Draxin from neocortical neurons controls the guidance of thalamocortical projections into the neocortex

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The thalamocortical tract carries sensory information to the neocortex. It has long been recognized that the neocortical pioneer axons of subplate neurons are essential for thalamocortical development. Herein we report that an axon guidance cue, draxin, is expressed in early-born neocortical neurons, including subplate neurons, and is necessary for thalamocortical development. In \( \text{draxin}^{-/-} \) mice, thalamocortical axons do not enter the neocortex. This phenotype is sufficiently rescued by the transgenic expression of \( \text{draxin} \) in neocortical neurons. Genetic interaction data suggest that draxin acts through Deleted in colorectal cancer (DCC) and Neogenin (Neo1), to regulate thalamocortical projections \emph{in vivo}. Draxin promotes the outgrowth of thalamic axons \emph{in vitro} and this effect is abolished in thalamic neurons from \( \text{Dcc} \) and \( \text{Neo1} \) double mutants. These results suggest that draxin from neocortical neurons controls thalamocortical projections into the neocortex, and that this effect is mediated through the DCC and Neo1 receptors.

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In the mammalian brain, reciprocal connections between the neocortex and thalamus formed by the corticothalamic and thalamocortical axons are critical for the relay and processing of sensory information. During development, corticothalamic and thalamocortical axons concurrently grow into the subcortical telencephalon, where they meet to form the internal capsule and continue to extend in opposite directions to reach their targets. It has been known for over two decades that the guidance of thalamocortical projections is dependent on the neocortical subplate neurons, which pioneer the corticofugal pathway from the neocortex to the internal capsule. Regional chemical ablation of subplate neurons leads to the disrupted thalamocortical innervation of corresponding cortical regions. Tbr1 (refs 5,6), Coup-tf1 (ref. 7) and Fez-like8,9 transcription factor mutants provided further evidence for the importance of the subplate in thalamocortical development. Mutations in these factors lead to the defective formation of the subplate and misguidance of thalamocortical axons. Analysis of these factors lead to the defective formation of the subplate and misguidance of thalamocortical axons. Analysis of these factors lead to the defective formation of the subplate and misguidance of thalamocortical axons. Analysis of these factors lead to the defective formation of the subplate and misguidance of thalamocortical axons. Analysis of these factors lead to the defective formation of the subplate and misguidance of thalamocortical axons. Analysis of these factors lead to the defective formation of the subplate and misguidance of thalamocortical axons. Analysis of these factors lead to the defective formation of the subplate and misguidance of thalamocortical axons. Analysis of these factors lead to the defective formation of the subplate and misguidance of thalamocortical axons. Analysis of these factors lead to the defective formation of the subplate and misguidance of thalamocortical axons. Analysis of these factors lead to the defective formation of the subplate and misguidance of thalamocortical axons.

Results

Thalamocortical phenotypes in draxin−/− mice. To examine axon guidance defects in draxin−/− mice, we analysed serial paraffin-embedded coronal sections of wild-type and draxin−/− brains using immunostaining against neurofilament (NF). Corticofugal and thalamocortical axons pass through the internal capsule by forming fasciculated axon bundles in wild-type mice (Fig. 1a, upper panels). In draxin−/− mice, the number of NF-positive axons increased in the external capsule (Fig. 1a, arrowheads), while the thickness of axon bundles reduced in the internal capsule (Fig. 1a, arrow). In addition, the density of NF-positive axons was robustly lower throughout the neocortex of draxin−/− mice than in that of wild-type mice (Fig. 1a). To visualize corticofugal and thalamocortical axons, we performed axonal tracing experiments by injecting 1,1′-dioctadecyl-3,3′,3′-tetramethylinodocarbocyanine perchlorate (DiI) into the neocortex and dorsal thalamus (Fig. 1b). In draxin−/− mice, some corticofugal axons did not enter the internal capsule and instead they grew into the external capsule (Fig. 1b, left panels). Thalamocortical axons of draxin−/− mice grew into the internal capsule but the majority of them either stalled or turned laterally towards the external capsule (Fig. 1b, right panels). We also examined the trajectories of corticofugal axons using immunostaining against TAG-1 (also known as Cntn2)13,14. Consistent with the DiI analysis, TAG-1-positive corticofugal axons entering the internal capsule were reduced in draxin−/− mice (Supplementary Fig. 1a, arrow). In addition, we observed that some of the TAG-1-positive axons in draxin−/− mice entered the internal capsule and reached the thalamus (Supplementary Fig. 1a, arrowhead). These results suggest that draxin is required for the normal guidance of corticofugal axons from the neocortex to the internal capsule. Furthermore, two-colour fluorescent tracing showed that in draxin−/− mice, thalamocortical and corticofugal axons were associated in an ectopic tract within the external capsule, in addition to the internal capsule (Supplementary Fig. 1b, arrowheads). These results indicate that in draxin−/− mice, thalamocortical and corticofugal axons followed an ectopic route through the external capsule instead of projecting to the neocortex and internal capsule, respectively. Next, we performed immunostaining against serotonin (5-HT) and the NF-positive axons increased in the external capsule (Fig. 1c, arrowheads), while the thickness of axon bundles reduced in the internal capsule (Fig. 1a, arrow). In addition, the density of NF-positive axons was robustly lower throughout the neocortex of draxin−/− mice than in that of wild-type mice (Fig. 1a). 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We observed that their expression patterns were severely disrupted in various sensory cortices, including the somatosensory, visual and auditory cortices, of draxin−/− mice (Fig. 1c, arrowheads), indicating that most thalamocortical axons did not innervate the neocortex properly. To examine the regional specificity of thalamocortical projection deficits in draxin−/− mice, we analysed serial coronal sections from the entire neocortex using immunostaining against netrin-G1, which is a marker for thalamocortical axons17. We found that netrin-G1-positive thalamocortical axons were notably decreased throughout the neocortex of draxin−/− mice (Supplementary Fig. 1c,d), suggesting no regional defects in the thalamocortical phenotype.

