Slits Are Chemorepellents Endogenous to Hypothalamus and Steer Thalamocortical Axons into Ventral Telencephalon

Thalamocortical axons (TCAs) originate in dorsal thalamus, extend ventrally along the lateral thalamic surface, and as they approach hypothalamic level make a lateral turn into ventral telencephalon. In vitro studies show that hypothalamus releases a chemorepellent for TCAs, and analyses of knockout mice indicate that Slit chemorepellents and their receptor Robo2 influence TCA pathfinding. We show that Slit chemorepellents are the hypothalamic chemorepellent and act through Robos to steer TCAs into ventral telencephalon. During TCA pathfinding, Slit1 and Slit2 are expressed in hypothalamus and ventral thalamus and Robo1 and Robo2 are expressed in dorsal thalamus. In collagen gel cocultures of dorsal thalamus and Slit2-expressing cells, axon number and length are decreased on the explant side facing Slit2-expressing cells, overall axon outgrowth is diminished, and axons turn away from the Slit2-expressing cells. Thus, Slit2 is an inhibitor and chemorepellent for dorsal thalamic axons. Collagen gel cocultures of dorsal thalamus with sections of live diencephalon, with and without the hypothalamus portion overlaid with Robo2-fc-expressing cells to block Slit function, identify Slits as the hypothalamic chemorepellent. Thus, Slits are chemorepellents for TCAs endogenous to hypothalamus and steer TCAs from diencephalon into ventral telencephalon, a critical pathfinding event defective in Slit and Robo2 mutant mice.

Keywords: axon guidance, axon inhibition, dorsal thalamus, hypothalamus, internal capsule, neocortex, robo

Introduction

The directed growth of axons along stereotypic pathways is a crucial early step in establishing the appropriate axonal connections that underlie the proper functioning of the adult nervous system. The projection of thalamocortical axons (TCAs), which originates in dorsal thalamus and relays sensory information from the periphery to the neocortex, forms a prominent axon tract in the mammalian forebrain called the internal capsule that passes through the mammalian ventral telencephalon. During development, TCAs extend ventrally from dorsal thalamus along the lateral surface of the diencephalon. As TCAs approach the hypothalamus, they make a sharp lateral turn in ventral thalamus to enter the ventral telencephalon and extend dorsolaterally through the internal capsule toward their major target, the neocortex.

Both axonal scaffolds and secreted molecular activities have been implicated in the control of TCA pathfinding. For example, neurons in the medial aspect of the ventral telencephalon have been proposed to form axonal scaffolds that direct TCAs into the ventral telencephalon (Mein and Godement 1996; Molnar et al. 1998; Braisted et al. 1999; Tuttle et al. 1999). In support of this proposal is the finding in mice deficient for the basic helix-loop-helix transcription factor Mash-1 that a failure of TCAs to extend into the ventral telencephalon is correlated with the absence of a discrete population of ventral telencephalic cells that extends axons into dorsal thalamus early in development (Tuttle et al. 1999). Other studies have provided evidence that secreted molecular activities have a prominent role in guiding TCAs. For example, in vitro axon guidance assays and analyses of mutant mice have provided evidence that the ventral telencephalon releases a diffusible attractant activity for dorsal thalamic axons (Braisted et al. 1999) and that the chemoattractant netrin-1 accounts at least in part for this activity (Braisted et al. 2000). In vitro studies have also shown that the hypothalamus, a ventral diencephalic structure, releases a robust chemorepellent activity for dorsal thalamic axons that is proposed to help deviate TCAs from their ventrally directed trajectory and steer them laterally into the ventral telencephalon (Braisted et al. 1999).

Here we provide evidence from in vitro axon guidance assays and in vivo expression analyses that members of the Slit family of secreted chemorepellents, acting through their receptors, Robo1 and Robo2, are the chemorepellent activity for TCAs secreted by ventral diencephalon (hypothalamus). Three mammalian Slit genes have been identified, Slit1, Slit2, and Slit3 (Itoh et al. 1998; Nakayama et al. 1998; Brose et al. 1999; Li et al. 1999; Wang et al. 1999), as well as 2 Robo genes, Robo1 and Robo2. Slit2 has been shown to inhibit or repel several axonal populations, including those from olfactory bulb (Nguyen Ba-Charvet et al. 1999), hippocampus (Nguyen Ba-Charvet et al. 1999; Wu et al. 1999), spinal motoneurons (Brose et al. 1999), and retinal ganglion cells (Erskine et al. 2000; Niclou et al. 2000; Ringstedt et al. 2000). Our results help provide a definitive model for the mechanism of TCA pathfinding in mice deficient in Slits (Bagri et al. 2002; Andrews et al. 2006) or Robo2 (López-Bendito et al. 2007).

