Semaphorin 3A is a chemoattractant for cortical apical dendrites

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The apical dendrites of pyramidal neurons integrate inputs from various cortical layers and are central to information processing. Here we show that the growth of apical dendrites towards the pial surface is regulated by a diffusible chemoattractant present at high levels near the marginal zone. A major component of this signal is semaphorin 3A (Sema3A), which was previously characterized as a chemorepellant for cortical axons. Soluble guanylate cyclase is asymmetrically localized to the developing apical dendrite, and is required for the chemoattractive effect of Sema3A. Thus the asymmetric localization of soluble guanylate cyclase confers distinct Sema3A responses to axons and dendrites. These observations reveal a mechanism by which a single chemotropic signal can pattern both axons and dendrites during development.

Pyramidal neurons convey information from the cerebral cortex to various cortical and subcortical targets[1,2]. These neurons are characterized by an apical dendrite that extends towards the pial surface and integrates information from superficial cortical layers[3]. Despite the functional importance of apical dendrites in cortical information processing, the mechanisms that regulate their orientation are not known. One possibility is that correct orientation involves the directed growth of the dendritic process towards the pial surface. Extracellular signals can influence dendritic growth and branching[4–6], so the directed dendritic growth might also be regulated by extracellular signals. We carried out a series of experiments to determine whether the directed growth of apical dendrites was regulated by signals in the extracellular environment. We were particularly interested in exploring the possibility that chemotropic

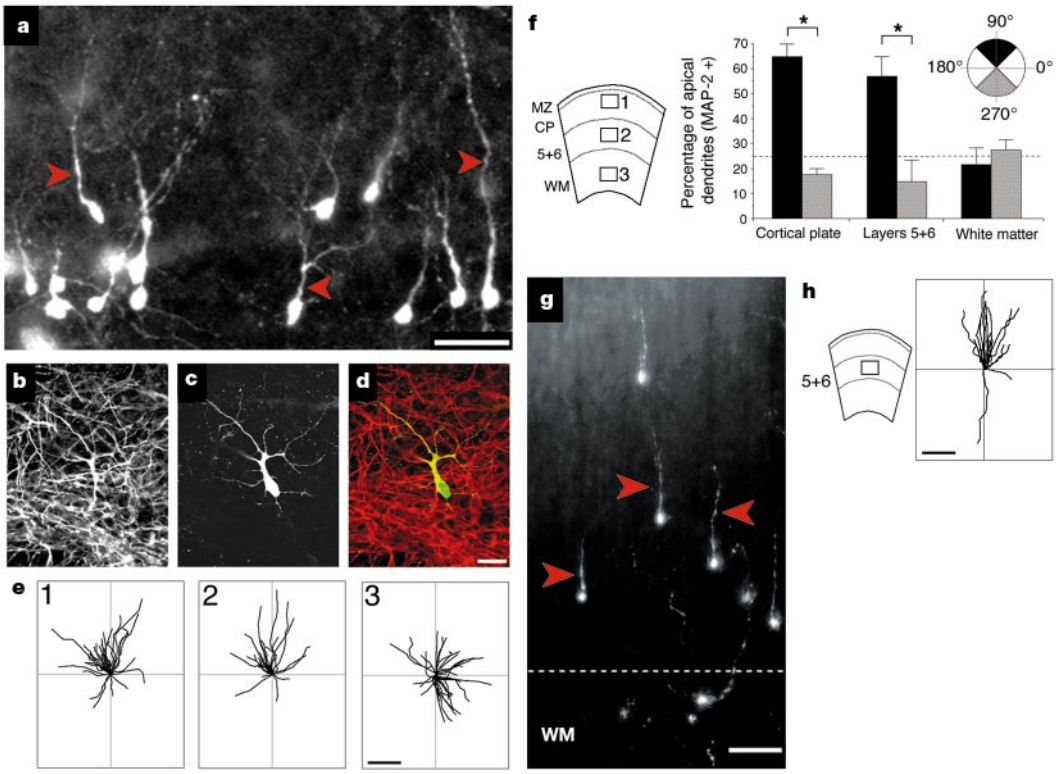

Figure 1 An extracellular signal present in the cerebral wall directs apical dendrite outgrowth towards the marginal zone. **a**, Examples of live E15 GFP neurons growing over a P3 cortical slice, photographed at five days *in vitro*. The apical dendrites (arrowheads) of GFP(+) pyramidal neurons are directed towards the marginal zone of the slice (top). **b**–**d**, Double immunofluorescence directed against MAP-2 (red channel, **b**) and GFP (green channel, **c**) indicates that the morphologically identified apical dendrites are immunopositive for the dendritic protein, MAP-2 (yellow on the merged image, **d**). **e**, Apical dendrite orientation plots of GFP neurons plated over different regions of a representative cortical slice (numbers refer to the inset in **f**: 1, cortical plate (CP); 2, layers 5 and 6; 3, white matter (WM). **f**, Apical dendrite orientation histogram of E15 GFP neurons cultured on P2 cortical slices for five days. In this and other histograms, the horizontal dashed line indicates expected distribution for random orientation. Asterisk, $P < 0.001$ according to a χ^2 test. **g**, E15 GFP neurons transplanted into rat cortex at P1 differentiate into pyramidal neurons with an apical dendrite directed towards the pial surface (arrowheads). **h**, Apical dendrite orientation plot of E15 GFP neurons transplanted into rat P1 cortex, examined at P5. Most apical dendrites are directed towards the pial surface (78.6%; $n = 28$). Scale bars: **a**, 80 μm; **b**–**d**, 25 μm; **e** (1–3) 100 μm; **g**, 60 μm; **h**, 100 μm.

signals, such as those described for axon guidance[7,8], might be involved in the patterning of dendrites.

