学位論文
Doctoral Thesis

Draxin inhibits olfactory bulb and cortical axonal outgrowth through the netrin receptor DCC

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Contents

Summary

Olfactory bulb (OB) projection neurons receive sensory input from olfactory receptor neurons and precisely relay it through their axons to the olfactory cortex. Thus, olfactory bulb axonal tracts play an important role in relaying information to the higher order of olfactory structures in the brain. Several classes of axon guidance molecules influence the pathfinding of the olfactory bulb axons. Draxin, a recently identified novel class of repulsive axon guidance protein, is essential for the formation of forebrain commissures and can mediate repulsion of diverse classes of neurons from chickens and mice. In this study, we have investigated the draxin expression pattern in the mouse telencephalon and its guidance functions for OB axonal projection to the telencephalon. We have found that draxin is expressed in the neocortex and septum at E13 and E17.5 when OB projection neurons form the lateral olfactory tract (LOT) rostrocaudally along the ventrolateral side of the telencephalon. Draxin inhibits axonal outgrowth from olfactory bulb explants *in vitro* and draxin-binding activity in the LOT axons *in vivo* is detected. The LOT develops normally in *draxin-/-* mice despite subtle defasciculation in the tract of these mutants. Furthermore, we found that draxin binds specifically and with subnanomolar affinity to the netrin receptor DCC, in a region of DCC distinct from its netrin-binding domain. *In vitro*, neurites from cortical and olfactory bulb explants of *DCC* knockout mice show a dramatic reduction in binding of draxin, and their outgrowth is significantly less inhibited by draxin, when compared with neurites from explants of wild type mice. These results unexpectedly identify DCC as a receptor for draxin in axon growth and guidance.

List of Abbreviations

µg:	Microgram
µm:	Micrometer
mm:	Millimetre
nM:	Nanomolar
mM:	Milimolar
DCC:	Deleted in Colorectal cancer
DSCAM:	Down Syndrom Cell Adhesion Molecule
Eph:	Erythropoietin producing hepatocellular
FCS:	Fetal calf serum
DMEM:	Dulbeccos modified eagles medium
BMP:	Bone morphogenetic protein
β-gal:	Beta-galactosidase
AP:	Alkaline phosphatase

List of Publications

1. **Ahmed G**, Shinmyo Y, Naser IB, Hossain M, Song X, Tanaka H. Olfactory bulb axonal outgrowth is inhibited by draxin. **BBRC.** 398:730-734 Aug, 2010.
2. Su Y, Zhang S, Islam SM, Shinmyo Y, Naser IB, **Ahmed G**, Tanaka H. Draxin is involved in the proper development of the dI3 interneuron in chick spinal cord. **Dev Dyn**. 239:1654-1663, Jun, 2010.
3. Zhang S, Su Y, Shinmyo Y, Islam SM, Naser IB, **Ahmed G**, Tamamaki N, Tanaka H. Draxin, a repulsive axon guidance protein, is involved in hippocampal development. **Neuroscience Res.** 66: 53-61, Jan, 2010.
4. Su Y, Naser IB, Islam SM, Zhang S, **Ahmed G**, Chen S, Shinmyo Y, Kawakami M, Yamamura K, Tanaka H. Draxin, an axon guidance protein, affects chick trunk neural crest migration. **Dev. Growth & Differentiation** 51: 787-96, Dec, 2009.
5. Naser IB, Su Y, Islam SM, Shinmyo Y, Zhang S, **Ahmed G**, Chen S, Tanaka H. Analysis of a repulsive axon guidance molecule, draxin, on ventrally directed axon projection in chick early embryonic midbrain. **Dev. Biol.** 332(2): 351-359, Aug, 2009.
6. Islam SM, Shinmyo Y, Okafuji T, Su Y, Naser IB, **Ahmed G**, Zhang S, Chen S, Ohta K, Kiyonari H, Abe T, Tanaka S, Nishinakamura R, Terashima T, Kitamura T, Tanaka H. Draxin, a repulsive guidance protein for spinal cord and forebrain commissures. **Science** 323(5912): 388-393, Jan, 2009.

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Introduction

1-1. Literature review

1-1-1. Axon guidance cues and their receptors function in nervous system development

The function of the nervous system depends on the establishment of precise and intricate neuronal connections. Neurons are connected through their axons and dendrites. Axons, originated from the same site, often travel long distance along specific pathways to reach their targets in a fasciculated manner. The axonal pathfinding is not random rather directional and tightly regulated by the guidance proteins. During such directional navigation, exquisite and motile structure of axonal tip, known as growth cone, functions as a sensor to explore the presence of guidance cues in the surrounding extracellular matrix and the surfaces of other cells. The guidance cues are classified into two broad classes: long range (diffusible) and short range (non-diffusible or membrane bound). Molecules in these categories are further subdivided as attractive or repulsive/inhibitory based on their influences on growth cones. However, a particular guidance cue can function as attractant for some neurons while repellent for others. These molecules opponent function for growth cone elongation or retraction depend on the expression of their receptor/s at growth cone. Past two decades have witnessed staggering advancement in axon guidance research. Several axon guidance proteins and their receptor have been identified within this period. Thus far, four conserved families of axon guidance cues, the netrin, semaphorin, ephrin and Slit proteins (Figure 1) mediate their guidance effects via receptors of the DCC or UNC5, Neuropilin/Plexin, Eph and Robo families, respectively (Tessier-Lavigne and Goodman, 1996; Dickson, 2002).

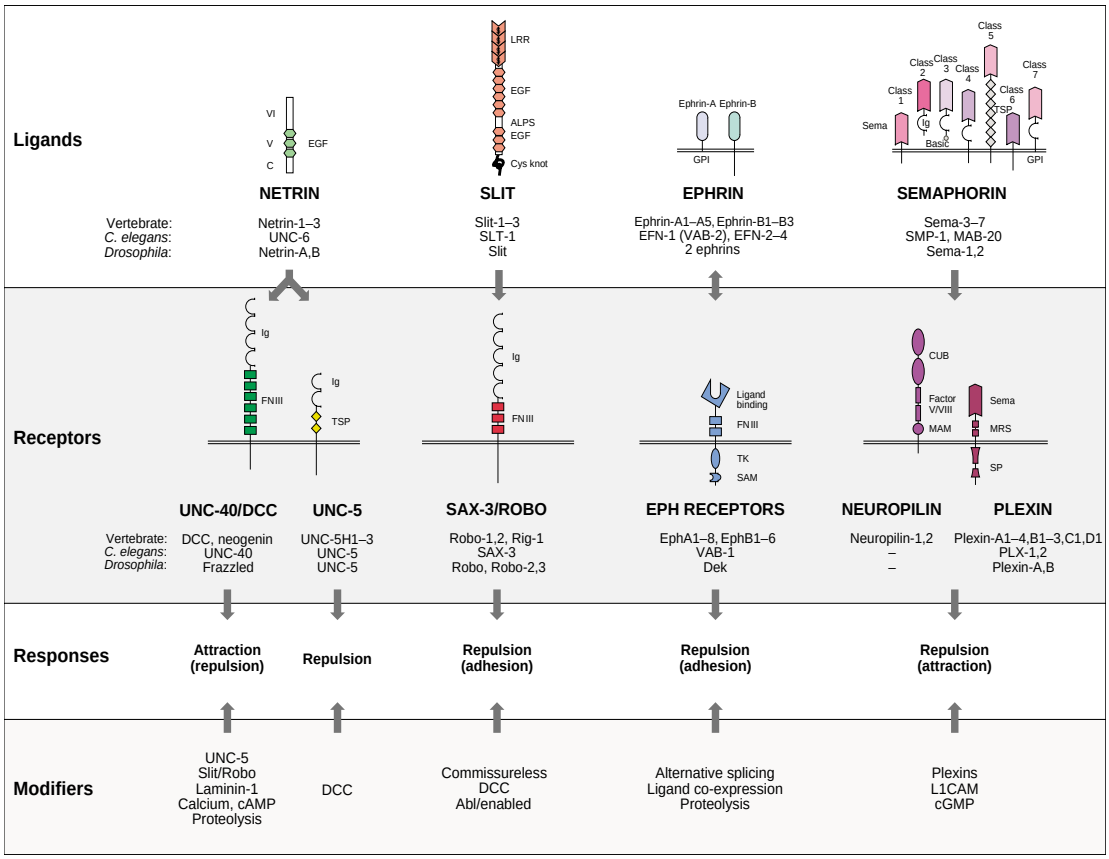

Figure 1. Summary of the four conserved families of axon guidance cues and their respective receptor/s. ALPS-agrin-laminin-perlecan slit domain; C- c terminus of netrin; CUB- C1/Uegf/BMP-1 domain; DCC-deleted in colorectal cancer; EGF-epidermal growth factor; FNIII-fibronectin type III domain; GPI- glycosylphosphatidyl–inositol anchor; Ig- immunoglobulin domain; LRR-leucine-rich repeat; MAM- meprin/A5 antigen motif; MRS-Met tyrosine kinase–related sequence; RK- arginine/lysine-rich basic domain; SAM- sterile alpha motif; SP- 'sex and plexins' domain; TK- tyrosine kinase domain; TSP-thrombospondin domain; VI and V- homology to laminin domains VI and V, respectively (Yu and Bargmann, 2001)

Netrin-DCC/Unc5 mediated axon guidance and cell migration

Axon guidance and cell migration research gets momentum from two great discoveries in the early 1990s. In a pioneer research, Hedgecock, et al. (1990) identified three genes (*unc5, unc6, unc40*) in *Caenorhabditis. elegans* that involved in axon guidance and cell migration. Mutations of these genes produced uncoordinated phenotype due to the disruptions in axonal pathfinding and cell migration. Based on their data, they hypothesized that *Unc6* encoded a guidance molecule, which formed a gradient, and that *Unc5* and *Unc40* encoded the receptors through which Unc6 guidance function is observed. Later, in a groundbreaking study, Tessier-Lavigne group observed that embryonic rat ventral neural tube explants attracted the commissural axons from rat dorsal neural tube explants. They then identified and purified netrin-1, chicken orthologue of Unc6, which was expressed at the floor plate of spinal cord and attracted commissural axons from embryonic rat dorsal neural tube explants (Serafini, et al. 1994; Kennedy, et al. 1994). So far, four members (netrin-1/-4) of netrin family have been identified in vertebrates and of these, netrin-1 is the best-characterized member of the netrin family. Netrin-1 is a secreted protein and it is structurally similar to laminin. Netrin-1 comprises a globular domain (VI), three EGF repeat domains (V1-3) and a positively charged C terminal domain. C-terminal domain is conserved in all netrins.

DCC (Deleted in colorectal cancer), a mammalian orthologue of Unc40, was identified as a netrin-1 receptor and mediates netrin-1-induced axon outgrowth and attraction (Keino-Masu, et al. 1996). Through Unc5, netrin-1 showed its repulsive function for trochlear motor axon (Colamarino and Tessier-lavigne, 1995). Orthologues of DCC/Unc40 have been found in a wide variety organisms that include frazzled in *Drosophila melanogaster*, zDCC in Zebrafish, xDCC in *Xenopus laevis*, DCC/Neogenin

in humans (Chan, et al. 1996; Keino-Masu, et al. 1996; Kolodziej, et al. 1996). For short-range netrin-1-induced chemorepulsion requires Unc5 alone while long-range chemorepulsion is facilitated by the formation of receptor complex between DCC and Unc5 (Hong, et al. 1999). Both short and long-range netrin-1 induced chemoattractive functions are mediated by its receptor DCC alone (Wen and Zheng, 2006)

Netrin-1 through its receptor DCC guides spinal commissural axon to project towards the ventral midline. This guidance activity is so important that both *netrin-1* and *dcc* knockout mice showed severe defect in commissural axon projection to floor plate and complete agenesis of all forebrain commissures: corpus callosum, hippocampal, and anterior commissure (Serafini, et al. 1996; Fazeli, et al. 1997). Netrin-1 attracts thalmocortical axons in vitro and its function is important for the development of thalamocortical projection in vivo (Braisted, et al. 2000). Besides these, netrin-1 also attracts and induces axonal outgrowth of corticofugal, hippocampal, retinal ganglion, habenular neurons (Metin, et al. 1997; Finger, et al. 2000; Barallobre, et al. 2000; Barallobre, et al. 2005). Netrin-1, expressing at the ventral midline of developing rat hindbrain, also attracts the migrating pontine cells towards the midline. Pontine structure plays important role to connect the cerebrum with cerebellum. Both *netrin-1* and *dcc* knockout mice showed the absence of pontine nuclei (Yee, et al. 1999; Serafini, et al. 1996; Fazeli, et al. 1997). It was reported that in the postnatal forebrain netrin-1 has important role to regulate the migration of *dcc* expressing neuronal precursor cells to olfactory bulb (Murase and Horwitz, 2002). Down's syndrom cell adhesion molecule (DSCAM) has been shown recently as another netrin receptor that mediates netrin-1-induced spinal commisural axon pathfinding (Ly, et al. 2008; Liu, et al. 2009). Thus, netrin-1 through its functional receptors plays an important role in guiding both axons

and the migrating cells to its proper target.

Slits and their receptors role in axon guidance

Slit is a large secreted protein (~190 KDa), which is produced by midline glia, and this protein plays an important role in the development of commissural axon and its midline crossing (Rothberg, et al. 1988; Rothberg, et al. 1990). There are at least three *slit* genes in mammals: *slit1*, *slit2* and *slit3* and all of these are expressed by the midline cells (Brose, et al. 1999; Holmes, et al. 1998; Itoh, et al. 1998, Nakayama, et al. 1998, Yuan, et al. 1999). Slit functions through its receptor Robo (derived from `roundabout`; drosophila mutation from which this cue was obtained) (Battye, et al. 1999, Kidd, et al. 1999; Brose, et al. 1999, Li, et al. 1999). In mammals, four members of Robo family have been identified. They are *Robo1*, *Robo2*, *Robo3* (*Rig1*) and *Robo4* (also known as magic roundabout) (Kidd, et al. 1998; Yuan, et al. 1999a). Except *Robo4*, the other *Robos* are expressed in CNS neurons including commissural neurons. Robo belongs to Ig superfamily receptor family. Extracellular domain of Robo1-3 consists of five immunoglobulin-like (Ig) and three-fibronectin type III (FN3) repeats, a single transmembrane segment, and a cytoplasmic domain (Kidd, et al. 1998). Mammalian Robo4 contains only two Ig domains and two FN3 domains. Slit binds to the first two Ig domains, most conserved region among Robos, (Liu, et al. 2004; Kidd, et al. 1998). The binding site for *Drosophila Robos* in Slit is same (Howitt, et al. 2004).

In normal course of circumferential spinal commissural axonal guidance system, netrin-1 in floor plate attracts spinal commissural axons from dorsal side to grow ventrally to cross the floor plate and move on to the anterior side and never recross again. Two events regarding this axonal pathfinding intrigued the researchers. First, why post-

crossing spinal commissural axons, expressing DCC continuously, lose their directional sensitivity to netrin-1? Second, why the precrossing commissural axons, expressing Robos, are not repelled by the Slits in floor plate? Data from two studies clarified these issues reasonably.

