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Serotonin modulates the response of embryonic thalamocortical axons to netrin-1

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Modifying serotonin (5-HT) abundance in the embryonic mouse brain disrupts the precision of sensory maps formed by thalamocortical axons (TCAs), suggesting that 5-HT influences their growth. We investigated the mechanism by which 5-HT influences TCAs during development. 5-HT_{1B} and 5-HT_{1D} receptor expression in the fetal forebrain overlaps with that of the axon guidance receptors DCC and Unc5c. In coculture assays, axons originating from anterior and posterior halves of the embryonic day 14.5 dorsal thalamus responded differently to netrin-1, reflecting the patterns of DCC and Unc5c expression. 5-HT converts the attraction exerted by netrin-1 on posterior TCAs to repulsion. Pharmacological manipulation of 5-$HT_{1B/1D}$ receptors and intracellular cAMP showed the signaling cascade through which this modulation occurs. An *in vivo* correlate of altered TCA pathfinding was obtained by transient manipulation of 5-$HT_{1B/1D}$ receptor expression abundance in the dorsal thalamus by *in utero* electroporation. These data demonstrate that serotonergic signaling has a previously unrecognized role in the modulation of axonal responsiveness to a classic guidance cue.

Specific neurotransmitter systems may be the targets of disrupted development in neuropsychiatric disorders[1,2]. There are at least two potential outcomes of such disturbances, which relate to the pleiotropic nature of neurotransmitters: (i) direct disruption of neuronal signaling in the adult and (ii) perturbations of brain histogenetic processes that are mediated in part by biogenic amines before they act as classical neurotransmitters. For example, temporally restricted genetic deletion of the 5-HT_{1A} receptor[3] during development is sufficient to produce altered behavior in the adult. Similarly, transient pharmacological inhibition of the 5-HT transporter (SERT) during early development produces abnormal emotional behaviors in adult mice[4]. These findings suggest that atypical 5-HT function during a sensitive developmental period alters the formation and subsequent function of behaviorally relevant brain circuitry. There have been long-standing hypotheses regarding such nonclassical roles for 5-HT because of its very early expression in embryogenesis and the developing CNS. Some early actions of 5-HT include effects on the morphogenesis of CNS and craniofacial structures, as well as subtle effects on neuronal growth, cell migration and survival[5–9]. The reported effects vary widely, probably reflecting the fact that, as in the adult, 5-HT actions during development differ according to the receptor subtypes and downstream transduction mechanisms. Perhaps the most notable example of a prominent developmental disruption following altered 5-HT levels is the aberrant formation of the somatosensory map in the mouse barrel cortex in genetic mutants of monoamine oxidase A (MAOA)[10] and of SERT[11].

We recently documented the dynamic expression patterns of 5-HT_1 receptor subfamily members in the telencephalon of the prenatal mouse[12]. There were unexpected spatial and temporal expression patterns of 5-HT_{1B} and 5-HT_{1D} (*Htr1b* and *Htr1d*) receptor transcripts, particularly in the dorsal thalamus, where there is overlap with expression of the axon guidance receptors DCC, Unc5c (Unc5h3), and Robo1 and Robo2 (refs. 13,14). In transfected cell lines and in the adult brain, both 5-HT_{1B} and 5-HT_{1D} receptors are coupled to the inhibition of adenylate cyclase via $G_{i/o}$ proteins, which leads to an intracellular decrease of cAMP[15,16]. Because altering intracellular cAMP and cyclic guanine monophosphate can influence the axon guidance of isolated spinal and peripheral neurons by modulating their responsiveness to attractive or repulsive cues[17–20], we hypothesized that 5-HT could influence aspects of TCA development by modulating intracellular second messenger systems through $G_{i/o}$ coupled 5-HT_1 receptors. We tested this hypothesis using explant cultures and found that 5-HT converts the attraction exerted by netrin-1 on developing posterior dorsal thalamus axons to repulsion through a 5-$HT_{1B/1D}$ receptor–dependent mechanism that controls intracellular cAMP. We also report the disruption of TCA development *in vivo* by using *in utero* electroporation of siRNAs and expression plasmids, targeting specifically the developing dorsal thalamus, to transiently decrease or increase 5-$HT_{1B/1D}$ receptor expression, respectively.

RESULTS

Previously, we showed that 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D} and 5-HT_{1F} receptors are expressed transiently and in distinct patterns in the fetal dorsal thalamus[12]. *Htr1b* and *Htr1d* transcripts are expressed in the dorsal thalamus by embryonic day 14.5 (E14.5), the age used to harvest

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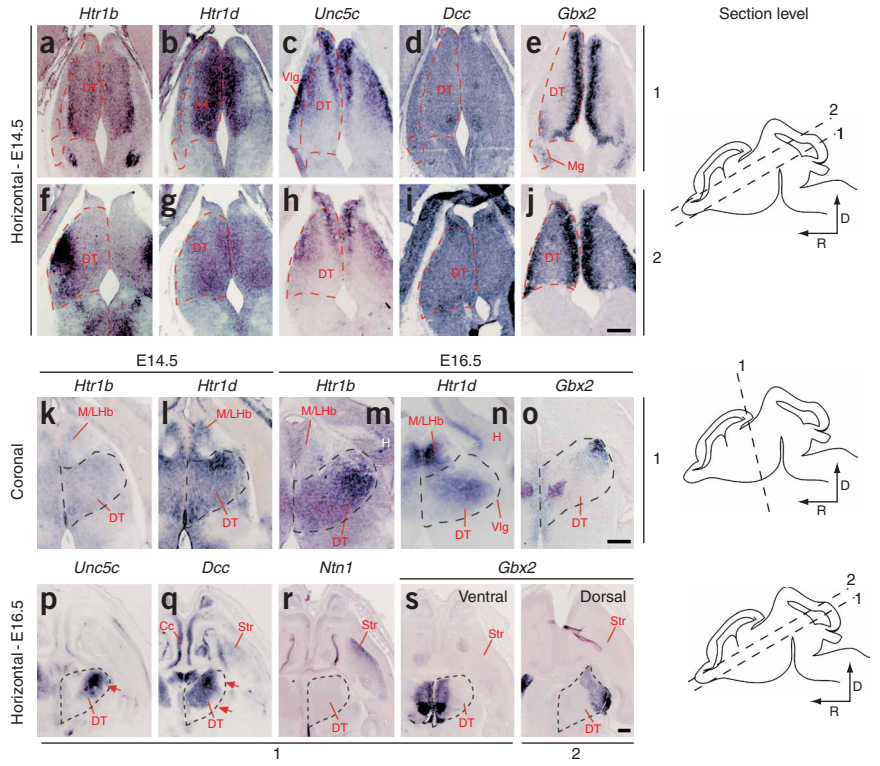

Figure 1 Bright-field micrographs illustrate the distribution of *Htr1b*, *Htr1d*, *Dcc* and *Unc5c* receptor transcripts and *Gbx2* transcription factor transcripts in embryonic dorsal thalamus (DT). (**a**–**j**) *In situ* hybridization performed with DIG-labeled riboprobes in the horizontal plane (at two different ventrodorsal levels, right) showed that *Htr1b* (**a**,**f**), *Htr1d* (**b**,**g**) and *Dcc* (**d**,**i**) receptor transcripts were expressed throughout the DT at E14.5, with a slight anterior-low to posterior-high gradient. *Unc5c* (**c**,**h**) expression was restricted to anterior and mediolateral regions of the DT at this age. The *Gbx2* (**e**,**j**) expression pattern delineates the boundaries (red dotted line) of the DT. (**k**–**s**) Sections in the coronal plane showed *Htr1b* (**k**,**m**) and *Htr1d* (**l**,**n**) expression in the DT at E14.5 and E16.5; expression patterns appeared more regionalized by E16.5 as DT nuclei were forming and present, like *Gbx2* (**o**), a medial to lateral gradient in the posterior dorsal thalamus. (**p**,**q**) *Unc5c* and *Dcc* expression in the DT at E16.5 on horizontal sections; both receptors were expressed in the anterior DT, but compared with *Unc5c* (**p**), *Dcc* expression also included the posterior DT (**q**, red arrows); netrin-1 was expressed in a anterior-high to posterior-low gradient in the striatum (**r**; see also **Supplementary Fig. 4**). At E16.5, expression of *Gbx2* was restricted ventrally to the medial DT and dorsally to the lateral DT (**s**). Cc, cingulate cortex; H, hippocampus; M/LHb, medial/lateral habenula; Str, striatum; Vlg, ventral lateral geniculate nucleus; Mg, medial geniculate nucleus; D, dorsal; R, rostral. Scale bars, 150 μm.

tissue for our *in vitro* coculture experiments, and at E16.5, when TCAs actively grow in the basal forebrain toward the dorsal pallium[21]. There is *Htr1b* expression in the dorsal thalamus at E14.5, which increases at E16.5, particularly in the most lateral regions, where the ventrolateral, ventral posterolateral and posteromedial thalamic nuclei are forming (**Fig. 1**). Similarly, there is abundant expression of *Htr1d* mRNA in the dorsal thalamus, peaking in intensity at E16.5 (**Fig. 1b**,**g**,**i**,**n**).