Regulation of dendrite orientation by a chemoattractant

To study the effect of the cortical environment on dendritic patterning, we modified a previous approach used to evaluate the influence of extracellular signals on axon guidance in the cortex[9]. We dissociated embryonic day 15 (E15) cortical neurons from green fluorescent protein (GFP)-expressing transgenic mice[10] and plated them onto neonatal cortical slices in culture. After five days *in vitro*, we visualized the morphology of plated neurons using anti-GFP immunofluorescence. Morphological analysis indicated that about 70% of GFP-positive neurons differentiated into pyramidal neurons characterized by one major apical dendrite and several basal dendrites (Fig. 1a). The dendritic nature of the apical processes was confirmed by double immunofluorescence against GFP and the dendritic marker microtubule associated protein-2 (MAP-2; Fig. 1b–d). Analysis of apical dendrite orientation of GFP-expressing pyramidal neurons (GFP neurons) at various locations across the cerebral wall revealed that around 65% of the apical dendrites of the GFP neurons growing over the cortical plate (CP; Fig. 1e, left, f) and over layers 5 + 6 (Fig. 1e, middle, f) were oriented towards the pial surface, whereas only about 15% were directed towards the white matter. In contrast, the apical dendrites of GFP neurons growing over the white matter were randomly oriented (Fig. 1e, right, f). Thus an extracellular signal present in the developing cerebral wall is able to orient apical dendrite outgrowth towards the pial surface.

To confirm that such dendrite orientation signals were also present *in vivo*, we transplanted E15 cortical GFP neurons into rat cortex on the day of birth, and analysed the morphology of transplanted cells after five days. Many of the transplanted cells in the cortex differentiated into pyramidal neurons (Fig. 1g) and most of them (78.6%; $n = 28$) extended apical dendrites oriented towards the pial surface (Fig. 1h).

To determine whether the dendrite orientation signal was diffusible, we performed a slice co-culture experiment in which the white matter of one cortical slice was placed next to the marginal zone of a second slice for 2–3 h before E15 GFP neurons were plated over the slices (Fig. 2a–d). Normally apical dendrites of neurons plated over the white matter are oriented randomly (Figs 1e, right, 2c, inset 2). However, when the marginal zone of a second slice was placed next to the white matter of the first slice (Fig. 2c), apical dendrites growing on the white matter of the first slice were directed towards the marginal zone of the second slice (Fig. 2c, inset 1, d). The ability of a slice to influence dendrite orientation in an adjacent slice indicates that the dendrite orientation signal is diffusible. The fact that apical dendrites on either side of the slice culture interface are directed towards the marginal zone indicates that the apical dendrites are attracted by a signal present at high levels near the marginal zone.

Using a similar co-culture configuration we have shown that a diffusible chemorepellant directs cortical axons away from the marginal zone[9]. As the axon typically emerges before the apical dendrite, one interpretation of the co-culture experiment is that the factor released by the marginal zone instructively orients axon outgrowth and that the apical dendrites grow 'by default' in an opposite direction. To test whether the cortical signal could exert its effect on apical dendrite orientation independently of the initial axon trajectory, we performed a modified co-culture experiment. We grew GFP neurons on a cortical slice for one day before a second slice was placed adjacent to the white matter of the first slice (Fig. 2e). As cortical neurons growing over the white matter extend randomly oriented axons within a few hours after plating[9], this experiment would reveal the influence of a second slice on dendritic orientation after initial axon extension. In this experiment the apical dendrites of GFP neurons growing over the white matter of the first slice were predominantly oriented towards the marginal zone of the second slice (Fig. 2e, inset 2, f). Thus the cortical signal can act as an attractant for apical dendrites independently of initial axon trajectory.

To determine whether the growing apical dendrite can respond to changes in the distribution of extracellular guidance cues, we analysed apical dendrites that grew from one slice onto a second