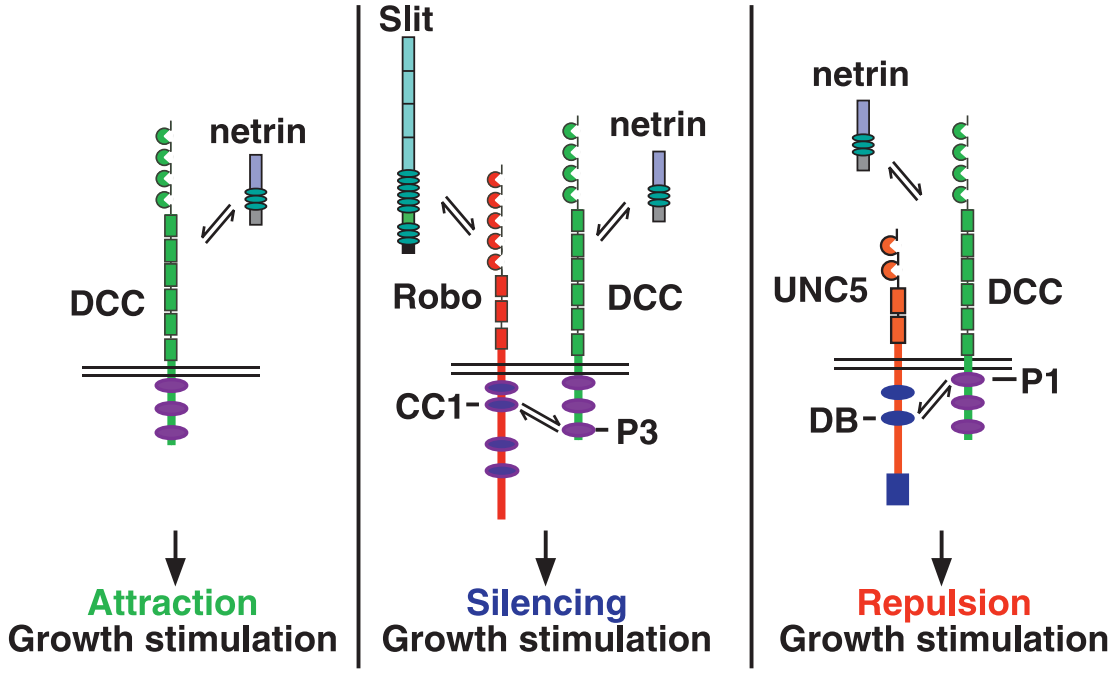

Figure 2. Receptor cross-talk determine the biological outcome of netrin-DCC interaction. Switching on the Slit-Robo signaling leads turning off the netrin-DCC attractive signaling by forming a receptor complex between the cytoplasmic domain of Robo and DCC. On the other hand, expression of Unc5 in DCC expressing neurons shifts the netrin-mediated attraction to repulsion by direct coupling of the cytoplasmic domains of DCC and Unc5 (Stein and Tessier-Lavigne, 2001).

First issue was resolved by a study conducted by Stein and Tessier-Lavigne (2001). In their study, they showed that the addition of slit-2 in vitro silenced the netrin-1 mediated turning of Xenopus spinal neuron though the netrin-induced axonal elongation effect was normal (Stein and Tessier-Lavigne, 2001). Based on their observations, they

concluded finally that the Robo expressing post-crossing spinal commissural axonal growth cones might be activated by Slits which in turn silence the netrin-induced attraction by forming a receptor complex between the cytoplasmic domain of Robo and DCC (Figure 2).

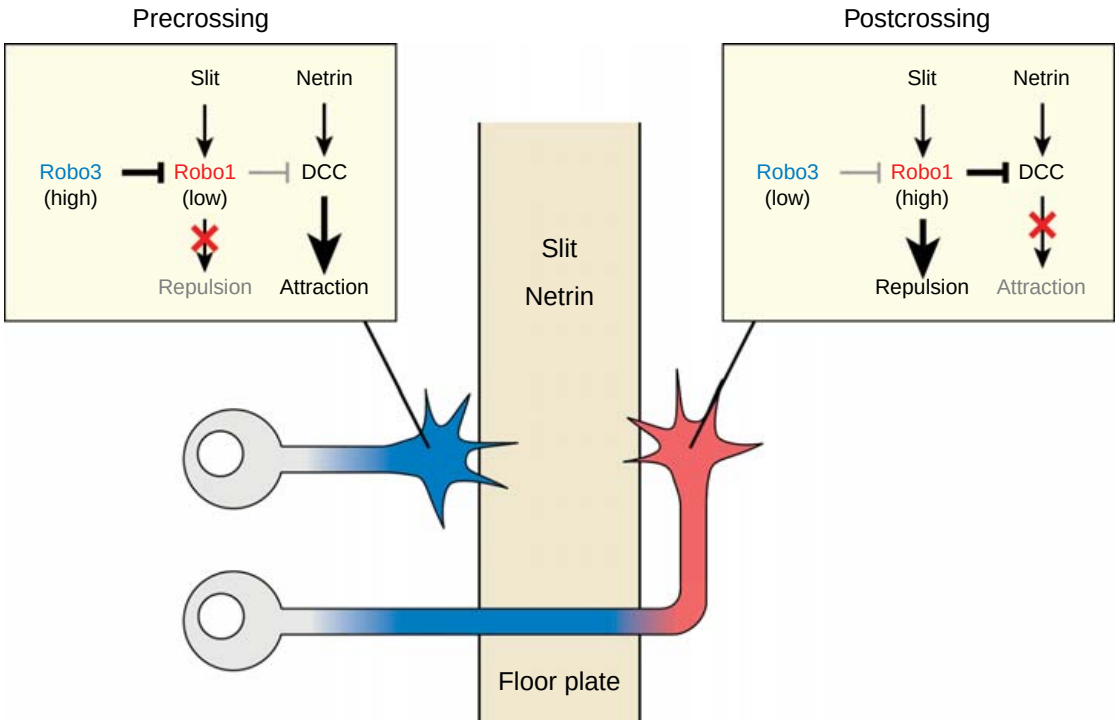

Figure 3. Robo3 is a negative regulator of Slit-Robo signaling. In precrossing axons high level of Robo3 is thought to suppress Robo1 mediated repulsion which in turn activate netrin-1 induced attraction. But in postcrossing axons Slit-Robo1 mediated repulsion activated due to the lower level of Robo3 and this event in turn silence the netrin-mediated attraction (Dickson and Gilestro, 2006).

Second event was addressed from a study, which showed that commissural axons in *Robo3-/-* knockout mouse embryos failed to cross the midline and made longitudinal turn on the ipsilateral rather than the contralateral side (Sabatier, et al. 2004). This phenotype is in parallel with a human syndrome called horizontal gaze palsy and progressive scoliosis (HGPPS) in which aberrant ipsilateral projections of major

ascending and descending axon pathways occurred due to a failure of these axons to cross the midline in the hindbrain which in turn results from the mutations in the human *Robo3* gene (Jen. et al. 2004; Bosley, et al. 2005). Like Robo1 and 2, expression of Robo3 in the spinal commissural axons is spatially controlled but in opposite manner. Levels of Robo3 in the precrossing axons are higher than the postcrossing axons. Furthermore, precrossing commissural axons from *Robo3*$^{-/-}$ mice are prematurely repelled by Slit in explant assays. Consistent with this, phenotype of *Robo3*$^{-/-}$ embryos is partially rescued by removing genetically either *Robo1* or both *Slit-1* and *Slit-2* (Sebatier, et al. 2004). These studies revealed that Robo3 (Rig-1) is a negative regulator of Robo 1 and 2 and inhibits Slit-Robo signaling in precrossing axons and thereby allows these axons to cross the midline (Figure 3).

Compared to *Drosophila* mutant, *Slit* triple knockout mice showed mild defects in abnormal axon crossing at midline or stalling (Long, et al. 2004). These data suggest the conserved role of slit proteins in midline axon guidance. Besides these, Slits function as a chemorepulsive axon guidance cue for other axons including olfactory bulb, hippocampal and spinal motor axons (Brose and Tessier-Lavigne, 2000). In addition, the chemorepulsive activity of the Slits has not only been shown in axon guidance but also in the targeted migration of neuroblasts within the rostral migratory stream towards the olfactory bulb (Hu, 1999; Wu, et al., 1999) and GABAergic neurons from the ganglionic eminence into the cortex (Zhu, et al., 1999). Surprisingly, Slit2 has also been shown to induce axon branching in sensory neurons (Wang, et al., 1999).

Semaphorins and their receptors in neural circuit formation

Semaphorins are large conserved family of axon guidance cues. Members of this family are either secreted or membrane bound and can repel and/or attract the growth cones of

the axons. There are 8 classes of semaphorins. Class II (invertebrates), class III (vertebrates), and class V (viral) are secreted. In contrast, membrane-associated form includes class I (invertebrates), classes IV-VII (vertebrates). All semaphorins contain a ~500 amino acid conserved sema domain at their amino terminal end. Sema domain confers biological activity to the semaphorins (Raper, 2000). Among all the semaphorins vertebrate class III semaphorins were best characterized. These semaphorins directly bind to the neuropilins (1 and 2), which in turn binds to the plexins to transduce the signals. However, some membrane-associated semaphorins can bind to the plexins and can transduce the signal without forming receptor complexes with neuropilins (Raper, 2000). In addition, neural cell adhesion molecules L1, the receptor tyrosine kinase Met, and the catalytically inactive receptor tyrosine kinase OTK have been identified as an important receptor complex components for Sema 3A, Sema4D, and *Drosophila* Sema1a respectively (Castellani, et al. 2000; Giordano, et al. 2002; Winberg, et al. 2001).

Secreted semaphorins were identified as chemorepulsive guidance cues for several classes of axons in the forebrain including hippocampal, pontocerebellar and olfactory as well as sympathetic, sensory and motor neurons from peripheral nervous system. Some semaphorins (Sema3B and 3C) also induce axonal outgrowth from cortical and olfactory bulb explant in vitro (Bagnard, et al. 1998; De Castro, et al. 1999). Semaphorin 7A, a membrane-anchored semaphorin, promotes axonal outgrowth from olfactory epithelium, olfactory bulb, cortical, and DRG explants (Pasterkamp, et al. 2003). The importance of semaphorin function was pronounced by observing the several defects in the projection of sensory axons, cortical neurites orientation or distorted odor map in sema3A deficient mice (Behar, et al. 1996; Taniguchi, et al. 2003). Severe abnormality in peripheral nerve projection was also observed in semaphorinIII/D deficient mice (Kitsukawa, et al. 1997).

Semaphorin3F mutant mice also showed several defects in axonal projection in the hippocampus, midbrain, forebrain and in the PNS (Sahay, et al. 2003). These reports indicated the functional involvement of semaphorins in complex axonal wiring of various regions in CNS and PNS.

The Eph and Ephrin guidance system

Among all the axon guidance receptors the Eph receptor tyrosine kinases have been studied well. Ephrins are the membrane bound ligand of Eph receptors. Ephrins are divided into two classes based on their membrane tethering nature. GPI anchored ephrins are designated as type A ephrins whereas type B ephrins have transmembrane and short cytoplasmic domain. Generally, ephrin As (A1-A5) classes ligand bind to Eph A (A1-A8) receptors and ephrin Bs (B1-B3) bind to EphB (B1-B6) receptors. Only exception is EphA4, which binds ligands from both type A and type B classes.

The Eph-Ephrin system has been studied well for their important roles in the formation of topographical maps within the CNS. In chicken retinotectal map formation, this systems function has been analyzed extensively. In retinotecatal system retinal axons are navigated to the tectum along the anterior/posterior and medial/lateral axes. Ephs and ephrins are expressed in gradient in complimentary manner in retina and tectum. Normally, nasal retinal axons containing lower EphA3 receptor are not repulsed properly by the higher concentration ephrin A2 and A5 in the posterior tectum and therefore terminate at posterior side. In contrast, temporal retinal axons having higher EphA3 are less repulsed by the lower concentration of ephrin A2 and A5 in the anterior side of the tectum and are therefore effectively innervated at the anterior side. Along the dorso/ventral axis, dorsal retinal axons containing lower EphB are attracted to the lateral side of the tectum where the concentration of ephrinB is lower than the medial side and

ventral retinal axons are chemoattracted to the medial side of the tectum where ephrinBs concentration is high (O'Leary and Wilkinson, 1999). In addition to topograph formation, ephrin-Eph has been implicated in various types of developmental events such as cell migration (Poliakov, et al. 2004) angiogenesis (Wang, et al. 1998) Vascular remodeling (Adams, et al. 1999) and neural tube closure (Holmberg, et al. 2000).

Apart from the above classical axon guidance cues and their receptors, another type of chemorepelent for chicken retinal axons named as repulsive guidance molecule (RGM) was identified (Monnier, et al. 2002). Later, neogenin was reported to be a functional receptor for RGM (Rajagopalan, et al. 2004). Although RGM knockout mouse showed defect in neural tube closure but the retinal topography in these mice were normal (Niederkofler, et al. 2004).

Morphogens act as an axon guidance cues

Morphogens are secreted signaling molecules that are involved in cell fate specification and tissue patterning. Morphogens usually have concentration gradient and work directly at a distance. Thus far, three families of morphogen have been identified. They are Wingless/Wnt, Hh and Dpp/BMP/TGF β (Teleman, et al. 2001). During the development of vertebrate nervous system, members of the above morphogen families play a critical role in the specification of diverse neural cell fates and tissue patterning (Ingham and McMahon, 2001; Lee, et al. 1998; Jessel, 2000; Muroyama, et al. 2002). The involvement of morphogens in axon guidance was started to emerge from evidences, obtained from several studies. The notion was developed from the observation of *netrin-1* and *DCC* knockout mice phenotype. In these mutants, spinal cord commissural axonal projection towards ventrally was normal till the first third of the whole trajectory and more ventrally most of these axons became misrouted though some axons still manage to

reach and cross the floor plate (Serafini, et al. 1996; Fazeli, et al. 1997). This suggested two possibilities: first, from roof plate, unidentified guidance cue might control the dorsal migration of spinal cord commissural axons, second, floor plate might have additional attractant that is responsible to attract some of the axons to the floor plate of these mutants. Subsequent studies (Augsburger, et al. 1999; Butler and Dodd, 2003; Charron, et al. 2003) indeed identified morphogens from roof and floor plate, which work as additional guidance cues to regulate the spinal commissural axonal pathfinding (Figure 4).

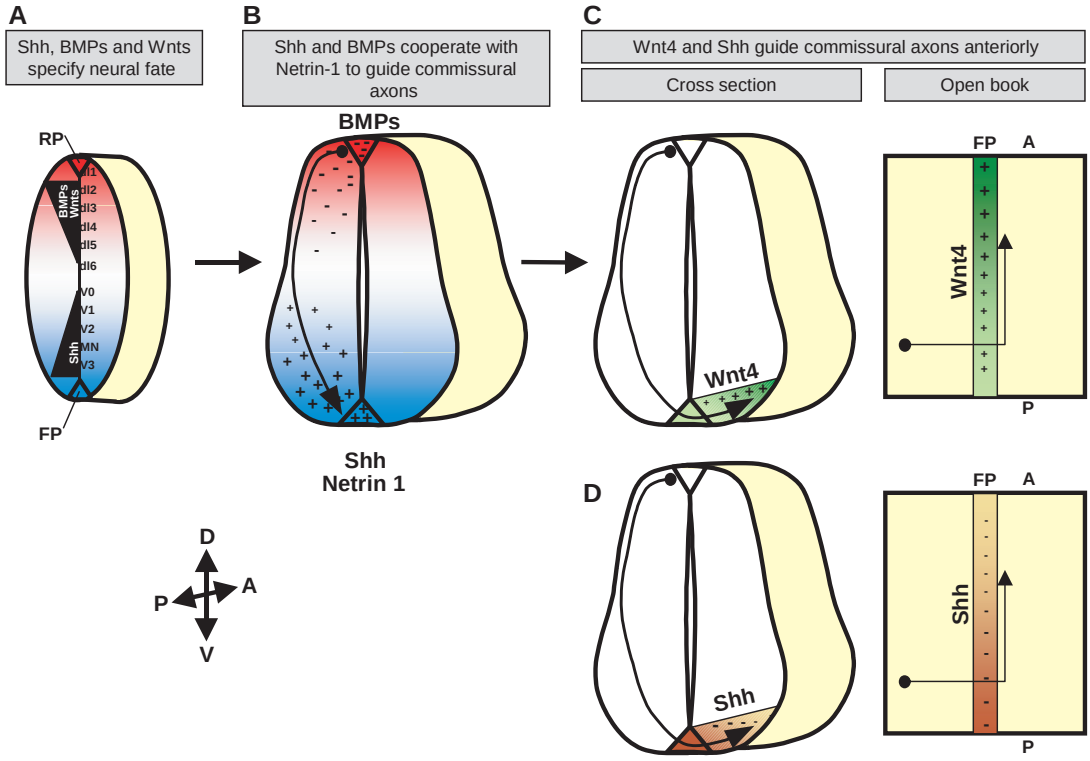

Figure 4. Morphogens specify the neural cell fate and guide commissural axons. Members of three morphogen families Shh, BMPs and Wnts, first induced to pattern

neural progenitors in the spinal cord, and then appear to be reused as guidance cues for commissural axons. (A) Shh, BMP and Wnt protein concentration gradients, in early neural tube, contribute neural cell fate specification in the ventral and dorsal spinal cord. (B) BMPs (red) from roof plate repel the commissural axons to grow ventrally and

combinatorial effect Netrin 1 and Shh (blue) from the floor plate attract these axons towards ventral midline (C,D) Post-crossing commissural axons are attracted anteriorly by a Wnt4 gradient (C, green) and repelled from the posterior pole by a Shh gradient (D, orange). A and B, and left panels in C and D, are cross section representations of the developing spinal cord; right panels in C and D are open book representations. V0-3, ventral interneuron sub-populations; dI1-6, dorsal interneuron sub-populations; MN, motoneurons; RP, roof plate; FP, floor plate; D, dorsal; V, ventral; P, posterior; A, anterior (Charron and Tessier-Lavigne, 2005).