The expression patterns of 5-HT receptor transcripts in the dorsal thalamus overlap with those of receptors for several guidance cues, including DCC, Unc5c (ref. 13) and Robo1 and 2 (ref. 22). To compare directly *Htr1b* and *Htr1d* expression with netrin-1 receptor expression in the dorsal thalamus, we carried out *in situ* hybridization using riboprobes complementary to the 3′ untranslated region (UTR) of the *Dcc* and *Unc5c* transcripts at E14.5 (**Fig. 1c**,**d**,**h**,**i**) and E16.5 (**Fig. 1p**,**q**). The transcription factor gastrulation brain homeobox 2 (Gbx2)[23] was used to delineate the boundaries of the developing dorsal thalamus at E14.5 and E16.5 (**Fig. 1e**,**j**,**o**,**s**). Analysis at different dorsoventral levels showed that *Gbx2* transcript was not uniformly expressed throughout the dorsal thalamus, but in combination the analyses provided an accurate demarcation. *In situ* hybridization carried out in the horizontal plane at two different ventrodorsal levels showed that at E14.5, *Dcc* and *Unc5* transcripts overlap broadly in the anterior dorsal thalamus, although *Unc5c* transcripts are much more abundant (**Fig. 1 c**,**h**). In contrast with *Gbx2*, which was expressed over the whole rostrocaudal extent of the dorsal thalamus at E14.5 (**Fig. 1e**,**j**), *Unc5c* expression formed a distinct rostral-high to caudal-low gradient (**Fig. 1c**,**h**) throughout the ventrodorsal axis. At both ventrodorsal levels of the dorsal thalamus shown in **Figure 1**, *Dcc* was expressed in a very slight anterior-low to posterior-high gradient (**Fig. 1d**,**i**). At E16.5, both *Dcc* and *Unc5c* were intensely expressed in the most anterior aspect of the dorsal thalamus, whereas *Dcc* expression appeared to be stronger than that of *Unc5c* in the posterior dorsal thalamus (**Fig. 1p**,**q**). These mapping data indicate that the expression patterns of the *Htr1b*/*Htr1d*, *Dcc* and *Unc5c* transcripts broadly overlap at E14.5 and E16.5 in the posterior dorsal thalamus. These transcript patterns for DCC and Unc5c are consistent with previously published studies (refs. 13,24,25 and the Brain Gene Expression Map database, http://www.stjudebgem.org).

Effect of netrin-1 on dorsal thalamus axons

To examine the influence of specific guidance cues on directed dorsal thalamus axonal outgrowth, we employed an *in vitro* assay using E14.5 dorsal thalamus explants, which we cocultured with aggregates of HEK-293 cells that stably expressed soluble forms of netrin-1 or slit-2 (ref. 26,27) in a matrix that allowed for three-dimensional outgrowth of axons (see Methods and ref. 13). Given the distinct patterns of receptor expression that we identified, the dorsal thalamus was subdivided into the anterior and posterior regions to assess potential differences in responsiveness to guidance cues. We used quadrant-based quantitative analysis of the orientation of dorsal thalamus axon outgrowth (**Fig. 2** and Methods). Not every axonal response in this assay was identical, which was expected given that the tissue contains different developing thalamic nuclei that show distinct projection patterns *in vivo*. The initial analysis showed that fibers emerging from explants of the posterior half (**Fig. 2a**) of the dorsal thalamus were attracted toward the source of netrin-1 (**Fig. 2d**), but were repelled away from a source of slit-2 (**Fig. 2f**). Parental HEK-293 cells do not influence the directionality of dorsal thalamus axon outgrowth (**Supplementary Fig. 1** online). In contrast, axons growing out of explants isolated from the anterior half of the dorsal thalamus are repelled from sources of either netrin-1 or slit-2 (**Fig. 2c**,**e**). Statistical analysis of the data obtained from over four independent experiments showed that the attractive effect of netrin-1 on axons from the posterior dorsal thalamus was significantly different from the mainly repulsive effect that netrin-1 exerts on axons from the anterior dorsal thalamus (**Fig. 2g**).

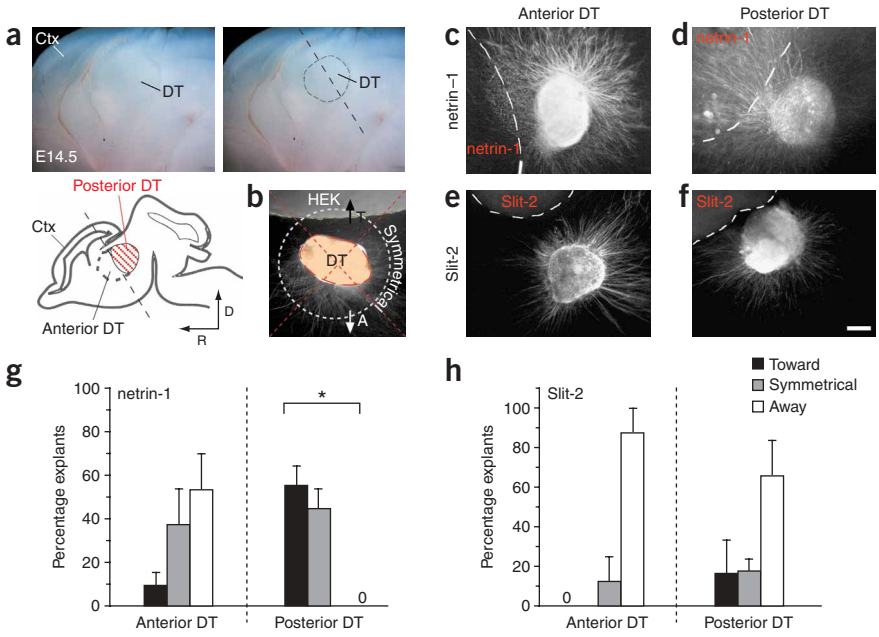

Figure 2 Analysis of attractive and repulsive effects of netrin-1 and slit-2 on DT axons *in vitro*. (**a**) Sagittal view of the prosencephalon at E14.5 illustrates the DT region dissected for the explant assays (dotted circle), which includes the mediolateral extent of the DT and most medially, part of the epithalamus (the pretectal area is avoided). The anterior and posterior halves of the tissue were obtained by a single bisecting cut (dotted line). Ctx, cortex; D, dorsal; R, rostral. (**b**) Method used to quantify axon outgrowth from DT explants, as either toward (T) or away (A) from the source of soluble guidance cues (HEK cells) or 'symmetrical' around the DT explant (no preferential direction; dotted circle). (**c**–**f**) DT explants at E14.5 + 3 DIV immunostained for Tuj1 show that axons from the anterior DT are repelled away (**c**), whereas axons from the posterior DT are attracted toward (**d**) a source of netrin-1. (**e**, **f**) Slit-2 repels axons originating from either anterior (**e**) or posterior (**f**) DT. Scale bar, 100 μm. (**g**) Quantification over four independent experiments shows that the response of anterior DT axons to netrin-1 was significantly different than the response of posterior DT axons (*, $\chi^2 = 16.57$, $P < 0.0001$, d.f. = 2, χ^2; $n = 20$ and 19 explants, respectively). (**h**) In contrast, the response to slit-2 was not significantly affected by the origin of explants (anterior versus posterior, $\chi^2 = 2.80$, $P = 0.2779$, d.f. = 2, χ^2; $n = 16$ and 26 explants, respectively; values ± s.e.m.).

Consistent with this difference between anterior and posterior segments, in experiments in which we isolated more finely dissected anterior, middle and posterior dorsal thalamus, we obtained differentially repelled, nonpreferential and attractive responses of dorsal thalamus axons to netrin-1 cues, respectively (**Supplementary Fig. 1**). Conversely, there was no significant difference between the responses of anterior and posterior dorsal thalamus axons to slit-2 (**Fig. 2h**). The distinct outgrowth responses to netrin-1 by the anterior and posterior halves of the dorsal thalamus are consistent and predictable in the context of our mapping data and with previously published reports showing that DCC alone mediates netrin-1 attraction, whereas DCC-Unc5c complexes mediate netrin-1 repulsion[28]. Moreover, the data are consistent with the intense DCC immunoreactivity observed on axons and growth cones originating from posterior dorsal thalamus explants (**Supplementary Fig. 1**).

5-HT modulation of posterior axon response to netrin-1

On the basis of the *Htr1b* and *Htr1d* transcript expression we observed in the dorsal thalamus, we next tested the possibility that 5-HT modulates the response of dorsal thalamus axons to netrin-1 and/or slit-2. Dorsal thalamus explants were exposed to increasing concentrations of 5-HT. High-performance liquid chromatography analysis showed that the basal serum-free culture medium did not contain 5-HT (**Supplementary Fig. 1**). In the concentration range tested (3 nM–30 μM), 5-HT did not significantly influence the direction or outgrowth rate of axons when dorsal thalamus explants were cocultured with parental HEK-293 cells (**Supplementary Fig. 1**), nor did it modulate the repulsive effect of either netrin-1 on anterior dorsal thalamus axons or slit-2 on anterior and posterior dorsal thalamus axons (data not shown). However, when explants from posterior dorsal thalamus, grown in the presence of netrin-1–expressing HEK-293 cells, were treated with 30 μM 5-HT, axons were repelled from, instead of being attracted to, the source of netrin-1 (**Fig. 3**). Dose-response analysis showed that nanomolar concentrations of 5-HT were sufficient to alter the responsiveness of axons to netrin-1, with the optimal response at 30 μM (data not shown).

5-HT$_{1B/1D}$ and cAMP modulate axon response to netrin-1

The expression of *Htr1b* and *Htr1d* transcripts in the dorsal thalamus at the time the explants are generated suggests that these receptors are likely to mediate the 5-HT effect on directed axon outgrowth that we observed *in vitro*. Consistent with this, immunohistochemical staining of thalamic explants after 3 days *in vitro* (DIV) showed that 5-HT$_{1D}$ receptor protein was distributed along dorsal thalamus axons and growth cones (**Fig. 3c**,**d**), similar to 5-HT$_{1B}$ immunoreactivity shown previously[29].

