¹). Sema3A increased pJNK1 in control shRNA-treated neurons by about 30−40% (Fig. 5h,i). Notably, Sema3A did not elevate pJNK1 when TAOK2 was knocked down (Fig. 5h,i). These results indicate that TAOK2 is required for Sema3A to induce pJNK1. Together, these data suggest that the interaction of TAOK2 with the Sema3A-Nrp1 signaling complex activates JNK1 phosphorylation to mediate neuronal differentiation in cortical pyramidal neurons.

**Sema3A-Nrp1 modulates basal dendrite formation through TAOK2**

Little is known about the molecular pathways that determine the formation of basal dendrites in pyramidal neurons. Therefore, we decided to analyze whether TAOK2 regulates basal dendrite formation downstream of Sema3A-Nrp1. To this end, we examined whether TAOK2 overexpression is sufficient to restore the defect in dendrite arborization observed in neurons from Nrp1Sema−− mice.14,15 These mice exhibit markedly reduced branching and growth of basal dendrites in layer V cortical neurons.4,13 In contrast, wild-type neurons in situ treated with Sema3A increase their dendritic complexity.17 We overexpressed TAOK2 in neurons dissociated from the E13.5 cortex of Nrp1Sema−− and wild-type mice. At DIV7, Sema3A treatment of Nrp1Sema−− neurons did not restore the reduced dendritic arborization complexity to wild-type levels, confirming the inability of the Nrp1Sema−− receptor to bind Sema3A (Fig. 6a,b). However, TAOK2

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**Figure 5** TAOK2 and Sema3A modulate the activity of JNK1. (a) TAOK2 downregulation (right) decreases pJNK1 immunoreactivity (arrowheads) in cortical neurons compared to that in control neurons (left). Inset: pJNK1 signal in transfected neurons. (b) Quantification of pJNK1 immunoreactivity in the longest neurite of control and TAOK2-downregulated cortical neurons (control, n = 72 cells, two cultures; Taok2 shRNA 1, n = 29 cells, two cultures; ***P < 0.0001 by t test). (c) TAOK2 downregulation decreases JNK1 phosphorylation (46 kDa) in Ht22 cells. (d–f) Sema3A treatment (2 µg ml⁻¹) increases pJNK1 immunoreactivity in cortical neurons. (d) Control neuron (top) and a neuron treated with Sema3A for 2 h (bottom). (e) Quantification of pJNK1 immunoreactivity from the longest neurite of control and Sema3A-treated cortical neurons (control, n = 71 cells, three cultures; Sema3A 30 min, n = 42 cells, two cultures; Sema3A 2 h, n = 97 cells, three cultures; Sema3A 6 h, n = 64 cells, three cultures; P < 0.0001 by one-way ANOVA, post hoc Dunnett test **P < 0.01 and NS, not significant). (f) Immunoblot of cultured cortical neuron lysates shows that Sema3A 6 h treatment increases pJNK1 immunoreactivity (46 kDa) compared to control. (g) Immunoblot of cortex lysates from Nrp1Sema−/− and −/− P7 littermate mice shows less pJNK1 immunoreactivity (46 kDa) in the −/− mouse than in the +/− littermate. (h) Western blotting showing that isolated cortical neurons infected with Taok2 shRNA 1 lentivirus failed to increase pJNK1 in the presence of Sema3A 6 h. (i) Quantification of pJNK1 (46 kDa) immunoreactivity from h (n = 2 experiments per triplicate; P < 0.0001 by one-way ANOVA, post hoc Dunnett test **P < 0.01, *P < 0.05). FAK and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are used as the loading controls; panJNK1, antibody labeling total JNK1. Mean ± s.e.m. Scale bars, 10 µm.
overexpression ameliorated the defective dendritic arborization in Nrp1Sema−− neurons (Fig. 6a,b). Sholl analysis of the dendritic arbors located between 10 µm and 100 µm from the cell soma showed that dendritic arborization was restored to wild-type levels in the Nrp1Sema−− TAOK2-overexpressing neurons (Fig. 6b).

