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Slit Promotes Branching and Elongation of Neurites of Interneurons But Not Projection Neurons from the Developing Telencephalon

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Proper neuronal migration and establishment of circuitry are key processes for laying down the functional network of cortical neurons. A variety of environmental guidance cues, attractive or repulsive, have been shown to guide cell migration and axon arborization. One of these, Slit, appears to possess contrarian properties; it can either inhibit axon outgrowth or promote branching and elongation. The object of the present study was to assess the effect of Slit on MGE and neocortical neurons in culture and in the developing ventricle. When cocultured with a Slit source, E13.5 MGE explants displayed inhibited neurite outgrowth while GABA neuron dispersion away from Slit was increased. Similar inhibition of neurite outgrowth was seen in dissociated cells from E13.5 MGE, these cells were identified to be interneurons based upon their GABA staining. In contrast, E13.5 interneurons, after culture for another 5 days, were responsive to Slit by neurite branching and elongation. Projection neurons, identified by lack of GABA staining, did not respond to Slit, either by branching or elongation. Furthermore, GABA interneurons but not pyramidal neurons, appeared to avoid neocortical areas close to an implanted source of Slit in the ventricular wall. These results lead us to suggest that interneurons but not projection neurons are responsive to the chemorepellant effect of Slit. However, more mature interneurons appear to respond to Slit by neurite arborization. These results demonstrate a selective response to Slit by GABAergic neurons during neocortical development.

Key Words: **slit; ganglion eminence; neocortex; axon branching; GABA; projection neuron.**

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INTRODUCTION

The two principal neuronal types of the cerebral neocortex are the excitatory projection neurons, which project to distant targets, and the inhibitory interneurons, which maintain local circuits. Projection neurons make up the vast majority with over 80% of the population and they utilise glutamate as their primary neurotransmitter (Conti *et al.*, 1987). Inhibitory interneurons comprise no more than 20%, and although they are a diverse group with different cell body shapes, they typically contain the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Hendry *et al.*, 1987; Meinecke and Peters, 1987). These two fundamentally distinct neuronal populations arise from diverse embryological origins. Glutamatergic neurons arise predominantly from the neocortical ventricular neuroepithelium; from this structure, they migrate radially to invade the cortical plate into layer specific arrangements (see review, Parnavelas, 2000). GABAergic interneurons appear to emerge from the subpallial telencephalon where the dome-shaped medial (MGE) and lateral ganglion eminences (LGE) are now recognized to be the principal sources of cortical interneurons (Anderson *et al.*, 1997; Parnavelas *et al.*, 2000; Anderson *et al.*, 2001; Marin *et al.*, 2001).

Recent studies have identified the subpallial MGE and LGE to be the main sources of neopallial interneurons with quite distinct migratory routes and possibly under differential regulation of genes (e.g., *Nkx2.1, Mash1, Dlx1/2*) that control basal ganglia patterning

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(Lavdas *et al.*, 1999; Anderson *et al.*, 2001). The MGE is the major contributor of interneurons that eventually settle in the striatum, hippocampus and neocortex (Marin *et al.*, 2000; Pleasure *et al.*, 2000; Anderson *et al.*, 2001); and perhaps even the exclusive source of layer I Cajal-Retzius cells, and GABAergic cells of the somatostatin sub-class (Lavdas *et al.*, 1999; Anderson *et al.*, 2001).

The molecular mechanisms that guide the migration of interneurons in the striatal-cortical pathway are not fully understood but thought to be under influence of chemotropic factors present in the developing basal ganglia and neocortex. Netrin, an extracellular protein with chemorepellant and chemoattractant properties (Kennedy *et al.*, 1994; Serafini *et al.*, 1996), is known to be expressed at high levels in the basal telencephalon but does not appear to be involved in interneuron migration (Metin *et al.*, 1997; Anderson *et al.*, 1999). On the other hand, the semaphorin/neuropilin signalling system has been shown to channel interneurons expressing neuropilins to the neocortex, presumably by repelling these neurons following contact with striatal cells expressing class 3 semaphorins (Marin *et al.*, 2001). Slit is a family of secreted proteins with chemorepellant activity (Brose *et al.*, 1999; Kidd *et al.*, 1999; Li *et al.*, 1999) and is expressed in regions associated with interneuron genesis and migration viz. MGE, LGE, and cortical plate (Nguyen Ba-Charvet *et al.*, 1999; Yuan *et al.*, 1999; Whitford *et al.*, 2002). Explant culture studies using Slit have demonstrated repellant activity on migratory interneurons from MGE explants and may be a prime candidate for driving striatal-cortical migration (Zhu *et al.*, 1999). Slit is also capable of repelling neuronal migration in neocortical explants, which are primary sources of projection neurons (Hu, 1999).

Besides promoting cell migration in the vertebrate nervous system, Slit is also a potent molecule for repelling axonal growth (Brose *et al.*, 1999; Li *et al.*, 1999; Nguyen Ba-Charvet *et al.*, 1999; Ringstedt *et al.*, 2000), and the cleaved 140 kDa amino-terminal fragment is a strong promoter of axon branching and elongation in dorsal root ganglia neurons (Wang *et al.*, 1999). The question then arises whether neurons from neocortical and subcortical origins (both shown to respond to Slit by migrating away) have similar or differential axonal responses to Slit by repulsion, elongation or branching. The question is pertinent because projection and inhibitory neurons in the neocortex pursue different developmental agendas during and after cessation of cell migration, in keeping with their separate morphological and functional traits (Jones, 1990). Projection neurons establish characteristic arborization patterns that are layer-specific and their axons typically navigate across white matter to long-distance targets (Feldman, 1984). Migrating projection neurons often extend their axons to subcortical targets even before the cell body has completed its migration (Schreyer and Jones, 1982; Schwartz *et al.*, 1991), suggesting complex cellular responses to extracellular guidance cues. In contrast, interneurons generally have a different axo-dendritic structure with no apical dendrite; they lack dendritic spines and their axons arborize in the vicinity of their dendritic fields (Fairen *et al.*, 1984; Naegele and Barnstable, 1989).

The objective of the present study was to evaluate the response of the developing MGE and neocortex to Slit protein. We found that in culture, early and late neurons respond differently to Slit. Whereas early neurons in explants tend to migrate away and their processes repelled by Slit, late neurons in dissociated cultures respond to Slit by increased branching and neurite elongation. These bifunctional responses were confined only to the interneuron subpopulation present cultures of the MGE and neocortex, suggesting that projection neurons do not utilize Slit for their migration and arborization activities.

RESULTS

Slit Protein Expression in Culture Cells and Conditioned Medium

HEK 293T cells stably transfected with *xSlit* (tagged with *myc* epitope) or control plasmid were used. To visualize Slit expression, a monoclonal anti-myc antibody was used to detect Slit-myc protein in Western blots of cells lysates and CM. (Fig. 1A). Slit protein was seen both in lysates and in CM, implying retention in cells as well as secretion into the media. CM collected after 48 h culture showed an upper band equivalent to the full-length Slit-myc as previously reported (Chen *et al.*, 2000), but an additional lower band was also detected. This, and other bands that may be present but not carrying the myc epitope, suggest proteolytic cleavage of the parent protein. In control transfected cells (Fig. 1B), no staining was seen with anti-myc whereas Slit-transfected cells showed ubiquitous staining (Fig. 1C). Higher power views of whole mounted (Fig. 1D) and sectioned (Fig. 1E) cell aggregates showed Slit-myc staining in both cytoplasm and surface membranes.