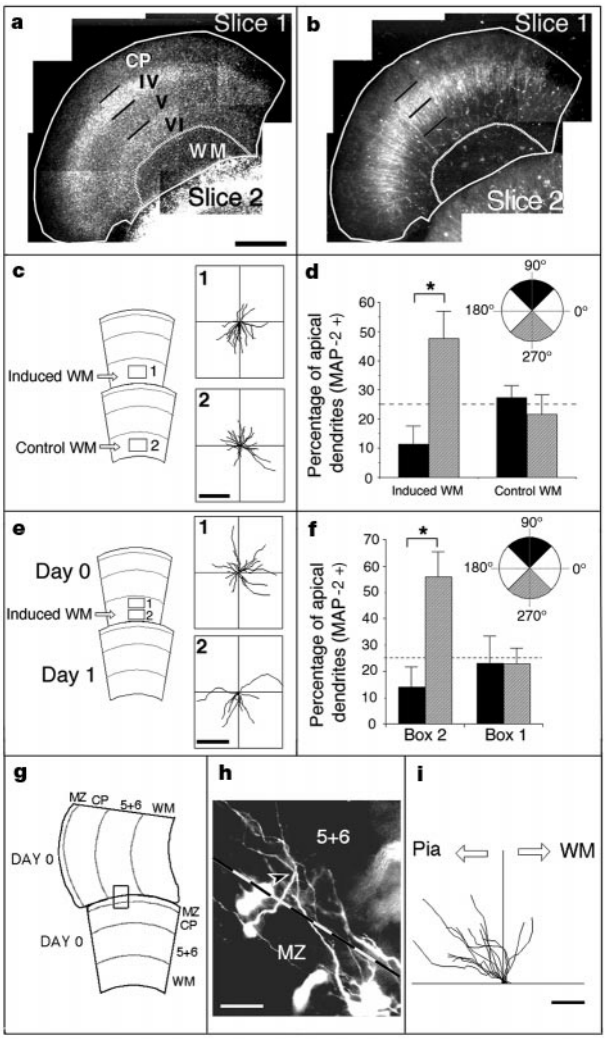

Figure 2 Apical dendrites are attracted by a diffusible signal near the marginal zone. **a**, **b**, The cytoarchitecture of cortical slices is preserved after six days in culture as revealed by nuclear staining using Hoechst 33258 (**a**) and dendritic MAP-2 labelling (**b**). **c**, Apical dendrite orientation plot of E15 GFP neurons cultured for four days over the induced white matter (inset 1) or the control white matter (inset 2) of P3 slices co-cultured as shown. **d**, Apical dendrite orientation histograms for the experiment shown in **c**. **e**, Apical dendrite orientations of GFP neurons cultured over P3 cortical slice in a delayed co-culture experiment where E15 GFP neurons were plated over a P3 slice on the first day (Day 0). Twenty-four hours after plating, a second cortical slice was placed next to the first slice (Day 1). Insets 1 and 2 illustrate the apical dendrite orientation of GFP neurons growing distant from (300–600 μm, inset 1) or close to (0–300 μm, inset 2) the slice–slice interface. **f**, Apical dendrite orientation histogram for the experiment depicted in **e**. **g**–**i**, Turning of apical dendrites towards the pial surface of an orthogonal slice. P3 slices were co-cultured for five days as shown in **g**, with the marginal zone of one slice in direct contact with the lateral aspect of a second slice. The box indicates the position of the GFP neurons shown in **h** whose apical dendrites turn towards the pial surface of the second slice (arrowhead). **i**, Trajectories of apical dendrites ($n = 15$) growing from the marginal zone of one slice into layers 5 and 6 or the cortical plate of a second slice positioned as depicted in **g**. Arrows indicate the direction to the pial surface and the white matter of the second slice. Scale bars: **a**, **b**, 600 μm; **c**, 200 μm; **e**, 200 μm; **h**, 25 μm; **i**, 40 μm.

slice positioned orthogonally (Fig. 2g). The majority (87%; $n = 15$) of apical dendrites that crossed from one slice to the orthogonal slice turned towards the pial surface of the second slice (Fig. 2h, i; average turning angle, 50.3° ± 9.2°). These observations indicate that growth of apical dendrites towards the pial surface is a chemotropic response.

Sema3A is a chemoattractant for apical dendrites

Cortical axons are directed towards the white matter by the diffusible chemorepellant Sema3A[9], which is expressed in the cortical plate during development[12–14,21] (see Supplementary Information). In our analysis of the SEMA3A null mice[16] we noticed that many of the cortical pyramidal neurons had abnormal morphologies[9], raising the possibility that Sema3A may be involved in regulating apical dendrite orientation. Therefore, we labelled cortical projection neurons in wild-type and SEMA3A null mice by injecting fluorescent carbocyanine dyes (DiI or DiA) into the white matter of cortical slices (Fig. 3a inset). In contrast to wild-type neurons, many of the neurons in SEMA3A null mice had identifiable apical dendrites that were not directed towards the pial surface (Fig. 3b–e). Other aspects of dendritic growth and branching were not notably affected in these mice. Thus Sema3A function appears to be necessary for correct orientation of apical dendrites.

If apical dendrite orientation is regulated by a gradient of Sema3A in the cortex, then abolishing that gradient by providing excess Sema3A should disrupt apical dendrite orientation. We cultured E15 GFP neurons on cortical slices for four days in the presence of exogenous recombinant alkaline phosphatase-tagged Sema3A (Sema3A–AP). Whereas incubation of the slices with supernatant from control-transfected 293T cells did not affect the orientation of apical dendrites (Fig. 3f inset 1, g), incubation with supernatant from Sema3A–AP-transfected 293T cells completely disrupted apical dendrite orientation (Fig. 3f inset 2, g).

To determine whether a localized source of Sema3A could direct apical dendrite outgrowth, we placed control or Sema3A-expressing 293T cell aggregates next to the white matter of cortical slices over which we plated E15 GFP neurons. The GFP neurons extended apical dendrites towards the Sema3A-expressing aggregates (Fig. 3h inset 2, i), but were unaffected by control 293T cell aggregates (Fig. 3h inset 1, i). Thus, a localized source of Sema3A is sufficient to attract apical dendrites. Importantly, this effect of Sema3A on apical dendrite orientation is opposite to the chemorepulsive effect of Sema3A on cortical axons[9].

The repulsive effect of Sema3A on axons is mediated by the high-affinity semaphorin receptor Neuropilin-1 (refs 9, 11, 15). To determine whether the directed growth of apical dendrites towards the pial surface was also mediated by Neuropilin-1, we examined the subcellular distribution of Neuropilin-1 using an affinity-purified antibody[9,11]. Neuropilin-1 is present on both axons and dendrites of cortical neurons (Fig. 4a–c). To examine the role of Neuropilin-1 in dendrite orientation we incubated GFP slice overlay cultures with an affinity-purified function-blocking antibody to Neuropilin-1 (anti-Np1)[9,11]. In control cultures about 70% of the dendrites of GFP neurons were oriented towards the pia, and less than 10% were directed towards the white matter (Fig. 4d–h). In anti-Np1-treated slices, only about 45% of the dendrites were directed towards the