Morphogen BMP7, expressed in gradient in roof plate, repel spinal commissural axons in vitro and disrupting the function of BMP7 by means of inhibitor of BMP activity, BMP function-blocking Ab, and genetice inactivation, relieve the repellent activities (Augsburger, et al. 1999). Further study showed that BMP7 in association with GDF7, made a heterodimer complex, which in turn several fold increase the chemorepellent potency of BMP7 in vitro. Genetic ablation studies of BMP7 and GDF7 showed that the expression of BMP7 and GDF7 in the roof plate is essential for the proper commissural axonal growth in vivo (Butler and Dodd, 2003).

In addition to netrin-1, sonic hedgehog (Shh) from floor plate attracts spinal commissural axons from dorsal spinal cord explant in vitro (Charron, et al. 2003). The chemoattractant activity of the Shh was blocked by the addition of cyclopamine, an inhbitor of Smo, suggesting the activity of Shh is mediated through Smo. Conditional inactivation of *Smo* in spinal commissural neurons showed defective axonal trajectory in the ventral spinal cord. These data indicated that Shh through Smo guide spinal commissural axons in vitro and in vivo. Although Shh guides commissural axon through Smo, Shh does not bind Smo directly. It was unknown about the receptor of Shh through which it mediates signal until the discovery of Boc [biregional Cdon (cell-adhesion-

molecule-related/downregulated by oncogene) -binding protein]. Shh binds to Boc with high affinity and *Boc* conditional knockout mice showed commissural axons misprojection near the floor plate. Knocking down the Boc expression by siRNA in the commissural neuron disrupts axonal ability to turn towards the Shh expressing cells. These results indicated that Boc mediates Shh signaling to guide the commissural axons (Okada, et al. 2006). Shh not only attracts the axons but also functions as a repellent for some other axons. Trousse et al. (2001) showed that the ectopic expression of Shh at the chiasm border impaired the retinal ganglion cell axonal growth and prevented these axons to cross the midline. When they cultured the retinal explants in presence of recombinant Shh they found that the number and length of the retinal axons were markedly reduced. Also bath application of Shh caused quick arrest growth cones of growing axons from retinal explant. There was a possibility that the effect of Shh on retinal explant was due to the modulation of intracellular second messenger. Indeed in later study it was shown that the addition of Shh to retinal explant in vitro reduce the intracellular level of cAMP, which was one of the responsible factor for the axonal growth inhibition (Song and Poo, 1999). Shh and other repellents like Slit and semaphorins express in the spinal cord floor plate. These repellents have a defined role to help the spinal commissural axons to cross the floor plate and Shh may have some role to modulate the activity of theses repellents. In fact, recently, it was shown in a study that Shh in the floor plate can induce the repulsion of semaphorins in the midline and thereby enable the commissural axons to cross the midline (Parra and Zou, 2009). Taken together, these report suggest that Shh can function as both positive and negative regulator in axon guidance in context dependent manner.

Spinal cord commissural axons after crossing the floor plate migrate longitudinally

towards the anterior side to the brain. It was unknown which molecules guide these post-crossing axons to move towards brain. By in vitro and in vivo loss of function studies, Lyuksyutova et al. (2003) have shown that the Wnt4, expressing anterior high and posterior low gradient, attracts the postcrossing axons through Frizzled 3 (Fz3) receptor to grow towards anteriorly. One important finding revealed in this study that Wnt4-Fz3 mediated anterior/posterior guidance of postcrossing axons does not use Wnt canonical signaling pathway since LRP6, coreceptor for Fz3 in Wnt canonical pathway, mutant mice show normal pathfinding of the post crossing commissural axons.

Thus, the above studies established the fact that the classical axon guidance cues along with the morphogens play a significant role in proper development of nervous system.

1-1-2. Olfaction and the Development of LOT

When we travel scenic places, the visual sensation from beautiful things leave a long lasting memory within us. Likewise when invisible aroma from delicious cuisine, flower or nice perfume tickles our nostrils the senses of smell also dominate our memory. Virtually, smell and visual perception create a deep impact in our memory about persons, places, things, and foods. Past two decades have seen the great advancement in understanding the mechanism of sensing the odorants. The collaborative research of Richard Axel and Linda Buck made initial breakthrough to unravel the sensing scenario how sensory neurons from olfactory epithelium detect the odorants and how this information is transmitted through the olfactory bulb to the higher olfactory center in the brain.

The process of sensing the odorants is known as olfaction. Much of the animal's

behaviors depend on their olfaction. The olfaction system is conserved in vertebrates and the olfactory bulb plays a critical role in transferring sensory information from olfactory receptor neurons (ORNs) to the brain. ORNs line the nasal cavity and receive odor signals. In rodent each ORN expresses only one of the 1000-1300 different olfactory receptor genes (Malnic, et al. 1999; Zhang and Firestein, 2002). ORNs, expressing the same receptor gene, are distributed randomly in the nasal cavity but their axons sort together and converge into the same glomeulus in the olfactory bulb (Mombaerts, et al. 1996). In the glomerulus of main olfactory bulb (MOB), axons from ORNs synapse with the dendrites of mitral/tufted cells (Figure 5). The primary axons of the projection neurons from the MOB form a fasciculated axonal tract, known as Lateral Olfactory Tract (LOT), which carries the sensory information ahead. The LOT runs ipsilaterally through a narrow region of the ventro-lateral part of the telencephalon, beneath the pial surface (Figure 6). The axons of the LOT send off the collateral brancehes to the target areas in the olfactory cortex, anterior olfactory nucleus, piriform cortex, olfactory tubercles, entorhinal cortex, and several amygdaloid nuclei (Derer, et al. 1977; Schwob and Price, 1984; Shipley and Ennis, 1996; Zou, et al. 2001).

Proper development of LOT is essential for the transmission of the sensory information to the olfactory cortex. In mice, LOT formation starts at embryonic day E12.5 when the mitral and tufted cell axons leave the OB and finishes at E13.5 (Pini, 1993; Sugisaki, et al. 1996). Previous studies indicated that the olfactory epithelium, septum and cortex derived chemorepulsive axon guidance molecules, such as Semaphorins and Slits (1 and 2), repelled axonal outgrowth from OB explants *in vitro*

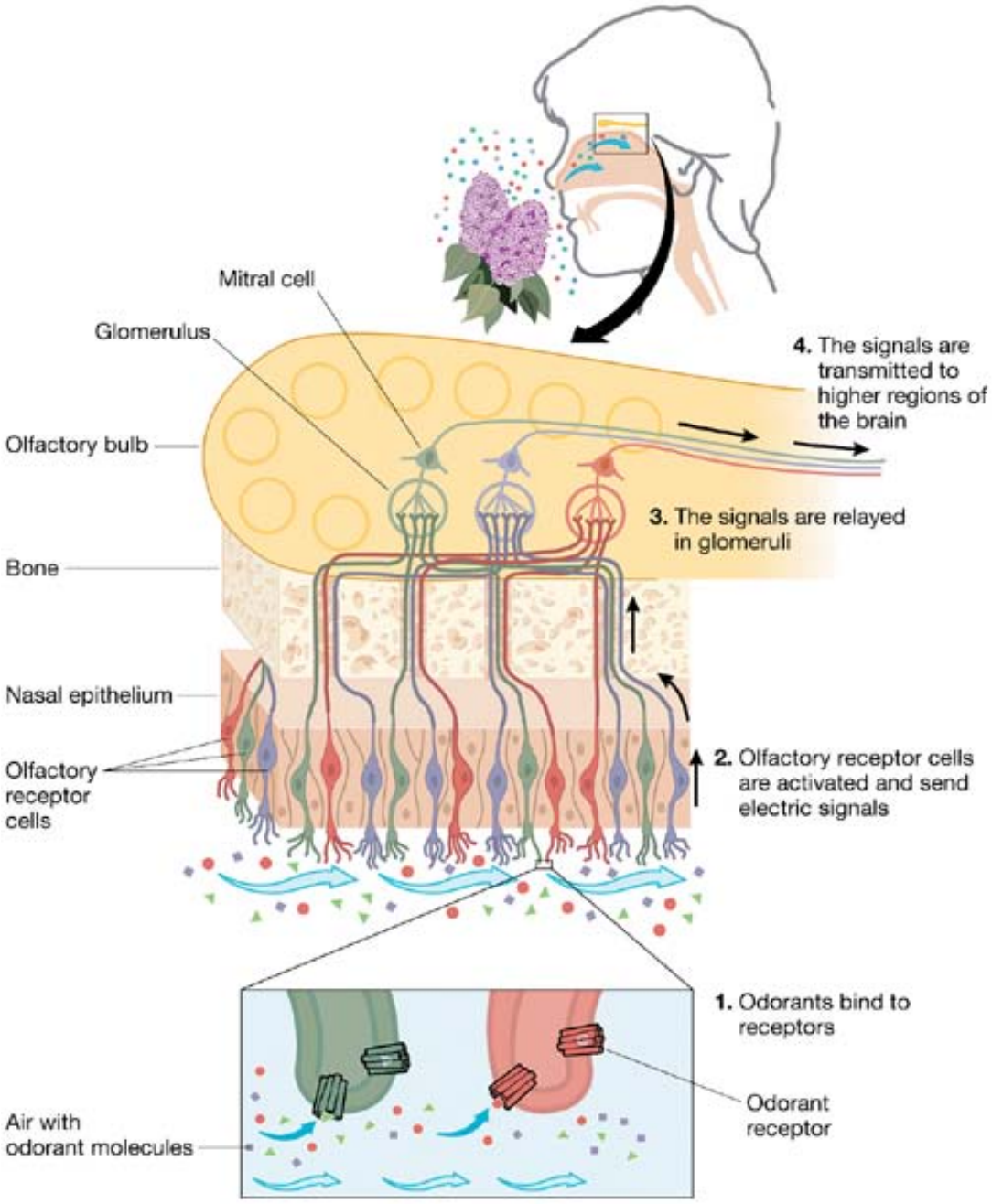

Figure 5. The human olfactory system. Olfactory receptor neurons expressing specific type receptor for odorant detect the odorant in the environment and transmit the information through their axons to the intermediate target, glomerulus of olfactory bulb. Here , these axons transfer the information to the dendrites of mitral/tufted cells, which in turn relay the signal to the higher regions of brain through their axons (Rinaldi, 2007).

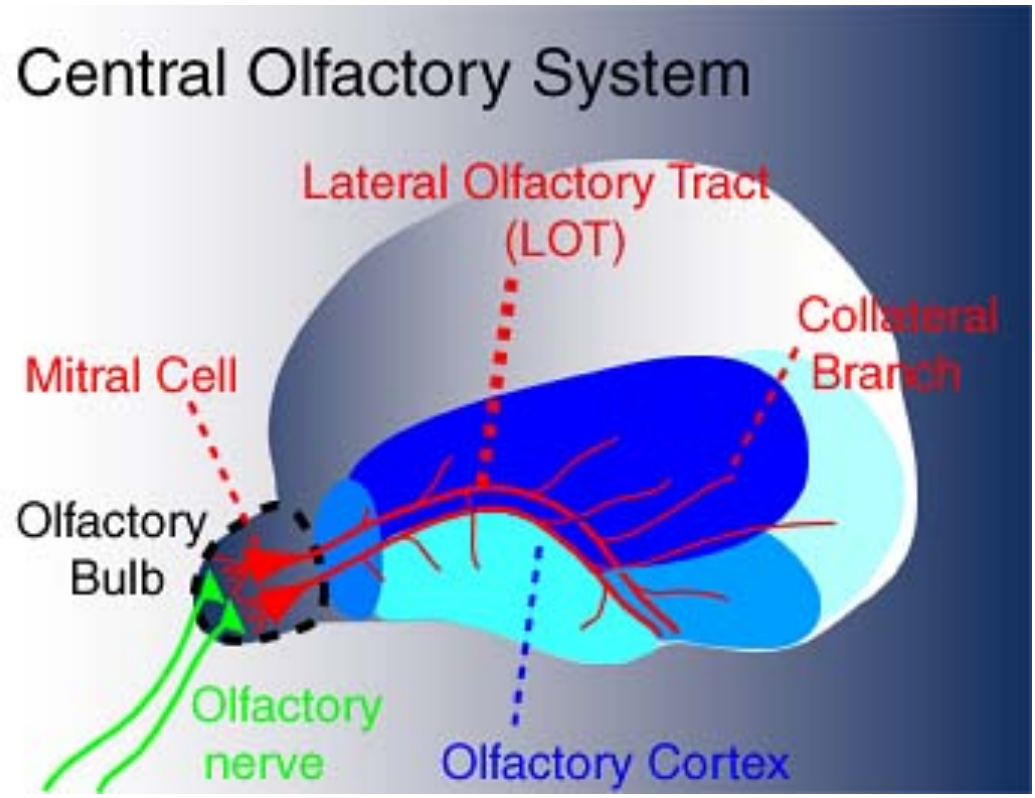

Figure 6. Central olfactory system. The dendrites of mitral/tufted cells in olfactory bulb form synapses with the axons of olfactory sensory neurons. Later the axons of mitral/tufted cells form LOT which sends the collateral branches to the different regions in brain (Adopted from the homepage of Dr. Tatsumi Hirata, NIG, Mishima, Japan).

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(De Castro, et al. 1999; Nguyen-Ba-Charvet et al. 1999; Li, et al. 1999). Slits regulate LOT formation through their receptors, Robos. Although severe disorganized LOT formation was observed in Slits or Robos double knockout mice, a subset of OB axons was found to be in the correct position (Nguyen-Ba-Charvet, et al. 2002; Fouquet, et al. 2007).

Apart from the classical guidance cues mediated mechanism, another short-range guidance cue called LOT cue has also been implicated in the development of LOT. By performing organotypic co-culture of the olfactory bulb with various parts of the

telencephalon, Sugisaki, et al. (1996) showed that the OB`s axonal pathfinding was guided by early generated neurons, called LOT cells. These cells are precisely located in the future pathway of LOT trajectory (Sugisaki, et al. 1996) and are recognized by monoclonal antibody lot1 (Sato, et al. 1998). By pharmacological ablation of lot cells, it was shown in this study that mitral cell axons stopped growing. These experiments suggested that lot cells make a scaffold on which the mitral cell axons grow to form the LOT. In the early developmental stages lot cells generate from diverse area of necortex and finally migrate tangentially and ventrally to the presumptive lot area (Tomioka, et al. 2000). Two classical guidance molecules have been implicated in the migration of lot cells. Semaphorin3F secreted from the ganglionic eminence (GE) repels the lot cells from invading the GE and ventral area. In *Sema3F* and *Nrp2* mutant mice significant number of lot cells penetrated deep medial region, which in otherwise wildtype mice restricted only on the surface. This data suggest that sema3F pushes lot cells on the surface to align them along the neocortex-GE boundary by its repulsive action (Ito, et al. 2008). On the hand, netrin-1 from the ventral brain attracts the LOT cells ventrally to array them at the border of neocortex and GE. *Netrin-1* and *DCC* knockout mice exhibited inappropriate distribution of lot cells, which lead partial disruption of LOT projection. These data suggested that netrin-1 regulates the migration of lot cells and LOT projections by distributing the guidepost neurons in their correct spatial postion (Kawasaki, et al. 2006).

The underlying molecular mechanism involved in the LOT development has been elucidated significantly but not fully by the function of both short and long-range axon guidance cues and their receptors. This suggests that the other unknown guidance molecules exist to contribute LOT development.