Why do corticofugal and thalamocortical axons misproject in draxin−/− mice? The ventral telencephalon contains the corridor and globus pallidus cells, which are essential for thalamocortical axon pathfinding. Corridor cells express Islet-1 and Ebf1, whereas globus pallidus cells express Nkx2-1 and Lhx6 (ref. 20). To determine whether the ventral telencephalon is defective in draxin−/− mice, we performed double immunostaining against Islet-1 and Nkx2-1, and in situ hybridization using Ebf1 and Lhx6 probes. We observed that their expression was not affected in draxin−/− mice (Fig. 2a and Supplementary Fig. 2a,b), suggesting that the formation of the ventral telencephalon is normal in draxin−/− mice.

Subplate neurons play an important role in the development of subsequent corticofugal and thalamocortical axons. Therefore, we examined the formation of the subplate in draxin−/− mice using immunostaining against MAP2, Tbr1 and chondroitin sulfate proteoglycan6,21. Expression patterns of these markers were comparable in the subplate of wild-type and draxin−/− mice (Fig. 2b and Supplementary Fig. 2c), indicating the normal differentiation and distribution of subplate neurons. Furthermore,
immunostaining for the layer-specific markers Brn2 (layers II/III, V), Ctip2 (layer V) and Foxp2 (layer VI)\(^2\) showed that the formation of neocortical layers was not affected in draxin\(^{-/-}\) mice (Supplementary Fig. 2d). We next investigated the spatiotemporal relationship between corticofugal and thalamocortical axons using double immunostaining against TAG-1 and calretinin (also known as Calb2), which are markers for neocortical\(^1\) and thalamic axons\(^2\), respectively.

Axons from neocortical pioneer neurons reach the pallial–subpallial boundary (PSPB) at embryonic day 13.5 (E13.5) and lie at the lateral striatum until E15.5 (ref. 24). This waiting period enables the arrival of reciprocal thalamocortical axons at the PSPB\(^2\). At E13.5 and E14.5, the projection patterns of TAG-1-positive axons in draxin\(^{-/-}\) mice were indistinguishable from those in wild-type mice (Fig. 2c). This result indicates that neocortical pioneer axons project normally towards the internal capsule and pause in the lateral striatum of draxin\(^{-/-}\) mice. At E14.5, calretinin-positive thalamocortical axons progressed through the PSPB to reach the neocortex in wild-type mice (Fig. 2c). In contrast, the thalamocortical axons of draxin\(^{-/-}\) mice did not reach the PSPB at E14.5 and instead they misrouted towards the external capsule by E15.5 (Fig. 2c, arrowheads). At E15.5, TAG-1-positive axons did not extend into the internal capsule of draxin\(^{-/-}\) mice (Fig. 2c, arrow).

To clearly visualize the trajectories of corticofugal axons in draxin\(^{-/-}\) mice, we injected DiI into the neocortex at E14.5 and E15.5. Although projection patterns from the neocortex of wild-type and draxin\(^{-/-}\) mice at E14.5 were similar, some DiI-labelled axons misprojected towards the external capsule at E15.5 in draxin\(^{-/-}\) mice (Fig. 2d, arrowhead). Thus, the pathfinding errors of thalamocortical axons precede those of corticofugal axons in draxin\(^{-/-}\) mice, suggesting that draxin plays a direct role in guiding thalamocortical projections from the internal capsule to the PSPB.
Figure 2 | Aberrant thalamocortical projections precede aberrant corticofugal projections. (a) Coronal sections from E13.5 brains of wild-type and draxin−/− mice stained with Islet1 and Nkx2-1 antibodies. The corridor and the globus pallidus were formed normally in draxin−/− mice (n = 8 for each genotype). Scale bar, 500 μm. (b) Coronal sections from E16.5 brains of wild-type and draxin−/− mice stained with a MAP2 antibody. MAP2 expression was not affected in the neocortex of draxin−/− mice (n = 8 for each genotype). Scale bar, 300 μm. (c) Coronal sections from E13.5–E15.5 brains of wild-type and draxin−/− mice stained with TAG-1 and calretinin antibodies. Arrowheads and the arrow indicate misprojected thalamocortical and corticofugal axons, respectively (n = 8 for each genotype). Scale bar, 500 μm. (d) Coronal sections from E14.5 and E15.5 brains of wild-type and draxin−/− mice with a Dil injection in the neocortex. In draxin−/− mice, Dil-labelled axons from the neocortex correctly projected towards the internal capsule at E14.5, but some Dil-labelled axons misprojected towards the external capsule at E15.5 (arrowhead, n = 5 for each genotype). Scale bar, 500 μm. CFA, corticofugal axons; Co, corridor cells; CP, cortical plate; GP, globus pallidus; IC, internal capsule; IZ, intermediate zone; MZ, marginal zone; SP, subplate; TCA, thalamocortical axons.