To determine whether 5-HT$_{1B/1D}$ receptors mediate the modulation of netrin-1 responsiveness by 5-HT, we used a potent 5-HT$_{1B/1D}$ agonist, L694,247 (ref. 30), and antagonist, BRL-15572 (ref. 31). Cotreating posterior dorsal thalamus explants with 5-HT and BRL-15572 at 30 μM completely blocked 5-HT modulation of netrin-1 responsiveness of dorsal thalamus axons (**Fig. 3b**,**e**). A dose-response study showed that concentrations as low as 300 nM in the culture medium potently blocked the 5-HT modulation of the response to netrin-1 (data not shown). BRL-15572 alone had no significant effect on the response of dorsal thalamus axons to netrin-1 (data not shown). L694,247 mimicked the effect of 5-HT in changing the response of posterior dorsal thalamus axons to netrin-1 from attraction to repulsion (**Fig. 3f**).

Our results demonstrate that activation of 5-HT$_{1B/1D}$ receptors on posterior dorsal thalamus neurons switches the response of TCAs to netrin-1 from attraction to repulsion. 5-HT$_{1B/1D}$ receptors are coupled to the inhibition of adenylate cyclase via $G_{i/o}$ proteins, and their activation leads to an intracellular decrease of cAMP[15,16]. On the basis of a previous analysis of isolated spinal neurons[18], we hypothesized that 5-HT$_{1B/1D}$ receptors modulate the response of TCAs to netrin-1 by changing the intracellular abundance of cyclic nucleotides in dorsal thalamus neurons. To test this directly, we modulated the abundance of cAMP using a well-characterized pharmacological strategy[18,20]. Bath application of 20 μM Rp-cAMPS (a nonhydrolyzable analog of cAMP and antagonist of protein kinase A, PKA[32]) converted

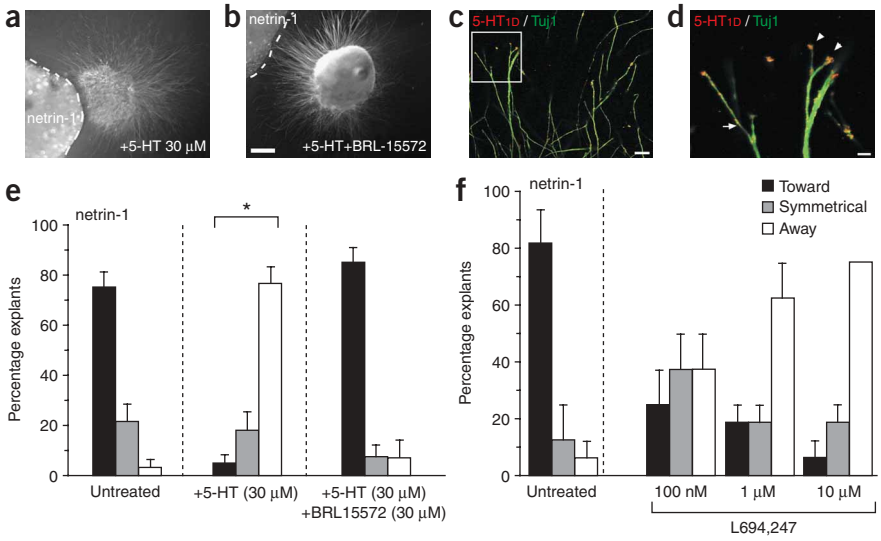

Figure 3 5-HT1B/1D receptors mediate 5-HT modulation of DT axons response to netrin-1. (**a**,**b**) Tuj1 immunostaining of posterior DT explants at E14.5 + 3DIV. When 5-HT (30 μM) was added to the culture medium, axons were repelled from the source of netrin-1 (**a**); this effect was inhibited by a 5-HT_{1D} antagonist (BRL-15572, **b**). Scale bar, 100 μm. (**c**) Confocal image showing 5-HT_{1D} immunostaining on axons and growth cones of DT neurons *in vitro* at E14.5 + 3 DIV. Scale bar, 20 μm. (**d**) Higher magnification of the growth cone area boxed in **c**. Scale bar, 5 μm. (**e**) Quantification over four independent experiments showed that 5-HT (30 μM) had a significant effect in switching the response of posterior DT axons to netrin-1 from attraction to repulsion (*, $\chi^2 = 44.00$, $P < 0.0001$, d.f. = 2, χ^2). In cultures treated with both 5-HT and the 5-HT_{1D} antagonist BRL-15572 (30 μM), the response of posterior DT axons to netrin-1 was not significantly different from untreated cultures ($n = 4$ independent experiments; $\chi^2 = 3.57$, $P = 0.2083$, d.f. = 2, χ^2; untreated explants: $n = 35$; 5-HT–treated explants: $n = 34$, 5-HT + BRL-15572–treated explants: $n = 30$; values ± s.e.m.). (**f**) Dose-response study of the effect of the 5-$HT_{1B/1D}$ agonist L694,247 showed that concentrations as low as 100 nM mimic the effect of 5-HT in changing the response of posterior DT axons to netrin-1 from attraction to repulsion.

the response of posterior dorsal thalamus axons to netrin-1 from attraction to repulsion (**Fig. 4**). In contrast, Sp-cAMPS (20 μM), an analog of cAMP that activates PKA[32], blocked the effect of 5-HT on the posterior dorsal thalamus axon response to netrin-1 (**Fig. 4e**). Sp-cAMPS alone had no direct effect on the response of dorsal thalamus axons to netrin-1 (**Fig. 4c**). These observations are consistent with our hypothesis that the ability of 5-HT to modulate the response of posterior dorsal thalamus axons to netrin-1 is mediated by a 5-$HT_{1B/1D}$–induced intracellular decrease of cAMP in dorsal thalamus neurons.

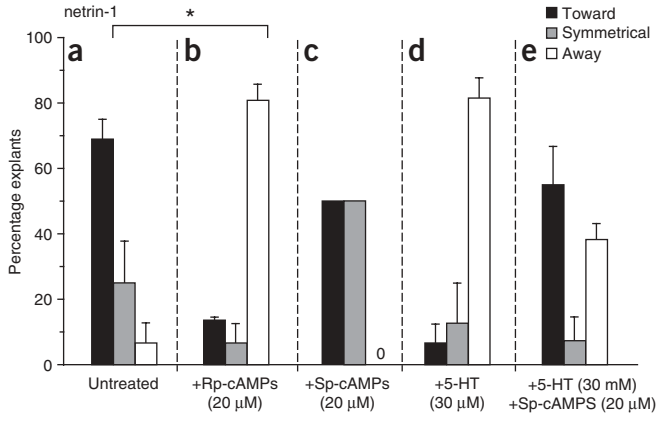

Figure 4 Modulation of the posterior DT axon response to netrin-1 is mediated by a 5-$HT_{1B/1D}$–mediated change of intracellular cAMP. (**a**–**e**) The DT axon response to netrin-1 was measured under the following conditions: untreated (**a**), 20 μM Rp-cAMPs (**b**), 20 μM Sp-cAMPs (**c**), 30 μM 5-HT (**d**) and 30 μM 5-HT + 20 μM Sp-cAMPs (**e**). Rp-cAMPs, like 5-HT, switched the DT axon response to netrin-1 from attraction to repulsion (*, $\chi^2 = 24.07$, $P < 0.0001$, d.f. = 2, χ^2; untreated explants: $n = 24$; Rp-cAMPS–treated explants: $n = 19$). Sp-cAMPs alone had no significant effect on the DT axon response to netrin-1 ($\chi^2 = 4.23$, $P = 0.1256$, d.f. = 2; Sp-cAMPS–treated explants: $n = 13$), but significantly blocked the effect of 5-HT (responses from untreated and [5-HT + Sp-cAMPS]-treated explants were not significantly different; $\chi^2 = 5.53$, $P = 0.0747$, d.f. = 2; values ± s.e.m.).

5-$HT_{1B/1D}$ expression influences TCA pathfinding *in vivo*

Our *in vitro* studies indicated that 5-HT is a potent modulator of posterior dorsal thalamus axon responsiveness to netrin-1, and suggested that 5-$HT_{1B/1D}$ activation in the dorsal thalamus may influence TCA pathfinding *in vivo*. To test this possibility, we used targeted *in utero* electroporation to the dorsal thalamus to selectively alter the expression of both 5-$HT_{1B/1D}$ receptors. 5-$HT_{1B/1D}$ siRNAs or control siRNA were injected into the third ventricle at E12.5 (**Fig. 5**) with the pCAG-EYFP plasmid (encoding enhanced yellow fluorescent protein), which labels all transfected cells (see ref. 33). The EYFP label facilitates visualization of the complete axonal projection of transfected dorsal thalamus neurons. The technical difficulty of targeting the dorsal thalamus *in utero* at E12.5 creates variability between brains in terms of the precise location of the electroporated plasmids. We therefore identified, while blinded to treatment and before any analysis, those brains in which only the dorsal thalamus was labeled ($n = 25$). Potential alterations of the TCA pathway were investigated using netrin-G1 immunostaining, which specifically labels TCAs at E16.5 and E18.5 (ref. 34; and see Methods for details). When control siRNAs and EYFP reporter plasmids are electroporated at E12.5, $EYFP^+$ cells are present throughout the dorsal thalamus at E18.5 (**Fig. 5b**). Labeled axons ($EYFP^+$) that originate from electroporated dorsal thalamus neurons projected normally through the internal capsule and toward the cortex (**Fig. 5d**), as previously described[35,36].