1-2. Background and Specific aims

Background

Although the identified classical axon guidance cues, as well as morphogens, have been found to regulate a wide variety of guidance decisions, it is expected that others await identification, and will help address the immense complexity of the nervous system. From this perspective, we screened a cDNA library made from enriched motoneurons, floor plate and roof plate cells, to identify new guidance cues. In our search, we identified a novel axon guidance molecule, draxin, which shares no sequence homology with other known guidance molecules (Islam, et al. 2009). Draxin is expressed strongly in various parts of the brain and spinal cord. In vitro, draxin can repel spinal commissural axons whose outgrowth is stimulated by netrin-1; *in vivo*, genetic deletion of *draxin* results in mild guidance defects of those axons, and in a dramatic loss of all forebrain commissures (Islam, et al. 2009). In another recent study, draxin inhibited chicken tectal axonal outgrowth *in vitro* and *in vivo* (Naser, et al. 2009). We also showed previously that the hippocampus size in *draxin* knockout mice is smaller than the wild type (Zhang, et al. 2010).

Olfactory topography is well studied. In olfaction, development of precise axonal tract emanating from the second order projection neurons i.e. mitral/tufted cells in olfactory bulb is important to faithfully transmit the sensory information to the different regions of brain. It has long been known that the olfactory bulb axonal tract avoid to enter the neocortex and midline structure septum. Thus far, neocortex and septum derived chemorepellents Slits (1 and 2) and SemaphorinIV through their respective receptors Robos (1 and 2) and Neuropilin (2) repel the growth of olfactory bulb axons in vitro. On the hand, SemaphorinA from the mesenchyme precursor attract the LOT axons.

But none of the single knockout mice of these molecules or their receptors exhibit detectable phenotype. However, double knockouts *slit1/2* and *Robo1/2* mice showed severe disorganized LOT though a subset of LOT axons were seen in their correct position (Nguyen-Ba-Charvet, et al. 2002; Fouquet, et al. 2007). This suggests that other guidance molecule with similar function may compensate the function of missing proteins.

Specific Aims

a) Since draxin is highly expressed in the neocortex and septum during the stages of LOT development (Islam et al. 2009), I explored the role of draxin in the LOT development.

b) Although our in vitro and in vivo studies clearly demonstrated the guidance function of draxin in different axons of chicken and mice tissues, the mechanism of draxin-mediated action cannot be addressed without the identification of its receptor. Therefore, my second aim was to identify the receptor for draxin to elucidate draxin signaling.

2. Materials and Methods

2-1. Mice

For *draxin* mRNA expression analysis, embryonic stages of *draxin* heterozygous mice were used. *Draxin* homozygous and wild type littermate mice were used for phenotype analysis. The procedure to generate *draxin* knockout mice /β-galactosidase (β-gal) knockin and knockout mice was described previously (Islam, et al. 2009). To breed homozygous *DCC* mutant embryos, *DCC* heterozygous mutant mice (Fazeli, et al. 1997) were mated. All mice were obtained from a colony in an animal center in Kumamoto University. The day of the vaginal plug was designated as embryonic day 0.5 (E0.5). All animal procedures were conducted in accordance with institutional guidelines.

2-2. Plasmids

Rat *DCC* (Keino-Masu, et al., 1996) subcloned into the expression vector pCEP4 (Invitrogen) was used in draxin binding experiments. Full-length cDNAs encoding rat-NCAM, and extracellular domain of rat *Robo 1-Fc* were a kind gift from Drs. Kouichi Itoh (Tokushima Bunri University, Japan) and F. Murakami (Osaka University, Japan), respectively. The extracellular domain of rat *DCC* was subcloned into pEF-Myc-His vector (Invitrogen). Different types of human *DCC* cDNA fragments encoding *DCC-ecto-hGH-His, DCC-Fn(1-6)-hGH-His,* and *DCC-IgG(1-4)-hGH-His* (Geisbrecht et al., 2003) were a generous gift from Dr. D. J. Leahy (The Johns Hopkins University, USA).

2-3. Antibodies

The mouse anti-chick draxin monoclonal and rabbit polyclonal antibodies were characterized previously (Islam, et al. 2009). Mouse anti-chick draxin monoclonal antibody was used to detect chick draxin-AP in conditioned medium by western blot, and to perform immunoprecipitations using brain lysates. Rabbit anti-chick draxin polyclonal

antibody was used to detect bound draxin by immunocytochemistry at 2.5 μg/ml and also in immunoprecipitation assays to pull down the DCC ectodoamin.

Different types of first antibodies such as rabbit polyclonal anti-chicken NCAM (in house), rabbit polyclonal anti-human Fc (Sigma), rabbit polyclonal anti-his (Santa Cruz), mouse monoclonal anti-human DCC (Calbiochem), goat polyclonal anti-human DCC (Santa Cruz), rabbit anti-hGH (Santa Cruz) were used to detect the expression of NCAM (cell overlay assay), ectodomain of Robo-1-Fc (IP assay), bound netrin-1-his (cell overlay assay), expression of DCC (cell overlay assay and IP assay with soluble version of DCC), expression of DCC (IP with full length DCC, section immunohistochemistry, in neurites in collagen gel culture, and dissociated cortical neurons), expression of different types of extracellular fragments of DCC tagged with hGH by western blot respectively. Anti-neuron-specific beta-tubulin (Tuj1) antibody (R&D systems) was used to label the neurites emanating from the explants.

2-4. X-gal Staining and Histological Analyses

To determine the *draxin* mRNA expression pattern in the neocortex, X-gal staining was performed on a 25 μm cryostat section according to standard protocol (Nagy, et al. 2003). Section immunohistochemistry was performed following a protocol previously described in detail (Okafuji and Tanaka, 2005). The following primary antibodies were used for immunostaining: rabbit polyclonal anti-draxin (mouse protein), rat anti-L1 (Chemicon), and mouse anti-neurofilament (2H3) (Developmental Studies Hybridoma Bank). For whole-mount immunostaining, embryonic mouse brains were dissected and fixed with cold ethanol for 10 min. Then the brains were treated with 0.03% hydrogen peroxide in methanol for 30 min at room temperature to quench endogenous per-oxidase activity, and immersed in *5%* skimmed milk in 10 mM Tris-HCL, pH 7.5, 150 mM sodium chloride,

0.1% Tween 20 (TBST) for 6 h to block nonspecific binding of antibodies. The specimens were then incubated with rabbit anti-neuropilin antibody (2 pg/mL) (Kawakami et al., 1995) in TBST for 6 h, and with biotinylated anti-rabbit immunoglobulin G (IgG) antibody (1:400; Jackson Laboratories) according to the protocol of (Shimamura and Takeichi, 1992). Bound antibodies were detected with a Vectastain ABC elite kit (Vector Laboratories), and visualized with diaminobenzidine.

2-5. Production of Draxin and Control-Alkaline Phosphatase Conditioned Medium

293T cells were transfected with cDNA encoding chick *draxin-AP* and empty *AP* tag vector (Islam, et al. 2009) using Lipofectamine-2000 (Invitrogen), conditioned for 5 days and concentrated using an Amicon Ultra centrifugal device (Millipore). Production of proteins was checked by western blot using anti-chicken draxin monoclonal antibody.

2-6. Binding Assay and Determination of Dissociation Constant, K_d, for draxin-DCC Binding

Cell overlay assay and other binding experiments were performed as previously described (Keino-Masu, et al. 1996; Islam, et al. 2009). Briefly, 293T cells were first transfected with cDNAs using Lipofectamine-2000 (Invitrogen). After 40 hours, cells were incubated with 30 nM of draxin-AP or control-AP conditioned medium for 90 min at room temperature. Cells were then washed extensively, fixed and heated at 65°C for 100 min. Bound draxin-AP was visualized with BCIP/NBT substrate (Flanagan and Cheng, 2000).

For double staining of receptors and bound draxin, 293 cells were seeded on PEI (Polyethyleneimine, 0.001%) coated coverslips and allowed to grow overnight in an incubator maintaining 37°C temperature plus 5% CO2. Later, these cells were transfected with respective cDNAs using lipofectamine 2000. After being washed one time with cold

HBAH buffer [HBSS, BSA (0.5mg/ml), 0.1% (w/v) NaN3, 20 mM HEPES (pH 7.0)], transfected cells were then incubated either with 30 nM draxin-AP or with chicken netrin-1-his (R&D systems) at 2μg/ml concentration for 90 minutes at room temperature. Later, cells were washed with HBAH buffer and were fixed briefly either with 4% PFA in PBS (for draxin binding) or with ice-cold methanol (for netrin-1 binding). After fixation, cells were washed with PBS and incubated with antibodies to DCC, NCAM, His and polyclonal anti-draxin antibody for an hour at room temperature. After 3 washes, bound draxin or netrin-1 and receptor expression were visualized using a Cy3 (Jackson immuno research laboratories) and Alexa 488 (Molecular probes) conjugated secondary antibodies respectively.

For quantitative analysis of draxin-AP binding, *DCC* and vector transfected cells were incubated with concentrated draxin-AP protein at different dilutions for 90 min at room temperature. Cells were lysed in 1% Triton X-100 in 10 mM Tris-HCl (pH 8.0) after being washed extensively with HBAH buffer. The cell lysates were centrifuged at 15,000 rpm for 1 minute. In order to inactivate endogenous phosphatase, the supernatant was heated at 65^{o}C for 10 minutes. The lysate was then incubated with 1 M diethanolamine (pH 9.8), 1 mM $MgCl_2$ and p-nitrophenyl phosphate (Sigma) for 1 hour and the AP activity was determined by OD at 405 nm. The net bound draxin-AP was calculated by subtracting the value of vector transfected cells from *DCC* transfected cells. Saturation binding curves and Scatchard analyses were performed, and the K_d value was determined as previously described (Cheng and Flanagan, 2000).

To determine draxin binding to neurites in mouse explants experiments explants from the olfactory bulb of stage E14.5 embryos and the cortex of stage E17.5 embryos of wild type and *DCC* KO littermate mice were dissected and cultured on poly-L-lysine

(0.01%) (Sigma) and laminin (10.0 μg/ml) (GIBCO) coated dishes for 48 hours in neurobasal medium (GIBCO) supplemented with B27 (GIBCO), glutamax-I and penicillin/streptomycin (GIBCO). Cultured explants were then incubated with either draxin-AP or control-AP conditioned medium for 90 minutes at room temperature and AP staining was performed as described above.

Draxin-AP binding to unfixed brain sections was performed using the same conditioned medium as previously described (Feiner, et al. 1997). Briefly, brains were embedded in OCT compound, and fresh frozen sections were cut. The sections were postfixed in ice-cold methanol at −20°C for 7–10 min. Care was taken not to allow the sections to dry out. Sections were then washed with phosphate-buffered saline (PBS), blocked with PBS containing 10% FCS for 15 min, and incubated either with draxin-AP or control-AP conditioned medium for 1 hr at room temperature. After incubation with the proteins, the sections were rinsed with PBS and fixed with 60% acetone, 3% paraformaldehyde, and 20 mM HEPES (pH 7.0) for 3 min and washed with PBS. Endogenous AP activity was heat inactivated by incubating the sections at 65°C for 3 hr, and sections were processed for AP color substrate NBT/BCIP and bound draxin-AP signal was visualized under the microscope.

2-7. Immunoprecipitation Assay

293T cells were transfected either with rat *DCC* or empty vector using Lipofectamine 2000. About 48 hours of post-transfecteion, transfected cells were washed with HBAH buffer and incubated with 1nM draxin-AP conditioned medium for 2 hours at room temperature with occasional gentle stirring. Later, cells were lysed with lysis buffer (50 mM Hepes pH 7.6, 150 mM NaCl, 0.1% Triton-X-100) supplemented with protease inhibitors (1 mM PMSF, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin). As a negative control

cell lysates from vector transfected cell, which were incubated with draxin-AP, and *DCC* transfected cells, which were not incubated with draxin-AP, were taken. Lysates were incubated with anti-chick draxin pAb (1 μg/ml) in IP buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1.5 mM CaCl2, 1.5 mM MgCl2, 0.1% Triton-X-100, 0.1% Octyglucoside, 0.1% CHAPS, 5% glycerol and 0.1% BSA) for 12 hours at 4°C and complexes were retained and washed extensively on protein G sepharose beads (GE Healthcare). Immunoprecipitated DCC protein was separated by 7.5% SDS-PAGE and detected by western blot using goat polyclonal anti-DCC antibody (Santa Cruz).

2-8. In-solution Binding Assay

Concentrated draxin-AP and DCC-ecto-Myc-His fusion protein conditioned medium was mixed together and incubated with anti-chick draxin pAb (1 μg/ml) in IP buffer at 4°C for 12 hours. Then the IP assay and detection by western blot using mouse monoclonal anti-DCC (Calbiochem) were performed as above.

For pull down of draxin-AP by DCC-ecto-Myc-His protein, the indicated conditioned media were mixed together and the pull down assay was performed as above using ProBond resin (Invitrogen). Bound draxin-AP was detected by western blot with anti-chick draxin mAb. As a negative control, draxin-AP was mixed with BSA in IP buffer and the assay was carried out as above. For the pull down assay of draxin-AP by Robo1-ecto-Fc, protein G sepharose was used instead of ProBond resin.

2-9. Tissue Immunoprecipitation

Brains from E17 mouse embryos were dissected and lysed in lysis buffer supplemented with protease inhibitors as described above. The mixtures were kept on ice for 30 minutes and centrifuged at 15000 rpm at 4°C for 20 minutes. Membrane fractions were

collected and lysates were incubated with concentrated draxin-AP conditioned medium and anti-chick draxin mAb for 12 hours at 4°C. Notably, mouse monoclonal anti-chick draxin was unable to detect endogenous mouse draxin protein; hence, it was used in the IP assay using E16 mouse brain lysates to pull down endogenous DCC in the presence of draxin-AP conditioned medium. Immunoprecipitates were recovered and analyzed as described above.

2-10. Explant Cultures

Explants from E14.5 olfactory bulbs (OB) of wild type mice were dissected as described previously (Nguyen-Ba-Charvet, et al. 2002) and cultured in collagen gels for 48 hours in the presence of either draxin-AP or control-AP conditioned medium mixed with our culture medium composed of Neurobasal medium (GIBCO) supplemented with B27 (GIBCO), glutamax-I and penicillin/streptomycin (GIBCO) at a 1:1 ratio. After culture, explants were stained with anti-neuron-specific β-tubulin (Tuj1) antibody (R&D systems) in the collagen gels. Photographs were taken using an inverted fluorescence microscope (Keyence, Biorevo) and the longest neurite length per explant was measured using ImageJ software. Averages were determined from the counted maximal neurite lengths from all of the explants.

For co-culture experiment, E14.5 mouse OB explants were dissected from wild type mice and were co-cultured with 293 cell aggregates transfected either with an empty vector or with cDNA encoding mouse draxin, as described previously (Islam, et al. 2009). Explants were placed at a distance from the aggregates. The aggregates and explants were cultured for 48 hours in collagen gel with our culture medium. The 293 cell transfectants and aggregates were prepared as described previously (Ohta, et al. 1999). After culture, explants were stained with Tuj1 antibody and photographs were taken

using a Leica inverted microscope. Explants were subdivided into four equal quadrants. The quadrant nearest the aggregates was designated as the proximal side, and the opposite quadrant was labeled distal. Neurite length in these two quadrants was measured by ImageJ software and the Proximal/Distal ratio was determined.

For checking the sensitivity of draxin on *DCC-/-* neurites, explants from E17.5 olfactory bulb (OB) and cortex of both wild type and *DCC* KO littermate mice were dissected as described previously (Nguyen Ba-Charvet, et al. 1999; Shu, et al. 2001) and cultured in collagen gels for 48 hours in the presence of either draxin-AP or control-AP conditioned medium (100 nM) mixed with Neurobasal medium (GIBCO) supplemented with B27 (GIBCO), glutamax-I and penicillin/streptomycin (GIBCO). After culture, explants were stained with anti-neuron-specific beta-tubulin (Tuj1) antibody (R&D systems) in the collagen gels. Photographs were taken in a microscope (Keyence, Biorevo) and longest neurite length per explant was measured using ImageJ software. Averages were determined from counted maximal lengths of all explants.