Effect of Slit Protein on Neurite Outgrowth from MGE Explants

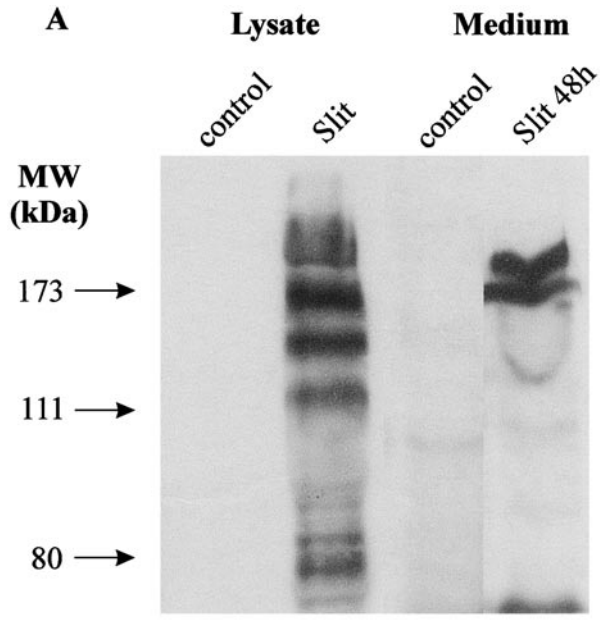
Explants from E13.5 MGE primordia were co-cultured with Slit or control cell aggregates in a three-dimensional collagen gel and stained with anti-β-tubulin to visualize neurite outgrowth (Figs. 2A and 2B). In addition, staining with anti-GABA antibody was used to reveal interneuron cell bodies and processes (Figs. 2A′ and 2B′). In explants ($n = 50$) cocultured with Slit cell aggregates, asymmetric neurite outgrowth was observed, with fewer and shorter neurites in the proximal aspect (facing the Slit cell aggregates) compared to the distal aspect (Fig. 2B). This effect was not seen in explants ($n = 50$) cocultured with control cell aggregates (Fig. 2A). Staining with anti-GABA antibody revealed processes emanating from the explant, and also cell bodies that appear to have dispersed into the collagen gel in Slit cocultures (inset, Fig. 2B′). Compared to the controls (Fig. 2A′), the number of GABA-positive processes facing Slit aggregates appear to be reduced (Fig. 2B′). Quantification of neurite length and interneuron dispersion was carried out for 10 Slit and control cocultures, comparing cell bodies and processes of proximal versus distal aspects of the explant (middle third of explant; Fig. 2C). Following staining with β-tubulin, the longest neurite found in the proximal and distal aspect of each of 10 explants was measured. The results showed longer neurites in the distal surface (249 ± 9 μm; mean $\pm$ SEM) compared to the proximal (85 ± 6 μm; Student's t test, $P < 0.001$), suggesting that Slit has the effect of inhibiting neurite outgrowth. Identical analysis of explants cultured in the presence of control cell aggregates showed no significant difference between neurite lengths of proximal compared to distal surfaces (Fig. 2C). Analysis of GABA cell dispersion from explants in Slit cocultures showed a reduction in the number of dispersed GABA interneurons in the proximal aspect of the explant, but not from the distal, dorsal, or ventral surfaces (Fig. 2B′). Scoring from 10 explants (with co-cultured Slit aggregates) showed greater numbers of dispersed interneurons from the distal (18 ± 3) compared to proximal (3 ± 1, Student's t test, $P < 0.001$) aspects. In contrast, GABA interneuron disperson from control explants showed no difference between proximal and distal surfaces (Fig. 2D). Thus, in addition to repulsion of neurite outgrowth, Slit caused an increased in GABA interneuron dispersion from cocultured MGE explants, except from the proximal surface of the explant closest to a Slit gradient.

Effect of Slit on Branching and Elongation of MGE and Cortical Neurites

To study the response of growing neurites to Slit, we used a low density *in vitro* cell culture system that allows the morphology of individual neurons to be unambiguously identified for quantification in terms of neurite length and branching (Wang *et al.*, 1999). For these experiments, both MGE and neocortical promordia were used. Dissociated cells from E13.5 MGE and neocortex were grown in the presence or absence of CM from Slit or control cells for 24 h before analysis. In the first set of experiments, MGE and neocortical cells were plated and immediately exposed (0 DIV) to CM for 24 h. In the second set, cells were cultured in standard media for 5 days (5 DIV) before the media was replaced with CM for 24 h.

In control CM, 0 DIV MGE neurons were all positive for TuJ1, a marker for both axonal and dendritic β-tubulin (Fig. 3A). These neurons were mostly unipolar or bipolar with extended processes (Fig. 3A), and invariably stained for GABA (Fig. 3A′). After 24 h in Slit CM, 0 DIV MGE neurons also stained for TuJ1 and GABA, but they tend to be more rounded and displayed stunted neuronal processes (Figs. 3B and 3B'). Similar results were encountered with 0 DIV neocortical interneurons (results not shown). Although the number of branch points per neuron in Slit CM was fewer in comparison to control CM (Fig. 4A), it was not statistically significant ($n = 60$, $P > 0.05$). However, the longest neurite and total accumulated neurite length of MGE interneurons were significantly reduced in Slit compared to control CM (longest neurite; $P < 0.05$; and accumulated neurite length; $P < 0.05$) (Figs. 4B and

FIG. 1. Expression of Slit in HEK 293 cells and secretion into conditioned medium. (A) Western blotting with antibody against c-myc epitope shows presence of Slit-myc protein only in transfected, but not in control cells. Multiple fragments, possibly representing cleavage products, can be seen in cell lysates. Conditioned medium from 48-h cultures contains an upper band equivalent to the size of the predicted Slit protein from cDNA, with an additional lower band just underneath. (B, C) Staining of cultured cell lines with anti-myc antibody shows staining only in Slit-transfected (C) but not in control (B) cell line. (D, E) Higher power views of whole-mounted (D) and sectioned (E) cells show that Slit protein is localized in both cytoplasm and cell membrane. Scale bars: B, C, 50 μm; D, E, 10 μm.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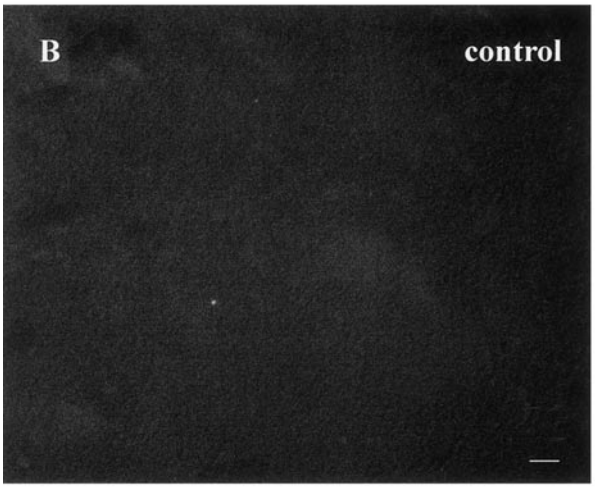
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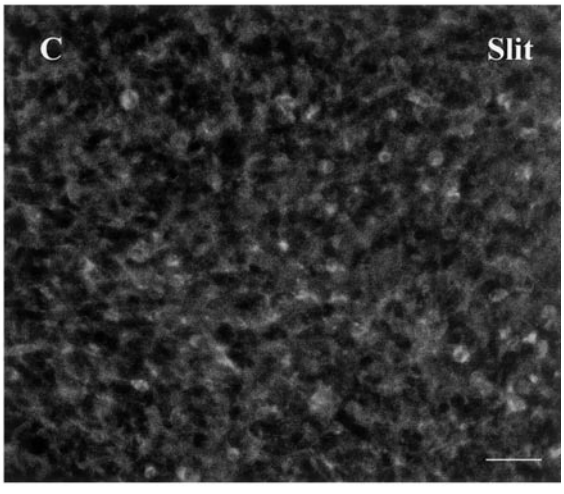
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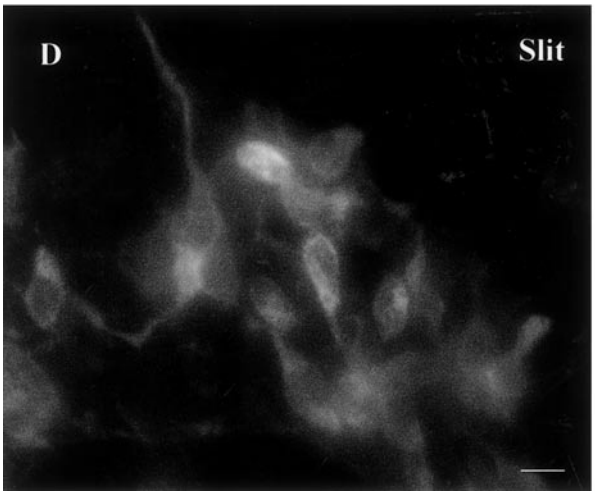
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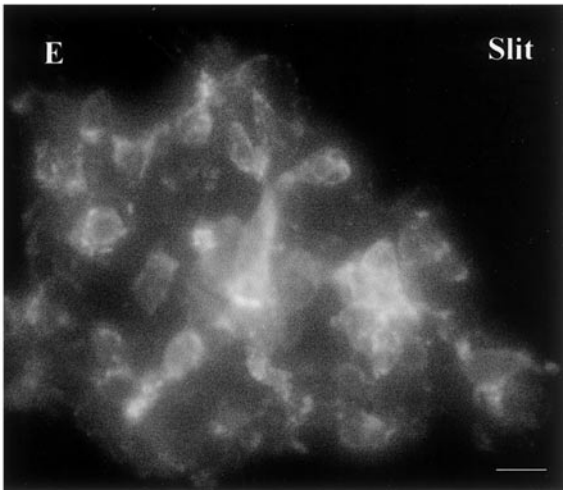
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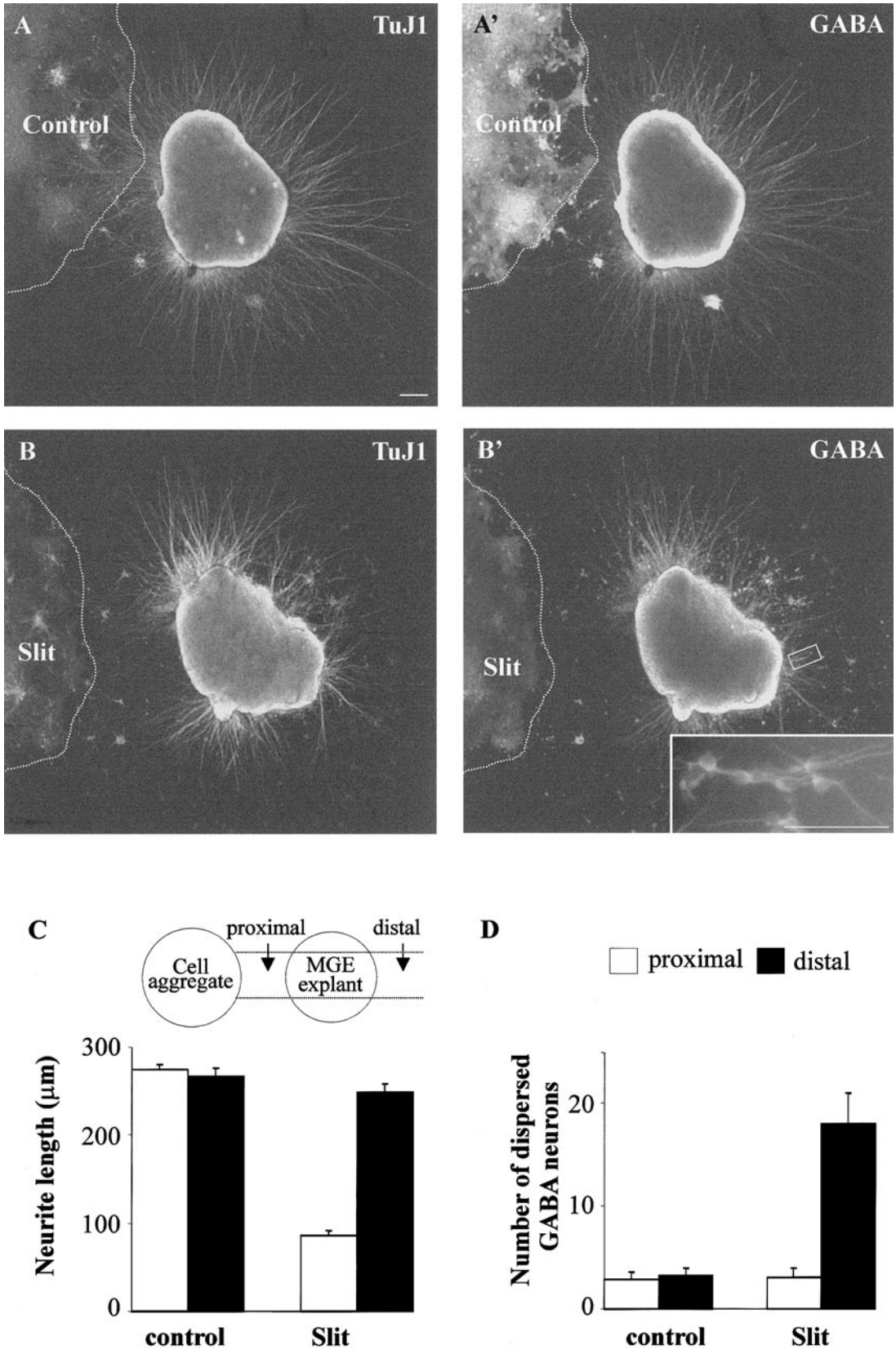
4C). A similar picture was also seen with neocortical interneurons exposed to Slit and control CM (Figs. 4B and 4C). Generally, the GABAergic neurons in 0 DIV cortical cells had shorter neurites compared to MGE. No assessment was undertaken for GABA-negative neurons in the 0 DIV cortical group as they generally lacked processes at this stage. These results suggest that after 0 DIV, MGE and neocortical interneurons respond negatively to Slit by reducing neurite length.