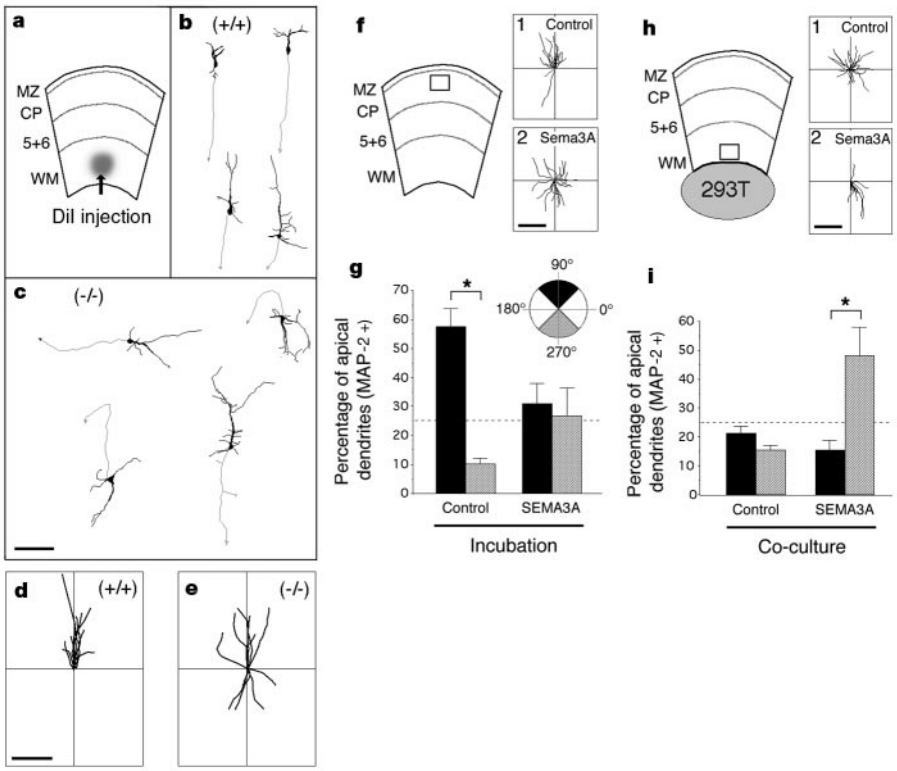

Figure 3 Sema3A is necessary and sufficient for appropriate apical dendrite orientation. **a**, Location of carbocyanine dye injection into the white matter of fixed cortical slices from E20 mice to label projection neurons retrogradely (reconstructed in **b** and **c**). **b**, **c**, Examples of cortical neuron morphologies in the cortical plate (two upper neurons) or in layers 5 and 6 (two bottom neurons) of wild-type (+/+) (**b**) or SEMA3A null (–/–) (**c**) mice. The pia is at the top. **d**, **e**, Apical dendrite orientation plot of cortical projection neurons in E20 wild-type (+/+) (**d**) or SEMA3A null (–/–) mice (**e**). **f**, **g**, Incubation with excess of recombinant Sema3A–AP disrupts the orientation of apical dendrite outgrowth in the cortical plate. **f**, Apical dendrite orientation plot of E15 GFP neurons cultured for four days over the cortical plate of a P3 slice in the presence of control supernatant or supernatant from Sema3A–AP expressing cells (Sema3A–AP concentration 8 nM). **g**, Apical dendrite orientation histogram for the experiment shown in **f**. **h**, **i**, A localized source of Sema3A is sufficient to attract apical dendrites. **h**, Apical dendrite orientation plot of E15 GFP neurons cultured for four days over the white matter of a P3 slice next to an aggregate of 293T cells transfected with a control vector (inset 1) or a plasmid expressing Sema3A (inset 2). **i**, Apical dendrite orientation histogram for the experiment depicted in **h**. Scale bars: **b**, **c**, 100 μm; **d**, **e**, 120 μm; **f**, **h**, 200 μm.

pia, and over 20% were directed towards the white matter (Fig. 4h). The marked reduction in the bias of apical dendrites to grow towards the pial surface indicates that the response of the dendrites to the cortical guidance signal is mediated at least in part by Neuropilin-1. The residual bias in the growth of apical dendrites towards the pial surface might simply reflect incomplete blocking over a four-day culture period, or might reflect a component of the dendritic response that is Neuropilin-1-independent. In either case, the ability of anti-Np1 to attenuate the dendrite orientation response is consistent with the experiments that show that this process is regulated by Sema3A.

Role of soluble guanylate cyclase in dendrite patterning

How could Sema3A act as a chemorepellant for the axon and a chemoattractant for the apical dendrite of the same neuron? One possibility is that axons and dendrites express different Sema3A receptors, but the Neuropilin-1 localization experiments indicate that at least one critical component of the receptor complex is present on both axons and dendrites. Another possibility is that a signal transduction component necessary for Sema3A signalling may be asymmetrically localized to the axon or apical dendrite. In that case, the signal transduction component must specify the polarity of the cellular response to Sema3A. Asymmetric localization of soluble guanylate cyclase (SGC) could in principle serve such a function, as the chemorepulsive response of *Xenopus* spinal cord axons to Sema3A can be converted into chemoattraction by