Further immunohistochemical staining for the CAM-like adhesion molecule L1, which labels subpopulations of both thalamocortical and corticofugal axons at this stage of brain development[37], showed that $L1^+$ corticothalamic axons followed a normal trajectory on the electroporated side of the brain (data not shown). After control siRNA electroporation in the dorsal thalamus at E12.5, the paths followed by netrin-$G1^+$ axons around the cortico-striatal boundary and in the cortex at E18.5 were identical on the control (**Fig. 6**) and electroporated (**Fig. 6b,c**) sides. The overlap of netrin-$G1^+$ axon pathways on the control and electroporated sides is shown (**Fig. 6d**). Quantification of netrin-G1 immunofluorescent intensities along the

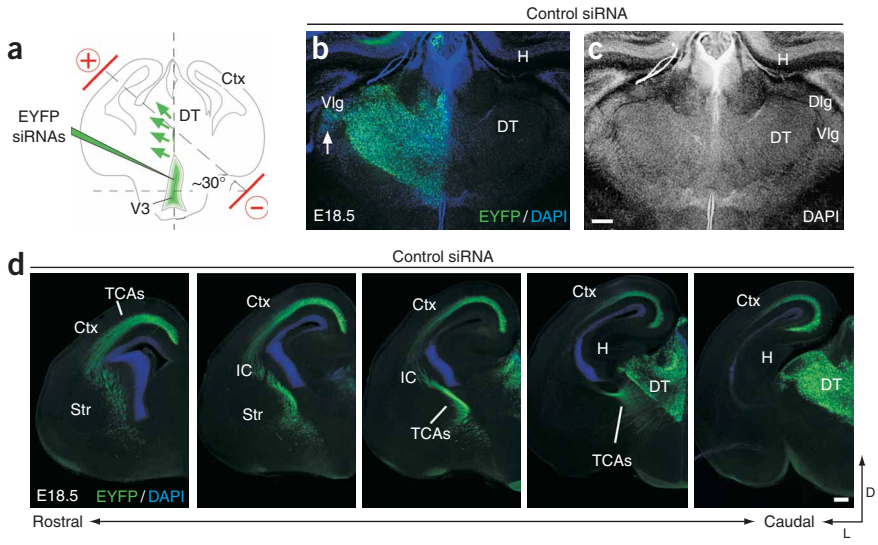

Figure 5 *In utero* electroporation of E12.5 DT neurons to examine TCA pathway formation *in vivo*. (**a**) Schematic representation of the electroporation protocol. Solutions containing 5-$HT_{1B/1D}$ overexpression plasmids or siRNAs and pCAG-EYFP reporter plasmids were injected into the third ventricle at E12.5 and an electric pulse was applied through the DT with the cathode apposed on the dorsomedial side of the brain, forming a 30° angle with the horizontal plane. (**b**) Brain electroporated in the DT at E12.5, harvested at E18.5 and stained for EYFP; neurons throughout the DT expressed the EYFP reporter protein (green). Some cells can be seen populating the Vlg at E18.5 (arrow). (**c**) DAPI staining reveals that *in utero* electroporation by itself does not alter the normal development of the DT, as assessed by cell density and cytoarchitecture. Scale bar, 200 μm. (**d**) Coelectroporation of a control siRNA and EYFP reporter plasmid at E12.5 in the DT allowed the subsequent tracking of TCAs at E18.5 ($EYFP^+$, green) throughout the rostrocaudal extent of the brain; control siRNA or electroporation by itself did not alter the normal projection patterns of TCAs. Scale bar, 200 μm. L, lateral. Ctx, cortex; IC, internal capsule; V3, third ventricle; Dlg, dorsal lateral geniculate nucleus.

mediolateral axis of the cortex (see Methods) confirmed that netrin-$G1^+$ axons distribute identically on both sides of control siRNA–electroporated brains (**Fig. 6e**).

In contrast to control siRNA, electroporation of siRNAs that specifically reduce *Htr1b*/*Htr1d* expression in the dorsal thalamus (see **Supplementary Fig. 2** online) produced an expansion of the netrin-$G1^+$ axon pathway into more lateral-ventral regions of the dorsal pallium at E18.5 ($n = 5$ out of 5 brains; **Fig. 6f–h**). Furthermore, the subpopulation of shifted netrin-$G1^+$ axons on the electroporated side were also $EYFP^+$ (**Fig. 6h**), indicating that these axons originated from dorsal thalamus neurons that had reduced expression of *Htr1b*/*Htr1d*. Measures of netrin-G1 immunofluorescent intensity along the TCA pathway (at the level shown in **Supplementary Fig. 3** online) confirmed that axons originating from dorsal thalamus neurons electroporated with *Htr1b*/*Htr1d* siRNA expand ventrolaterally in the cerebral wall as compared with the control side (**Fig. 6j**). We obtained the exact opposite growth pattern of TCAs after overexpression of 5-$HT_{1B/1D}$ receptors by electroporation of expression plasmids into the dorsal thalamus at E12.5. In these brains, the pathway of netrin-$G1^+$ axons was more restricted dorsomedially in the dorsal pallium (**Fig. 6k,l**; $n = 5$ out of 6 brains), with similar patterns observed at multiple rostrocaudal levels. Measures of immunofluorescent intensity on each side of the brain (**Fig. 6o**) confirmed that netrin-G1 immunoreactivity was decreased laterally in the cerebral wall. A detailed examination of the corticostriatal boundary showed that $EYFP^+$ axons (which originated from dorsal thalamus neurons overexpressing 5-$HT_{1B/1D}$ receptors) made a sharp turn in the internal capsule and followed a somewhat more medial course in the cortex compared with the axons of nonelectroporated neurons (netrin-$G1^+$ only; **Fig. 6m**). Such a ventrolateral reduction (after overexpression) or expansion (after siRNA treatment) of TCA pathways was never observed when control siRNA or control expression plasmids were electroporated in identical dorsal thalamus regions ($n = 8$ out of 8; **Fig. 6a–e**); therefore, disruption of TCA pathfinding can be specifically attributed to alteration of 5-$HT_{1B/1D}$–mediated signaling.

To understand the possible basis for these shifts in dorsal thalamus axon trajectory, we carried out a more detailed analysis of the patterns of netrin-1 expression in the ventral forebrain, where dorsal thalamus axon sorting occurs. Netrin-1 expression in the internal capsule at E14.5 and E16.5 (**Fig. 7** and **Supplementary Fig. 4** online) showed a very complex tridimensional gradient (**Fig. 7e**) that is compatible with a potential role in the 'sorting', or proper ventrodorsal and mediolateral positioning, of TCAs before they reach the cortex (see ref. 13). In experiments in which TCAs from the anterior and posterior dorsal thalamus at E14.5 were labeled with the fluorescent lipophilic probes DiI and DiA, respectively, we observed that in all of the brains analyzed ($n = 10$), anterior dorsal thalamus axons grew preferentially following a dorsomedial course in the internal capsule, along the lower–netrin-1 part of the gradient. Conversely, posterior dorsal thalamus axons followed a more ventrolateral course, along the higher–netrin-1 part of the gradient (**Fig. 7d**). The growth trajectory was also highly dependent on the medial or lateral placement in either the anterior or posterior dorsal thalamus, as medial axons in either region tended to project more anteriorly than those originating from lateral regions (**Fig. 7d** and **Supplementary Figs. 4** and **5** online). Thus, the changes in the TCA pathway that we observed at E18.5 after manipulation of 5-$HT_{1B/1D}$ expression levels could be due to an altered response of dorsal thalamus axons to netrin-1 in the internal capsule, consistent with our observation *in vitro*. We therefore examined, after *in utero* dorsal thalamus electroporations at E12.5, TCA pathways in the internal capsule at E16.5, when axons are still actively growing through the region. After *Htr1b*/*Htr1d* siRNA electroporation (**Fig. 8**), a subpopulation of TCAs ($EYFP^+$ axons; **Fig. 8b,c**) spread ventrally in the internal capsule and cerebral wall, compared with brains electroporated with control siRNA (**Fig. 8a**). In contrast, when 5-$HT_{1B/1D}$ receptors were overexpressed ($EYFP^+$ axons; **Fig. 8d,e**), TCAs followed a more dorsally restricted path in the internal capsule and coursed more medially in the cerebral wall compared with normal (not shown). Therefore, increasing and reducing expression of 5-$HT_{1B/1D}$ receptors in the dorsal thalamus (compare **Fig. 8b,d** and **8c,e**) produce opposite effects on TCA pathways in the internal capsule at E16.5 (**Fig. 8e–g**). Note that in both situations, $EYFP^+$ axons were still distributed in the internal capsule, albeit abnormally, within the normal boundaries of the TCA pathway.