Results from the second set of experiments showed that after 5 DIV, cultured MGE interneurons exposed to Slit had a different response. Whereas control interneurons from this group showed occasional neurite branching and longer neurites (compared to 0 DIV), Slit-treated interneurons by far showed more frequent branching and had longer neurites (Figs. 3D and 4A–4C). Many of these were multipolar (38% of Slit-treated interneurons had 3 or more neurites per cell versus 13% in control-treated interneurons). Statistical comparison of control and Slit interneurons ($n = 60$ in each 5 DIV group) revealed statistically significant differences in branching frequency ($P < 0.01$), longer neurites ($P < 0.001$) and greater accumulated neurite length ($P < 0.001$) in the Slit population (Figs. 4A–4C).

In the neocortical group, TuJ1-positive neurons (after 5 DIV) were separately assessed in their response to Slit depending on whether they were GABA-positive or GABA-negative. Interestingly, these two groups of neurons showed different responses to Slit. Like the MGE, GABA-positive cells from the neocortex responded to Slit by greater branching and neurite extension (Figs. 3F and 4A–4C). GABA neurons from the neocortex, exposed only to control CM were bipolar in morphology and clearly had shorter neurite lengths (Fig. 3E). Statistical comparison of the Slit and control treated groups revealed significantly increased number of branch points per neuron ($P < 0.05$), greater neurite length ($P < 0.001$) and increased accummulated neurite length ($P < 0.001$) in the Slit group (Figs. 4A–4C). In contrast, GABA-negative (but TuJ1-positive) neurons showed no statistical difference to Slit compared to controls for the three parameters considered (Figs. 4A–4C). Indeed, GABA-negative neurons showed extensive branching in both control and Slit CM (compare Figs. 3G, 3H with 3I, 3J), exceeding even the branching of any Slit treated GABA-positive neurons (Fig. 4A). We judged these to be projection neurons based upon their negative GABA-immunoreactivity, extensive arborization, and spiny-like dendritic structures. We conclude that interneurons, but not projection neurons from neocortical cultures, respond to Slit by neurite branching and elongation. Although GABA-positive cells from the neocortex have a similar morphology to MGE interneurons (after 5 DIV), there was a greater degree of branching per cortical interneuron compared to MGE (4.62 ± 0.31 versus 2.65 ± 0.14, mean $\pm$ SEM; $P < 0.001$). Hence, cortical interneurons showed a greater response to Slit by neurite elongation and branching.

Previous experiments have indicated secreted RoboN to be an effective competitive blocker of xSlit activity in the repulsion of cells in the LGE and septum (Zhu *et al.*, 1999; Wu *et al.*, 1999), and of mitral cell axons in the olfactory bulb (Hirata *et al.*, 2001). To test whether neurite growth and branching in cortical interneurons are similarly inhibited by RoboN, we have exposed cultured cortical interneurons to CM containing both Slit and secreted RoboN (Fig. 5A). Whereas GABAergic interneurons exposed to control CM showed minimal branching and neurite length (Figs. 5B, 5E, and 5F), the opposite was seen with Slit CM (Figs. 5C, 5E, and 5F). Interneurons exposed to varying concentrations of Slit/RoboN CM displayed a dose-dependent response, with reductions in Slit-mediated activity seen at increasing RoboN concentrations (Figs. 5D, 5E, and 5F) (one-way ANOVA, $P < 0.05$).