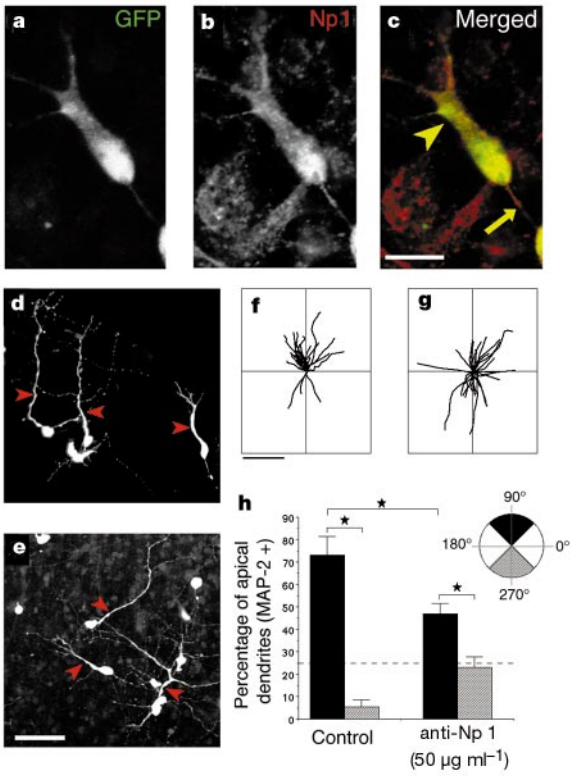

Figure 4 The Sema3A receptor Neuropilin-1 is present on apical dendrites, and is required for correct apical dendrite orientation. **a**–**c**, Double immunofluorescence against GFP (**a**) and Neuropilin-1 (Np1) (**b**) on a GFP neuron cultured for one day over the cortical plate of a P3 cortical slice shows that Neuropilin-1 is present on both the axon (arrow in **c**) and the apical dendrite (arrowhead in **c**) of developing cortical neurons. In the merged image (**c**), GFP is in green and Neuropilin-1 is in red. **d**, **e**, Examples of GFP neurons growing over the cortical plate of a P3 cortical slice for four days in the absence (**d**) or presence (**e**) of affinity-purified anti-Np1. **f**, **g**, Apical dendrite orientation plots of E15 GFP neurons growing over the cortical plate of postnatal cortical slices in the absence (**f**) or presence (**g**) of anti-Np1. **h**, Apical dendrite orientation histogram for the experiment described in **f**, **g**. Scale bars: **a**–**c**, 15 μm; **d**, **e**, 50 μm; **f**, **g**, 200 μm.

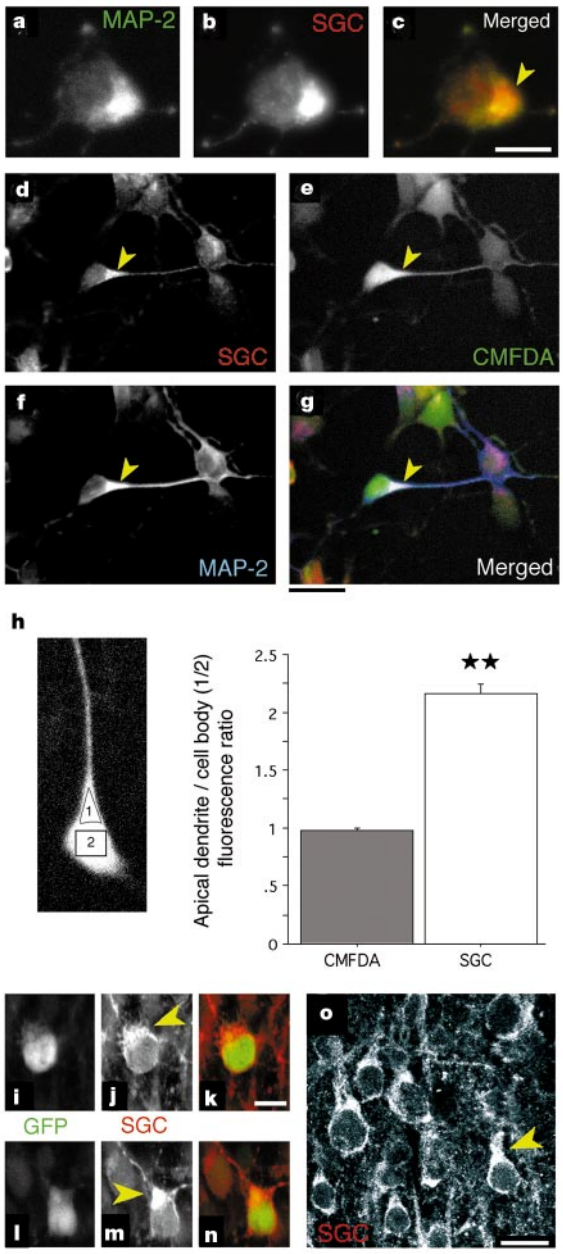

Figure 5 Soluble guanylate cyclase is asymmetrically localized to the apical dendrite in developing pyramidal neurons. **a**–**c**, MAP-2 (**a**) and SGC (**b**) immunofluorescence in cortical neurons 3 h after plating. The merged image (**c**) shows that SGC (red) co-localizes with MAP-2 (green) in the region of the cell where the apical dendrite is generated (arrowhead in **c**). **d**–**g**, Localization of SGC in pyramidal neurons in culture 48 h after plating. Cells were labelled with antibodies to SGC (**d**) and MAP-2 (**f**), and the distribution of the cytoplasm was visualized by loading with CMFDA (**e**). In the merged image (**g**), SGC is in red, MAP-2 is in blue and CMFDA is in green. **h**, Quantification of relative SGC immunofluorescence in apical dendrites and cell bodies of pyramidal neurons. Average fluorescence signals for CMFDA and SGC were quantified in regions 1 and 2, which represent the apical dendrite and cell body, respectively, and plotted as a fluorescence ratio. Double asterisk, $P < 0.01$ (Wilcoxon rank non-parametric test for paired values). **i**–**n**, SGC localization in two morphologically undifferentiated E15 GFP neurons (**i**–**k** and **l**–**n**) that were cultured for 12 h over the cortical plate of a P3 slice. SGC (arrows in **j** and **m**) is asymmetrically localized in a cap oriented towards the pial surface (arrows) before an apical dendrite is clearly apparent. **o**, Subcellular distribution of SGC in layer 5 neurons of P3 cortex showing its enrichment in the apical dendrites of large pyramidal neurons. Scale bars: **a**–**c**, 10 μm; **d**–**g**, 30 μm; **i**–**n**, 10 μm; **o**, 15 μm.

artificially elevating intracellular cyclic GMP (cGMP) levels[17].

To explore the possibility that SGC might be asymmetrically localized in cortical neurons, we used immunofluorescence to determine the subcellular distribution of SGC. In dissociated neurons beginning to undergo morphological differentiation in culture, SGC was localized to the pole of the cell where the apical dendrite is generated (Fig. 5a–c). Strikingly, a cap of SGC co-localized with MAP-2 was apparent even before the neurons had extended a morphologically distinguishable apical dendrite (Fig. 5a–c), indicating that SGC might be important in the early patterning of the apical dendrite. To confirm that the apparent asymmetric localization of SGC was not simply due to increased cytoplasm at one pole of the cell, we triple-labelled cortical neurons with anti-SGC, anti-MAP-2 and the vital dye CMFDA (a cytoplasmic marker). Whereas CMFDA immunofluorescence was uniformly distributed over the cell body and apical dendrite (Fig. 5e, g, h), SGC and MAP-2 immunofluorescence was localized to the apical dendrite (Fig. 5d, f–h). SGC immunofluorescence in undifferentiated GFP neurons 12 h after being plated over the cortical plate of a postnatal day 2 (P2) slice showed asymmetric caps of SGC oriented towards the pial surface before the formation of apical dendrites (Fig. 5i–n). We also found that SGC was localized to the base and proximal segment of apical dendrites of layer 5 pyramidal neurons in P3 cortical slices, indicating that the asymmetric subcellular distribution of SGC is characteristic of cortical neurons *in vivo* (Fig. 5o).