DISCUSSION

The present study demonstrates that TCA responsiveness to netrin-1 is modulated by 5-HT through $G_{i/o}$-coupled 5-$HT_{1B/1D}$

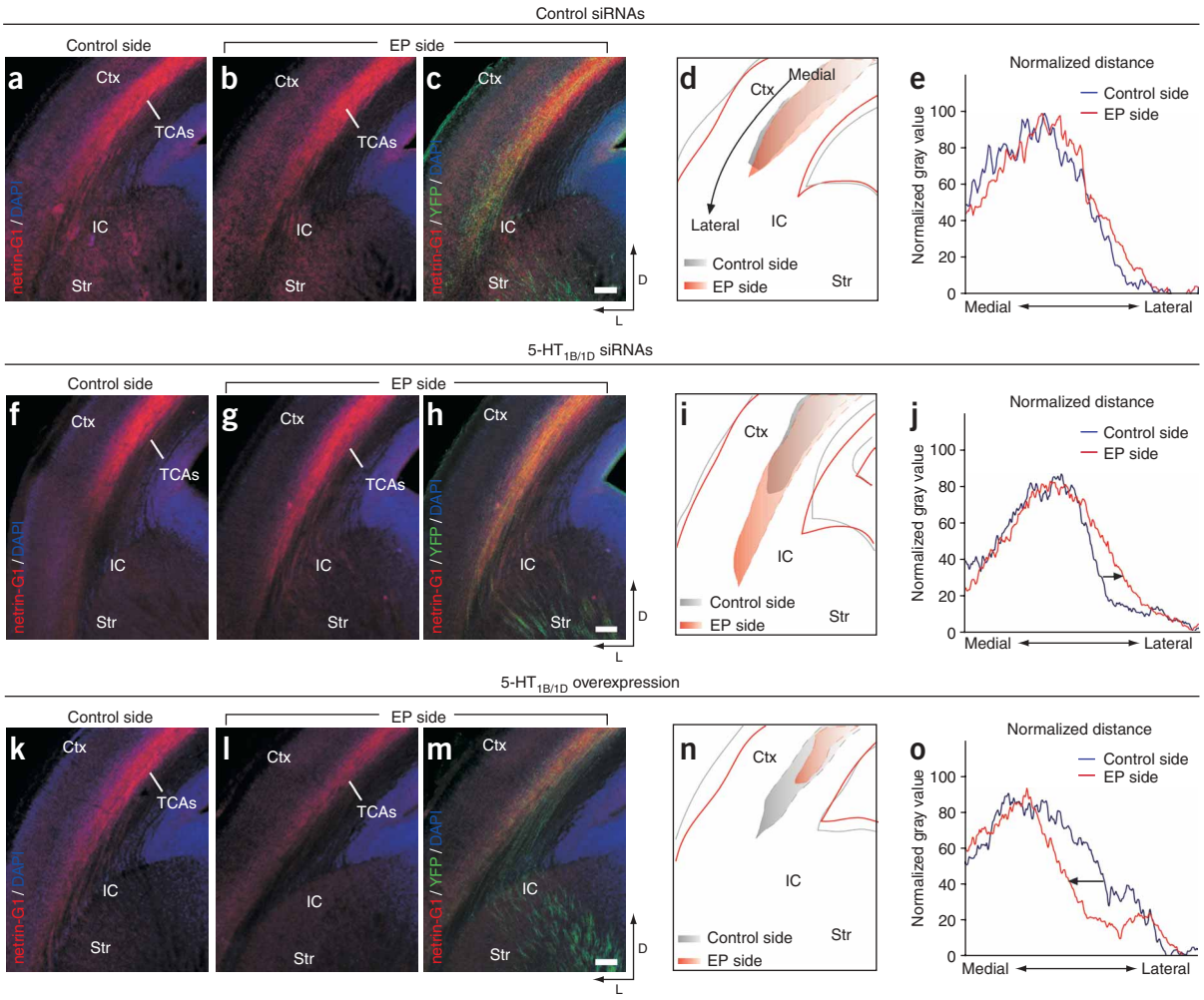

Figure 6 Modifying 5-HT$_{1B/1D}$ receptor expression *in vivo* alters the topography of a subpopulation of TCAs. (**a**–**e**) After *in utero* DT electroporation of control siRNA at E12.5, netrin-G1$^+$ axons at E18.5 extended ventrolaterally in the cortex along similar pathways on the nonelectroporated (control, **a**) and electroporated (EP, **b**) sides. (**c**) Netrin-G1$^+$ and EYFP$^+$ axons overlapped on the electroporated side (**d**). Netrin-G1 immunofluorescent quantification (**e**) showed that TCA pathways on the control side (gray in **d**, blue curve in **e**) and control siRNA-electroporated side (red in **d**, red curve in **e**) overlapped in the cortex. (**f**–**h**) 5-HT$_{1B/1D}$ siRNA electroporation at E12.5 in the DT induced a ventrolateral shift/expansion of netrin-G1$^+$/EYFP$^+$ axons on the electroporated side of the cortex (**g**,**h**) at E18.5 compared with the control side (**f**). (**i**,**j**) Schematic representation (**i**) and quantification (**j**) of netrin-G1 immunofluorescent distribution illustrating the ventrolateral shift/expansion on the electroporated side (red curve, **j**) of the cortex. (**k**–**m**) pCAG–5-HT$_{1B/1D}$ electroporation at E12.5 reduced the number of netrin-G1$^+$ axons projecting ventrolaterally in the cortex. Netrin-G1$^+$/EYFP$^+$ axons in the cortex at E18.5 (**l**,**m**) appeared to be shifted dorsally on the electroporated side compared with the control side (**k**) of the same brain. (**n**) Schematic representation of netrin-G1$^+$ axon pathways shown in **k** (gray) and **l** (red). (**o**) Quantification illustrating the ventrolateral decrease of netrin-G1$^+$ fibers on the electroporated side (red curve) of the cortex. Scale bars, 100 μm.

receptor subtypes through the alteration of intracellular cyclic nucleotide levels. *In vivo*, we observed consistent, though modest, disruptions of TCA pathway topography following *in utero* alteration of 5-HT$_{1B/1D}$ receptor expression. The lack of 5-HT modulation of either netrin-1 (on anterior dorsal thalamus axons) or slit-2 repulsive cues in general suggests a highly selective mechanism through which specific G protein–coupled receptors affect intracellular translation of axon guidance receptor activity on defined subsets of axons. These data provide a potential mechanism for altered TCA patterning after prenatal increases in 5-HT[10,11]. The results also suggest that long-term changes in behavior following developmental disruption of 5-HT receptor signaling could occur as a result of altered circuit formation, rather than through alterations later in life in the 5-HT modulation of fast synaptic transmission.

Anterior and posterior thalamic axon response to netrin-1

Detailed expression mapping revealed some unexpected data regarding the differential expression of netrin-1 and 5-HT receptors in subregions of the fetal dorsal thalamus (see also ref. 12). Individually, these patterns are consistent with previous studies that show a widespread expression of *Dcc* throughout the dorsal thalamus[13,24] and a more restricted *Unc5c* expression to anterior dorsal thalamus domains (ref. 25, **Supplementary Fig. 6** online and http://www.stjudebgem.org). We interpreted the distinct outgrowth responses of anterior dorsal thalamus axons to netrin-1 (compared with posterior dorsal thalamus axons) as being related to the repellant activity mediated through the coexpressed Unc5c and DCC receptors, as has been shown previously[28]. These differential responses may be an important mechanism for the initial sorting of dorsal thalamus axons. For example, anterior and

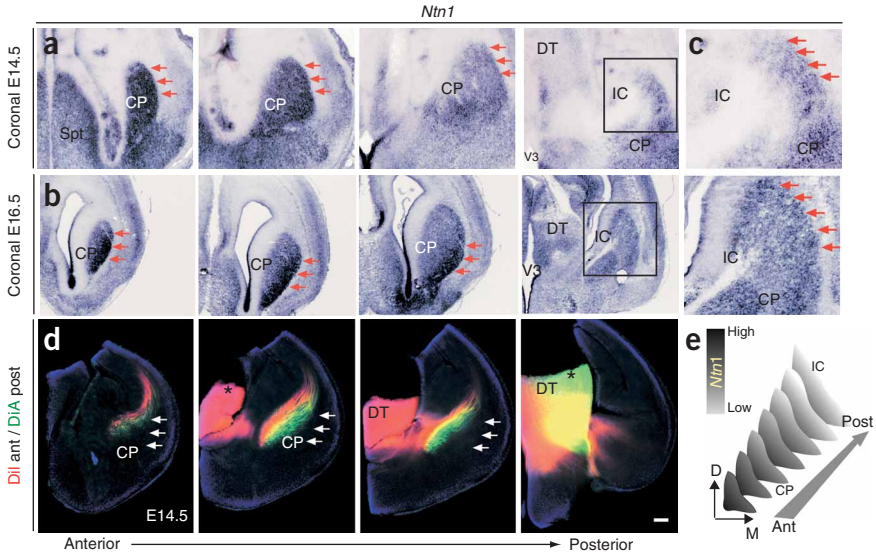

Figure 7 *Ntn1* (netrin-1) transcript expression in the striatum/IC area at E14.5 and E16.5. (**a**) At E14.5, *Ntn1* showed a graded expression throughout the forebrain; *Ntn1* was strongly expressed in the rostroventral domain of the striatum and, more caudally, showed a ventrolateral (pial side) high to dorsomedial (ventricular side) low gradient in the IC (indicated by red arrows). This three-dimensional gradient is schematized in (**e**). (**b**) A similar distribution was observed at E16.5. (**c**) Higher magnification of areas boxed in **a** and **b** show that netrin-1 expression was graded (high ventrolateral to low mediodorsal) in the IC proper. (**d**) TCAs from the anterior and posterior DT at E14.5 (sites of injection are indicated by asterisks) were labeled with DiI (red) and DiA (green), respectively. In all brains analyzed ($n = 10$), anterior DT axons (red) grew preferentially following a dorsomedial course in the IC, along the low netrin-1 part of the gradient. Conversely, posterior DT axons (green) followed a more ventrolateral course, along the higher-*Ntn1* part of the gradient. Scale bar, 100 μm. CP, caudate putamen; Spt, septum; M, medial.

posterior dorsal thalamus axons follow different routes through the internal capsule to reach distinct cortical areas. The graded expression of netrin-1 throughout the rostrocaudal extent of the striatum and internal capsule (ventrolateral (pial side) high to dorsomedial (ventricular side) low) seen with detailed transcript mapping is consistent with the finding that *Dcc/Unc5c*–expressing axons from the anterior dorsal thalamus grew preferentially along the lower netrin-1 part of the gradient; these axons followed a more dorsomedial course in the internal capsule that may have been due partly to a repulsive influence of the more dense ventrolateral source of netrin-1 (**Fig. 7** and **Supplementary Fig. 4**). Conversely, the attractive influence of netrin-1 on posterior dorsal thalamus axons, which was consistent with previous reports (see ref. 13), could account in part for their more ventrolateral and more widespread outgrowth throughout the striatum (**Fig. 7** and **Supplementary Fig. 4**, and see ref. 38).