Draxin expression during thalamocortical development. To investigate the expression pattern of draxin during the development of corticofugal and thalamocortical axons, we performed β-galactosidase (β-gal) staining on the brains of draxin+/− mice, in which the second exon containing the ATG start codon was replaced with the β-gal gene. At E14.5, draxin was strongly expressed in the neocortex (Fig. 3a,c) and was weakly expressed in the ventral telencephalon and thalamus (Fig. 3a,b). In the ventral telencephalon, draxin expression was observed in the corridor cells (Supplementary Fig. 3a). draxin expression was also observed in the zona limitans intrathalamica (the border between the dorsal and ventral thalamus; Fig. 3a,b, arrows), the ventricular zones of the ventral thalamus (Fig. 3a,b, asterisks) and the amygdala (Fig. 3a, arrowhead). Furthermore, β-gal staining at E17.5 clearly showed draxin expression in the early-born neurons, deep cortical plate, subplate and marginal zone of the neocortex (Fig. 3d). Double immunostaining against β-gal and TAG-1 or L1 at E14.5 revealed that draxin is strongly expressed in the corticofugal neurons, but not in the thalamocortical neurons (Fig. 3e,f). Consistent with this result, we confirmed with in situ hybridization that draxin messenger RNA is strongly expressed in the neocortex but not in the dorsal thalamus (Supplementary Fig. 3b). We next examined the distribution of draxin proteins in wild-type mice at E14.5 using a draxin antibody (Supplementary Fig. 3c). Double immunostaining against draxin and TAG-1 or L1 at E14.5 revealed the presence of draxin proteins in corticofugal and thalamocortical axons (Fig. 3g,h). These results suggest that draxin proteins on thalamocortical axons are mainly provided by diffusion from other regions including the corticofugal neurons.

Importantly, the thalamocortical phenotype of draxin−/− mice is very similar to that of conditional mutant mice lacking corticofugal axons. This fact suggests that draxin may be involved in establishing reciprocal interactions between corticofugal and thalamocortical axons. As draxin is strongly expressed in early-born neurons of the neocortex, draxin secreted from the neocortical neurons may be essential for thalamocortical axon development. To test this hypothesis, we performed rescue experiments using the Z/draxin transgenic mice, in which draxin and EGFP are co-expressed as a result of Cre-mediated recombination (Supplementary Fig. 4a–c). Induction of draxin expression in the neocortex of draxin−/− (Ctx-draxin) mice was achieved by crossing Z/draxin homo/draxin−/− mice with Emx1Cre+/−;draxin−/− mice, which show specific Cre expression in the dorsal telencephalon, but not in the ventral telencephalon and the thalamus (Fig. 4a). Immunostaining against
These results indicate that draxin compared with that of wild-type mice (Supplementary Fig. 4d,e). Draxin immunoreactivity in the neocortex of Ctx- mice (Fig. 4c). Consistent with this result, we observed weak expression of draxin -gal expression compared with that in Ctx- mice. In draxin knockout mice, TAG-1-positive axons did not extend into the internal capsule (Fig. 4e, arrow). In contrast, we found that TAG-1-positive axons in Ctx- mice extended into the internal capsule (Fig. 4e, arrowhead). In addition, we observed corticofugal axons entering the internal capsule of Ctx- mice using immunostaining against EGFP at E17.5 (Fig. 4e, arrowhead). These results demonstrate that corticofugal projections into the internal capsule were sufficiently rescued in Ctx- mice. Thus, these data suggest that draxin from the neocortical neurons is sufficient for the normal development of corticofugal and thalamocortical axons.

Draxin has an outgrowth-promoting effect on thalamic axons. We hypothesized that draxin secreted from neocortical neurons controls the guidance of thalamocortical axons through the draxin receptors expressed in thalamic neurons. To test this hypothesis, we examined the distribution of draxin receptors in the brain at E14.5 with a binding assay based on the alkaline phosphatase (AP)-tagged draxin protein (draxin-AP). Draxin-AP binding was observed in the intermediate zone of the neocortex and in the internal capsule (Figs 5a and 6c). In contrast, control-AP binding was not observed in the brain sections (Supplementary Fig. 5a). These results suggest that draxin binds to corticofugal and thalamocortical axons, because at E14.5 the intermediate zone of the neocortex and the internal capsule mainly contain neocortical and thalamocortical axons, respectively. Next, we performed a draxin-AP-binding assay on dissociated neurons of the neocortex and the dorsal thalamus, to assess potential differences in the draxin-binding affinity of these neurons. The dorsal thalamus was subdivided into anterior and posterior regions. We found that draxin-AP bound to the majority of neurites extending from the dissociated neurons of the neocortex, anterior dorsal thalamus and posterior dorsal thalamus (Fig. 5b). There were no significant differences among the growth cones of these neurons regarding draxin-AP binding (Supplementary Fig. 5b). These results suggest that draxin binds to neocortical and thalamic axons with a similar affinity.