To further confirm the functional interaction of TAOK2 and Nrp1 in vivo, we acutely knocked down the expression of Nrp1 using an shRNA construct and overexpressed TAOK2 simultaneously in layer II–III cortical neurons by in utero electroporation. The Nrp1 shRNA sequence was previously reported to downregulate Nrp1 expression in the cortex[11], and we confirmed that it efficiently knocked down the expression of Nrp1-mCherry in HEK-293T cells (Supplementary Fig. 4a,b). E15 embryos were in utero electroporated with the Nrp1 shRNA plasmid or a control shRNA plasmid, together with F-GFP and rTAOK2 cDNA constructs, and studied at P7. We evaluated the dendritic morphology in neurons of the layer II–III cortex from control and Nrp1 shRNA–expressing brains. The knockdown of Nrp1 impaired the formation of basal dendrites in the upper cortical layers (II–III; Fig. 6c), as has been previously reported[16]. Notably, TAOK2 overexpression ameliorated the basal dendrite arborization deficit in Nrp1 shRNA-expressing neurons (Fig. 6c). TAOK2-overexpressing neurons showed a significantly increased primary basal dendrite number in neurons with Nrp1 knockdown; this number was not significantly different from that of controls (Fig. 6d). However, using Sholl analysis, we found that the basal dendritic branching (secondary branching) was not restored to control levels (Fig. 6e). Sholl analysis also demonstrated that Nrp1 downregulation did not affect the apical dendrites of transfected neurons (Fig. 6f). These results reflect a partial restoration of the formation of basal dendrites following TAOK2 expression in Nrp1-deficient neurons. Our results thus provide evidence that Nrp1 and TAOK2 constitute a pathway that regulates basal dendrite development in pyramidal neurons of the developing cortex.

TAOK2 modulates basal dendrite formation through JNK
Activated JNK1 has been shown to preferentially localize to the longest neurite, presumably the axon, of cultured hippocampal neurons[31]. However, pJNK1 has also been reported to be involved in the regulation of microtubule dynamics in axons, as well as dendrites, in both hippocampus and cortex[34]. Furthermore, JNK1-deficient mice exhibit a progressive loss of microtubules in both axons and dendrites[34]. Until now, it has remained unclear whether pJNK1 modulates microtubules in basal or apical dendrites.

To assess the localization of pJNK1 in cortical pyramidal neurons, we examined its subcellular localization in DIV7 mouse cortical neurons dissociated at E17. We analyzed cells that displayed a pyramidal morphology bearing a thick 'apical' dendrite and several thin 'basal' dendrites. We observed that pJNK1 was enriched in developed axons (Fig. 7a) as previously reported[31]. Of note, we also found that the basal dendrites showed a significant increase in the intensity of pJNK1 immunoreactivity, normalized to tubulin or MAP2 immunoreactivity, compared with that in the apical dendrite (Fig. 7a–c). Thus, the compartmentalization of pJNK1 might function in the preferential elongation of basal versus apical dendrites.