The localization of SGC to the developing apical dendrite together with evidence implicating cGMP in Sema3A signalling suggested that SGC might be important for the oriented growth of apical dendrites towards the pial surface. Therefore, we performed slice overlay assays in the presence of ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), a specific inhibitor of SGC[18]. Whereas apical dendrite orientation was unaffected in vehicle (DMSO)-treated slices (Fig. 6a, f, k), ODQ treatment (1 μM) completely disrupted oriented dendritic outgrowth (Fig. 6b, g, k). The differentiation of the apical dendrite, however, was not affected by ODQ treatment (Fig. 6b), indicating that SGC is not required for dendritogenesis but is specifically required for oriented dendritic outgrowth. To determine whether the effects of SGC were mediated by the downstream effector cGMP-dependent protein kinase (PKG), we treated slice overlay cultures with Rp-8-pCPT-cGMPS (8-(4-Chlorophenylthio-)guanosine-3′-5′ cyclic monophosphoro-

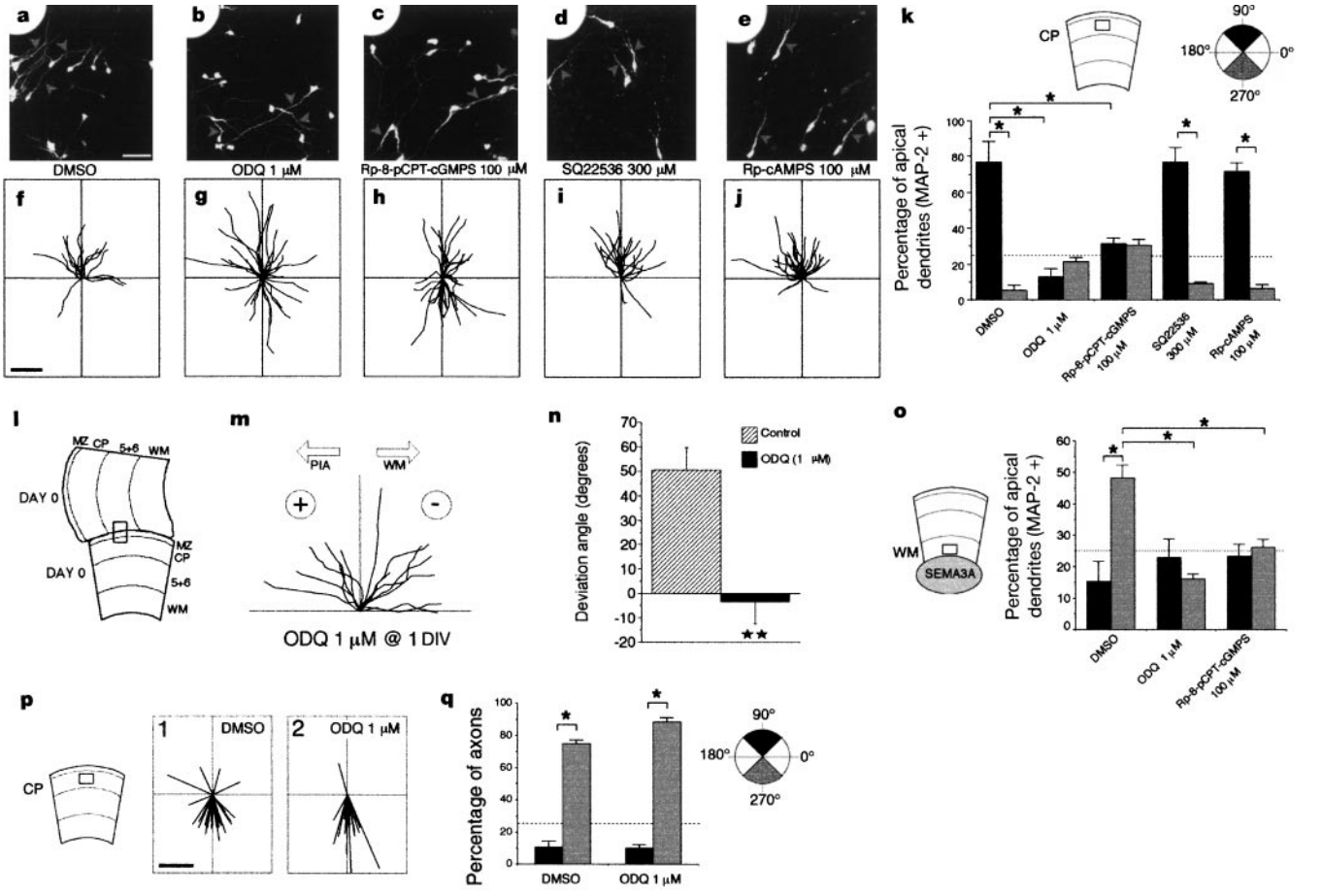

Figure 6 Inhibition of soluble guanylate cyclase and protein kinase G disrupts apical dendrite orientation. **a**–**j**, Photomicrographs and apical dendrite orientation plots of E15 GFP neurons cultured over the cortical plate of P2 cortical slices for three days in the presence of DMSO (**a**, **f**; control vehicle at a 1:1,000 dilution), 1 μM ODQ, an inhibitor of SGC (**b**, **g**), 100 μM Rp-8-pCPT-cGMPS, an inhibitor of all protein kinase G isoforms (**c**, **h**), 300 μM SQ22536, an inhibitor of all isoforms of adenylate cyclase (**d**, **i**), 100 μM Rp-cAMPs, an inhibitor of protein kinase A (**e**, **j**). ODQ- and Rp-8-pCPT-cGMPS-treated cells extend apical dendrites (arrowheads), but they are randomly oriented. **k**, Apical dendrite orientation histogram for the experiment shown in **a**–**j**. **l**–**n**, Effect of ODQ on the ability of apical dendrites to reorient in response to an orthogonally positioned slice (shown in **l**). **m**, Trajectories of apical dendrites of GFP neurons plated in the lower slice (**l**) that cross into the orthogonal slice exposed to 1 μM ODQ after one day *in vitro* (DIV). **n**, Comparison of average turning angles of apical dendrites (measured at 5 DIV) in the absence or presence of ODQ. Positive deviation angles represent deflections towards the pial surface of the second slice. Double asterisk, $P < 0.01$ (Mann-Whitney non-parametric test). **o**, Apical dendrite orientation histogram of GFP neurons cultured for four days over the white matter of P2 slices placed next to an aggregate of Sema3A-expressing 293T cells in the presence of indicated pharmacological agents. **p**, **q**, Inhibition of SGC activity by ODQ does not affect directed axon outgrowth towards the white matter. **p**, Axon orientation plots of cortical neurons cultured on P2 slices for 3 h in the presence of control vehicle (DMSO, 1:1,000; inset 1) or 1 μM ODQ (inset 2). **q**, Axon orientation histogram for the experiment described in **p**. Scale bars: **a**–**e**, 60 μm; **m**, 150 μm; **p**, 40 μm.

thioate, Rp isomer; 100 μM), a specific inhibitor of all three PKG isoforms (Iα, Iβ and II)[19]. As with SGC, inhibition of PKG disrupted the oriented growth of apical dendrites towards the pial surface (Fig. 6c, h, k). Apical dendrite orientation appears to involve the cGMP-dependent pathway specifically, as incubation with a general adenylate cyclase inhibitor, SQ22586 (9-(tetrahydro-2′-furyl)-adenine; 300 μM)[20] or with the protein kinase A inhibitor Rp-cAMPS (adenosine 3′-5′-cyclic monophosphorothioate, Rp-isomer; 100 μM) did not affect apical dendrite orientation (Fig. 6d, i, k and Fig. 6e, j, k, respectively). In further support of a role for SGC in dendritic guidance, we found that the ability of apical dendrites to turn towards the pial surface of an orthogonal cortical slice was completely disrupted in ODQ-treated cultures (Fig. 6l–n).