To examine the effect of draxin on the outgrowth of thalamic axons, we cultured dissociated thalamic neurons with different concentrations of draxin-AP proteins. Thalamic neurons were cultured on coverslips coated with poly-L-lysine (PLL) and L1, because L1 promotes significantly higher neurite outgrowth than PLL or laminin/PLL. This enabled us to evaluate the effects of draxin more easily (Supplementary Fig. 5c). Interestingly, draxin inhibited neurite outgrowth at high concentrations but promoted neurite outgrowth at lower concentrations (Fig. 5c). To further investigate the potential contribution of draxin to thalamocortical axonal outgrowth, dissociated thalamic neurons were cultured on HEK293T cells stably expressing draxin (draxin-293 cells). We observed 86% increase in neurite outgrowth of thalamic neurons cultured on draxin-293 cells compared with that on control-293 cells (Supplementary Fig. 5d). These data raise the possibility that draxin has an outgrowth-promoting effect on thalamic axons at physiological concentrations. To test this possibility, we cultured thalamic neurons obtained from CAG-EGFP mice on a enhanced green fluorescent protein (EGFP) and in situ hybridization for draxin showed the specific expression of EGFP and draxin transcripts in the dorsal telencephalon of Ctx- mice (Fig. 4b). Furthermore, we investigated the expression levels of draxin in the neocortex of Ctx- and wild-type mice at E14.5. We found that draxin expression in the neocortex of Ctx- mice was lower than that in wild-type mice (Fig. 4c). Consistent with this result, we observed weak draxin immunoreactivity in the neocortex of Ctx- mice compared with that of wild-type mice (Supplementary Fig. 4d,e). These results indicate that draxin expression was specifically induced in the neocortex of Ctx- mice, and that Ctx- mice have physiological expression levels of draxin mRNA and its proteins. To examine the trajectories of thalamocortical axons in Ctx- mice, we performed immunostaining against calretinin and injected DiI into the neocortex. We found that thalamocortical axons extended into the neocortex of Ctx- mice (Fig. 4d, open arrowheads). In addition, immunostaining with a 5-HT antibody also revealed that thalamocortical projections targeting the neocortex were sufficiently rescued in Ctx- mice (Fig. 4d, open arrowheads).
neocortical feeder layer prepared from wild-type, Ctx-\(e\)draxin\(-/\) and Ctx-\(e\)draxin\(-/-\) mice. In this study, Emx\(^{+/+}\);Z/draxin hetero; draxin\(-/-\) mice are referred to as Ctx-draxin mice and Emx\(^{1+}/+\); Z/draxin hetero; draxin\(-/-\) mice are referred to as draxin knockout (KO) mice. (b) Immunostaining against EGFP and in situ hybridization for draxin in coronal sections of Ctx-draxin mice showed the specific expression of EGFP and draxin mRNA in the neocortex. Scale bars, 500 µm. (c) draxin mRNA expression detected with in situ hybridization in the neocortex of draxin KO, Ctx-draxin and wild-type mice. Scale bar, 100 µm. (d) Coronal or tangential sections of draxin KO and Ctx-draxin mice stained with calretinin or 5-HT antibodies. Coronal sections of draxin KO and Ctx-draxin mice with Dil injected into the dorsal thalamus. The thalamocortical phenotype was rescued in Ctx-draxin mice (\(n=5\) for each condition). Open arrowheads indicate thalamocortical axons in the neocortex. Scale bars, 500 µm. (e) Coronal sections of draxin KO and Ctx-draxin mice stained with TAG-1 or EGFP antibodies. In draxin KO mice, TAG-1-positive axons did not extend into the internal capsule (arrow). This corticofugal phenotype was rescued in Ctx-draxin mice (arrowhead, \(n=4\) for each condition). Scale bars, 500 µm.

Figure 4 | Draxin expression in the neocortex is critical for corticofugal and thalamocortical development. (a) Schematic of the Z/draxin transgene. Specific expression of draxin in the neocortex of draxin\(-/-\) mice (Emx\(^{Cre/+}\); Z/draxin hetero; draxin\(-/-\)) is achieved by crossing Z/draxin hetero; draxin\(-/-\) mice with Emx\(^{Cre/+}\); draxin\(-/-\) mice. In this study, Emx\(^{Cre/+}\); Z/draxin hetero; draxin\(-/-\) mice are referred to as Ctx-draxin mice and Emx\(^{1+}/+\); Z/draxin hetero; draxin\(-/-\) mice are referred to as draxin knockout (KO) mice.

DCC and Neo1 are draxin receptors in thalamic neurons. We previously reported that draxin binds to the netrin-1 receptors DCC, UNC5s, DSCAM and Neo1 (ref. 30). DCC has been proposed as a functional receptor for draxin in neurons from the neocortex\(^{30}\) and the dorsal horn of the spinal cord\(^{31}\). All of these receptors are expressed in the dorsal thalamus\(^{32-35}\). However, the thalamocortical phenotype observed in draxin\(-/-\) mice has not been previously reported in mutants of these receptors. Thus, there may be redundancies in the receptors that mediate draxin signalling in thalamocortical neurons. To assess the ligand–receptor relationship between draxin and these receptors in thalamocortical projections in vivo, we investigated genetic interactions by generating compound mutant mice (Table 1). The mutations of Dcc\(^{36}\), Unc5a\(^{37}\), Unc5b\(^{38}\) and Dscam\(^{39}\) are very strongly hypomorphic or null alleles, whereas the mutation of Neo1 is a hypomorphic allele\(^{40,41}\). Previous studies have demonstrated that Neo1 protein levels are reduced by \(~90\%\) in Neo1\(^{G0/G0}\) mice\(^{42}\). We confirmed that the amount of Neo1 protein was dramatically reduced in the thalamus of Neo1\(^{G0/G0}\) embryos at E14.5 (Supplementary Fig. 6a). Thalamocortical projections in the internal capsule of various mutants were analysed by immunostaining against calretinin (Fig. 6a) and L1 (Supplementary Fig. 6b) at E15.5. draxin\(-/-\) mice had a severe phenotype, as most thalamocortical axons did not enter the neocortex, whereas the thalamocortical projections of
Draxin+/−, Dcc−/− or Neo1Gt/Gt mice did not reveal any significant defects. Mild pathfinding defects of thalamocortical axons were observed in 36% of draxin+/−;Dcc+/− mice and 33% of draxin+/−;Neo1Gt+ mice. In addition, all draxin+/−;Dcc−/− mice and 75% of draxin+/−;Neo1Gt/Gt mice mimicked the thalamocortical phenotype of draxin−/− mice. In contrast, no significant defects were observed in the thalamocortical projections of draxin/ Unc5a, draxin/Unc5b or draxin/Dscam compound mutant mice. These results suggest that DCC and Neo1 might play critical roles in the draxin-mediated signalling pathway in vivo. Importantly, double mutants for Dcc and Neo1 displayed a severe phenotype, which resembled that of draxin−/− mice, indicating the compensatory functions of DCC and Neo1 in draxin signalling. Furthermore, we performed immunostaining against TAG-1, to examine the phenotypic similarities in corticofugal projections between draxin−/− and compound mutant mice. We observed no significant defects in TAG-1-positive corticofugal axons of draxin+/−, Dcc−/− or Neo1Gt/Gt mice at E15.5 (Fig. 6b). In contrast, TAG-1-positive axons of draxin+/−;Dcc−/−, draxin+/−;Neo1Gt/Gt or Neo1Gt Gt;Dcc−/− mice did not extend into the internal capsule (Fig. 6b), which was very similar to the phenotype observed in