Figure 6 TAOK2 counteracts the dendritic arborization deficit in neurons expressing a deficient receptor for Nrp1 or with Nrp1 downregulation. (a) rTAOK2 overexpression ameliorates the dendritic arborization deficit in primary cortical Nrp1Sema−− neurons. (b) Sholl analysis of the dendritic arbors, showing no statistical differences between wild-type (WT) neurons and Nrp1Sema−− plus rTAOK2-overexpressing cultured neurons at a distance between 10 µm and 100 µm from the cell soma (n = 20 cells per condition, two cultures, ***P < 0.001 by t test). (c) rTAOK2 overexpression reverses the basal dendritic arborization deficit in Nrp1 shRNA-transfected neurons from layer II–III cortex. (d) rTAOK2 overexpression restores the number of primary dendrites in Nrp1 downregulated neurons (control, n = 16 cells, two brains; Nrp1 shRNA, n = 47 cells, three brains; Nrp1 shRNA plus rTAOK2, n = 35 cells, three brains; P < 0.0001, by one-way ANOVA, post hoc Dunnett test **P < 0.01 and NS, not significant). (e) Sholl analysis of the dendritic arbors, showing significant differences between Nrp1 shRNA–expressing neurons and Nrp1 shRNA + rTAOK2–expressing neurons (P < 0.0001 by one-way ANOVA, post hoc Dunnett test **P < 0.01). (f) Sholl analysis of the apical dendrite, showing no differences between Nrp1 downregulation, Nrp1 shRNA + rTAOK2 overexpression, and control conditions. Mean ± s.e.m. Scale bars, 10 µm.
Figure 7 Activated JNK1 ameliorates deficient basal dendrite formation after TAOK2 downregulation. (a) Cultured cortical pyramidal neuron immunolabeled for tubulin (green) and pJNK1 (red). Activated JNK1 is enriched in the axon (arrowheads). (b) The apical dendrite (1) and basal dendrites (2, 3, 4) outlined in a. (c) Ratio of pJNK1 and tubulin or MAP2 fluorescence intensities, showing higher ratios of pJNK1 to tubulin and pJNK1 to MAP2 in basal dendrites than in apical dendrites. Values are normalized to the mean of apical dendrites (tubulin, n = 23 cells, three cultures; MAP2, n = 20 cells, three cultures; ***P = 0.0051, *P = 0.0154 by t test). (d) Upper cortical layer (II-III) transfected neurons, showing underdeveloped basal dendrites after shRNA-mediated knockdown of TAOK2 compared with control-transfected neurons. MKK7-JNK1 overexpression ameliorates the basal dendrite deficit following Taok2 shRNA-mediated downregulation; MKK7-JNK1 overexpression alone increases the number of basal dendrites compared with that in control-transfected cells. (e) The number of primary dendrites increases after MKK7-JNK1 overexpression (control, n = 19 cells, three brains; Taok2 shRNA 1, n = 23 cells, three brains; Taok2 shRNA 1 + MKK7-JNK1, n = 32 cells, three brains; MKK7-JNK1, n = 42 cells, three brains; ***P < 0.0001 by one-way ANOVA, post hoc Dunnett test **P < 0.01 and NS, not significant). (f) Sholl analysis of the dendritic arbor from upper cortical layer transfected neurons (***P < 0.0001 by one-way ANOVA, post hoc Dunnett test **P < 0.01, *P < 0.05). (g) Sholl analysis of apical dendrites from cells in f. Quantifications for control and Taok2 shRNA 1–transfected cells are the same as in Figure 3b–d. Mean ± s.e.m. Scale bars, 10 µm (a,d) and 5 µm (b).