To determine whether the chemoattractive effect of Sema3A is also regulated by SGC- and PKG-dependent mechanisms, we performed slice-overlay assays with Sema3A-expressing 293T cells placed next to the white matter in the presence of various pharmacological inhibitors. Control treatments did not affect the growth of apical dendrites towards the Sema3A-expressing cells, but inhibition of SGC (1 μM ODQ) or PKG (100 μM Rp-8-pCPT-cGMPS) completely disrupted the oriented outgrowth of the apical dendrites towards the Sema3A source (Fig. 6o). Thus the chemoattractive effect of Sema3A on apical dendrites requires SGC and PKG function.

Finally, we examined the effects of inhibiting SGC on directed axon outgrowth to determine whether cGMP signalling is specifically involved in apical dendrite orientation. ODQ treatment did not affect the directed growth of cortical axons towards the white matter (Fig. 6p, q). Thus, inhibition of SGC does not have a general disruptive effect on all chemotropic responses, and specifically affects the oriented growth of apical dendrites.

Discussion

These experiments provide the first evidence that different compartments of a neuron can respond in opposite ways to the same chemotropic signal: apical dendrites of pyramidal neurons grow towards a source of Sema3A, whereas their axons are repelled by the same ligand. Sema3A is expressed in the cortex, but the pattern of expression changes during development, so it may be involved in regulating several developmental events. At the onset of cortical neurogenesis, Sema3A is expressed primarily within the ventricular zone, which is consistent with the proposal that Sema3A may prevent early cortical efferents (such as those from subplate neurons) from entering the ventricular zone[21,22]. At late embryonic and early postnatal stages, Sema3A is expressed within the cortical plate[12–14,21] (see Supplementary Information). Although Sema3A appears to be expressed in all cortical layers at these stages, the higher density of neurons in the superficial cortical plate may allow a gradient of Sema3A to be established. We propose that a polarized cellular response to Sema3A causes cortical neurons to extend efferent axons towards the white matter and apical dendrites towards the pial surface.

The differential response of axons and dendrites to Sema3A appears to be mediated by the asymmetric localization of SGC to the developing apical dendrite. Our results indicate that localization of SGC to the dendrite confers upon it a chemoattractive response, which is consistent with the observation that elevation of cGMP levels can allow Sema3A to act as a chemoattractant[17]. The generation of a polarized guidance response by asymmetric localization of an intracellular signalling component is similar to a mechanism that has been proposed for chemotaxis in *Dictyostelium* and mammalian leukocytes[23,24]. In these cells, the receptor for a chemotactic molecule is distributed uniformly over the cell surface and a chemotactic response is triggered by the asymmetric distribution of one component of the signal transduction machinery. Asymmetric localization of signal transduction components may therefore be a general mechanism for generating polarized cellular responses[25,26].