FIG. 2. Neurite outgrowth and GABA interneuron dispersion from E13.5 MGE explants is inhibited by a gradient source of Slit. (A) MGE coculture with control-transfected cells shows symmetrical neurite outgrowth (stained with β-tubulin) from the circumference of the explant. (A′) Following staining with GABA, neuronal processes appear to emanate symmetrically from all surfaces of the explant in coculture with control cell aggregates (some autofluorescence is detected here). (B) MGE coculture with Slit cell aggregates shows asymmetric neurite outgrowth with fewer and shorter neurites in proximal compared to the distal surface. (B′) In coculture with Slit aggregates, GABA-positive cell processes are fewer in the proximal explant surface facing Slit, but are exuberant in distal, dorsal and ventral surfaces. In addition, dispersed GABA cell bodies are seen outside the explant except for the proximal surface where hardly any GABA-positive cell bodies are present. Inset shows higher magnification of GABAergic neuron (arrowhead) present in boxed area. (C) Comparison of neurite lengths from 10 explants cocultured with Slit and control aggregates. Neurites found in the distal aspect in the presence Slit aggregates were significantly longer ($P < 0.001$). No difference between proximal and distal was seen in control aggregate cultures. (D) Examination of 10 explants cocultured with Slit or control aggregates. There were significantly reduced numbers of GABA interneurons in the proximal compared to the distal surface in the presence of Slit ($P < 0.001$). In contrast, no difference was seen in control explants. Scale bar, 50 μm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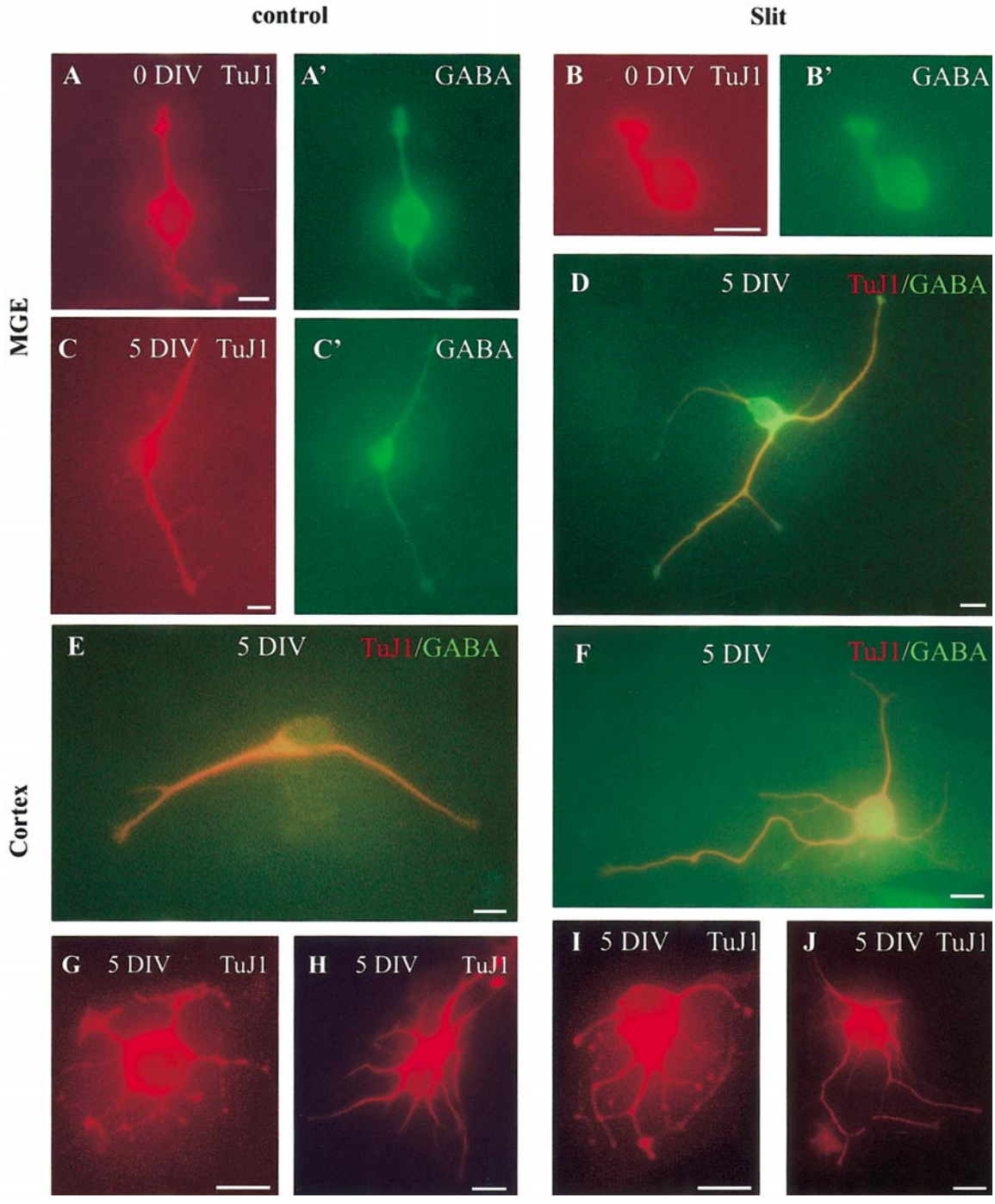


FIG. 3. Slit-conditioned media selectively promotes branching and elongation of GABA-positive but not GABA-negative neurons in dissociated MGE and neocortex after 5 DIV. In control CM, 0 DIV neurons from E13.5 MGE stained with TuJ1 (A) and GABA (A′) have few cell processes and they extend relatively short neurites. After 5 DIV in control CM, MGE GABA-positive neurons display longer neurites and increased branching (C, C′) but still remained unipolar or bipolar. A similar picture was encountered with GABA-positive neurons from E13.5 neocortex, exposed to control CM after 5 DIV, showing increased neurite length (E) but hardly any branching. In this group of neocortical cultures, GABA-negative (but TuJ1-positive) neurons (G, H) generally show greater arborization and spiny-like dendritic structures compared to GABA-positive neurons (E). Interneurons exposed to Slit show a contrasting picture. 0 DIV GABA-positive neurons from MGE after exposure to Slit showed reduced neurite length (B, B′). The situation was reversed among 5 DIV GABA-positive neurons from MGE where exposure to Slit led to extensive branching and increased neurite length (D). Similarly, GABA-positive cells from 5 DIV neocortical cultures also showed increased branching and neurite extension in response to Slit (F). On the other hand, GABA-negative (but TuJ1-positive) neurons exposed to Slit (I, J) continue to display the same level of arborization as the controls (G, H). Scale bars, 10 μm.

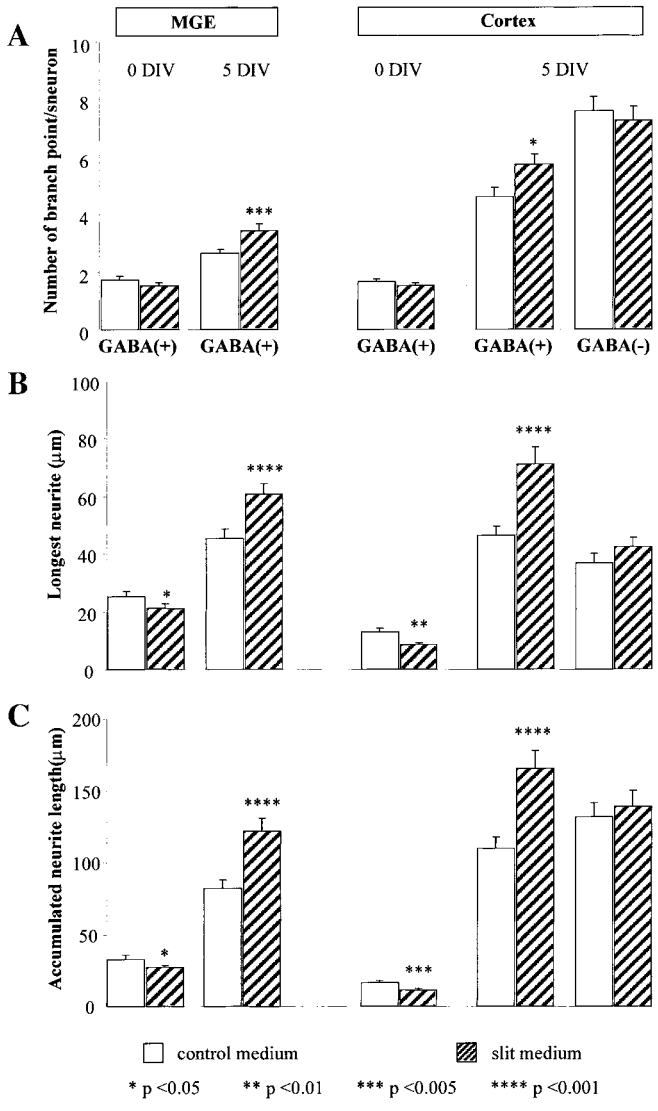


FIG. 4. Quantification of neurite outgrowth and branching by dissociated MGE and neocortical neurons following exposure to Slit or control CM. Neurite outgrowth (B, C) is inhibited by Slit CM in E13.5 GABAergic neurons from both MGE and neocortex (after 0 DIV) but Slit did not appear to affect the number of branch points per neuron in 0 DIV interneurons. Interneurons from 0 DIV neocortex generally possessed shorter neurites. In the 5 DIV group, GABAergic neurons from both MGE and neocortex showed significantly increased branching (A), greater single neurite length (B) and total accummulated length of all neurites (C) in response to Slit. In contrast, GABA-negative neurons from cortical cultures did not display a similar response to Slit. Values represent mean ± SEM, $n = 60$.