To further investigate the relationship between TAOK2 and JNK1 in basal dendritic arborization, we determined whether the addition of pJNK1 was able to abrogate the basal dendrite phenotypes caused by TAOK2 loss of function. It had previously been shown that fusing MKK7 to JNK1 (MKK7-JNK1) renders JNK1 constitutively active, thus mimicking the activity of pJNK1 (refs. 36,37). We electroporated E15 mouse embryos in utero with Taok2 shRNA 1 or control shRNA together with F-GFP and MKK7-JNK1 cDNA constructs, and analyzed them at P7. TAOK2 downregulation impaired the formation of primary dendrites in layer II-III neurons (Fig. 7d,e). Notably, the number of primary dendrites of transfected neurons was markedly increased when MKK7-JNK1 was expressed alone or with Taok2 shRNA 1 (Fig. 7d,e). Sholl analysis showed significant differences in the dendritic arborization between neurons expressing Taok2 shRNA 1, neurons expressing Taok2 shRNA 1 + MKK7-JNK1 and neurons expressing MKK7-JNK1 at a distance between 15 µm and 55 µm from the cell soma (Fig. 7f). However, neurons expressing Taok2 shRNA and MKK7-JNK1 produced basal dendrites that were not as elaborated as those produced from control shRNA–transfected neurons (Fig. 7f). This result reflects a partial restoration of the branching of basal dendrites in Taok2 shRNA–transfected neurons after the expression of MKK7-JNK1. Notably, MKK7-JNK1 overexpression with or without TAOK2 downregulation did not affect apical dendrite morphology as compared with that of control-transfected neurons (Fig. 7g), further supporting a preferential basal dendrite compartmentalization of pJNK. We also found that the active JNK1 rescued the callosal axon deficit in the midline after TAOK2 downregulation (Supplementary Fig. 5). Together, these experiments provide evidence that TAOK2 and JNK1 modulate neuronal differentiation in cortical pyramidal neurons, and collectively, these results delineate a molecular pathway whereby TAOK2 governs the morphogenesis of pyramidal neurons in the developing cortex.

DISCUSSION
Here we describe a new molecular pathway that preferentially modulates the formation of basal dendrites in cortical pyramidal neurons and that also functions in axonal projection. Extensive work has addressed the actions of several molecules in dendrite arborization (for review, see ref. 38). However, little is known about the molecular differences that may direct the formation of different domains within the same dendritic tree. Our work aimed to understand the mechanisms responsible for the delineation of basal and apical dendrites during pyramidal neuron development in the embryonic cortex.

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Over a decade ago, electron microscopy studies in cultured neurons indicated that axons contain microtubules of uniform polarity, whereas dendrites contain microtubules of mixed polarity. These data support the concept of the molecular homogeneity of the dendritic tree. However, it was recently found that apical dendrites from hippocampal CA1 and cortical layer V pyramidal neurons ubiquitously have polarized microtubule arrays, suggesting that the morphogenesis of dendritic subtypes (for example, basal versus apical dendrites) may rely upon distinct cellular and molecular pathways. Accordingly, it was shown that post-Golgi membrane trafficking is polarized toward the apical dendrites of pyramidal neurons and that the disruption of Golgi polarity produces neurons with symmetric dendritic arbors, which lack a single longest principal, or apical, dendrite.

The Sema3A-Nrp1 signaling cascade is coupled to TAOK2-JNK1

In previous reports, activation of the Nrp1 receptor by Sema3A appeared to regulate neuronal polarization. It was also shown that Sema3A-Nrp1 signaling through PlexinA4 controls the basal dendritic arborization of cortical neurons. As the Nrp1 receptor is likely to be uniformly distributed on all dendritic processes, as well as the axon, one question that arises is how this ubiquitous receptor specifically controls the formation of basal dendrites.

We now show that TAOK2 downregulation specifically impairs the formation of basal dendrites, without affecting apical dendrites. These data strongly suggest that TAOK2 is a mediator of the Sema3A–Nrp1 pathway that sustains basal dendrite formation. Our biochemical analyses demonstrate that TAOK2 and Nrp1 form a complex, and that acute Nrp1 downregulation specifically impairs the formation of the basal dendrite. Importantly, we report that the deficit in basal dendrite formation following Nrp1 downregulation can be counteracted by TAOK2 overexpression.

Our data also show that TAOK2 appears to be uniformly distributed in the growth cones of cultured developing cortical neurons, without any preference for dendritic or axonal neurites. It is possible that our methods could not detect a differential subcellular distribution of TAOK2. Alternatively, the subcellular compartmentalization of molecules that sustain basal dendrite formation may occur downstream of TAOK2.