Materials and Methods

Animals

Rat embryos were obtained from timed-pregnant females (Harlan Sprague-Dawley) and staged. The day of in utero death was designated as embryonic day 0. All animal work was done in accordance with the regulations governing the use of animals in research and were carried out under the auspices of animal protocols approved by our institutions animal use and care committee.

In Situ Hybridization

Timed-pregnant rats were anesthetized, and embryo heads were fixed by immersion in 4% paraformaldehyde (PFA). After cryoprotection, the heads were sectioned coronally at 20 μm in a cryostat and processed
for in situ hybridization using S35 radiolabeled riboprobes, as previously described (Ringstedt et al. 2000).

**Preparation of Explants**

E15 embryos were removed by Cesarean section from timed-pregnant rats anesthetized with sodium pentobarbital and placed in cold L-15 medium supplemented with 0.6% glucose (Sigma, St Louis, MO; L15-glucose). To prepare explants of dorsal thalamus and ventral diencephalon, brains were removed, embedded in 3% low melting point agar in L15-glucose, and then sectioned coronally at 200 μm using a vibratome. Sections in which the internal capsule bridges the diencephalon and ventral telencephalon were collected, and the ventral telencephalon was removed. The ventral portion of the diencephalic piece contains the ventral thalamus and hypothalamus and was used as the "hypothalamus" explant. Dorsal thalamus was isolated from sections similar to those used for the hypothalamic explants as previously described (Braisted et al. 1999), and its lateral-medial axis was positioned parallel to the test tissue.

**Preparation of Aggregates of Slit2-Expressing Cells**

The 293T cells were transfected with a pSecTagB expression vector containing cDNA for human Slit2 tagged with a myc epitope Slit2-myc or Robo2-fc using SuperFect Transfection Reagent (Qiagen, Valencia, CA). The pSecTagB without the insert was used for mock transfections. Subconfluent cells were transfected, agar blocks containing cells were prepared, and small cubes were cut from the agar and used for cocultures as described in Braisted et al. (2000).

**Preparation of Collagen Gel Cocultures**

Slit2-myc Expressing Cells

Collagen was prepared from adult rat tails, and cocultures were suspended in collagen in 4-well dishes (Nunc, Rochester, NY) as described (Braisted et al. 1999). Dorsal thalamic explants and Slit2-myc or vector (mock)-transfected 293T cell aggregates were positioned approximately 150-500 μm apart.

Slit Function Blocking Experiments

Dorsal thalamic explants were placed adjacent to the lateral aspect of hypothalamic explants in collagen gel cocultures as described above. To block Slit function, the hypothalamus was overlaid with a block of Robo2-fc-expressing 293T cells or mock-transfected cells as a control.

**Dil Labeling of Cocultures**

Collagen gel cocultures were fixed with 4% Pfa plus 1% glutaraldehyde at 4 °C, rinsed and then crystals of the axonal tracer 1,1'-dioctadecyl 3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Carlsbad, CA; Honig and Hume 1989a, 1989b) were placed into the dorsal thalamic cultures. Cultures were stored to allow the DiI to diffuse along the full length of the axons.

**Immunocytochemistry**

Collagen gel cocultures were fixed and incubated 3–4 days at 4 °C in mouse monoclonal anti-ß-tubulin antibody (Amersham, Piscataway, NJ), prior to secondary processing.

**Analysis of Axon Outgrowth in Cocultures**

All analyses were done blind to the nature of the test explant.

**Semi-quantitative Analysis**

After 1.5 days in vitro, cocultures were photographed using phase-contrast optics. Axon outgrowth was assigned to 1 of 3 categories: explants that had more axon outgrowth 1) "toward," 2) "away" from the test explant or 293 cell aggregates, or 3) explants that had similar amounts of axon outgrowth on both these sides and were therefore scored as "symmetric."
Slits, Robos, and Thalamocortical Axon Pathfinding

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Slit2 Is a Chemorepellent for Dorsal Thalamic Axons In Vitro

To test whether Slits act as growth inhibitors or chemorepellents for TCAs, we cocultured in 3-dimensional collagen gels explants of E15 rat dorsal thalamus at a distance from aggregates of 293T cells transiently transfected either with the human slit2-myc cDNA or with the vector cDNA as a control (referred to as mock). When dorsal thalamus is cocultured with mock-transfected cells, 27% of the explants have more outgrowth on the side facing toward the cells and none have more outgrowth on the side facing away from the cells (Fig. 4A). In contrast, when dorsal thalamus is cocultured with Slit2-expressing cells, axon outgrowth is strongly biased away from the cells (Fig. 4B). 61% of the explants have more axon outgrowth on the side facing away from the cells and only 22% have more axon outgrowth on the side facing toward the cells (Fig. 4C, D). Differences between cocultures with mock-transfected or Slit2-expressing cells are statistically significant, \( P < 0.0001 \). These results indicate that Slit2 inhibits and/or repels dorsal thalamic axons in vitro.