Effect of Exogenous Slit on Neocortical Development

Previous studies conducted *in vitro* have demonstrated that Slit is capable of promoting migration of neurons from explants of ganglionic eminence and cortical subventricular zone (Zhu *et al.*, 1999; Hu, 1999). To test whether or not a Slit is able to perform a similar role *in vivo*, Slit cell aggregates were injected into the developing lateral ventricles of the telencephalon at E13.5. Pups were reared until P1 and P14, and then analysed to study the effect on cell migration from a localized source of Slit. In six pups (out of 34 live births) containing Slit aggregates, an ectopic mass of cells may be seen embedded in the ventricular wall after bisbenzamide staining for cell nuclei (Fig. 6A, circular outline). These cells were immunoreactive for anti-myc (Fig. 6D, inset) but did not stain for NeuN, a neuron-specific cell marker (Fig. 6A), suggesting active production of Slit-myc protein. Under low magnification, the density and arrangement of NeuN-positive cells in parasagittal sections appeared to be unaffected (Fig. 6A). To assess the effect of an ectopic Slit source on cortical neuron development, three regions at varying distances were selected for examination after staining for pyramidal and nonpyramidal specific antigens. Higher magnification views (Fig. 6A, numbered boxes) showed no difference in the density and distribution of pyramidal neurons (stained with Tbr1) in regions that are rostral (box 1), immediately above (box 2), or caudal (box 3) to the Slit mass (Fig. 6B). However, GABAergic interneurons were rarely seen in the region above the Slit mass (Fig. 6, box C2) compared to rostral and caudal regions (Fig. 6, boxes C1, C3). Quantification of GABAergic interneurons was performed for three experimental and three control animals, comparing neurons stained with GABA, NeuN, and bisbenzimide in the three different areas (Fig. 7A, boxes 1–3). Whereas NeuN-positive neurons as a fraction of bisbenzimide-stained cells showed no statistical differences (Fig. 7C), there were statistically fewer GABA interneurons in the region (area 2) above the Slit mass (Fig. 7B). Brains injected with control cell aggregates did not exhibit a difference in the distribution of GABAergic neurons. (Fig. 7B). To rule out the possibility that fewer GABA interneurons was the result of generalized cell death in the P14 neocortex, TUNEL staining for apoptotic cells was carried out, comparing two different regions in relation to the Slit mass (Fig. 6D, boxes 1,2). The results showed no difference in the number of apoptotic cells (Fig. 6E, arrows in box E2), indeed more dead cells were observed in the region (box E2) further away from Slit. This would suggest that given an *in vivo* environment, an ectopic Slit source mass is correlated with a scarcity of interneurons in the vicinity but has no effect on pyramidal neurons.

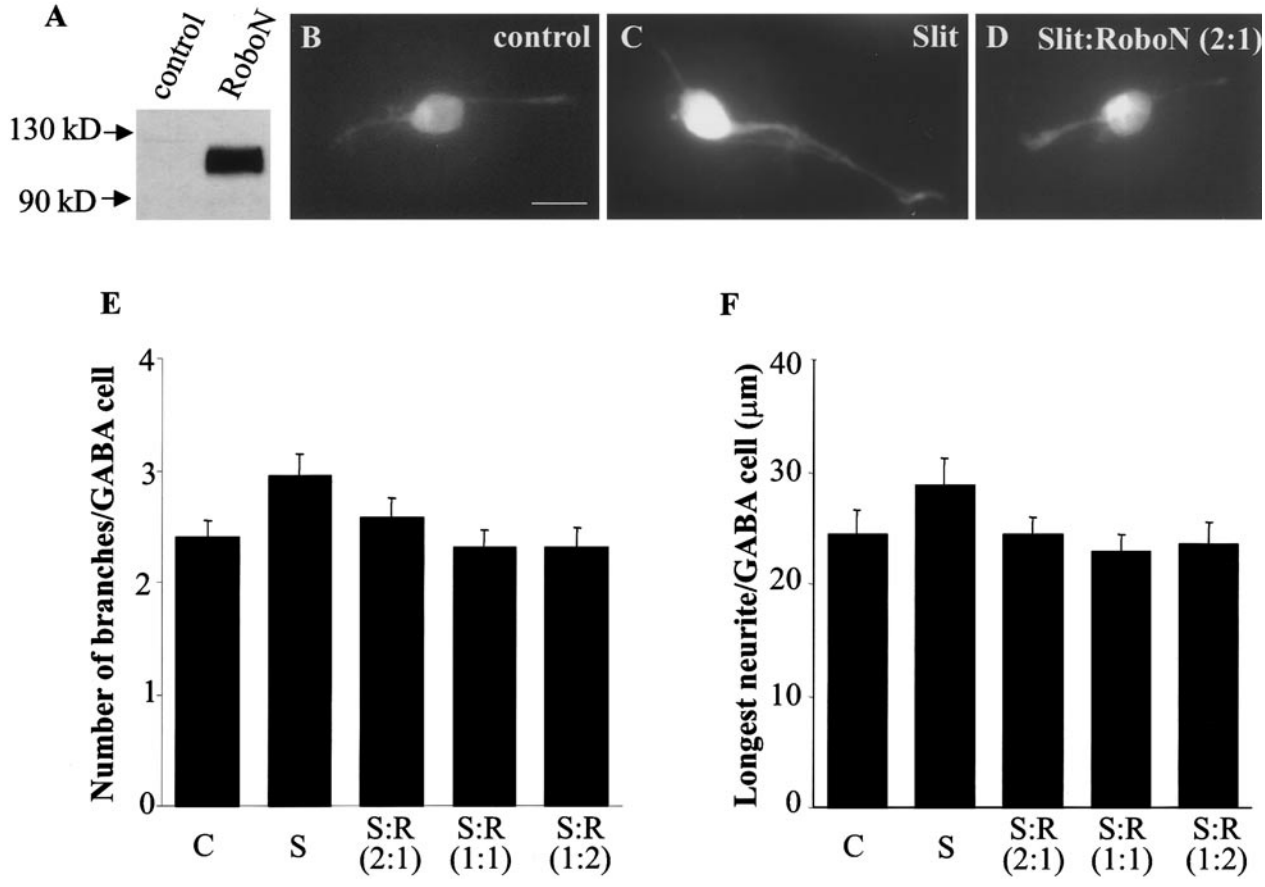
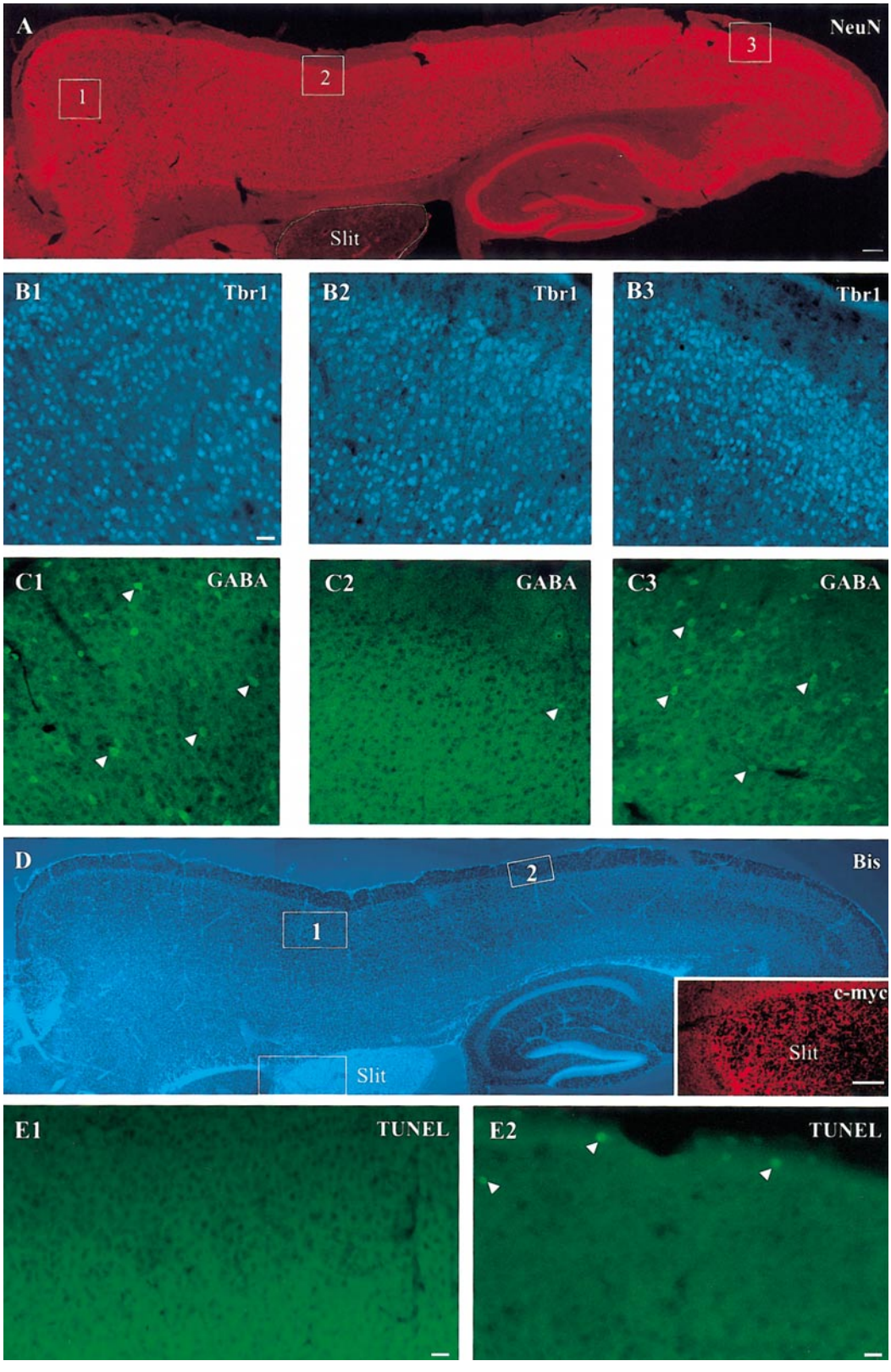