This work implicates activated JNK1 as an effector of TAOK2 that modulates basal dendrite morphogenesis. It has been shown that pJNK1 is enriched in axons of hippocampal neurons in culture. We also found that pJNK1 was preferentially localized to the longest neurite, presumably the axon, of DIV2 cultured cortical neurons. It has also been reported that pJNK1 is involved in the regulation of microtubule dynamics in both axons and dendrites of the hippocampus and cortex and that JNK1 is essential for dendrite morphogenesis. However, the function of pJNK in the different domains of the dendritic tree has not been clear.

In vivo experiments examining the loss of function of the MAP kinase phosphatase 1 (MKP-1), which deactivates JNK1, show reduced dendritic arborization of cortical pyramidal neurons, as well as impaired axonal branching. In agreement with these findings, we found that pJNK1 was preferentially enriched in the basal dendrites and the axons of cortical pyramidal neurons. Moreover, overexpression of the constitutively active JNK1 counteracted defects in basal dendrite formation and axonal projection after TAOK2 downregulation. Therefore, it is plausible that the subcellular compartmentalization of pJNK1 may explain, at least in part, the specific action of TAOK2 in basal dendrite formation and axonal projection (Supplementary Fig. 6). Our results demonstrate that Sema3A/Nrp1-mediated activation of pJNK1 requires TAOK2 (Fig. 5h,i), which indicates that the three proteins act in the same pathway. Although additional independent pathways could activate JNK, the existence of such pathways does not preclude the importance of the observed TAOK2-mediated activation of JNK1 in regulating dendrite morphogenesis.

TAOK2 and autism spectrum disorders

Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental syndrome for which there is not a clear neurobiological etiology. Although the genetic underpinnings of ASD remain elusive in most cases, a unifying model for ASD has recently been suggested. This model proposes that ASD results from a developmental disconnection of brain regions that are involved in higher-order associations. Emerging literature, which shows functional and anatomical cortical hypoconnectivity in autistic patients, supports this model. The cellular basis for dysfunctional circuits, however, remains poorly understood.

Several genes have been described as contributing to ASD and, and it has been proposed that aberrant synaptic protein synthesis may represent one pathway leading to an autistic phenotype. Recently, a recurrent microdeletion or reciprocal microduplication of chromosome 16p11.2 has been identified that carries substantial susceptibility to autism and appears to account for approximately 1% of cases. One of the genes from the affected region encodes for TAOK2. Therefore, our findings of immature basal dendrite development and axonal projections deficits following TAOK2 downregulation support the hypothesis that underdeveloped neuron morphology contributes to the disconnection of brain regions that may underlie the autistic phenotype.
Association between microdeletion and microduplication at en...
We used primarily Taok2 shRNA 1 and 2 throughout the experiments. The Taok2 shRNA 1–resistant construct (pCMV human TAOK2) is from imaGenes (Berlin, Germany), clone IRS1p097003140D. The sequence for the Nrp1 shRNA is AGAGAAGCCAACCATTA (ref. 16). The shRNAs sequences used in this study were inserted into a piCilencer vector. A pSilencer vector containing a random-sequence hairpin insert was used as a control for the shRNAs. The Venus (pCAGIG) and mCherry (pCAGIG) plasmids were kindly provided by Z. Xie (Boston University). The F-GFP (pCAGIG-GAP 43-GFP) construct was gift from A. Gartner (University of Leuven, Belgium). The Myc-TAOK2 (pCMV rat Taok2) was kindly provided by M. H. Cobb (University of Texas Southwestern Medical Center). The Nrp1-mCherry (plasmid 21934) and MKK7-JNK1 (plasmid 19726) are from Addgene (Cambridge, MA).

**Lentiviral production.** Production of plentilo3.7 Taok2 shRNA. Taok2 shRNA was cloned into the plentilo 3.7 vector (Addgene plasmid 11795) as previously described. Briefly, complimentary 5’ phosphorylated oligonucleotides FC_TAOK2 sh3pfl and FC_TAOK2 sh3pflr with the sequences TGGCGAGAGCACTTGGAATAGAAAATTCAGAGATATTCTTATCAGCTCTCCTGT TTTTC and TCGGAGAGACAGGACCTGAATAAGAAGAATCCTCTGAG AATTCTTATTCAGCTGACCGA, respectively, were annealed, digested with Xhol, and ligated into the plentilo 3.7 vector that had been digested with Hpal and XhoI. Proper insertion and orientation of the sequence downstream of the U6 promoter was confirmed using a sequencing primer with the sequence CAGTGGGAGAAAAGATAGAC.