Two recent reports claim that during fetal development, TCAs from anterior and posterior dorsal thalamus preferentially grow anteriorly and posteriorly in the internal capsule, respectively[38,39]. This simplified model does not agree with previously described projection patterns[40–42], which illustrate a more complex organization. Our own DiI and DiA

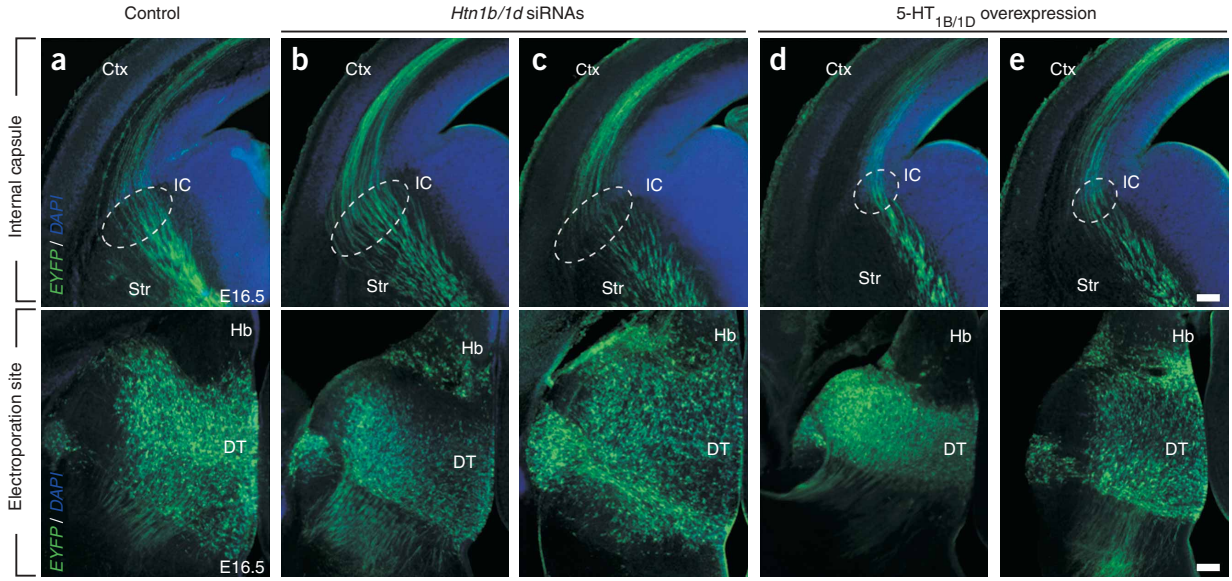

Figure 8 Modifying 5-$HT_{1B/1D}$ receptor expression *in vivo* alters TCA distribution in the internal capsule. Embryos were electroporated at E12.5 in the dorsal thalamus and harvested at E16.5. (**a**) When control siRNAs (or pCAG plasmid) were electroporated in the DT (lower), $EYFP^+$ TCAs were distributed in a stereotypical manner in the IC where they spread out (dotted circle) before turning sharply into the cortex. (**b**,**c**) In 5-$HT_{1B/1D}$ siRNA–electroporated brains, $EYFP^+$ TCAs spread across a larger domain ventrolaterally in the IC (dotted circle) and took a slightly more ventrolateral course when turning in the cortex compared with similarly electroporated controls. (**d**,**e**) After 5-$HT_{1B/1D}$ overexpression in the DT, $EYFP^+$ TCAs followed a more dorsally restricted course in the IC (dotted circle), and a more medial course in the cortex, than similarly electroporated controls. Differences in TCA pathfinding after 5-$HT_{1B/1D}$ siRNA and overexpression were observed across embryos with similar electroporation patterns in the DT (compare **a**,**c**,**e** lower with **b**,**d** lower). Scale bars, 100 μm.

double-labeling also showed a complex pattern, in which the anterior-posterior organization of TCAs in the internal capsule depended on the anterior-posterior and the medial-lateral origin of the axons in the dorsal thalamus. Thus, labeled axons in the antero- or posteromedial aspect of the dorsal thalamus project anteriorly, whereas those in the antero- or posterolateral aspects of the dorsal thalamus project to more posterior cortical areas. This suggests that complex guidance mechanisms occur in the internal capsule to regulate the pathway of TCAs from various sites in the dorsal thalamus. It is important to note that in our *in vitro* assays, anterior and posterior dorsal thalamus explants were composed of mixed populations of neurons of medial and lateral origin in the dorsal thalamus. This is consistent with the observation that *in vitro* the overall response of anterior and posterior dorsal thalamus axons to netrin-1 was not uniform, but rather included subpopulations of explants whose axons are attracted, repelled and unresponsive to netrin-1. These were all included in the statistical analyses of responses.

We did not expect major disruption of the TCA pathway to result from altering 5-HT signaling *in vivo*, as there are other guidance cues expressed in the basal forebrain that also influence the pathways followed by TCAs, which are suggested by the modest disruption of TCA pathfinding in the striatum of netrin-1 (*Ntn1*) knockout mice[13]. However, it is difficult to interpret the phenotype in these knockout mice, as our data showed that netrin-1 also is expressed in the medial dorsal thalamus prenatally.

5-HT, pleiotropic signal changing attraction to repulsion

We hypothesized that 5-HT, acting through 5-HT$_{1B/1D}$ receptors, may promote a regional modulation of TCA guidance. This was supported by several observations. First, 5-HT axons are present in the vicinity of growing TCAs, reaching the basal telencephalon by E14–15, in close apposition with TCAs along the internal capsule[7]. Moreover, 5-HT and its metabolites accumulate at nanomolar concentrations by E16.5 in the forebrain (**Supplementary Fig. 1**). The physiological concentration of 5-HT in the synaptic cleft is in the millimolar range (1–5 mM)[43]. After release, the extrasynaptic concentration of 5-HT can still reach 10 μM[44]. Therefore, physiologically relevant concentrations of 5-HT in and around the synaptic cleft are much higher than those we determined by HPLC in the whole forebrain, where the amine is diluted. The effective concentration of 5-HT that modulated netrin-1 was well within this range.

What is the molecular basis for this modulation? A recent study has shown that PKA stimulation increased DCC translocation in commissural neurons *in vitro*[45]. We colocalized 5-HT$_{1D}$ and DCC receptors along dorsal thalamus axons and at growth cones. Therefore, it is possible that in growing dorsal thalamus axons, 5-HT$_{1B/1D}$–mediated cAMP-dependant inhibition of PKA might modulate DCC receptor translocation at the membrane. As noted above, DCC alone mediates attraction, whereas a DCC-Unc5c complex mediate repulsion[28]. We showed that *Unc5c* is expressed in a marked anterior-high to posterior-low gradient in the dorsal thalamus (see **Supplementary Fig. 6**), whereas *Dcc* is expressed throughout the dorsal thalamus. Thus, if signaling through 5-HT$_{1B/1D}$ receptors inhibits DCC translocation, it could increase the proportion of DCC-Unc5c receptor complexes present at the surface of posterior dorsal thalamus axons and switch responses to netrin-1 from attraction to repulsion. This hypothesis also is consistent with anterior dorsal thalamus explants showing a basal repulsive response to netrin-1 that is not modulated by 5-HT.

5-HT$_{1B/1D}$ receptors modulate TCA topography *in vivo*

Because of the widespread distribution of 5-HT receptor family members in forebrain regions outside of the dorsal thalamus, conditional gene targeting would be necessary to manipulate gene expression in the dorsal thalamus without altering expression elsewhere in the forebrain; however, such mouse lines are not available for 5-HT$_{1B/1D}$ receptors. We developed an alternative strategy of *in utero* electroporation selectively into the dorsal thalamus, using siRNA and overexpression plasmids to manipulate gene expression. Because 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors have similar spatiotemporal expression patterns in the dorsal thalamus and signal through the same pathway, the impact of altered 5-HT signaling was maximized by reducing the expression of both receptors simultaneously. A technical challenge of this method is the inherent difficulty of reproducibly targeting the identical areas of the dorsal thalamus in different embryos. This required the electroporation of more embryos, and before any analysis of axonal patterning, identification (blind to the manipulation) of those cases in which the electroporated region included only the dorsal thalamus. Qualitative analysis revealed that changing 5-HT$_{1B/1D}$ receptor expression caused altered distribution of TCAs ventrodorsally in the internal capsule and subcortical white matter. Unlike gene-targeting studies, in which altered patterning of several forebrain structures through which TCAs normally navigate confounds the analysis, our results showed that a selective alteration of 5-HT signaling in dorsal thalamus neurons led to shifted axonal topography through an otherwise normal terrain.