2-11. Growth Cone Collapse and Neurite Outgrowth Assay

Cortical neurons from mouse embryo at E16.5 were dissociated by trypsinization as described previously (Hata, et al. 2006) and seeded at low density in 4 well dishes containing coverslips coated with poly-L-Lysine (100 μg/ml) and laminine (20 μg/ml).

For neurite outgrowth assay, neurons were cultured in neurobasal medium (B27, glutamax, penicillin-streptomycine) having either equal amount of draxin-AP or control-AP conditioned medium for 48 hours. Later, neurons were briefly fixed and stained with goat polyclonal anti-DCC (Santa Cruz) following a protocol described above.

For growth cone collapse experiment, neurons were seeded in a dish as above and

cultured in neurobasal medium. After 60 hours of post seeding, neurons were incubated with draxin-AP (100 nM) and control medium alone for 1 hour at 37°C to induce collapse. Cultures were fixed later by 4% paraformaldehyde/10% sucrose for an hour. After washing well with PBS, growth cones were stained phalloidin-Alexa 568 (Invitrogen) and images were acquired on a microscope (Nikon, Model Eclipse, E600) for scoring the collapse. To calculate the percentage of collapse, the total number of growth cones divided the number of collapsed growth cones. Data were collected from at least two independent experiments and expressed as means ± SEM. In each independent experiment, at least 40 neurons were counted per group.

3. Results

Results section has been divided into two sections: Part A and Part B. In part A, I have shown the data that demonstrate draxin's function in olfactory bulb axonal outgrowth. Part B deals with the data, which suggest that draxin's neurite outgrowth inhibitory effect is mediated by DCC.

3-1. Part A

3-1-1. Expression Pattern of draxin in the Cortex and Septum

Earlier studies (Pini, 1993; De Castro, et al. 1999; Nguyen-Ba-Charvet, et al. 1999; Li, et al. 1999; Nguyen-Ba-Charvet, et al. 2002) demonstrated that the olfactory bulb (OB) axonal pathfinding is controlled by the orchestrated action of chemorepellents and chemoattractants from the neocortex and septum. To check whether draxin influences the formation of the OB axonal tract, we first examined the draxin expression pattern in the developing telencephalon at E13.0 and E17.5. Between these stages, the OB axons develop to form a complete trajectory. To assess the *draxin* expression, we used *draxin* knockout mice; these mice were generated by replacing the second exon, containing ATG start codon, with a lacZ-neo selection cassette (Islam, et al. 2009). We therefore examined the expression of *draxin* using β-galactosidase staining of heterozygous mice. Moreover, the *draxin* expression pattern inferred from this β-galactosidase staining follows precisely the pattern of draxin transcripts detected previously by *in situ* hybridization (Zhang, et al. 2010). Strong *draxin* expression in terms of β-galactosidase staining was observed in the neocortex and septum of *draxin* heterozygous mouse embryos at E13 (Fig. 7A). However, at E17.5, the expression became weaker in the septum while it remained stronger in the neocortex (Fig. 7B). In the mouse neocortex, robust *draxin* expression persists throughout embryogenesis and in

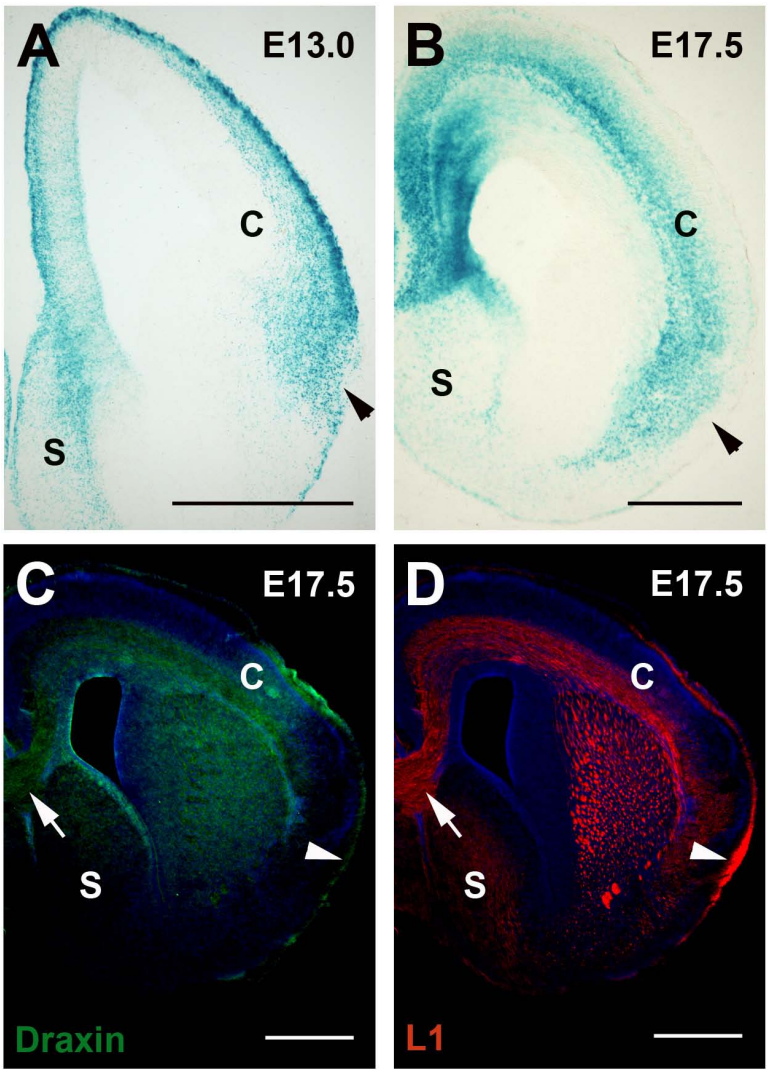

Figure 7. Expression of *draxin* is observed in the mouse neocortex and septum. (A, B) β-galactosidase staining was performed on 25 μm coronal cryostat sections of E13.0 (A) and E17.5 (B) *draxin* heterozygous mouse embryos to check the *draxin* mRNA expression pattern. *draxin* expression was observed strongly in the cortex and weakly in the septum (A and B). (C, D) Immunocytochemistry was performed against draxin (C) and L1 (D) on E17.5 wild type mouse telencephalon coronal sections. Arrowheads in (A, B) indicate the location of the LOT. Draxin protein expression was observed in the corpus callosum axons (arrow in C and D) but not in the LOT (arrowhead in C and D). S and C stand for septum and cortex, respectively, in A-D. Scale bars =500 μm for A-D.

the postnatal period up to P5 (data not shown). By performing section immunohistochemistry with anti-draxin staining, we previously showed the presence of draxin protein in the forebrain commissures at E17.5 (Islam, et al. 2009). Therefore, we wanted to examine whether draxin protein is also present in the LOT. Since L1 is abundantly expressed in LOT axons (Fouquet, et al. 2007), we performed double immunostaining with antibodies against draxin and L1. Draxin protein expression was not merged with L1 in the LOT (Fig. 7C and D), suggesting that draxin is not present in LOT axons. These results confirm *draxin* expression in the cortex and septum at different embryonic developmental stages when LOT formation occurs.

3-1-2. Draxin Inhibits Axon Outgrowth from Olfactory Bulb (OB) Explants

To examine whether draxin influences the outgrowth of OB axons, we cultured OB explants from E14.5 wild type mouse embryos in collagen gels, in the presence of either chicken draxin fused with alkaline phosphatases (draxin-AP) or control-AP conditioned medium. Robust neurite outgrowth was observed from the OB explants when cultured in control-AP conditioned medium (Fig. 8A and C), whereas neurite outgrowth was significantly diminished when the explants were incubated with draxin-AP conditioned medium (Fig. 8B and C). We also co-cultured OB explants from E14.5 wild type mouse embryos at a distance from 293 cell aggregates expressing mouse draxin in collagen gels for 40-48 hours. We used mock-transfected 293 cell aggregates as a control. We observed symmetrical neurite outgrowth from explants cultured with control 293 cell aggregates (Fig. 8D and F). In contrast, when co-cultured with cell aggregates expressing draxin, the extent of neurite outgrowth from the explant was significantly better from the distal side of the cell aggregates rather than from the proximal side (Fig. 8E and F). The

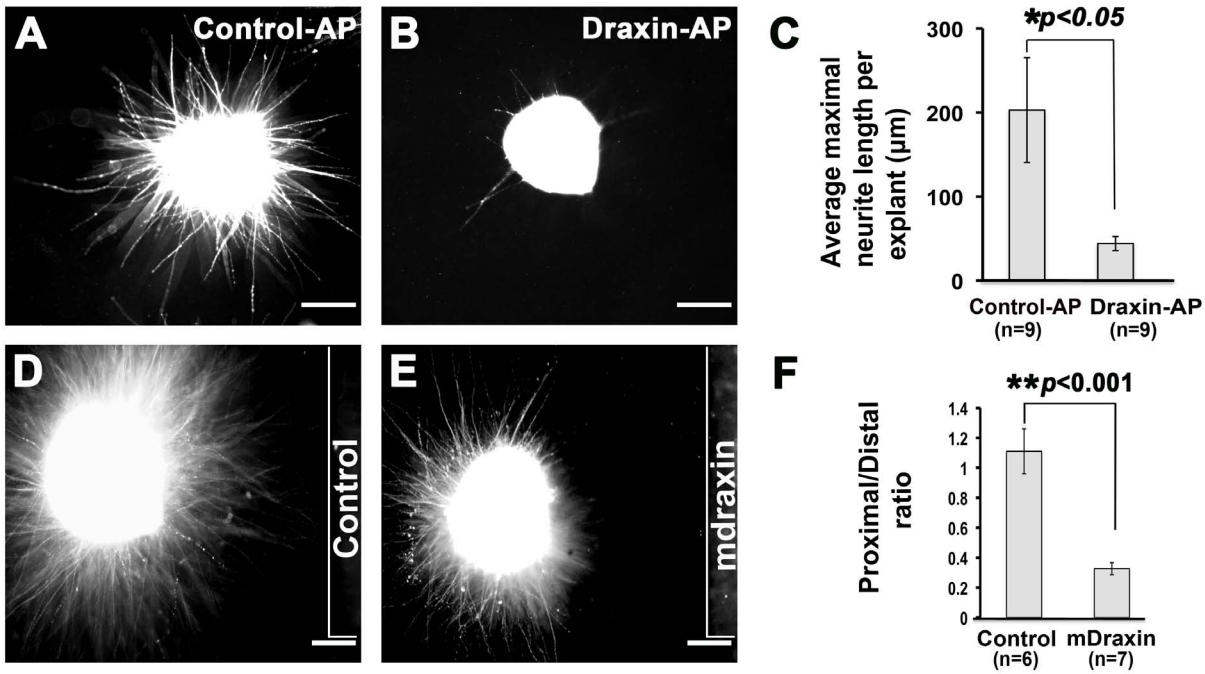

Figure 8. Draxin inhibits neurite outgrowth from olfactory bulb explants. (A, B) Olfactory bulb explants from wild type E14.5 mice were cultured in collagen gel in the presence of either control-AP (A) or draxin-AP (B) conditioned medium. (C) After 48 hours, explants were fixed and stained with Tuj-1 antibody. The length of the longest neurite was measured using ImageJ software and the average maximal length was quantified. Compared to control-AP (A, C), neurite outgrowth was significantly (*$p<0.05$, determined by Student's t-test) inhibited by draxin-AP (B, C) conditioned medium. (D, E) Explants, dissected out from E14.5 wild type mouse olfactory bulbs, were co-cultured in collagen gel with mock transfected (D) and mouse *draxin* transfected (E) 293 cell aggregates. In control cases, neurites emanating from the explants grew out radially (D), while the growth of neurites in the proximal side of draxin expressing cell aggregates was markedly inhibited. (F) After fixation, explants were stained with tuj-1 antibody; the explants' neurites lengths from the proximal and distal quadrants of the aggregates were measured and the proximal/distal ratio was quantified. The difference in the proximal/distal ratio of neurite outgrowth between control and draxin expressing cell aggregates was significant (**$p<0.001$ assessed by Student's t-test). The number of explants is indicated by n (C and F). Error bars indicate the mean ± SEM. Scale bars = 200 µm for A, B, D, E.

effect of draxin on the OB axon outgrowth demonstrated here was similar to that observed in our earlier studies with other type of axons (Islam, et al. 2009; Naser, et al. 2009).

3-1-3. Draxin Binds to the Lateral Olfactory Tract (LOT) in vivo.

Diffusible axon guidance molecules function through their receptors (Tessier-Lavigne and Goodman, 1996; Dickson, 2002). Since draxin is a secreted repulsive axon guidance protein, its function should be mediated through some receptor or receptor complex. To investigate the draxin receptor distribution pattern in LOT axons, unfixed horizontal brain sections from E17.5 wild type mouse embryos were incubated either with draxin-AP or with control-AP conditioned medium and a binding assay was performed (Fig. 9A and B). We observed that draxin-AP bound evenly in the entire tract of the LOT, which in turn implies the presence of a draxin receptor in the LOT *in vivo*.

3-1-4. The LOT is Apparently Normal in draxin-/- mice

To examine whether draxin function observed *in vitro* corroborates with the *in vivo* phenotype, we analyzed the development of the LOT in *draxin* deficient mice. Whole brains from E17.5 wild type (wt) and *draxin* knockout littermate mice were dissected and whole-mount immunostaining with anti-neuropilin 1 antibody was performed to visualize the OB axonal trajectory. Since neuropilin1 and 2 are extensively expressed in the entire tract of the LOT [6], whole-mount staining would determine whether the integrity of the LOT in *draxin* knockout mice is maintained. Although the LOT in *draxin* mutants formed and reached the telencephalon normally, axons were slightly defasciculated in the rostral part of the tract (Fig. 10A and arrow in B). Brain sections from P0 wt and *draxin* deficient mice were stained with anti-neurofilament antibody to check whether OB axons in draxin knockout mice precisely reached the telencephalon. The LOT in both wt and

mutant mice was normal (Fig. 10C and D). These data suggest that although draxin inhibited axonal outgrowth *in vitro*, other molecules with similar functions might compensate for its function *in vivo*.

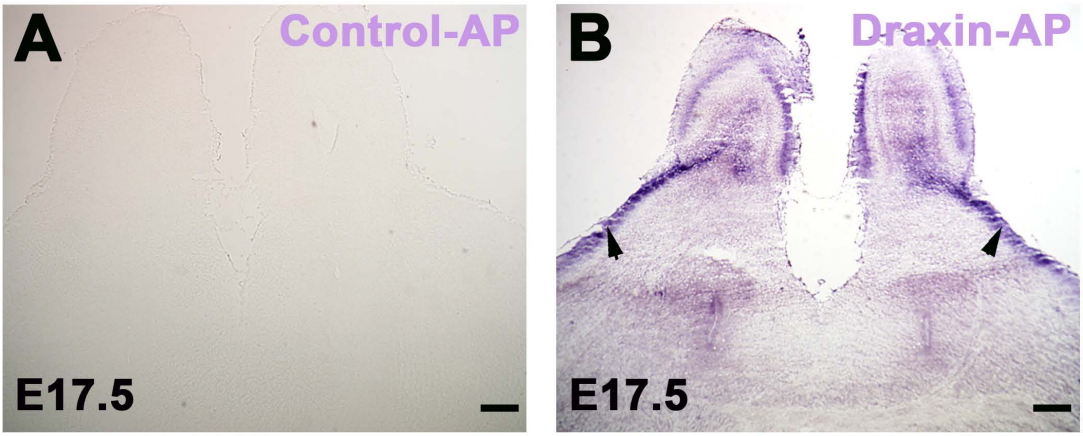

Figure 9. Draxin binds to the LOT. (A, B) A binding assay was performed on horizontal sections of E17.5 wild type mouse embryos brains. Draxin-AP bound to the LOT axons (arrowheads in B). Control-AP proteins did not bind to the sections (A). Scale bars = 200 μm for A, B.