FIG. 5. Inhibition of Slit-mediated neurite outgrowth and branching by RoboN. HA-tagged RoboN from transfected HEK cells is secreted in the CM and detectable in Western blots using antibodies to HA (A). GABAergic interneurons exposed to CM containing control (B), Slit (C) or Slit/RoboN (D) showed different responses in neurite outgrowth. Comparisons of interneuron branching (E) and neurite outgrowth (F) showed that both characteristics are attenuated with increasing concentrations of RoboN in the CM.

DISCUSSION

In this study, we describe the *in vitro* effect of *Xenopus* Slit, an orthologue of mSlit2, on developing neurons from cultured MGE and neocortical primordia. Previous experiments with this molecule have shown that it has repulsive activity (on a wide range of tissues including cortical interneurons, olfactory bulb axons, in-

FIG. 6. Repulsion of interneurons, but not pyramidal neurons, close to an implanted Slit mass in the developing neocortex. Following injection of Slit cell aggregates into the E13.5 lateral ventricles, brains were perfused at P14 and parasagittal sections obtained. Cell nuclei staining with bisbenzamide (D) reveals an ectopic mass of implanted Slit cells beneath the ventricular ependyma. The same area did not stain for the neuronal marker NeuN (A). Inset in (D) shows Slit mass stained with myc antibody suggesting expression of Slit-myc protein. The general distribution of neurons, revealed by NeuN staining (A), suggests apparently normal density of neurons in the plane of section. Higher power views of the number boxed areas are presented in B, C. Staining with pyramidal neuron marker Tbr1 (B1–3) corresponding to the boxed areas shows no difference in staining density or distribution of pyramidal neurons. Staining for GABA (D) in the same areas reveals a conspicuous lack of interneurons (white arrows) in box C2, located immediately above the Slit mass. The distribution of GABAergic neurons in rostral (box C1) and caudal (box C3) regions appears to be unaffected. Detection of cell death using TUNEL showed no significant increase in number of dying cells when proximal (box 1) and distal (box 2) regions were compared (D). Only the distal box (E2) revealed dying cells (arrowheads). Scale bars: A, D, 200 μm; B, C, E1, 10 μm; E2, 5 μm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TUNEL

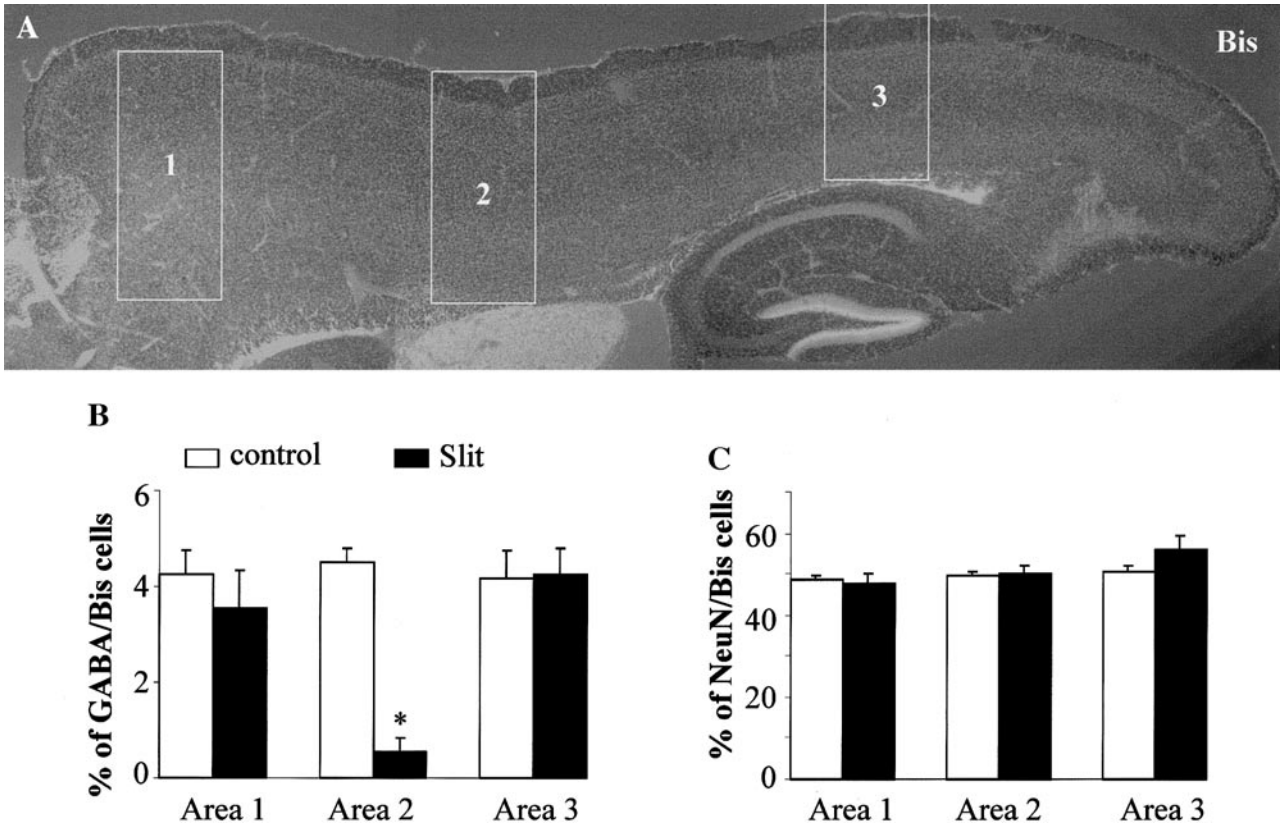

FIG. 7. GABAergic interneurons are reduced in number in a region closer to the implanted Slit mass. The number of cells stained with GABA, NeuN, and bisbenzimide were quantitated in three different boxed areas in relation to the Slit or control mass (A). Pool data from three control or Slit-implanted brains showed no significant differences in the number NeuN-positive neurons as a percentage of bisbenzimide-stained cells. In contrast, GABAergic neurons show a statistically significant reduction in Area 2, corresponding to the cortical space immediately dorsal to the Slit mass (B).

terneurons in the rostral migratory stream) that is equivalent to mSlit1 and mSlit2 (Wu *et al.*, 1999; Zhu *et al.*, 1999; Chen *et al.*, 2001). We also tested the *in vivo* effect of exogenous Slit on migrating neurons in the developing cortical environment. Our principal findings are (1) explant coculture of MGE with Slit aggregates is associated with reduction of neurite outgrowth from GABAergic interneurons while cell bodies appear to be repelled away from Slit; (2) dissociated E13.5 interneurons from MGE and neocortex showed minimal effect after 24 h culture in Slit media whereas similar cells, after 5 DIV, responded to Slit by neurite branching and elongation; (3) interneuron, but not pyramidal neuron, migration *in vivo* is affected by an exogenous source of Slit; (4) Slit-mediated neurite outgrowth is competitively attenuated with increasing concentrations of RoboN. In addition, we showed that dissociated pyramidal neurons in culture were unaffected by Slit CM. Taken together, our results reveal the interneuron populaton to be the principal target of xSlit activity during forebrain development. In addition, this work confirms Slit to be a multifunctional molecule and the nature of the interneuron response, be it neurite repulsion or branching and elongation, appears to be dependent on the developmental stage of the interneuron.