To further quantify the effects of Slit2 on dorsal thalamic axons and assess the possibility that it inhibits their growth, we measured the number and length of axons on the explant sides facing toward or away from the transfected cells. When dorsal thalamus is cocultured with Slit2-transfected cells, 65% fewer axons extend from the side facing toward the cells (Fig. 4E), and these axons are 48% shorter than those on the side facing away from the cells (Fig. 4F). In contrast, when dorsal thalamus is cocultured with mock-transfected cells, 11% more axons extend from the side facing toward the cells, and they are only 8% shorter than those on the side facing away from the cells (the 8% decrease in length is likely an artifact because many axons on the side facing toward the cells are cut in half and therefore their maximum length is limited to the distance between the explant and cells). The total number of axons extending from the dorsal thalamic explants is also decreased by 51% in cocultures with Slit2-transfected cells compared with mock-transfected cells (Fig. 4G). All comparisons between cocultures with mock-transfected or Slit2-expressing cells are statistically significant (Fig. 4). Taken together, these findings indicate that Slit2 inhibits dorsal thalamic axon growth in vitro.

To determine whether Slit2 also repels dorsal thalamic axons, we measured the angle of deviation of axons away from the transfected cells (Fig. 4H also Materials and Methods). The angle
of axon turning away from Slit2-transfected cells is significantly greater than their angle of turning away from mock-transfected cells (Fig. 4I). Taken together, these findings indicate that Slit2 acts as a chemorepellent for dorsal thalamic axons in vitro.

Slit-Dependent Repulsion of Dorsal Thalamic Axons by Hypothalamus

The findings presented above suggest that Slits are components of the hypothalamic repellent activity. Because the original experiments demonstrating that hypothalamus releases a repellent activity for dorsal thalamic axons were done using mouse tissues (Braisted et al. 1999), we performed similar experiments to verify that rat hypothalamus has a similar repellent effect. Living vibratome sections of the ventral diencephalon of E15 rats were cocultured in collagen gels with explants of E15 rat dorsal thalamus placed at a distance from the lateral surface of hypothalamus. Similar to mouse, rat dorsal thalamic axon outgrowth is biased away from hypothalamus (Fig. 5A): 50% of the explants have more axon outgrowth on the side facing away from hypothalamus, whereas only 12.5% had more outgrowth on the side facing toward hypothalamus (Fig. 5D).
To determine whether the endogenous hypothalamic repellent activity is due to Slits, we repeated these experiments but overlaid hypothalamus with an agarose block containing Robo2-fc-expressing 293T cells to block Slit function. First, we confirmed that Robo2-fc itself has no significant effect on the outgrowth bias because the distribution of axon outgrowth from explants of dorsal thalamus cocultured with mock-transfected 293T cells (Fig. 5B) is similar to that which we observe in cocultures with aggregates of Robo2-fc-expressing 293T cells (data not shown). However, exposure of hypothalamus to Robo2-fc results in a statistically significant reversal of the biased directional outgrowth of dorsal thalamic axons compared with hypothalamus alone ($P < 0.002$)—instead of being biased away from hypothalamus, dorsal thalamic axon outgrowth is biased toward hypothalamus overlaid with Robo2-fc-expressing cells (Fig. 5E): 54% of explants have more outgrowth toward hypothalamus and only 16% have more outgrowth away from hypothalamus (Fig. 5D). When hypothalamus is overlaid with mock-transfected cells, the overall outgrowth of dorsal thalamic axons is symmetric and shows a statistically significant ($P < 0.002$) difference compared with that in cocultures of hypothalamus overlaid with Robo2-fc (Fig. 5C,D).

In conclusion, these coculture experiments, and in particular the finding of a substantial, statistically significant block of the chemorepellent effect of hypothalamus by the Slit function blocking reagent, Robo2-fc, show that Slits are responsible for the chemorepellent activity endogenous to hypothalamus.