Why would altering 5-HT signaling induce shifts in TCA trajectories? The *in vitro* results demonstrate that 5-HT signaling, through 5-HT$_{1B/1D}$–mediated changes of intracellular cAMP levels, switched dorsal thalamus axon responses to netrin-1 from attraction to repulsion. The analysis of TCA pathways performed in netrin-1 knockout mice[13] suggested that netrin-1 is important for proper ventrodorsal sorting of growing TCAs, and netrin-1 also is likely to influence anteroposterior organization. We show that TCA response to netrin-1 is modulated by cAMP, suggesting that the sorting of TCAs in the internal capsule likely depends on the control, through 5-HT modulation, of intracellular cAMP levels in dorsal thalamus neurons. The experimentally induced shifts that we observed were consistent with the organization of dorsal thalamus axons in the internal capsule just before birth (**Supplementary Fig. 7** online; at midrostral and middle levels of the internal capsule, the axons arising from posterior dorsal thalamus are situated ventrolateral to the more dorsomedially located anterior dorsal thalamus axons). In the present study, decreased intracellular levels of cAMP in dorsal thalamus neurons as a result of 5-HT$_{1B/1D}$ overexpression resulted in an increased repulsive response of a subpopulation of TCAs that normally are attracted by netrin-1 in the internal capsule. In the coronal plane, these axons adopted a more dorsal trajectory to avoid the ventrolateral netrin-1–rich region. We consistently obtained this shift by 5-HT$_{1B/1D}$ overexpression in the dorsal thalamus. Conversely, in dorsal thalamus neurons in which 5-HT$_{1B/1D}$ expression is decreased by siRNA electroporation, a likely loss of negative regulation of cAMP levels in a subpopulation of TCAs would favor an attractive response to netrin-1, thus positioning them more ventrally.

We suggest that changes in netrin-1 responsiveness by 5-HT are due to an altered DCC/Unc5c ratio in individual axons. We cannot exclude, however, the possibility that altered 5-HT signaling disrupts the responsiveness of dorsal thalamus axons to other guidance cues, because changes in cAMP levels influence axon responsiveness to cues such as slit-2 and semaphorin 3A/C[45,46]. We note that in our explant assays, 5-HT was ineffective in modulating the responsiveness of dorsal thalamus axons to slit-2. There also is a possibility that altered 5-HT signaling could change TCA fasciculation, leading to the expanded and restricted pathways observed in the internal capsule.

However, if this was the case, we would expect a more dramatic phenotype: for example, as reported in the L1-knockout mice[37] where TCAs appear disorganized in the internal capsule. Notably, the changes in TCA topography reported here occurred within the normal boundaries of the TCA pathway, suggesting that disruption of 5-HT signaling does not grossly alter the initial trajectories of these axons (for example, their course through the 'corridor region' lying upstream of the internal capsule). *In vivo*, the site of 5-HT action in the modulation of TCA guidance is likely to reside in the ventral telencephalon, because this is the earliest region of overlap between 5-HT axons and netrin-1 (ref. 47). There is little innervation of the dorsal thalamus at this age, indicating that 5-HT released by serotonergic afferents signals via the 5-$HT_{1B/1D}$ receptors expressed on the growth cones of TCAs as they navigate through the internal capsule.

The data reported here have important implications in understanding the long-term consequences of 5-HT disruption in development. Previous studies show that dramatic increases in 5-HT levels during development[10,11] alter cortical barrel field organization. What could be the outcome of a subtle topographical shift of the TCA pathway as described here? We hypothesize that there will be subtle topographical mismatches after both decreased 5-$HT_{1B/1D}$ signaling (siRNAs), in which posterior dorsal thalamus axons would innervate more lateral and posterior domains of the somatosensory areas (S1), and increased 5-$HT_{1B/1D}$ signaling (overexpression), in which posterior dorsal thalamus axons would innervate more dorsal and anterior areas of S1 in the cortex. Although it will be technically challenging, this question may be addressed using markers of barrel fields (for example, SERT) in early postnatal pups with retro- and anterograde tracing of TCAs in the adult brain after *in utero* electroporation of 5-$HT_{1B/1D}$ receptors siRNAs and overexpression plasmids. There is a possibility that genetic and/or environmental modifications resulting in specific developmental alterations of 5-HT signaling are likely to generate subtle topographical defects of TCA circuit formation. If such defects have consequences for the function of adult TCA circuits, then the regulation of 5-HT signaling during development, via highly selective spatiotemporal control of differential 5-HT receptor expression, forms a potential basis for the neurodevelopmental etiology of mental health disorders in which 5-HT signaling is implicated.

METHODS

Animals. Timed-pregnant C57BL/6^J and CD-1 mice were purchased from the Jackson Laboratory. All research procedures using mice were approved by the Institutional Animal Care and Use Committee at Vanderbilt University and conformed to US National Institutes of Health guidelines. Unless otherwise noted, all reagents were purchased from Sigma.

***In situ* hybridization.** *In situ* hybridization for *Htr1b* and *Htr1d* transcripts were performed as described[12]. We obtained *Dcc* and *Unc5c* (*Unc5h3*) receptor gene sequences from the project 'Ensembl' mouse genome database (www.ensembl.org) using the following accession numbers: *Dcc* (ENSMUST00000073379) and *Unc5c* (ENSMUSG00000059921). We designed probes (510 base pairs (bp) for *Dcc* and 465 bp for *Unc5c*) to target unique sequences (verified using the National Center for Biotechnology Information BLAST server) in the 3′ untranslated regions of each receptor. We generated the *Ntn1* riboprobe from a 376-bp cDNA fragment (clone ID: 1139227; Invitrogen). The *Gbx2* probe was kindly provided by J. Rubenstein. Probes were synthesized and *in situ* hybridizations performed as described[12].

Explant assays. We used a coculture assay to monitor axonal growth from dorsal thalamus explants toward or away from a source of soluble guidance cues (HEK-293 cells stably expressing netrin-1 or slit-2 were a gift from J. Wu[26,27]). The dorsal thalamus[48] was dissected at E14.5 (**Fig. 2a**). We generated explants by cutting posterior and anterior halves of the dorsal thalamus into 16 pieces of approximately 300 μm in diameter. Hanging drops[49] of HEK-293 cells were embedded in Matrigel (35 μl, BD Bioscience) and four dorsal thalamus explants were carefully positioned approximately equidistant (200–400 μm) from the HEK-293 blocks. Cocultures were grown serum-free in Neurobasal medium supplemented with N2 and B27 (Invitrogen). After 72 h, cultures were fixed in 4% paraformaldehyde (PFA) and processed for immunohistochemistry. The attractive or repulsive effects of guidance cues were quantified by blind scoring using a virtual quadrant strategy (**Fig. 2b** and ref. 13). Explants from three or four independent experiments were scored.

Data were analyzed using SAS (Windows version 9, SAS Institute). Pearson χ^2 statistics were used to compare the distribution of the orientations of axons. All statistical comparisons were performed using two-sided tests at the 5% significance level. When sample sizes are small (any of the expected cell counts is less than 5), exact *P* values were calculated.

Explants immunohistochemistry. PFA-fixed explants were incubated overnight in primary antibody (2% BSA, 0.2% Tween-20 in PBS) using the following antibody dilutions: anti-DCC (Pharmingen, 1:500; mouse IgG directed against the intracellular domain of DCC), anti-Tuj1 (Covance, 1:500) and anti-5HT_{1D} (Abcam, 1:2,000, and a previously characterized rabbit polyclonal antibody, 1:10,000; the latter was a kind gift from A. Basbaum; both antibodies yielded similar staining patterns). Explants were washed, incubated overnight with Cy2,3-conjugated secondary antibodies (1:1,000), washed and imaged using an Axiocam CCD camera coupled to a Leica MZFLIII stereoscope.

***In utero* electroporation, localization of EYFP$^+$ and netrin-G1$^+$ axons.** We carried out *in utero* electroporation at E12.5, as described[33]. Briefly, the uterine horns of anesthetized (50 mg per kg of body weight, Nembutal, intraperitoneal) pregnant C57BL/6 mice (E12.5) were exposed. We injected solutions of expression plasmids or siRNAs (plasmid sources and details are listed in **Supplementary Methods** online) for each gene (2–3 mg ml^{-1} each for expression plasmids; 0.5 mg ml^{-1}each for siRNAs; 1 μl total), control plasmid (4 mg ml^{-1}) or siRNA (1 mg ml^{-1}) with pCAG-EYFP or PCAG-DsRed2 (1 mg ml^{-1}). DNA solutions were injected with a glass micropipette into the third ventricle. Tweezer-type electrodes were placed on each side of the fetal head forming a ~30° angle from the horizontal plane (**Fig. 5a**) and electronic pulses were charged with an electroporator (BTX). Natural reabsorption of embryos occurred with a frequency ~10–20%. We harvested electroporated embryos at E16.5 or E18.5. Brains were fixed with 4% PFA overnight, sectioned at 70 μm on a vibratome and processed for immunohistochemistry (**Supplementary Methods**). For each animal, we delimited a region for quantification from the medial cortex to the claustrum at each level and on each side of the brain (**Supplementary Fig. 3**).

Axonal tracing. After fixation in 4% PFA, E14.5 and E16.5 brains were bisected in the sagittal plane. We inserted small crystals of DiI and DiA (Invitrogen) with a fine needle into the anterior and posterior parts of the dorsal thalamus in either lateral or medial regions of the exposed surface. After storage in fixative about 2 weeks in the dark at 37 °C, we collected 100-μm slices and counterstained using DAPI (Molecular Probes).

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

A.B. participated in the design of the project, the writing of the manuscript and conducted and analyzed the experiments. M.T. conducted and participated in the analyses of crucial *in utero* electroporation experiments and helped to write the manuscript. L.W. performed the statistical analyses. P.R. contributed to the *in vivo* experiments and helped to write the manuscript. P.L. contributed to the design of the experiments, interpretation of results and writing of the manuscript.

COMPETING INTERESTS STATEMENT
The authors declare no competing financial interests.