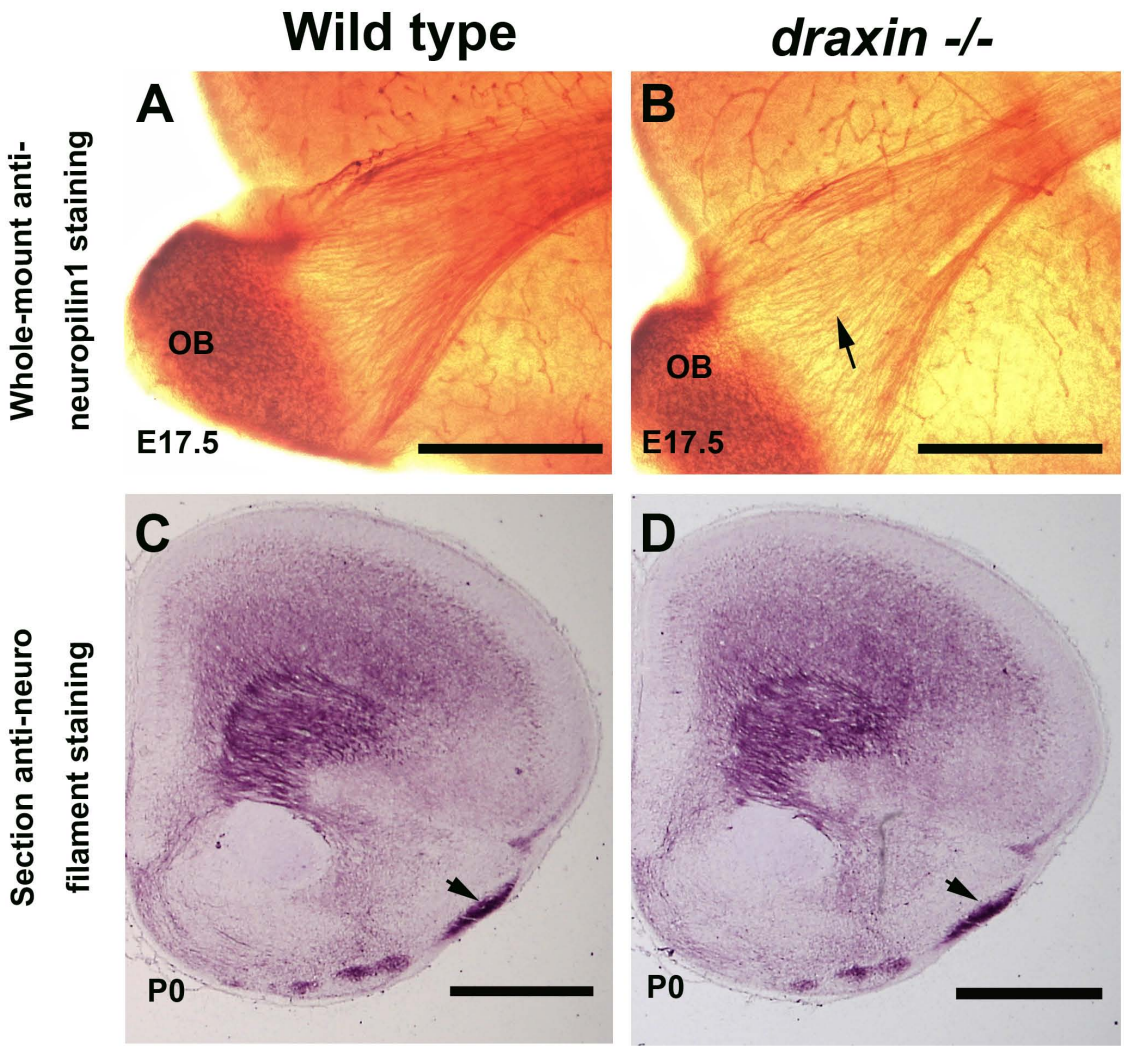

Figure 10. The LOT in *draxin* knockout mice. (A, B) The olfactory bulb axonal trajectory in E17.5 wild type (A) and *draxin* deficient (B) littermate mice was visualized by anti-neuropilin-1 antibody staining in whole mount. Although the tract in both wild type (4 of 4) and mutant (5 of 5) mice was apparently normal, mild defasciculation within the LOT was observed in all analyzed mutant mice (arrow in B) compared to wild type (A). OB in A and B stands for olfactory bulb. (C, D) Coronal brain sections from P0 wild type (10 of 10) and *draxin* knockout (10 of 10) littermate mice were stained with anti-neurofilament antibody. The appearance of the LOT (arrowhead in C and D) in wild type and draxin-deficient embryos was normal. Scale bars = 500 μm for A-D.

3-2. Part B.

3-2-1. Draxin Binding Merges with DCC Expression

We screened several candidate transmembrane proteins by transient expression in 293 cells and measured the binding of draxin-AP (alkaline phosphatase). Surprisingly, the netrin receptor DCC was found to be a promising candidate receptor, because draxin-AP bound to cells expressing DCC (Fig. 11A-C) but not to any of the other candidates tested. To examine whether DCC and bound draxin-AP co-localize, we assessed the draxin-AP binding and DCC expression of *DCC* transfected 293 cells by immunocytochemistry (Fig. 11D). We observed that draxin-AP binding completely overlapped with DCC expression in a manner similar to netrin-1 binding, but not with expression of NCAM, a member of the Ig superfamily. Thus, draxin binding to DCC-expressing cells is specific.

3-2-2. Draxin Specifically Interacts with DCC

To test whether draxin interacts directly with DCC, we performed immunoprecipitation assays. Full length *DCC* or empty vector transfected 293 cells were incubated with draxin-AP conditioned medium and cell lysate was subjected to immunoprecipitaion (IP) assay with anti-draxin anitibody. Cell lysate from *DCC* transfected cells was used as a negative control in IP assay. DCC was retained in the complex precipitated by draxin-AP, as detected by immunoblot using an anti-DCC antibody (Fig. 12A). To determine whether the interaction between DCC and draxin was direct, we performed IP assays using draxin-AP and the soluble DCC-ectodomain. DCC-ectodomain was specifically immuno-precipitated by draxin-AP (Fig. 12B). In addition, draxin-AP was pulled down by the DCC-ectodomain (Fig. 12C). The interaction between draxin and DCC appeared specific, because a soluble version of the Robo1 ectodomain (Robo1-ecto-Fc) did not precipitate.

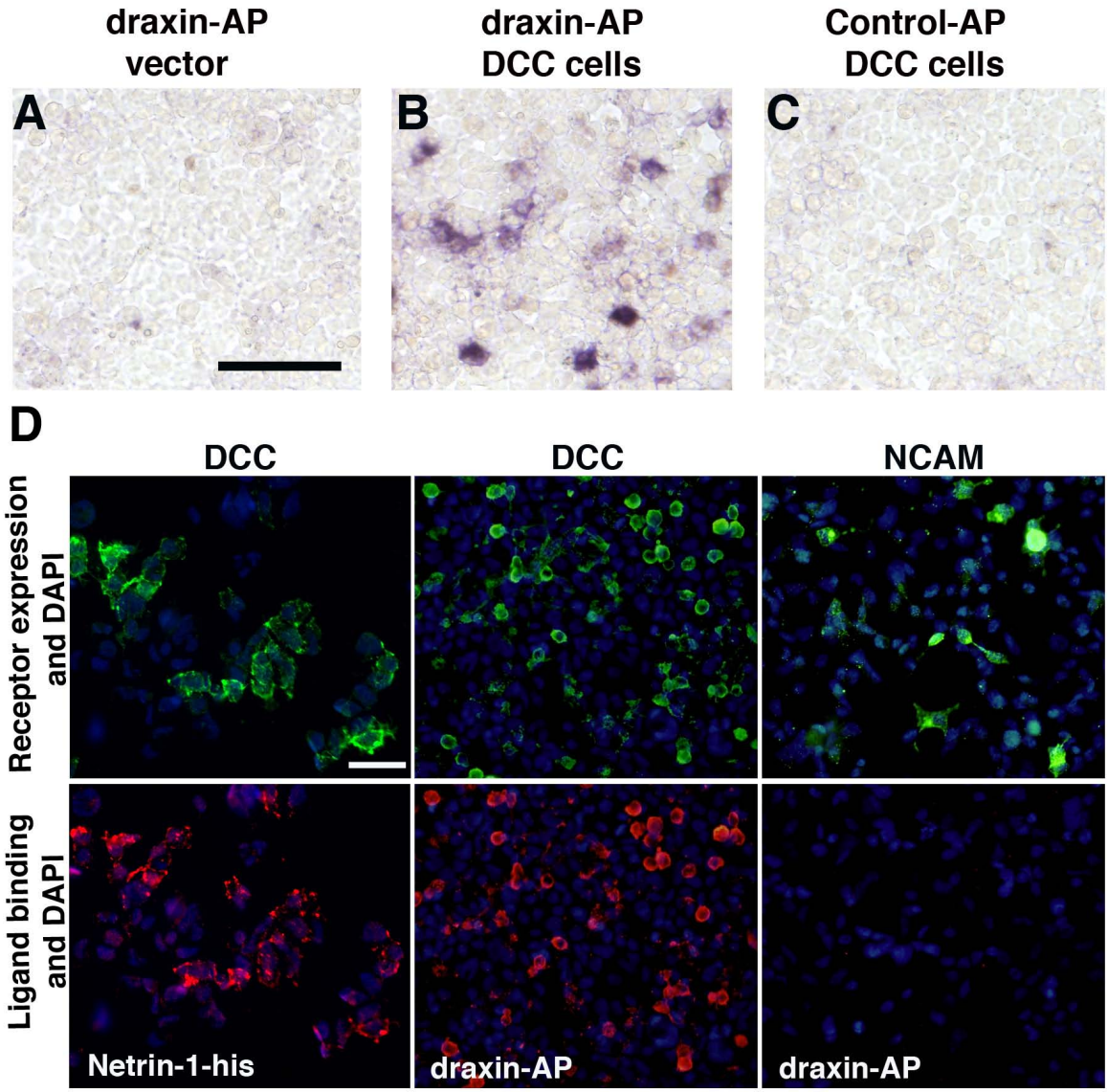

Figure 11. Draxin binds specifically to DCC expressing cells. 293 cells were transfected either with cDNAs encoding rat *DCC* (B, C) or with the empty vector (A) and 40 hours later cells were either incubated with draxin-AP (A, B) or control-AP (C) conditioned medium and AP staining was performed, Strong draxin-AP binding signal above background was observed only in *DCC* transfected cells. (D) (Bottom) 293 cells expressing DCC bound to both netrin-1 and draxin but draxin binding was not observed in NCAM expressing cells. (Top) Receptor expression was confirmed by immunocytochemistry. Only in case of NCAM-draxin cell overlay assay, staining of NCAM protein and bound draxin for the NCAM transfected cells were done separately

since both anti-NCAM and anti-draxin antibodies were rabbit polyclonal antibodies. Scale bar represents A-C, 100 μm and D, 50 μm.

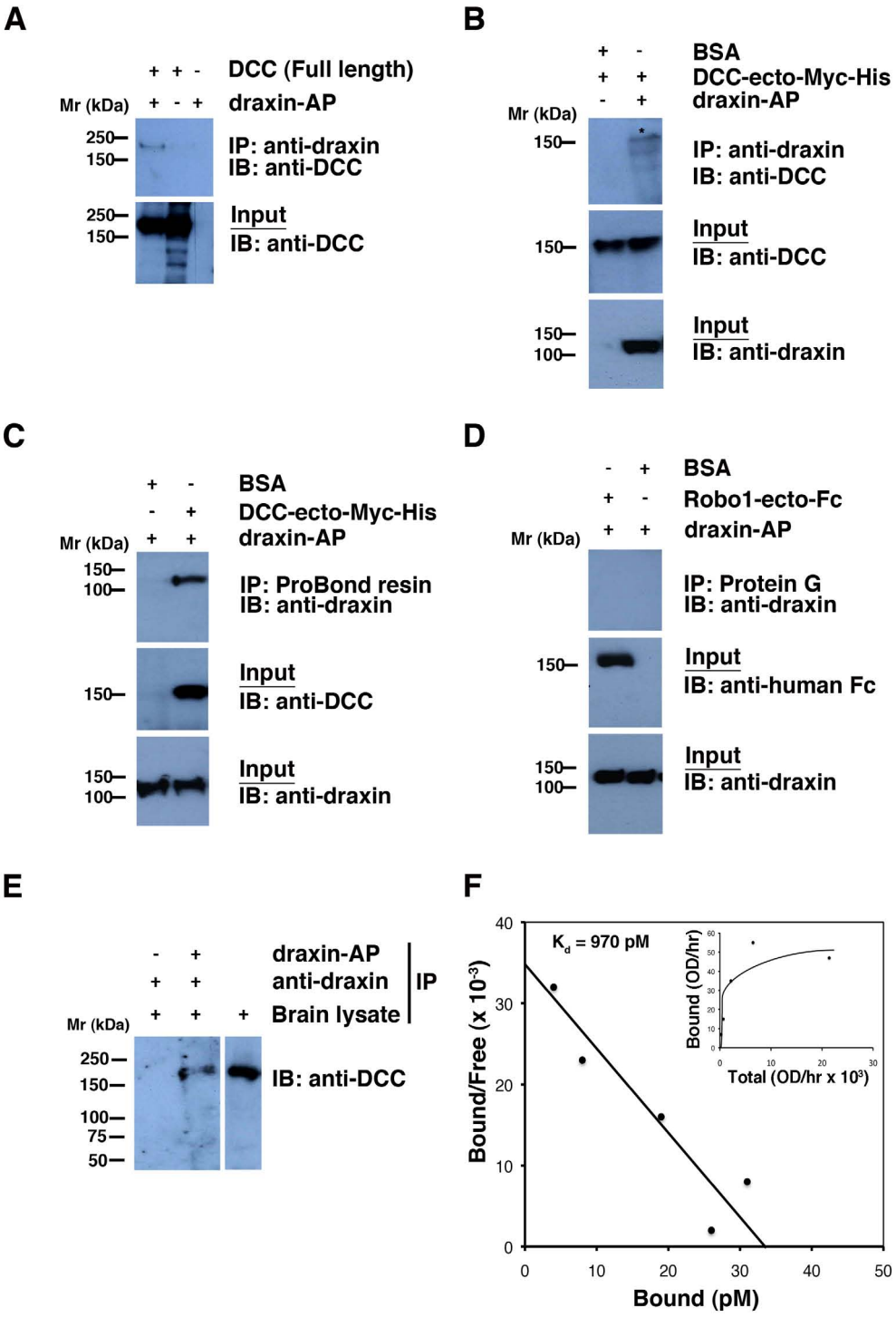

Figure 12. Draxin interacts with DCC. (A) DCC protein in cell lysates and

immunoprecipitates was detected by anti-DCC antibody. (B) Asterisk indicates the band of DCC-ecto-Myc-His, which was precipitated by draxin-AP. (C) DCC-ecto-Myc-His specifically pulled down draxin-AP. (D) Draxin-AP was not immunoprecipitated by Robo 1-ecto-Fc. (E) Endogenous DCC protein from E17 mouse brain lysates was immunoprecipitated by exogenous draxin-AP. The right lane is the brain lysate input. (F) DCC expressing or vector transfected 293 cells were incubated with different concentrations of draxin-AP containing medium and free and bound AP activities were measured as described under Materials and Methods. The apparent K_d was estimated as 970 pM

draxin-AP (Fig. 12D). Moreover, endogenous DCC from brain lysates was precipitated by draxin-AP (Fig. 12E). The binding affinity of draxin for DCC in transfected cells was determined from the binding curve, and the dissociation constant (K_d) was 970 pM (Fig. 12F), higher than that of the DCC-netrin-1 interaction in a similar assay (Keino-Masu et al., 1996). These biochemical data confirm specificity of the interaction between draxin and DCC.

3-2-3. Draxin Binds to the IgG Domain of DCC

We next sought to identify the draxin-binding domain of DCC by evaluating the binding of draxin to the soluble form of fragmented DCC proteins by pull down assay. Whereas netrin-1 binds the fibronectin-type-III-like (FnIII) region of DCC (Geisbrecht, et al. 2003; Kruger, et al. 2004), draxin preferentially bound the IgG (1-4) domain of DCC, rather than the Fn (1-6) domain (Fig. 13A and B).