**Production and titration of virus.** Lentiviral particles were made as previously described. Briefly, HEK-293T cells were plated on 10-cm dishes in 10% FBS DMEM and transfected at 95% confluency with 8 µg pCMV-VSV-G per dish. The culture medium was switched to 30% FBS DMEM 12 h after transfection and viral supernatant was collected 48 and 96 h later. Viral supernatant was filtered through a 0.45-µm cellulose acetate vacuum filter (Corning 431155) and concentrated by ultracentrifugation at 25,000g for 90 min. Viral pellets were resuspended in Dulbecco’s PBS + 0.1% glucose and stored at −20 °C. After blocking in goat serum (Zymed), neurons were incubated with primary antibody for 2 h to overnight at 4 °C. Membranes were washed for 30 min in TBS-T and incubated for 1 h at room temperature with horseradish peroxidase–conjugated secondary antibodies (GE) and then washed for 30 min in TBS-T. Immunoreactivity signals were detected by enhanced chemiluminescence (PerkinElmer).

**Immunoprecipitation.** HEK-293T cell lysates. For transient transfection, HEK-293 cells were cotransfected with equal amounts of overexpression plasmids carrying myc-Taok2 and mCherry-Nrp1 cDNA. The total amount of transfected DNA was between 3.5 and 4.0 µg per 35 mm plate. The cells were allowed to express the constructs for 24 h before lysis and analysis. Transfected cells were washed once with ice-cold PBS and immediately lysed in lysis buffer with protease inhibitors. The Bio-Rad assay kit was used to determine protein concentration. For TAOK2 and Nrp1 immunoprecipitation assays, lysates were incubated with protein A–Sepharose conjugated to anti-mCherry overnight at 4 °C. Lysates containing 0.5 mg of protein were used for each condition. The beads were then washed with RIPA buffer twice to remove nonspecific proteins and then washed five times with lysis buffer before boiling in Laemmli sample buffer. Following SDS-PAGE to separate the proteins, blots were incubated with anti-TAOK2.

**Cortical brain lysates.** Cortices from P0 Swiss Webster mice were dissected and homogenized in 350 µl of sterile-filtered 50 mM Tris-Cl pH 7.4, 0.120 mM NaCl, 0.5% NP-40 containing proteinase inhibitors (Roche) using a 26G syringe, followed by a 15-min centrifugation at 14,000 rpm at 4 °C and collection of the supernatant. Following a 60-min incubation with 1–2 µg of the corresponding antibodies, 20 µl of protein G–Sepharose (GE Healthcare) was added to the lysates and incubated for 45 min at 4 °C. The bound immune complexes were then collected at 8000 rpm for 3 min followed by one wash each in sterile-filtered 50 mM Tris-Cl, pH 7.4, 500 mM NaCl, 1% NP-40; and sterile-filtered 50 mM Tris-Cl pH 7.4, 120 mM NaCl, 0.5% NP-40. Samples were boiled for 5 min at 95 °C, run on a 10% SDS gel and analyzed with the same primary antibodies used for the immunoprecipitation.

**In utero electroperoration.** The Institutional Animal Care and Use Committee of the Massachusetts Institute of Technology approved all experiments. Pregnant Swiss Webster mice were anesthetized by intraperitoneal injections of ketamine 1% / xylazine 2 mg/ml (0.01 ml/g body weight), the uterine horns were exposed, and plasmids mixed with Fast Green (Sigma) were microinjected into the lateral ventricles of embryos. The shRNA plasmid concentration was two to three times that of mCherry, Venus, or F-GFP. Five current pulses (50 ms pulse, 950 ms interval; 35–36 V) were delivered across the heads of the embryos.