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1. Rehn, A.E. & Rees, S.M. Investigating the neurodevelopmental hypothesis of schizophrenia. *Clin. Exp. Pharmacol. Physiol.* **32**, 687–696 (2005).
2. Persico, A.M. & Bourgeron, T. Searching for ways out of the autism maze: genetic, epigenetic and environmental clues. *Trends Neurosci.* **29**, 349–358 (2006).
3. Gross, C. *et al.* Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. *Nature* **416**, 396–400 (2002).
4. Ansorge, M.S., Zhou, M., Lira, A., Hen, R. & Gingrich, J.A. Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. *Science* **306**, 879–881 (2004).
5. Haydon, P.G., McCobb, D.P. & Kater, S.B. Serotonin selectively inhibits growth cone motility and synaptogenesis of specific identified neurons. *Science* **226**, 561–564 (1984).
6. Whitaker-Azmitia, P.M., Druse, M., Walker, P. & Lauder, J.M. Serotonin as a developmental signal. *Behav. Brain Res.* **73**, 19–29 (1996).
7. Vitalis, T. & Parnavelas, J.G. The role of serotonin in early cortical development. *Dev. Neurosci.* **25**, 245–256 (2003).
8. Persico, A.M., Di Pino, G. & Levitt, P. Multiple receptors mediate the trophic effects of serotonin on ventroposterior thalamic neurons *in vitro*. *Brain Res.* **1095**, 17–25 (2006).
9. Janusonis, S., Gluncic, V. & Rakic, P. Early serotonergic projections to Cajal-Retzius cells: relevance for cortical development. *J. Neurosci.* **24**, 1652–1659 (2004).
10. Cases, O. *et al.* Lack of barrels in the somatosensory cortex of monoamine oxidase A–deficient mice: role of a serotonin excess during the critical period. *Neuron* **16**, 297–307 (1996).
11. Persico, A.M. *et al.* Barrel pattern formation requires serotonin uptake by thalamocortical afferents, and not vesicular monoamine release. *J. Neurosci.* **21**, 6862–6873 (2001).
12. Bonnin, A., Peng, W., Hewlett, W. & Levitt, P. Expression mapping of 5-HT1 serotonin receptor subtypes during fetal and early postnatal mouse forebrain development. *Neuroscience* **141**, 781–794 (2006).
13. Braisted, J.E. *et al.* Netrin-1 promotes thalamic axon growth and is required for proper development of the thalamocortical projection. *J. Neurosci.* **20**, 5792–5801 (2000).
14. Garel, S. & Rubenstein, J.L. Intermediate targets in formation of topographic projections: inputs from the thalamocortical system. *Trends Neurosci.* **27**, 533–539 (2004).
15. Adham, N., Romanienko, P., Hartig, P., Weinshank, R.L. & Branchek, T. The rat 5-hydroxytryptamine1B receptor is the species homologue of the human 5-hydroxytryptamine1D beta receptor. *Mol. Pharmacol.* **41**, 1–7 (1992).
16. Hamblin, M.W. & Metcalf, M.A. Primary structure and functional characterization of a human 5-HT1D–type serotonin receptor. *Mol. Pharmacol.* **40**, 143–148 (1991).
17. Ming, G.L. *et al.* cAMP-dependent growth cone guidance by netrin-1. *Neuron* **19**, 1225–1235 (1997).
18. Song, H.J., Ming, G.L. & Poo, M.M. cAMP-induced switching in turning direction of nerve growth cones. *Nature* **388**, 275–279 (1997).
19. Hopker, V.H., Shewan, D., Tessier-Lavigne, M., Poo, M. & Holt, C. Growth-cone attraction to netrin-1 is converted to repulsion by laminin-1. *Nature* **401**, 69–73 (1999).
20. Nishiyama, M. *et al.* Cyclic AMP/GMP–dependent modulation of Ca^{2+} channels sets the polarity of nerve growth-cone turning. *Nature* **423**, 990–995 (2003).
21. Lopez-Bendito, G. & Molnar, Z. Thalamocortical development: how are we going to get there? *Nat. Rev. Neurosci.* **4**, 276–289 (2003).
22. Marillat, V. *et al.* Spatiotemporal expression patterns of slit and robo genes in the rat brain. *J. Comp. Neurol.* **442**, 130–155 (2002).
23. Miyashita-Lin, E.M., Hevner, R., Wassarman, K.M., Martinez, S. & Rubenstein, J.L. Early neocortical regionalization in the absence of thalamic innervation. *Science* **285**, 906–909 (1999).
24. Gad, J.M., Keeling, S.L., Wilks, A.F., Tan, S.S. & Cooper, H.M. The expression patterns of guidance receptors, DCC and Neogenin, are spatially and temporally distinct throughout mouse embryogenesis. *Dev. Biol.* **192**, 258–273 (1997).
25. Przyborski, S.A., Knowles, B.B. & Ackerman, S.L. Embryonic phenotype of Unc5h3 mutant mice suggests chemorepulsion during the formation of the rostral cerebellar boundary. *Development* **125**, 41–50 (1998).
26. Li, H.S. *et al.* Vertebrate slit, a secreted ligand for the transmembrane protein roundabout, is a repellent for olfactory bulb axons. *Cell* **96**, 807–818 (1999).
27. Liu, G. *et al.* Netrin requires focal adhesion kinase and Src family kinases for axon outgrowth and attraction. *Nat. Neurosci.* **7**, 1222–1232 (2004).
28. Round, J. & Stein, E. Netrin signaling leading to directed growth cone steering. *Curr. Opin. Neurobiol.* **17**, 15–21 (2007).
29. Lotto, B., Upton, L., Price, D.J. & Gaspar, P. Serotonin receptor activation enhances neurite outgrowth of thalamic neurones in rodents. *Neurosci. Lett.* **269**, 87–90 (1999).
30. Geerts, I.S., Matthys, K.E., Herman, A.G. & Bult, H. Involvement of 5-HT1B receptors in collar-induced hypersensitivity to 5-hydroxytryptamine of the rabbit carotid artery. *Br. J. Pharmacol.* **127**, 1327–1336 (1999).
31. Price, G.W. *et al.* SB-216641 and BRL-15572–compounds to pharmacologically discriminate h5-HT1B and h5-HT1D receptors. *Naunyn Schmiedebergs Arch. Pharmacol.* **356**, 312–320 (1997).
32. Rothermel, J.D. & Parker Botelho, L.H. A mechanistic and kinetic analysis of the interactions of the diastereoisomers of adenosine 3′,5′-(cyclic)phosphorothioate with purified cyclic AMP-dependent protein kinase. *Biochem. J.* **251**, 757–762 (1988).
33. Torii, M. & Levitt, P. Dissociation of corticothalamic and thalamocortical axon targeting by an EphA7-mediated mechanism. *Neuron* **48**, 563–575 (2005).
34. Nakashiba, T. *et al.* Netrin-G1: a novel glycosyl phosphatidylinositol-linked mammalian netrin that is functionally divergent from classical netrins. *J. Neurosci.* **20**, 6540–6550 (2000).
35. Auladell, C., Perez-Sust, P., Super, H. & Soriano, E. The early development of thalamocortical and corticothalamic projections in the mouse. *Anat. Embryol. (Berl.)* **201**, 169–179 (2000).
36. Molnar, Z. & Blakemore, C. How do thalamic axons find their way to the cortex? *Trends Neurosci.* **18**, 389–397 (1995).
37. Wiencken-Barger, A.E., Mavity-Hudson, J., Bartsch, U., Schachner, M. & Casagrande, V.A. The role of L1 in axon pathfinding and fasciculation. *Cereb. Cortex* **14**, 121–131 (2004).
38. Vanderhaeghen, P. & Polleux, F. Developmental mechanisms patterning thalamocortical projections: intrinsic, extrinsic and in between. *Trends Neurosci.* **27**, 384–391 (2004).
39. Seibt, J. *et al.* Neurogenin2 specifies the connectivity of thalamic neurons by controlling axon responsiveness to intermediate target cues. *Neuron* **39**, 439–452 (2003).
40. Crandall, J.E. & Caviness, V.S., Jr. Thalamocortical connections in newborn mice. *J. Comp. Neurol.* **228**, 542–556 (1984).
41. Garel, S., Yun, K., Grosschedl, R. & Rubenstein, J.L. The early topography of thalamocortical projections is shifted in Ebf1 and Dlx1/2 mutant mice. *Development* **129**, 5621–5634 (2002).
42. Molnar, Z., Adams, R. & Blakemore, C. Mechanisms underlying the early establishment of thalamocortical connections in the rat. *J. Neurosci.* **18**, 5723–5745 (1998).
43. Bruns, D., Riedel, D., Klingauf, J. & Jahn, R. Quantal release of serotonin. *Neuron* **28**, 205–220 (2000).
44. Clements, J.D. Transmitter timecourse in the synaptic cleft: its role in central synaptic function. *Trends Neurosci.* **19**, 163–171 (1996).
45. Bouchard, J.F. *et al.* Protein kinase A activation promotes plasma membrane insertion of DCC from an intracellular pool: a novel mechanism regulating commissural axon extension. *J. Neurosci.* **24**, 3040–3050 (2004).
46. Kreibich, T.A., Chalasani, S.H. & Raper, J.A. The neurotransmitter glutamate reduces axonal responsiveness to multiple repellents through the activation of metabotropic glutamate receptor 1. *J. Neurosci.* **24**, 7085–7095 (2004).
47. Aitken, A.R. & Tork, I. Early development of serotonin-containing neurons and pathways as seen in wholemount preparations of the fetal rat brain. *J. Comp. Neurol.* **274**, 32–47 (1988).
48. Puelles, L. & Rubenstein, J.L. Forebrain gene expression domains and the evolving prosomeric model. *Trends Neurosci.* **26**, 469–476 (2003).
49. Fan, C.M. & Tessier-Lavigne, M. Patterning of mammalian somites by surface ectoderm and notochord: evidence for sclerotome induction by a hedgehog homolog. *Cell* **79**, 1175–1186 (1994).