Figure 5 | Outgrowth-promoting effects of draxin on thalamic axons. (a) Draxin-AP binding in coronal sections from E14.5 brains of wild-type mice. The draxin-AP signal was observed in the intermediate zone of the neocortex (arrowhead) and the internal capsule (arrow). Scale bar, 500 μm. (b) Draxin-AP bound to the majority of neurites extending from dissociated neurons of the neocortex (CTX), anterior dorsal thalamus (anterior DT) and posterior dorsal thalamus (posterior DT) of E14.5 wild-type mice. Arrows indicate draxin-AP binding to the growth cones of dissociated neurons. In contrast, control-AP did not bind to neurites from these neurons (arrowheads). Scale bar, 50 μm. (c) Dissociated cultures of thalamic neurons with different concentrations of draxin-AP proteins showed that neurite outgrowth was promoted by low draxin concentrations and inhibited by high draxin concentrations. Error bars are s.e.m. (n = 5 independent experiments). *P < 0.05 and **P < 0.01 by Welch’s t-test. Scale bar, 100 μm. (d) Dissociated cultures of thalamic neurons on neocortical neurons prepared from wild-type, draxin+/− and Ctx-draxin mice. Neurite outgrowth was reduced in thalamic neurons cultured on draxin−/− neocortical neurons compared with that in thalamic neurons cultured on wild-type neocortical neurons. This decreased neurite outgrowth was significantly rescued when thalamic neurons were cultured on Ctx-draxin neocortical neurons. Error bars are s.e.m. (n = 3 independent experiments). **P < 0.01 by one-way analysis of variance followed by Tukey’s honest significance test. Scale bars, 100 μm.
draxin−/− mice. Thus, our data revealed a high degree of similarity in thalamosomal and corticofugal phenotypes between draxin−/− and Neo1Gt/Gt, Dcc−/− mice.

We determined the binding affinity of draxin to Neo1 in transfected cells by generating a binding curve with a dissociation constant (Kd) of 620 PM (Supplementary Fig. 6c), which is comparable with the Kd of the draxin–DCC interaction30. To investigate whether DCC and Neo1 are the primary receptors of draxin in vivo, we performed a draxin-AP-binding assay on brain sections obtained from Neo1Gt/Gt, Dcc−/− mice at E14.5. We found that the draxin-AP signals was robustly reduced in the intermediate zone of the neocortex and the internal capsule in Neo1Gt/Gt, Dcc−/− mice (Fig. 6d) compared with that in wild-type mice (Fig. 6c, arrows). The amounts of neocortical and thalamocortical axons in these regions were not greatly affected in Neo1Gt/Gt, Dcc−/− mice at this stage (Fig. 6a, b and Supplementary Fig. 6b). These results indicate that draxin binding was reduced in the neocortical and thalamocortical axons of Neo1Gt/Gt, Dcc−/− mice, suggesting that DCC and Neo1 are the primary binding partners for draxin in vivo.

To explore whether DCC and Neo1 on thalamic axons are required for the outgrowth-promoting effects of draxin, which seems to be critical for thalamocortical development, we cultured dissociated thalamic neurons from Dcc−/−, Neo1Gt/Gt and Neo1Gt/Gt, Dcc−/− mice (Fig. 6d–g). There was no significant difference in the neurite outgrowth from thalamic neurons of wild-type (Dcc+/+, Neo1+/+), Dcc−/−, Neo1Gt/Gt and Neo1Gt/Gt, Dcc−/− embryos in the absence of draxin-AP proteins. However, in the presence of draxin-AP proteins, we observed 27% and 30% reductions in the neurite outgrowth from thalamic neurons in Neo1Gt/Gt and Dcc−/− embryos, respectively, compared with that in the littermate controls (Fig. 6e,f).

Moreover, the growth-promoting effect of draxin-AP was absent in thalamic neurons from Neo1Gt/Gt, Dcc−/− embryos (Fig. 6g). These results, taken together with our in vivo data, suggest that DCC and Neo1 are essential components of draxin-mediated pathway that controls the development of thalamocortical projections. In contrast to the outgrowth-promoting effect of draxin-AP, the inhibitory effect on neurite outgrowth induced by high concentrations of draxin-AP was not completely abolished in the thalamic neurons from Neo1Gt/Gt, Dcc−/− embryos (Supplementary Fig. 6d). This result suggests that additional receptor(s) may be necessary for the inhibitory effect of draxin.