3-2-4. Reduced Draxin-AP Binding was Observed on DCC-Lacking Neurites

DCC was reported to be expressed on both cortical and olfactory bulb axons (Gad, et al. 1997; Shu, et al. 2000) and both *DCC* and *draxin* KO mice show severe malformation of

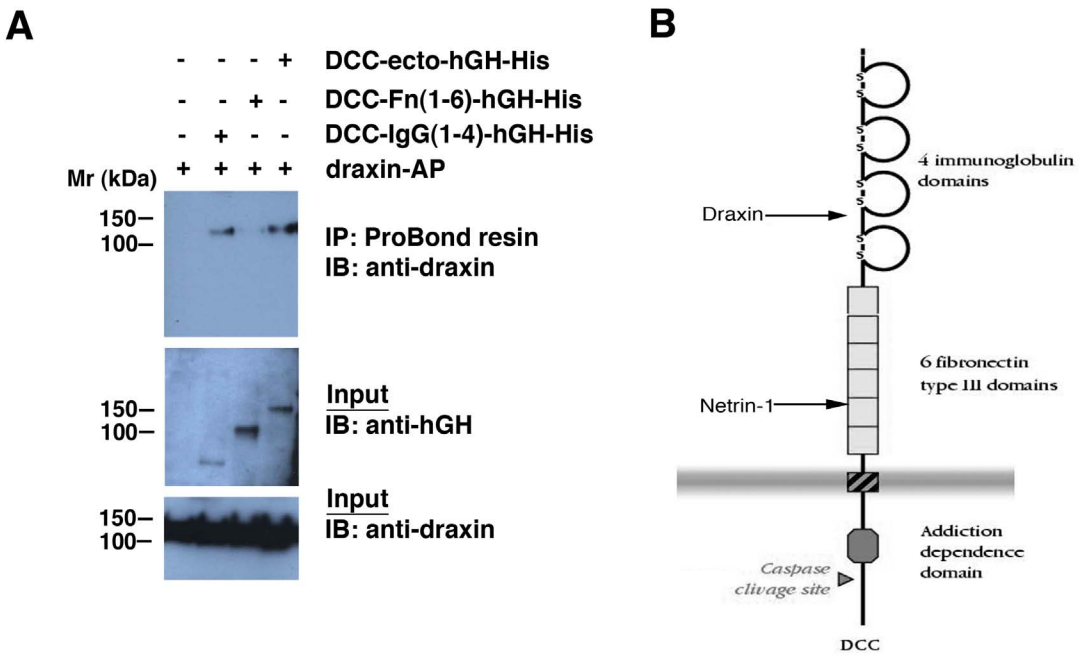

Figure 13. Binding domain of draxin in DCC. **(**A) Different types of fragmented DCC proteins conjugated with hGH and His were mixed with draxin-AP and pull down assay was performed. Draxin-AP only bound to the ectodomain and IgG domain of DCC. (B) Schematic diagram showing the different domains in DCC. Netrin is reported to bind the fibronectin domain while draxin preferably binds to the IgG domain.

forebrain commissures (Islam, et al. 2009; Fazeli, et al. 1997). To determine whether the interaction of draxin with DCC is functionally important, we first examined the binding of draxin-AP to the neurites of E17.5 cortical and E14.5 olfactory bulb (OB) explants from wild type (WT) and *DCC* knockout (KO) littermate mice. Cortical and olfactory bulb explants were cultured for 48 hours and being observed for good neurite outgrowth. These cultured explants were then incubated either draxin-AP or control-AP conditioned medium. After being extensively washed and fixed AP staining was performed according to the standard protocol (Flanagan and Cheng, 2000). We found that draxin-AP (Fig. 14C and 14G), but not control-AP (Fig. 14A and 14E), avidly bound to the neurites of cortical and OB explants from WT mice. In *DCC* KO explants, however, draxin-AP binding to neurites was markedly reduced (Fig. 14D and 14H) and no binding was observed with control-AP (Fig. 14B and 14F). Thus, DCC is required for draxin binding to the majority of neurites emerging from cortical and OB explants.

3-2-5. DCC Is Required for Neurite Outgrowth Inhibition by Draxin

We next tested the ability of *DCC* deficient cortical and OB axons to respond to draxin *in vitro*. Explants from E17.5 cortex and OB of *DCC* KO and WT littermate mice were cultured in the presence of either draxin-AP or control-AP conditioned medium. Robust neurite outgrowth from both cortical and OB explants of WT mice was observed in control-AP conditioned medium (Fig. 15A and 15E), and a similar degree of outgrowth was observed from explants of *DCC* knock-out mice in the presence of control-AP (Fig. 15B and 15F). Draxin-AP significantly inhibited neurite outgrowth from cortical and OB explants of WT mice to 38% and 16%, respectively, of control levels (Fig. 15C, 15G, 15I, and 15J), whereas neurite growth in *DCC* deficient cortical and OB explants was inhibited to 64% and 59% of control levels, respectively (Fig. 15D, 15H, 15I, and 15J).

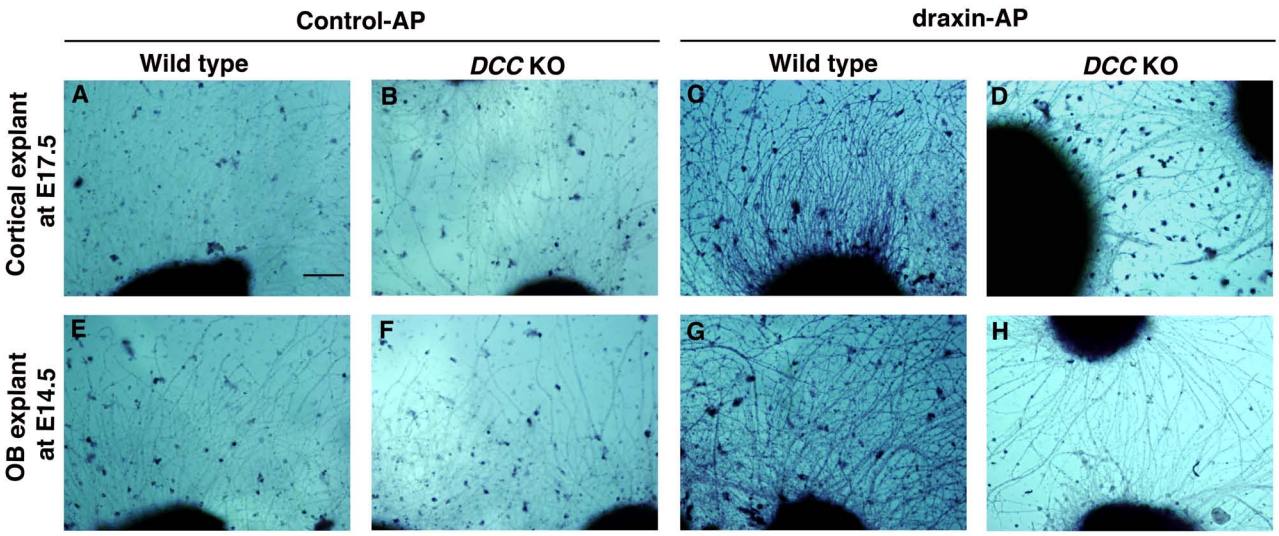

Figure 14. Draxin binding was reduced in *DCC* lacking neurites. E17.5 cortical (A-D) and E14.5 olfactory bulb (OB) explants (E-H) from wild type (A, C, E, G) and *DCC* KO (B, D, F, H) littermate mice were cultured on poly L Lysine/ laminin coated dish. 48 hours later neurites from explants were stained either with control-AP (A, B, E, F) or with draxin-AP (C, D, G, H) conditioned medium. Considerably less draxin-AP binding was observed in neurites of cortical (D) and OB (H) explants from *DCC* KO mice. Scale bar, 100 μm.

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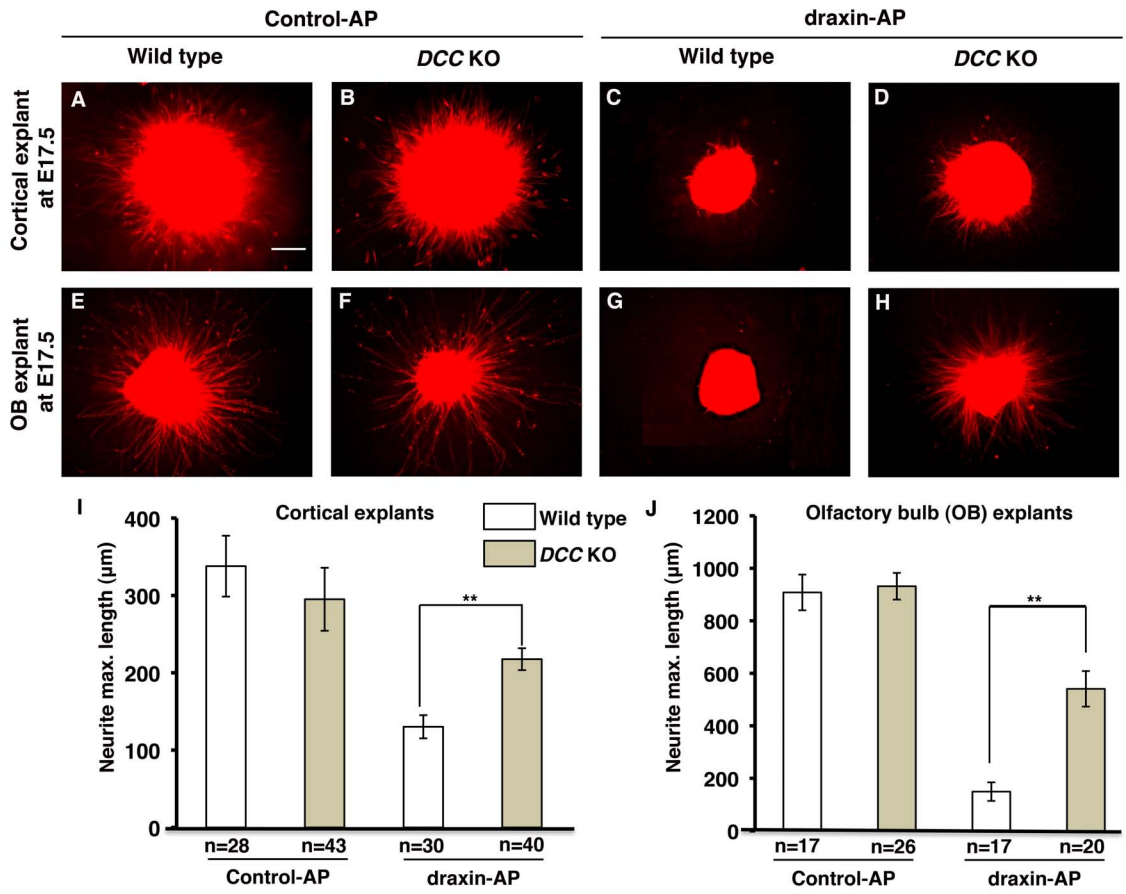

Figure 15. DCC Deficient Neurite Outgrowth is Significantly Less Affected by Draxin. Cortical (A-D) and OB (E-H) explants from E17.5 wild type (A, C, E, G) and *DCC* KO (B, D, F, H) mice were cultured in collagen gel in the presence of either control-AP (A, B, E, F) or draxin-AP (C, D, G, H) conditioned medium for 48 hours. Neurites were labeled using an antibody to class III β-tubulin and results were quantified (I, J) by measuring the maximum neurite length per explant from cortex (A-D) and OB (E-H). The total number of explants, *n* in (I, J), was derived from two independent experiments and average maximum neurite length was determined. Similar results were observed in other two independent experiments. Neurites of cortical (I) and OB (J) explants from *DCC* KO mice were significantly (**$p<0.0001$; *p* values were calculated by Student's *t* test) less inhibited by draxin-AP compared with that from wild type mice. Scale bars represent 200 μm. Error bar indicates mean ± SE.

These findings demonstrate that DCC is substantially required for draxin to inhibit neurite outgrowth

3-2-6. DCC Is Required for the Growth Cone Collapse Induced by draxin

Draxin not only inhibits neurite outgrowth but also induces growth cone collapse when added acutely and uniformly to growth cones in culture (Islam, et al. 2009). We therefore investigated whether DCC is required for this acute response. For this assay, we used E16.5 mouse cortical neurons, which express DCC and were inhibited by draxin (Fig. 16A and 16B). We found that growth cones in this culture system had a high baseline level of collapse (~35%), which was further increased (to ~85%) when treated with draxin-AP (Fig. 16C and 16D). This increase was markedly reduced (to ~ 50%) in cortical neurons derived from *DCC* KO mice (Fig. 16D).

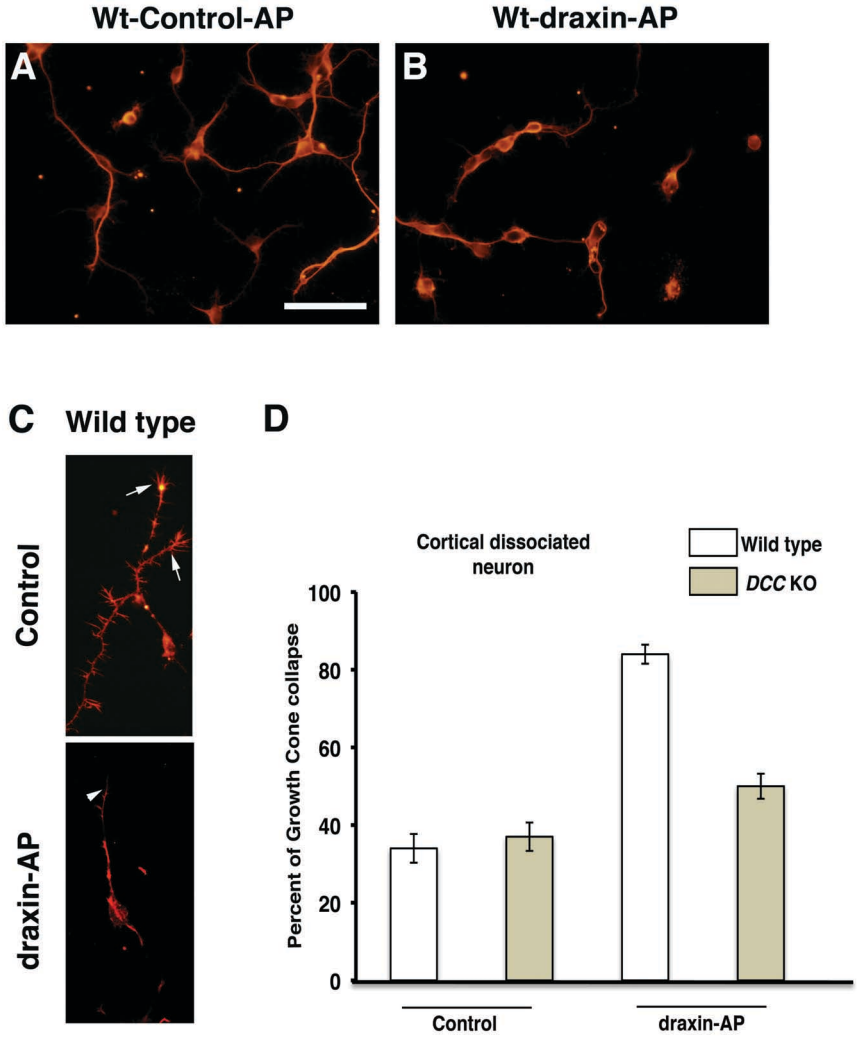

Figure 16. Draxin Induces Growth Cone Collapse Through DCC. Cortical neurons from E16.5 wild type mouse embryo were cultured on poly-L-Lysine/laminin coated dish either in presence of control medium (A) or draxin-AP (B) conditioned medium for 40 hours. Neurites were stained with anti-DCC antibody and inhibited markedly by draxin-AP. Growth cones of E16.5 cortical neurons from both wild type and *DCC* KO littermate mice were either treated with medium alone or draxin-AP 100 nM for 1 hour at 37°C to induce the collapse and were stained with phalloidin-Al 568 to visualize the growth cones. Representative pictures were shown in (C) and quantified results were shown in (D). Data were obtained from two independent experiments and growth cones of 40 neurons were counted in each group on average per experiment. Arrow and arrowhead in (C) indicates the intact growth cone and collapsed one respectively. Scale bar, 50μm.

4. Discussion

4-1. Part A.