**Cortical cultures.** Neurons were transfected by in utero electroperoration at E15 and the transfected cortices were dissected 2 d later. Cortical neurons were isolated in HBSS (Invitrogen) containing papain and DNase at 37 °C (Worthington). Papain was inhibited by the addition of ovomucoid (Worthington). Neurons were plated on poly-d-lysine and laminin pre-coated glass coverslips in Neurobasal/ B27 medium (Invitrogen), maintained in culture for 48 h, and then fixed for immunofluorescence analysis. Neurons treated with Sema3A were dissected from brains harvested at E17 and cultured as above. Sema3A (R&D Systems) was applied 48 h later at a final concentration of 2 µg/ml. Neurons infected with lentiviral particles (infected 3 h after plating) were treated with Sema3A for 6 h after 4 d in culture. Cells were either fixed as described for immunofluorescence studies or lysed with cold RIPA buffer supplemented with phosphatase and protease inhibitors (Roche) for western blot analysis.

**Immunofluorescence.** Dissociated neurons. Neurons were fixed with 4% formaldehyde at 37 °C for 2 min followed by fixation for 3 min in methanol at −20 °C. After blocking in goat serum (Zymed), neurons were incubated with the primary antibodies.

**Cortical sections.** Brains were removed and fixed overnight in 4% formaldehyde and thereafter transferred to 30% sucrose/PBS (4 °C, overnight). Brains were
embedded in OCT compound and sectioned in a cryostat. The 20–30 µm cryo-sections were incubated overnight at 4 °C with the primary antibodies.

Confocal imaging. Images were taken with a Zeiss LSM 510 confocal microscope. z-series images were collected with 1-µm steps. To perform 3D reconstructions on stacks of images of transfected cells, only z sections in the same focal plane as GFP were used for analysis and for producing figures. 3D reconstructions and z-stack analyses were produced using ImageJ software. Brightness and contrast were adjusted.

Quantitative phalloidin fluorescence determination in growth cones. Neurons were cultured for 2DIV after plating and then fixed as described above (see Immunofluorescence). F-actin was visualized by the binding of fluorescently labeled phalloidin. The mean intensity gray value of phalloidin in the growth cone area was measured using ImageJ.

Quantitative immunofluorescence of pTAOK2, pJNK, tubulin and MAP2 in neurites of cultured cortical neurons. Neurons were cultured for 2 DIV and 7 DIV after plating and then fixed as described (see Immunofluorescence). pTAOK2, pJNK, tubulin, and MAP2 were visualized by indirect immunofluorescence. The mean intensity gray value of a line drawn along the neurites was measured using ImageJ.

Sholl analysis. All GFP-positive image stacks from transfected cortical neurons were taken as described (see Confocal imaging). All Sholl analyses use cortices that displayed relatively low transfection efficiencies in order to be able to select and analyze isolated transfected neurons in the cortex. Sholl analysis was performed by drawing concentric circles centered on the cell soma using Adobe Illustrator CS3. The starting radius was 15 µm and the ending radius was 55–100 µm; the interval between consecutive radii was 5 µm. All analyses were performed in a blinded manner.

Statistical analysis. Compiled data are expressed as mean ± s.e.m. We used the two-tailed Student’s t test and one-way ANOVA, with post hoc Tukey or Dunnett tests, for statistical analyses. The P values in the Results are from t tests unless specified otherwise. P < 0.05 was considered statistically significant.

Corrigendum: Autism spectrum disorder susceptibility gene \textit{TAOK2} affects basal dendrite formation in the neocortex

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In the version of this article initially published, the scale bar length for Figure 7d was not given. The bar represents 10 $\mu$m. The error has been corrected in the HTML and PDF versions of the article.