Draxin regulates neurite outgrowth of neocortical neurons. In vitro binding assay showed that draxin bound not only to thalamic axons but also to neocortical axons with a similar affinity (Fig. 5b and Supplementary Fig. 5b). To test the effect of draxin on the outgrowth of neocortical axons, we cultured dissociated neocortical neurons from wild-type mice at E14.5. Neocortical neurons were cultured on coverslips coated with L1/PLL, because L1 promoted neurite outgrowth significantly better than PLL or laminin/PLL (Supplementary Fig. 7a). We found that the neurite outgrowth in neocortical neurons was promoted by draxin at low concentrations and inhibited at higher concentrations (Supplementary Fig. 7b). It was previously reported that Dcc and Neo1 are expressed in neocortical neurons33,44. To examine whether DCC and Neo1 are necessary for the draxin-mediated effects on neocortical neurons, we cultured dissociated neocortical neurons from Neo1Gt/Gt, Dcc−/− embryos, whereas the inhibitory effect of 100 nM draxin-AP was partially abolished (Supplementary Fig. 7c,d). Thus, draxin regulates the neurite outgrowth of both thalamic and neocortical neurons in a concentration-dependent manner. Furthermore, our data suggest that DCC and Neo1 are sufficient to mediate the growth-promoting effect of draxin in these neurons, whereas additional receptor(s) may be necessary for the inhibitory effect of draxin.

Discussion

The handshake hypothesis, proposed by Molnar and colleagues35, postulates that corticofugal and thalamocortical axons meet in the internal capsule and follow each other from the internal capsule to the thalamus and neocortex, respectively. In fact, the experimental data in mutant mice lacking different types of transcription factors such as Tbr1 (refs 5,6), Fez-like5,6,9, Pax6 (refs 5,46,47), Otx2 (refs 5,48) and Emx2 (refs 49,50) suggest that corticofugal and thalamocortical axons depend on each other for their guidance. Furthermore, it was shown in conditional mutant mice lacking corticofugal axons that descending corticofugal axons are essential for guiding thalamocortical axons across the PSPB11. In a complementary study using conditional mutant mice lacking thalamocortical axons, it has been shown that thalamocortical axons are necessary to guide corticofugal axons into the coroid and toward the thalamus25. Recently, it has been reported that the transmembrane protein Linx is expressed on corticofugal axons and is necessary for thalamocortical development11. Although numerous studies support the handshake hypothesis, molecular mechanisms governing the interaction of these axons remained unclear. In this study, we showed that draxin expressed in the neocortical neurons is critical for the normal development of corticofugal and thalamocortical axons.

Several guidance molecules regulate the projection of thalamocortical axons through various decision points3. During embryonic development, thalamocortical axons first grow towards the hypothalamus through the prethalamus and...
then they turn laterally towards the internal capsule51 (Fig. 7). Slit1- and Slit2-mediated repulsion from the hypothalamus is required for this turning behaviour of thalamocortical axons23,52,53. In contrast to the hypothalamus, corridor cells in the ventral telencephalon express the axonal growth-promoting factor neuregulin-1 and thus establish a permissive environment for thalamocortical axons18. In the mutant for NRG-1 or its receptor ErbB4, the majority of thalamocortical axons fail to progress normally through the corridor cells in the medial ganglionic eminence18. In contrast, in draxin−/− mice
neurons for this phenotype. In addition, our
suggesting the importance of
expression in the ventral telencephalon.
conditional knockout mice, to clarify the precise role of
thalamocortical development. This is also supported by the
expression in the corridor cells may not be essential for
normally and the formation of corridor cells was not impaired
Th, thalamus.
expressed in the corridor
Draxin functions as a long-range mediator of reciprocal
interactions between corticofugal and thalamocortical axons. Thus, we suggest that
mediated by the DCC and Neo1 receptors (Figs 5c and 6d–g).
Based on these results, we propose a model in which
draxin secreted from the corticofugal axons promotes the
growth of thalamocortical axons towards the neocortex and
induces them to cross the PSPB. Thalamocortical axons stalled or
grew slowly towards the external capsule in draxin
(Figs 2c and 7), probably due to the loss of outgrowth-promoting
effect of draxin generated by the corticofugal axons, although it is
unclear why thalamocortical axons grow slowly towards the
external capsule.

It should be noted that corticofugal and thalamocortical axons
associated in an ectopic tract within the external capsule of
draxin
mice (Supplementary Fig. 1b). This close association
of corticofugal and thalamocortical axons might be regulated by
other short-range surface-bound molecules. Thus, we suggest that
draxin functions as a long-range mediator of reciprocal
interactions between corticofugal and thalamocortical axons.
However, this observation might support that draxin provides a
permissive environment at the PSPB for the normal development of
corticofugal and thalamocortical axons. Importantly, draxin-
AP strongly bound to the thalamocortical axons in the brain
sections at E14.5, whereas we observed no specific AP signal in
the cell populations at the PSPB such as the corridor cells in the
medial ganglionic eminence (Figs 5a and 6c). This observation
suggests that draxin is more likely to have direct functions for the
guidance of thalamocortical axons. However, we cannot rule out
the possibility that draxin is indirectly involved in the
establishment of a permissive corridor at the PSPB. The
importance of axon–axon interactions, which do not depend on
axon–target interactions, has been well documented during the
establishment of anterior–posterior and dorsal–ventral axes within
the olfactory map. The neocortex contains distinct
populations of projection neurons that send axons to various
areas, such as the contralateral cortex, thalamus and the spinal
cord. Self-organization by axon–axon interactions may be a
critical mechanism for establishing complex neuronal circuits in the
neocortex.