It was earlier reported (Schwob and Price, 1984) that LOT axons avoid entering into the embryonic neocortex, and this led to speculation that the neocortex might have some repulsive factors that inhibit LOT axons from invading. Later, in another study, this hypothesis was confirmed by an *in vitro* experiment (Pini, 1993). The latter study also documented the presence of another repulsive factor in the septum that restricts the development of the LOT along the lateral side of the rostrocaudal axis of the brain.

Later studies (Nguyen-Ba-Charvet, et al. 1999; Li, et al. 1999) identified Slits as septum and cortex derived chemorepulsive molecules that play an important role in the development of the LOT. Though both Slit1 and Slit2 repel olfactory bulb (OB) axons *in vitro,* the LOT was formed normally in both of these *Slit* knockout mice. However, double *Slit1/2*-deficient mice showed disorganized LOTs, which suggested that the combinatorial effect of Slits control the development of the LOT (Nguyen-Ba-Charvet, et al. 2002). Slits repel OB axons through their receptors Robo1 and Robo2. However, LOT formation was profoundly disorganized either in single *Robo2-/-* or in *Robo1-/-*; *Robo2-/-* double mutant mice (Fouquet, et al. 2007). These data indicate that Slits, through their Robo receptors, regulate the precise formation of the LOT *in vivo* (Fouquet, et al. 2007). Although in Slit1; Slit2 or Robo1; Robo2 double deficient mice many axons from the medial OB defasciculated and seemed to cross the midline, there was still an axon bundle along the normal LOT trajectory (Nguyen-Ba-Charvet, et al. 2002; Fouquet, et al. 2007). This suggests that other unknown molecules present in the neocortex or septum of these mutants control the guidance of a subpopulation of OB axons.

In this study, we observed that *draxin* expression remained stronger in the mouse cortex from embryonic day E13 to E17.5. Like other diffusible axon guidance molecules that control the development of the LOT, draxin is also expressed strongly in the septum at E13 when the majority of the OB axons are developing to form the LOT. However, the level of expression gradually decreases at later developmental stages. *In vitro*, draxin inhibited OB neurite outgrowth. The presence of the draxin receptor throughout LOT axons *in vivo* suggests that draxin may regulate the development of these axons *in vivo* through its receptor. Although we could observe slight axonal defasciculation in the rostral part of the LOT in *draxin*-/- mice, the whole trajectory developed and normally reached the telencephalon. Similarly, this tract was also normal in *Slit1*, *Slit2*, *Robo1*, *neuropilin1*, and *neuropilin2* single knockout mice (Nguyen-Ba-Charvet, et al. 2002; Fouquet, et al. 2007; Chen, et al. 2000; Kitsukawa, et al. 1997).

One possible explanation for observing normal LOT development in *draxin* mutant mice, along with other single mutant mice, is that the guidance activity of missing proteins may be counterbalanced by the redundant functions of other proteins. Along the pathway of the OB axons, several studies (Sugisaki, et al. 1996; Hirata, et al. 1997; Sato, et al. 1998; Tomioka, et al. 2000) revealed the existence of a subset of neurons, called lot cells. These short-range cues also guide the OB axons to form their precise trajectory. There is a possibility that the functions of these short-range cues were not perturbed in these mutants, and hence the tract was found to be normal. Indeed, it was observed that the position of lot cells was not significantly changed in *Robo1/2* double knockout mice (Fouquet, et al. 2007). Thus, multiple diffusible factors, together with a variety of short-range cues, work in concert to regulate the LOT development *in vivo*.

4-2. Part B.

We have shown that DCC is a binding partner for draxin in both cell overlay and biochemical assays. Draxin binding was reduced in *DCC*-lacking neurites, and these neurites were substantially less sensitive to draxin *in vitro*. Furthermore, the degree of growth cones collapse of *DCC* KO cortical neurites in response to draxin was significantly lower than that of wild type neurons. Taken together, these data indicate that DCC is a functionally important receptor for draxin-mediated inhibition of cortical and olfactory bulb neurite outgrowth.

Because DCC is a receptor for netrin-1, it will be of interest to determine how DCC can mediate signaling cascades for two such unrelated ligands, especially since we previously showed that spinal commissural axons can simultaneously respond to netrin-1 with outgrowth and to draxin with repulsion. The answer may lie partly in the structure of DCC, which in principle could bind different ligands through different binding domains. In fact, our data show that the draxin-binding site on DCC is different from that of netrin-1. A growing body of literature indicates that the interaction of DCC with netrin-1 can lead to switching on or off of diverse intracellular processes (Ming, et.al. 1997; Li, et al. 2004; Moore, et al., 2008). There is a possibility that the downstream events that result from the DCC-draxin interaction are complementary to those triggered by DCC-netrin-1 binding. In addition, the DCC-draxin interaction might recruit other molecules as co-receptors; for example, DCC-netrin-1 can recruit UNC5 family members to mediate a repulsive action of netrin-1 (Hong, et al. 1999). The possibility of a co-receptor that can also bind draxin is suggested by the finding that the inhibitory effect of draxin was not completely abolished by removal of DCC; this would parallel the situation with netrin, since UNC5 family members can also bind netrin and by

themselves mediate repulsion, though a DCC-UNC5 co-receptor is a more potent mediator of repulsion (Keleman and Dickson, 2001).

While a conserved role for DCC in mediating netrin responses, both attractive and repulsive, has been well-documented in multiple species, in *C. elegans* the DCC homolog UNC-40 was also shown to collaborate with the Slit receptor Sax-3/Robo in mediating repulsion in response to SLT-1/Slit (Yu, et al. 2002); however, DCC family members do not appear to bind Slit proteins, and this effect appears instead to reflect a direct interaction of the receptors UNC-40/DCC with Sax-3/ (Yu, et al. 2002). Our results extend the principle of DCC involvement in mediating effects of distinct ligands, but indicate that it can also involve direct binding of the ligand, as we show here for draxin. Since commissural axons can be simultaneously stimulated to extend by netrin-1 and repelled by draxin, it will be of particular interest to determine how DCC can be involved at the same time in mediating these apparently opposite functions. The identification of DCC as a receptor for draxin provides an essential starting point to further elucidation of downstream components of DCC-draxin signaling.

6. Conclusion

6-1. Part A.

Several diffusible axon guidance proteins that function either as an attractant or a repellent for the olfactory bulb axons *in vitro* are expressed in orchestrated manner in the septum and neocortex of brain so that single genetic deletion either of them will not produce any significant phenotype. Thus, we propose a scheme, modified from De Castro et al. (1999), to show the expression pattern of diffusible cues that may act together to regulate the development of the LOT (Fig. 17)

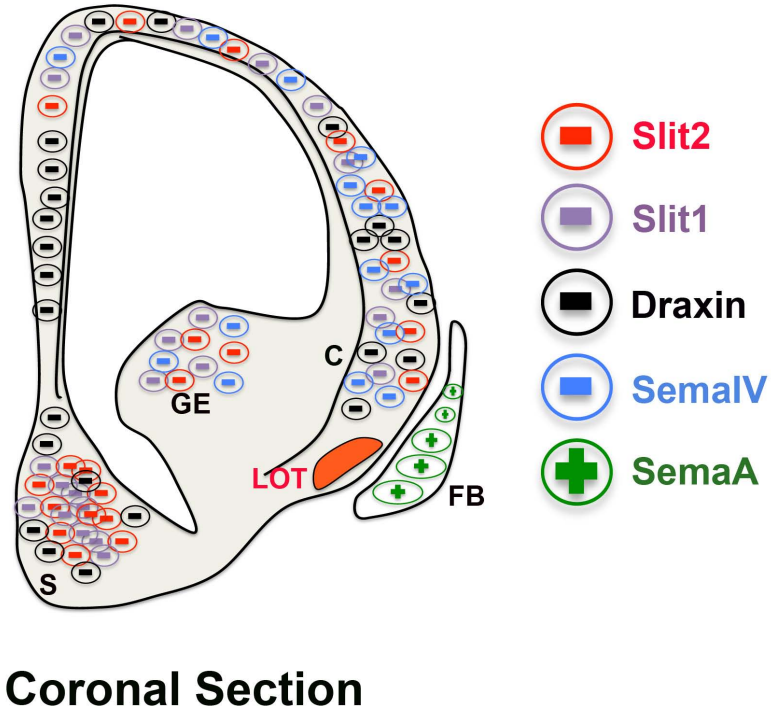

Figure 17. A schematic diagram, adopted and modified from de Castro et al. [7], representing a coronal brain section, shows the expression pattern of chemorepulsive and chemoattractive molecules that regulate the development of the LOT. Septum (S), cortex (C), and ganglionic eminence (GE) derived repulsive molecules and attractive molecules produced by mesenchymal precursors of the frontal bone (FB) restrict the LOT axons to grow ventrolaterally underneath the pial surface of the telencephalon.

6-2. Part B.

Our data here demonstrate that draxin binds to DCC and DCC is required receptor for draxin mediated neurite outgrowth inhibition. Since DCC deficient neurites growth was not rescued fully in presence of draxin, it is obvious that other receptor alone or in association with DCC may contribute draxin-induced inhibition (Fig. 18).

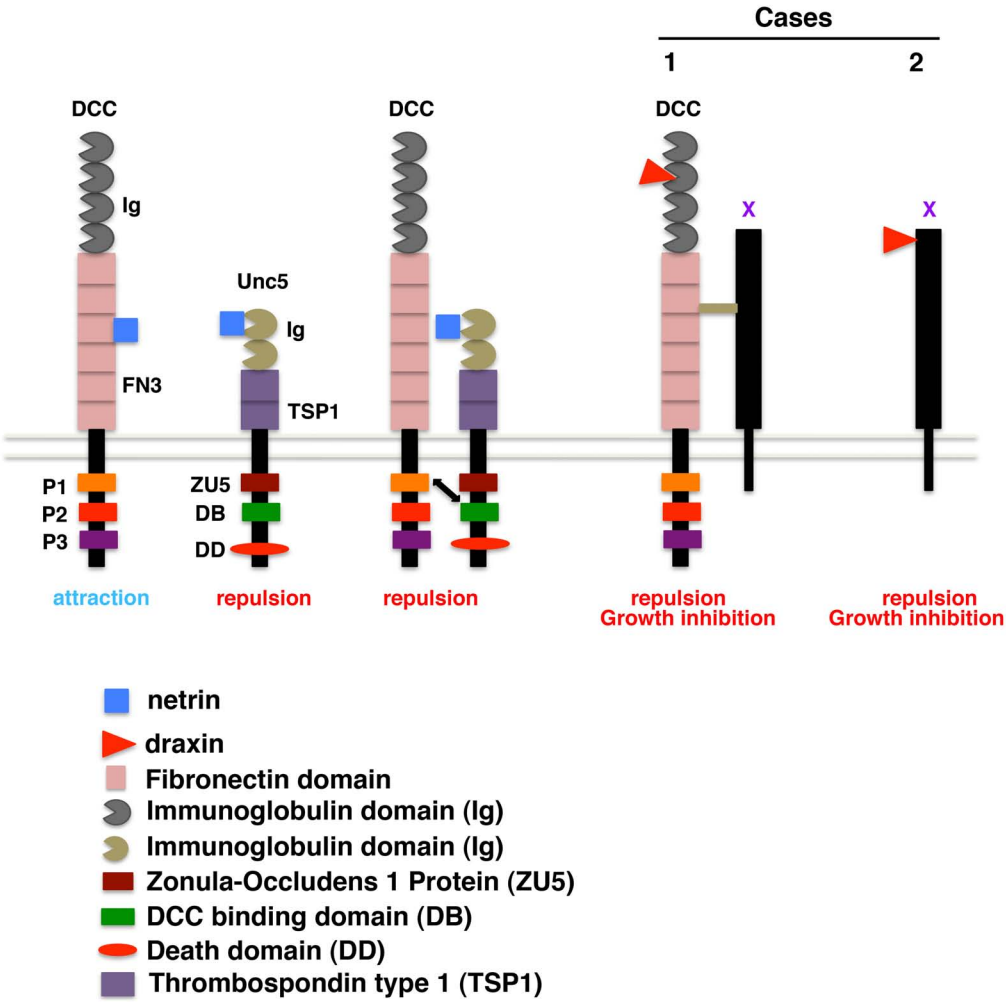

Figure 18. Schematic diagram showing the outcome of netrin and draxin mediated responses through their receptors. Netrin attractant activity is mediated by DCC alone whereas netrin mediated repulsion requires either Unc5 alone or Unc5 and DCC receptor complex. In cases of draxin-mediated repulsion, two possible mechanisms may separately or mutually be involved. Draxin either requires receptor complex between DCC and unknown receptor X or unknown receptor X alone for its repellent activity

7. Future direction

7-1. Part A.

It would be interesting to generate *draxin* knockout mice in either the *Slit* or the semaphorin null backgrounds to clarify the contribution of draxin in the formation of the OB axonal trajectory *in vivo*.

7-2. Part B.

Future research can be carried out in two different directions. First is to identify the involvement of other receptors in draxin function. Second is to clarify the downstream signaling cascades that draxin initiates through their receptors.

I. Identification of other receptors

Other possible receptors involved in draxin induced neurite outgrowth activity can be identified by two different approaches. They are candidate approach and proteomics approach.

Candidate approach

The most promising other candidate through which draxin may exert its function is Unc5 family members. After checking the binding of draxin with Unc5 receptors, it is important to check further whether, draxin induces receptor complex between DCC and Unc5. Positive data from these experiments lead to analyze the functional importance of the above binding. For this, it would be interesting to see whether knocking down the expression of UNC5B in wild type or *DCC* lacking cortical neurons by siRNA completely abolish the draxin effect. The data from this experiment would suggest two possibilities: first whether UNC5 is involved at all in draxin functioning, second either UNC5 alone or in concert with DCC mediates draxin's repulsive function.

Proteomics approach

Good antibodies against mouse draxin capable of detecting and pulling down the endogenous draxin from the mouse tissue is essential for this approach. Embryonic brain lysates can be first stimulated with or without draxin or netrin-1 protein. Later, using anti-draxin and anti-netrin-1 antibodies, pull-down experiments can be performed. Later, the recovered immunocomplexes can be analyzed by mass spectrometer. It is possible to identify a unique profile of interacting proteins, involve in draxin signaling, from the comparison between draxin and netrin-1interacting proteins profile. Finally these proteins will be analyzed functionally to evaluate their role in draxin-mediated inhibition.

II. Downstream signaling cascades

Involvement of Rho kinase

There is a possibility that draxin-DCC initiates signaling event opposite to the netrin-DCC signaling. Hence, in the case of draxin-DCC interaction it would be interesting to investigate the status of two different intracellular signaling cascades simultaneously that normally switch on through DCC-netrin or DCC-netrin-UNC5 interactions. Several reports indicate that axonal outgrowth stimulating and turning activity of netrin-1 through its receptor DCC leads to inhibit RhoA, member of Rho GTPases family of intracellular proteins. Therefore, the first possibility is to check whether draxin inhibits axonal outgrowth by increasing the Rho kinase activity. Dissociated cortical neurons can be cultured in the presence or absence of specific inhibitor of Rho kinase Y27632 in control or draxin conditioned medium. Positive data from this experiment can be confirmed further by doing other experiments like IP assay to check the level of active Rho from draxin treated neurons, growth cone collapse assay to see whether Rho kinase inhibitor would rescue draxin activity.

Involvement of Ca^{+2} mediated cAMP level

The conclusion of some research findings (Hong, et al. 2000; Nishiyama, et al. 2003) suggest that at a high ratio of cAMP to cGMP, netrin-1 works as an attractant through its receptor DCC by enhancing the Ca^{+2} influx on the other hand, lower ratio of cAMP to cGMP reduces Ca^{+2} influx and thereby induces repulsion. So, draxin`s neurite outgrowth inhibitory and growth collapse response through DCC might lead lowering influx of $Ca^{+2.}$ intracellularly. For this experiment, dissociated cortical neurons from wild type and *DCC* KO mice can be bath applied either with draxin-AP or control-AP conditioned medium and later calcium imaging can be performed using calcium indicator Fluo-4 (Invitrogen). Results from this experiment would clarify whether the above signaling event associated with the level of Ca^{+2} influx occurs when the neurons are treated with draxin.

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