Little is known about the mechanism by which cGMP signalling might modulate responses to Sema3A, but our pharmacological experiments indicate that PKG is likely to be an important downstream effector in this process. Also inhibition of SGC or PKG disrupts oriented dendritic growth but does not cause the dendrites to be repelled by Sema3A. Thus, SGC does not simply act as a polarity switch, and repulsion of axons by Sema3A must involve mechanisms that are not functional in dendrites. The polarity of chemotropic responses to Netrin-1 can be influenced by differences in Netrin-1 receptor complexes[27,28], indicating that differences in receptor complexes between axons and dendrites might also contribute to the differential responses to Sema3A. The details of the intracellular signalling mechanisms that generate diverse Sema3A responses remain to be understood. Our results provide compelling evidence that the patterning of dendrites can be regulated by chemotropic cues, and that the differential response of axons and dendrites to such cues can be an important determinant of neuronal morphology. □

Methods

Slice overlay assay

Early postnatal rat cortical slices (P1–P3) were cultured using an air-interface protocol[9]. Embryonic cortical neurons were isolated at E15 from timed pregnant transgenic mice expressing the enhanced green fluorescent protein (EGFP) under the control of a β-actin promoter[10]. After gentle trypsin-based dissociation of the cortex, GFP(+) cells were cultured on top of cortical slices for 4–6 days.

Immunofluorescence

Slices were fixed overnight with 4% paraformaldehyde and processed for immunofluorescence using a protocol where the three major steps (blocking of non-specific antigenic sites in 3% BSA plus 0.3% Triton X-100, incubation with primary antibody and incubation with fluorescent secondary antibodies) were performed overnight at 4 °C. The primary antibodies used were: rabbit polyclonal anti-GFP (1:3,000; Molecular Probes), mouse monoclonal anti-GFP (1:2,000, Molecular Probes), mouse monoclonal anti-MAP-2 (1:3,000, Sigma), rabbit polyclonal anti-β1-subunit of soluble guanylate cyclase (1:2,000, Calbiochem), and a rabbit polyclonal, affinity-purified, anti-Neuropilin-1 (1:600, A. Kolodkin and D. Ginty). The fluorescent secondary antibodies were goat anti-rabbit or goat anti-mouse Oregon Green-conjugated IgG (1:800, Molecular Probes), goat anti-rabbit or goat anti-mouse Cy3-conjugated IgG and donkey anti-mouse Cy5-conjugated IgG (1:800, Jackson ImmunoResearch). The secondary antibody incubations were performed in the presence of 3% goat or normal donkey serum. Cell nuclei were stained for 2 h using Hoechst 33258 (1:2,000, Molecular Probes).

Scoring of apical dendrite orientation

Apical dendrites were imaged and scored using a Nikon TE300 Eclipse microscope equipped with 20× and 40× ELWD objectives. GFP(+) neurons were imaged and analysed using IPLab Spectrum 3.2 (Scanalytics). To generate apical dendrite orientation plots, the trajectories of individual apical dendrites were plotted with the pial surface at the top (90°) in each plot (for example, Fig. 1e). Apical dendrites were scored as being directed towards the pia (45°–135°) or towards the ventricle (225°–315°) based on their orientation. To generate apical dendrite orientation histograms, the orientation of MAP-2(+) apical dendrites of GFP neurons was scored for a minimum of 150 neurons from six to twelve slices for each experimental condition.

In vivo transplantation of E15 GFP(+) cells

10,000–20,000 freshly isolated E15 GFP(+) cortical cells were transplanted using a Hamilton syringe into the cortex of hypothermia-anaesthetized rats at P1. After five days survival, pups were perfused using 4% paraformaldehyde, sectioned at 100 μm using a vibratome, and processed for immunofluorescence directed against MAP-2 and GFP.

SEMA3A knockout mice

A colony of SEMA3A knockout mice[16] was maintained using heterozygous animal breeding. Experimental litters were perfused at E20 and treated blind with regard to the genotype of the embryos (analysed by PCR using tail DNA).

Tracing experiments

The morphology of cortical projection neurons was reconstructed using fluorescent carbocyanine dye (DiI or DiA, Molecular Probes) injected into the white matter of fixed coronal slices from E20 wild-type ($n = 11$) and SEMA3A null ($n = 8$) mice. After 2–3 weeks of diffusion, 80–100 μm thick vibratome sections were studied using confocal microscopy.

Microscopy

Reconstruction of DiI- and DiA-labelled neurons (Fig. 3), and immunofluorescent localization of proteins (Figs 4, 5), was carried out using an LSM 510 Zeiss confocal microscope equipped with Helium-Neon and Argon lasers.

CMFDA labelling

Dissociated E15 cortical cells in suspension were centrifuged once and incubated for 30 min at 37 °C in culture medium containing 10 μM CellTracker Green CMFDA (5-chloromethylfluorescein diacetate, Molecular Probes). Cells were then incubated for 30 min in CMFDA-free culture medium at 37 °C. During this second incubation CMFDA undergoes a glutathione *S*-transferase-mediated reaction to produce membrane-impermeant glutathione–fluorescent dye adducts that can be detected by direct fluorescence using a 488-nm excitation line.

Production of 293T cell aggregates

293T cells (ATCC) were transfected using Lipofectamine+ (Gibco) according to a standard protocol[29]. Sema3A-expressing 293T cells were produced by co-transfection of a plasmid expressing Sema3A protein tagged wiht alkaline phosphatase[9] and an EGFP-expressing plasmid (Clontech) for a better visualization of the aggregate-slice boundary. Control-transfected 293T cells were transfected only with a plasmid expressing EGFP.

Production of recombinant Sema3A–AP protein

293T cells cultured in serum-free medium (Nephrigen-Celox) were transfected as described above, and after two days supernatant of control or Sema3A-AP-transfected 293T cells was collected and concentrated using a centrifugation-based dialysis device (Centriprep, Millipore). The final concentration of Sema3A–AP was determined using an alkaline phosphatase enzymatic assay[30].

Scoring of axon orientation

Cortical neurons were isolated from E18 rat cortex, dissociated, labelled with DiI and plated over P2 rat cortical slices[9]. They were co-cultured in the presence of control vehicle (DMSO, 1:1,000) or 1 μM ODQ and then axon orientation was determined[9].

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