We showed that some of the corticofugal axons did not enter
the internal capsule in draxin
mice (Fig. 1b and
Supplementary Fig. 1b) and this corticofugal phenotype was
rescued by the transgenic expression of draxin in the neocortex
(Fig. 4). A previous study showed that corticofugal axons enter

| Genotype | Normal n (%) | Mild n (%) | Severe n (%) |
|----------|--------------|------------|-------------|
| draxin+/− (n = 8) | 0 | 0 | 8 (100%) |
| draxin+/− (n = 8) | 8 (100%) | 0 | 0 | 0 |
| Dcc−/− (n = 8) | 8 (100%) | 0 | 0 | 0 |
| draxin+/−; Dcc+/− (n = 11) | 7 (64%) | 4 (36%) | 0 |
| draxin+/−; Dcc−/− (n = 8) | 0 | 0 | 8 (100%) |
| Neo1Gt/Gt (n = 8) | 8 (100%) | 0 | 0 | 0 |
| draxin+/−; Neo1Gt/+ (n = 9) | 6 (67%) | 3 (33%) | 0 |
| draxin+/−; Neo1Gt/Gt (n = 8) | 0 | 2 (25%) | 6 (75%) |
| Unc5a−/− (n = 8) | 8 (100%) | 0 | 0 | 0 |
| draxin+/−; Unc5a+/− (n = 8) | 8 (100%) | 0 | 0 | 0 |
| draxin+/−; Unc5a−/− (n = 8) | 8 (100%) | 0 | 0 | 0 |
| Unc5b−/− (n = 0) | Embryonic lethal (E10) |

*draxin+/−; Dcc−/−, draxin+/−; Neo1Gt/+ and Neo1Gt/+Dcc−/− mice exhibited severe thalamocortical projection defects that resembled those in draxin−/− mice.*

**Table 1** | Frequencies of thalamocortical phenotypes in the compound mutants.

**Figure 7** | Summary of the draxin−/− phenotype at E14.5. In draxin−/− mice, thalamocortical axons did not cross the PSPB, whereas early
corticofugal axons projected normally towards the internal capsule. CFA, corticofugal axons; Co, corridor cells; Hyp, hypothalamus; PreTh, prethalamus; PSPB, pallial-subpallial boundary. TCA, thalamocortical axons; Th, thalamus.
the internal capsule even in the absence of thalamocortical axons25. These facts suggest that draxin might autonomously regulate the entering of corticofugal axons into the internal capsule. Accordingly, we showed that draxin bound to the neocortical axons and regulated neurite outgrowth in vitro (Supplementary Figs 5b and 7). Thus, these results are consistent with the idea that draxin functions in an autocrine manner to regulate the normal progression of corticofugal axons. On the other hand, we cannot exclude the possibility that primary defects in thalamocortical projections cause secondary pathfinding errors in corticofugal axons, because the guidance of corticofugal and thalamocortical axons depend on each other. Thalamocortical axons misprojecting towards the external capsule could misguide corticofugal axons to the external capsule, resulting in the reduction of corticofugal axons that enter the internal capsule. Further studies are needed to clarify the role of draxin in corticofugal axon development.

It has been recently reported that Neo1 is a functional netrin-1 receptor, which acts in concert with DCC to direct commissural axons in the spinal cord55. Indeed, commissural axon guidance defects in Dcc−/− was comparable to those in Dcc−/− and Neo1 double mutants55. In the forebrain, netrin-1 is expressed in the ventral telencephalon and it is involved in the topographic establishment of thalamocortical projections33,34. However, netrin-1 is not expressed in neocortical neurons and no obvious defect is observed during thalamocortical axon pathfinding in the internal capsule of netrin-1−/− embryos. Importantly, Neog/Neo1−/− mice showed a severe thalamocortical projection phenotype similar to that observed in draxin−/− mice (Fig. 6). These facts suggest that draxin is a major ligand for Neo1- and DCC-mediated guidance of thalamocortical projections into the neocortex.

Our in vitro data suggest that low concentrations of draxin (10 nM) stimulate the neurite outgrowth of neocortical and thalamic neurons through the DCC and Neo1 receptors. We previously reported that 10 nM draxin inhibits neurite outgrowth from the neurons of the dorsal spinal cord12. How can draxin induce opposite growth behaviours in different neurons? A plausible mechanism, supported by previous studies on other axon guidance cues, is that different receptors or proteoglycans might be expressed on spinal cord neurons and neocortical/thalamic neurons. It has been shown that several axon guidance cues including netrin-1 and semaphorins (Sema) can act either as attractive or repulsive cues depending on the receptor complexes present on the growth cones56. For instance, netrin-1 binding to DCC induces attraction57, whereas this response is repulsive when both UNC5 and DCC are present58. In addition, the attractive and repulsive effects of SemA5 are controlled by different sulfated proteoglycans59. It should be noted that the effect of draxin on neurite outgrowth depends on its concentration (Fig. 5c and Supplementary Fig. 7b). We showed that DCC and Neo1 are sufficient to mediate the positive effects of draxin, but not its negative effects, suggesting that additional receptor(s) may be necessary for mediating the negative effects of draxin. Similar bimodal responses depending on the concentration have been observed in the case of ephrinA2 (ref. 60) and Shh61 on retinal ganglion cells, although extracellular and intracellular mechanisms underlying these bimodal effects remain unclear. In recent times, the crucial role of local protein synthesis has been shown in the concentration-dependent activities of axon guidance cues SemA3A and Sema3F62. To realize their repulsive guidance activities, low and high concentrations of Sema3A and Sema3F engage two distinct signal transduction pathways, a protein synthesis-dependent and -independent pathway. Importantly, DCC interacts with the translation machinery and functionally mediates translational regulation in response to netrin-1 (ref. 63). Thus, the concentration-dependent effects of draxin might require DCC-mediated local protein synthesis.