At a first approximation, these results appear to be paradoxical in terms of interneuron response to Slit protein. When exposed to a Slit source in explant cocultures, neurite outgrowth appears to be diminished. Later on, older (E13.5 + 5 DIV) interneurons from MGE respond to Slit protein by neurite branching and elongation. These contrarian activities exerted by Slit on a single tissue source (MGE) have not previously been demonstrated with other types of neurons that are known to respond to Slit. Interneurons from the subventricular zone are also repelled by Slit during their migration to the olfactory bulb (Wu *et al.*, 1999), but it is

not known if they can respond to Slit by axon branching. On the other hand, an impressive list of neurons in the central nervous system, including those in the lateral olfactory tract, hippocampal dentate gyrus, spinal motor column and commissural neurons, and retinal ganglion cells all appear to respond to Slit by chemorepulsion (Brose *et al.,* 1999; Li *et al.,* 1999; Nguyen Ba-Charvet *et al.,* 1999; Erskine *et al.,* 2000; Niclou *et al.,* 2000; Ringstedt *et al.,* 2000), while dorsal root ganglion neurons respond by axon branching (Wang *et al.,* 1999). Thus, while Slit is known to be capable of bifunctional effects, their occurrence *in vitro* at two developmental time points within a single tissue (MGE) is striking. Comparable results have also been obtained for cortical neurons which showed both repulsion and branching activity in response to exogenous Slit (Whitford *et al.,* 2002).

At this stage, it is not known whether the effects are attributable to two different types of responsive cells in MGE. However, it is worth noting that Slit may not be the only guidance molecule capable of mediating bifunctional effects, given that the axon outgrowth pathway is known to be paved with other molecules with bifunctional properties, including netrins and semaphorins (Tessier-Lavigne and Goodman, 1996; Van Vactor and Flanagan, 1999). So far we are unable to comment on the nature of the Slit protein responsible for neurite branching and elongation observed in this study. Whereas neurons in the dorsal root ganglia were found to respond specifically to the 140-kDa N-terminal fragment, and not full-length Slit (Wang *et al.,* 1999), we have not undertaken biochemical characterization of the Slit CM to ascertain the active fragment. It remains to be determined whether or not the branching and elongation promoting activity observed in our system is equivalent to the 140 kDa N-terminal fragment of mammalian Slit. A recent study indicates that an N-terminal fragment of Slit1 is effective in promoting cortical neuron branching (Whitford *et al.,* 2002).

Why do 5 DIV, but not 0 DIV, interneurons from E13.5 MGE and neocortex respond to Slit by neurite branching and elongation? Conversely, explants from E13.5 (equivalent to 0 DIV cells) when cocultured with Slit aggregates respond by neurite outgrowth inhibition and increased interneuron cell dispersion. One could speculate that MGE cells switch their responsiveness to Slit as they progress through different developmental stages, an effect of Slit previously reported in other systems *in vivo* (Kramer *et al.,* 2001; Stein and Tessier-Lavigne, 2001). At E13.5, the MGE is a major source of tangentially migrating interneurons destined for the striatum and neocortex (Marin *et al.,* 2000; Anderson *et al.,* 2001). Slit, shown previously to be an effective repellant for migrating interneurons from the LGE (Zhu *et al.,* 1999), is perhaps involved in guiding interneuron migration in the striatal-cortical pathway where Slit is expressed in the ganglionic eminence and intermediate zone (Nguyen Ba-Charvet *et al.,* 1999; Yuan *et al.,* 1999). Most, if not all, neocortical interneurons are derived from both MGE and LGE but dye-labeling and transplantation studies have identified early and late populations of cells from the MGE. Early-born (E11.5–E14.5) cells migrate primarily within the marginal and intermediate zones whilst later-born (E14.5–E16.5) cells migrate largely within the intermediate and ventricular zones (Lavdas *et al.,* 1999; Anderson *et al.,* 2001). Thus it is possible that at E13.5, interneurons in MGE explants belong to the early population and respond to Slit by moving away and inhibiting axonal outgrowth. By culturing E13.5 MGE and neocortical interneurons for a further 5 days, it is possible that interneurons change their Slit responsiveness from neurite inhibition to branching and elongation. Expression studies reveal that Slit and Robo is expressed in the cortical plate at about the time when neurons settle in their correct destinations and known to begin arborization (Bagri *et al.,* 2002; Whitford *et al.,* 2002). At this point, it is worthwhile observing that our culture conditions may also have selected for interneurons that no longer respond to Slit in the form of cell and neurite repulsion; instead an older interneuron population may have survived that is equivalent to E18.5 neurons (E13.5 + 5 DIV) that would normally have settled in their cortical destinations, and seen here to respond to Slit by neurite elongation and branching. In the developing dorsal root ganglia, axon branching in response to the amino-terminal fragment of Slit occurs only in neurons that would be old enough to collateralize *in vivo* (Wang *et al.,* 1999). Therefore a switch in Slit responsiveness may have occurred in MGE and cortical interneurons within a few days; indeed in the *Drosophila* embryo, migrating mesodermal cells convert Slit response from repulsion to attraction within a few hours (Kramer *et al.,* 2001).

The results of the present study suggest that interneurons destined for the neocortex undergo a Slit-driven cell migratory phase followed by elaboration of local circuitry by neurite branching and elongation. These Slit-mediated effects were attenuated in the presence of RoboN, a competitive blocker of Slit activity. Whether this reflects the *in vivo* situation remains to be determined. Slit2 is expressed in the striatal-cortical pathway of interneuron migration and in the intermediate zone beneath the cortical plate where MGE and LGE interneurons are known to penetrate (Nguyen

Ba-Charvet *et al.*, 1999). Slit1 is strongly expressed in the cortical plate and the Slit receptor Robo is also expressed in cells found in the cortical plate (Nguyen Ba-Charvet *et al.*, 1999; Yuan *et al.*, 1999, Bagri *et al.*, 2002; Whitford *et al.*, 2002). Therefore Slit and Robo expression appears at the appropriate time and place compatible with the promotion of interneuron migration and branching. A formal demonstration of these Slit-promoting activities *in vivo* will require further experiments, including loss of function studies (Bagri *et al.*, 2002).

In contrast, GABA-negative cells from dissociated E13.5 neocortex did not exhibit increased branching or elongation in Slit CM. Because these cells are Tuj1-positive and GABA-negative, we presume them to be pyramidal neurons. These contrasting results reaffirm that Slit is not a general branching molecule for neurons, in systems so far studied. Instead, recent studies suggest that neocortical projection neurons respond to other guidance and branching molecules. For example, corticospinal axons extend interstitial branches that collaterize in respond to polysialated neural cell adhesion molecule (PSA-NCAM) and removal of PSA affects corticospinal axon branching in the spinal cord (Daston *et al.*, 1996). Another distant target of projection neurons, the basilar pons, has been shown to release a diffusible chemotropic signal which directs the growth of collateral branches from layer 5 pyramidal axons (Heffner *et al.*, 1990; O'Leary *et al.*, 1991). More recently, it has been demonstrated that semaphorin 3A has a chemorepulsive effect on cortical projection axons, although paradoxically, it also attracts their apical dendrites (Polleux *et al.*, 2000). Such bifunctional properties have also been reported by others for Sema 3A (previously SemD) and Sema 3C (SemE) (Bagnard *et al.*, 1998). Taken together, it is reasonable to hypothesize that different molecular pathways are utilized for neurite branching and extension in the two separate populations of the neocortex.

A recent study on the effect of Slit-Robo pathway also reports increased dendritic growth and branching following *in vitro* exposure of cortical neurons to supernatant Slit (Whitford *et al.*, 2002). Using morphological criteria, these workers found equal branching responses by both pyramidal and nonpyramidal neurons to Slit. In the present study nonpyramidal neurons were identified using GABA antibodies and we demonstrate a clear distinction between pyramidal and nonpyramidal neuron response to Slit supernatant. There are at least two other reasons for the perceived discrepancy. First, our dissociated neurons were sourced from younger embryos (E13.5) compared to Whitford *et al.*, (2002)(E15.5). (It is likely that dissociated neuroblasts at E15.5 would comprise a greater mixture of deep and middle layer cortical neurons that may have differing sensitivities to Slit, some of which may survive and exhibit extensive branching while others may not. In contrast, the pyramidal neurons in our study were from earliest post-mitotic neurons from the deep layers of E13.5 cortex where neurons are mainly destined for layer VI and whose branching activity may depend upon the presence of other molecules. Secondly, neurons in our assay were kept longer in the presence of Slit (5 DIV compared to 3 DIV) and the longer culture period may have attenuated the branching response of our pyramidal neurons.