Discussion

The TCA projection originates in dorsal thalamus and relays sensory information from the periphery to neocortex. As TCAs navigate from dorsal thalamus to cortex, they make a sharp lateral turn near the ventral thalamic-hypothalamic border to enter the ventral telencephalon. These findings suggest that hypothalamus is repulsive and ventral telencephalon is attractive for TCAs. Supporting these hypotheses, dorsal thalamic axons are repelled at a distance in vitro by hypothalamus and attracted by ventral telencephalon (Braisted et al. 1999). We have previously provided evidence that netrin-1 is the chemoattractant for TCAs associated with ventral telencephalon (Braisted et al. 2000). Here we have investigated the chemorepellent activity for TCAs that is secreted by hypothalamus and provided evidence that the chemorepellent Slit2, and likely the related chemorepellent Slit1, induces this turning behavior exhibited by TCAs. The expression patterns of Slit1 and Slit2 and of their receptors, Robo1 and Robo2, are appropriate for this role (Fig. 1). We also find that in vitro, dorsal thalamic axons are both inhibited and repelled at a distance by aggregates of Slit2-expressing cells.
that block Slit function, the Robo2-fc, also block the repellent activity endogenous to hypothalamus. These findings indicate that the Slits are ventral diencephalic/hypothalamic chemorepellents for TCAs and control a crucial phase of TCA pathfinding by deviating TCAs from their ventral trajectory and steering them laterally into the telencephalon.

We show that the distribution of mRNAs encoding Slits and Robos in the diencephalon would fit a model where the Slits exert a chemorepellent effect on TCAs. During the period of TCA pathfinding, Robo1 and Robo2 are expressed by dorsal thalamic projection neurons that form the TCA projection. Over the same time frame, when TCAs extending from dorsal thalamus make a sharp turn in response to a hypothalamic chemorepellent activity, the chemorepellents Slit1 and Slit2 are expressed in hypothalamus as well as in ventral thalamus. TCAs that extend ventrally toward these ventral telencephalic structures, do not enter them, but instead follow a path along the lateral surface of ventral thalamus as they turn sharply into ventral diencephalon.

In cocultures of explants from dorsal thalamus with aggregates of Slit2-expressing 293T cells, dorsal thalamic explants show a strong bias in axon outgrowth away from the Slit2 source. Further, dorsal thalamic axons are longer and more numerous on the explant side away from the Slit-secreting cells. Because Slit2 also significantly reduced the total number of axons, this effect might be due to an inhibition of...
axon growth. Therefore, to demonstrate the chemorepellent activity of Slit2, we also measured the angle of deviation of the paths of axons from a hypothetical path of growth that would extend radially from the explant and find that on average the axons exhibit a significant turning away from the Slit-secreting cell aggregates. Thus, TCAs exhibit a true chemorepellent response to Slit2.

In cocultures of explants of hypothalamus and dorsal thalamus, we show that Robo2 receptor bodies (Robo2-fc), which bind Slits and prevent their function (Nguyen Ba-Charvet et al. 2001), strongly diminish the chemorepellent effect exerted by hypothalamus on dorsal thalamic axons. In addition, we find that overlaying hypothalamus with Robo2-fc-expressing cells diminishes its inhibitory effect on axon outgrowth from dorsal thalamic explants. Thus, taken together, these studies demonstrate that Slits are chemorepellents for dorsal thalamic axons and are responsible for the chemorepellent activity endogenous to hypothalamus that we observe in vitro.

Recent studies have analyzed mice deficient for Slits and Robos to assess their requirements for TCA pathfinding. Mice deficient in Slit2 show defects in TCA pathfinding, characterized by a failure of TCAs to turn properly from diencephalon into ventral telencephalon; instead many TCAs continue on a ventral trajectory and abnormally enter the hypothalamus. This aberrant phenotype is exaggerated in mice deficient in both Slit1 and Slit2 (Bagri et al. 2002). Mice deficient in Robo2 exhibit similar defects in TCA pathfinding as Slit deficient mice (López-Bendito et al. 2007). Mice deficient in Robo1 do not exhibit these TCA pathfinding defects, and in fact, TCAs reach cortex earlier in Robo1 knockouts than in wild-type mice (Andrews et al. 2006) but fewer TCAs reach cortex in mice deficient in both Robo1 and Robo2 (López-Bendito et al. 2007). Thus, our in vitro axon guidance assays demonstrate that Slit2 is a chemorepellent and growth inhibitor for TCAs and that the Slits are responsible for the chemorepellent activity on TCA axons that is endogenous to hypothalamus. These in vitro findings corroborate and extend the analyses of Slit and Robo...
mouse and provide definitive support for the mechanism underlying defects in TCA pathfinding by definitely identifying Slits as the hypothalamic chemorepellent. In conclusion, the Slits acting through the Robos have a prominent role as chemorepellents in TCA pathfinding from dorsal thalamus to cortex and cooperate with other diffusible and substrate-associated molecules, as well as guidepost cells and the scaffolds they establish, to ensure the proper development of TCA input.

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