**Methods**

**Mutant mice.** Mice were treated according to protocols approved by the Committee on Animal Research at the University of Kumamoto. To obtain draxin−/− mice, males were crossed with draxin+/− females, which were maintained in a mixed C57BL/6-CBA background12. We observed that the phenotypes in draxin−/− mice were identical in the F1 to F6 generations, which were backcrossed with C57BL/6N or ICR mice. Dcc56, Neo1 (MMRRC:030400-MU)57, Unsc (MMRRC:030749-MU)57, Unscb (MMRRC:030410-MU)58 and Dcam (BBR/C02590)59 mutant mice and genotyping methods have been described previously. Male and female mice between 3 and 6 months old were used for breeding. All embryos and newborns examined in this study were collected regardless of sex.

**Generation of draxin transgenic mice.** To generate the pZ/draxin expression construct, we cloned the floxed vector pCAG-loxP-βgeo-loxP, based on the pCALL construct50, was constructed using the pCAGGS vector60. Mouse draxin mRNA was amplified using primers 5’-CACAGAATGCAACGTTCGTC3’ and 5’-CCTCTCTAGGCC CatG, which amplified the 290-bp fragment. We selected one transgenic line that exhibited significant β-gal expression levels in the brain. Emb1-Cre knock-in mice (BBR/C01345) have been described previously27,28.

**Histological analyses.** In situ hybridization and immunohistochemistry were performed as previously described12. For immunohistochemistry, we prepared cryostat sections mounted onto glass slides. Primary antibodies were detected with Alexa-conjugated fluorescent secondary antibodies including netrin-1 and semaphorins (Sema) can act either as attractive or repulsive cues depending on the receptor complexes present on the growth cones. For instance, netrin-1 binding to DCC induces attraction, whereas this response is repulsive when both UNC5 and DCC are present. In addition, the attractive and repulsive effects of SemA5 are controlled by different sulfated proteoglycans. It should be noted that the effect of draxin on neurite outgrowth depends on its concentration (Fig. 5c and Supplementary Fig. 7b). We showed that DCC and Neo1 are sufficient to mediate the positive effects of draxin, but not its negative effects, suggesting that additional receptor(s) may be necessary for mediating the negative effects of draxin. Similar bimodal responses depending on the concentration have been observed in the case of ephrinA2 (ref. 60) and Shh61 on retinal ganglion cells, although extracellular and intracellular mechanisms underlying these bimodal effects remain unclear. In recent times, the crucial role of local protein synthesis has been shown in the concentration-dependent activities of axon guidance cues SemA3A and Sema3F62. To realize their repulsive guidance activities, low and high concentrations of Sema3A and Sema3F engage two distinct signal transduction pathways, a protein synthesis-dependent and -independent pathway. Importantly, DCC interacts with the translation machinery and functionally mediates translational regulation in response to netrin-1 (ref. 63). Thus, the concentration-dependent effects of draxin might require DCC-mediated local protein synthesis.

**Draxin-API binding assay.** Control-AP (pAPtag-5 vector) and draxin-AP constructs were transfectioned into HEK293T cells (Riken BRC)63. After 4–5 days, the conditioned media and media harvested from the Amicon Ultra-15 kDa molecular weight cutoff filter device (Millipore). In the conditioned media, draxin-AP was detected using western blotting with an anti-draxin antibody (1:1,000)64. Control- and draxin-AP
concentrations in the conditioned medium were determined with the SensoLyte pNPP Secreted Alkaline Phosphatase Reporter Gene Assay Kit (AnaSpec). Draxin-AP binding to the dissociated neurons and brain sections was investigated as previously described12,26. We quantified draxin-AP signals on the growth cones of dissociated neurons from the neocortex, anterior dorsal thalamic and posterior dorsal thalamic. For the visualization of draxin-AP binding, neurons were stained with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium for 20 min and was separated by SDS–PAGE. Neo1 (Santa Cruz Biotechnology, 1:100) and glyceraldehydes 3-phosphate dehydrogenase (Sigma, 1:1,000) antibodies were used. The nuclear orphan receptor COUP-TFI is required for the nuclearization of L1 and neurite outgrowth activity of netrin-G subfamily members. References 1. Lopez-Bendito, G. & Molnar, Z. Thalamocortical development: how are we going to get there? Nat. Rev. Neurosci. 4, 276–289 (2003). 2. 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Author contributions
Y.S., M.A.R. and H. Tanaka designed the study. Y.S. and M.A.R. performed most of the experiments and data analysis. G.A. and M.H. performed in vitro experiments. I.B.N. produced HEK293T cells stably expressing draxin. H. Takebayashi contributed to the generation of draxin transgenic mice. H.K. supported phenotypic analyses of draxin mutants. K.O. supervised analysis of the Kd value for draxin–Neom1 binding. Y.S. wrote the manuscript with help from all authors.

Additional information
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