There are other differences in the response of pyramidal and nonpyramidal neurons to a source of implanted Slit *in vivo*. The grey matter immediately dorsal to the implanted Slit mass appeared to have very few GABA-positive neurons, compared to rostral and caudal sites. In contrast, pyramidal neurons stained with Tbr1 did not appear to be affected, leading us to suggest that radial migration of pyramidal neurons may be independent of a Slit gradient. The choroid plexus in the lateral ventricle has been reported to be source of Slit, leading one to suggest that in this site, Slit may be more important for driving interneuron migration in the rostral migratory stream (Hu, 1999; Wu *et al.*, 1999). The observation of few GABAergic cells present in the vicinity of the implanted Slit may result from one of two possible causes: (1) due to chemorepulsion of incoming interneurons from the subpallium, or (2) increased interneuron cell death. TUNEL staining in the adult brain suggests there is no increased interneuron cell death. We would suggest that by implanting a source of cells secreting Slit, we have created a local chemorepulsive environment, comparable to that which normally propels interneurons away from the Slit-rich ganglionic eminences. Interneurons in all cortical layers appeared to be equally affected and as Slit a large secreted molecule, this raises the question of how it can affect interneurons situated some distance away in the superficial layers. Recent lineage-tracing studies indicate that almost all incoming interneurons from the basal telencephalon into the neocortex initially migrate close to the ventricular zone (Lavdas *et al.*, 1999; Anderson *et al.*, 2001), thus a localized source of Slit in the subependymal region would be able to influence interneurons destined for both deeper and superficial layers by repelling them away to surrounding cortical areas.

In conclusion, our results point to Slit as an important molecule for integrating the GABAergic interneurons into the developing neocortex by eliciting a different response both in terms of neuronal migration and neurite branching. Such differential responses, probably driven by appropriate cell-autonomous expression of

ROBO receptors, are key mechanisms for ensuring that different neuronal phenotypes sharing the same cortical environment are able to pursue separate developmental programs.

EXPERIMENTAL METHODS

Cell Culture and Slit Expression Detection

Human embryonic kidney (HEK) 293 cells stably-transfected with *Xenopus Slit* (*xSlit*) carrying the six-myc epitope tag or with control vector plasmid were used for the experiments (Li *et al.*, 1999). Cells were maintained in Dulbeco's modified Eagle's medium (DMEM, GIBCO, Life Technologies, Australia) containing 10% fetal bovine serum, 100 unit/ml penicillin and streptomycin at 37°C with 5% CO_2. For immunocytochemical staining, Slit or control cell aggregates were fixed in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 30 min at 4°C. Wholemount or 10-μm frozen sections of cell aggregates were incubated in a mouse anti-c-myc antibody (9E10, 1:200, Berkeley Antibody Company, Richmond, CA) and immunoreactivity was revealed with Cyanine3 (Cy3) conjugated donkey anti-mouse IgG (1:1000, Jackson ImmunoResearch, West Grove, PA). For Western blotting, Slit or control cell lysates were prepared with lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40, 50 mM DTT and 1 mM PMSF). The cell lysates and conditioned medium collected from Slit or control cells after 48 h culture were separated on 10% SDS–polyacrymade gel and transferred to PDVF membrane. The membrane was immunoblotted with mouse anti-c-myc antibody (1:2000) and HRP-conjugated sheep anti-mouse IgG (1:2000, AMRAD Biotech, Australia). The immunoblotting signals were detected using an enhanced chemilluminescense kit (Amersham Pharmacia, Piscataway, NJ).

Neurite Repulsion Assay

Slit or control cell aggregates were embedded in a rat tail collagen gel and cultured in DMEM with 7.5% fetal calf serum and 75 unit/ml penicillin and streptomycin. Pregnant mice (C57BL/6J and DBA/2) were anesthetized with pentobarbitone sodium (Nembutal 60mg/Kg, Rhone Merieux, Australia), and brains from embryonic (E) day 13.5 embryos were dissected out. The proliferative and surrounding regions of MGE (separated from the LGE) were trimmed into blocks of 600–800 μm and embedded in the collagen gel at a distance about 600–800 μm from the cell aggregates. Explants ($n = 50$) were cultured for up to 48 h before fixing in 4% paraformaldehyde and stained with mouse anti-β-tubulin (βIII) antibody (TuJ1, 1:200, Berkeley Antibody Co.) to reveal neuronal processes and anti-GABA (1:200, Sigma, St. Louis, MI) to reveal interneurons. Neurite length and number of dispersed GABAergic neurons in the proximal versus distal quarters were quantified for 10 explants cocultured with Slit cell aggregates.

In Vitro Neurite Elongation and Branching Assay

To examine the effects of Slit on neurite outgrowth from individual MGE or cortical cells, a low-density cell culture system was utilized (Wang *et al.*, 1999). MGE and neocortical tissues were dissected out from E13.5 embryos, triturated and then dissociated in 0.1% trypsin at 37°C for 30 min. The cell suspension (5×10^4 cells per ml) was then cultured and exposed to Slit or control cell conditioned medium (CM) according to two different regimes. One batch of tissues (MGE and cortex) were treated with Slit or control CM immediately after plating (0 days *in vitro*, DIV) and analyzed after 24 h in CM. The other batch was cultured in 1% fetal calf serum for 5 DIV before addition of Slit and control CM, and maintained for another 24 h before analysis. CM used for these experiments were collected from Slit or control cells approximately 48 h after plating. Following fixation, cells were immunoreacted for β-tubulin and GABA. Staining of β-tubulin and GABA was revealed by a Cy3 conjugated donkey anti-mouse and a fluroescein (FITC)-conjugated goat anti-rabbit IgG (1:200, Jackson ImmunoResearch) respectively. Fluorescent images of individual neurons were captured (x40 objective) with a Spot Digital Camera (Diagnostic Instrument Inc., Sterling Heights, MI) and the magnified images used for analysis of neurite length and branching. Only neurons with unambiguous extension of neurites from the cell soma were included in the analysis (Image-Pro-Plus software; Media Cybernetics, Silver Spring, MD). The first 20 neurons that fulfilled this criteria were analysed, this was repeated for 3 separate experiments making a total of 60 neurons per sample category. For each neuron, measurements were taken to record the number of neurite branch points, the longest neurite and the accumulated length of all neurites extending from the soma. Statistical analysis of data from Slit and control CM cultures was performed using Student's *t* test.

Inhibition of Slit Activity Using RoboN

To provide a competitive blocker of Slit-induced neurite branching, we made use of RoboN which contains the extracellular portion of Robo, the Slit receptor. RoboN carries a hemagglutinin tag and can bind to Slit1 and Slit2 but cannot transmit the signal to the intracellular compartments (Wu *et al.*, 1999). RoboN transfected in HEK cells were cultured in a serum-free medium as previously described (Wu *et al.*, 1999; Zhu *et al.*, 1999), and the CM containing secreted RoboN was added together with Slit CM (2:1, 1:1, 1:2 dilutions) onto cultured neurons from E16.5 neocortex. After 40h, cells were fixed and stained for GABA immunoreactivity followed by measurements of neurite length and branching.

Introduction of Slit into Lateral Ventricles

To assess the effect of introducing a local source of Slit in the developing neocortex, Slit and control cell aggregates were transplanted into the developing lateral ventricles of E13.5 telencephalons *in utero.* Pregnant mice were anesthetized and the uterus was exposed through a midline incision and bathed with 0.2% warm $MgSO_4$. The forebrains of individual embryos was identified and injected, through the placental wall, with 1 μl of Slit (or control) cell aggregates (2×10^4) using a hand-pulled glass pipette. Following suturing, the animals were allowed to recover and reared to term. Pups at postnatal (P) day 1 and 14 were anaesthetised and perfused with 4% paraformaldehyde, their brains dissected out and sectioned (50 μm) parasagittally using a cryostat. To identify neurons, mouse anti-NeuN (1:250; Chemicon International Inc., Temecula, CA,) was used. To distinguish between pyramidal and nonpyramidal neurons in cortical sections, polyclonal anti-Tbr1 (Hevner *et al.*, 2001) and rabbit anti-GABA (1:200; Sigma, St. Louis, MI) were used respectively. Immunoreactivities were revealed using fluorescent-conjugated secondary antibodies as above. To reveal cell nuclei, staining with 1μg/ml bisbenzamide (Molecular Probe, Inc., Eugene, OR) was carried out. To detect the presence of Slit-myc in implanted cell aggregates of postnatal brains, 50-μm parasagittal sections were stained with mouse anti-c-myc antibody (1:100 dilution). For the detection of apoptotic cell death, the TUNEL (TdT-mediated dUTP nick end labelling) technique was used according to manufacturer's instructions (Roche, Mannheim, Germany).

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