学位論文
Doctoral　Thesis

Analysis of draxin function in tectum and retinocollicular axon projection

（視蓋と網膜上丘軸索投射におけるドラキシンの機能解析）

ナセール エフテェカール ビン
Naser Iftekhar Bin

熊本大学大学院医学教育部博士課程生体医科学専攻神経分化学

指導教員

田中　英明　　教授
熊本大学大学院医学教育部博士課程生体医科学専攻神経分化学

２００９年３月

学　位　論　文

Doctoral　Thesis

Analysis of draxin function in tectum and retinocollicular axon projection

（視蓋と網膜上丘軸索投射におけるドラキシンの機能解析）

著 者 名　：　ナセール エフテェカール ビン
（単名）　Naser Iftekhar Bin

指導教員名　：　熊本大学大学院医学教育部博士課程生体医科学専攻神経分化学　田中英明　教授

審査委員名　：

脳発生学担当教授	嶋村　健児
細胞情報薬理学担当教授	中西　宏之
脳回路構造学担当教授	玉巻　伸章
知覚生理学担当教授	宋　　文杰

２００９年３月

Contents

Summary

Nervous system development depends on correct wiring of axons and dendrites. Axons travel long distances along specific pathways to reach their targets. Axon guidance proteins play the major role for the precise projection of axons to form the functional neural circuit. Several axon guidance molecules and their family members have been well characterized. However, the existence of unidentified guidance cues is likely due to the immense complexity of the brain. Draxin is a previously unknown axon guidance molecule that plays very important role in the formation of spinal cord and all three major commissures of the forebrain (Islam *et. al.* 2009). Draxin is expressed in dorsal high to ventral low gradient chick optic tectum and mouse superior colliculas. *In vitro* experiments show that draxin repels neurite outgrowth from chick dorsal tectum explants. *In vivo* overexpression resulted in inhibition or misrouting of axon growth in the chick tectum. Draxin also inhibit axon outgrowth from mouse retinal explants. *draxin* knock out mice shows mapping defect along the medio-lateral and anterior-posterior axis. Thus, draxin is a chemorepulsive axon guidance molecule that plays important role in guiding ventrally directed axon projection in chick early embryonic midbrain and required for the correct formation of the mouse retinocollicular projection.

List of Abbreviations

μg:	Microgram
μm:	Micrometer
mm:	Milimeter
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
IRES:	Intracellular ribosome entry sequence
EGFP:	Enhanced green fluorescent protein
Tm:	Transmambrane
SHH	Sonic Hedgehog

DIG: Digoxigenin

List of Publications

1. **Naser IB**, Su Y, Islam SM, Shinmyo Y, Zhang S, Ahmed G, Chen S, Tanaka H. 2009. Analysis of a repulsive axon guidance molecule, draxin, on ventrally directed axon projection in chick early embryonic midbrain. Dev Biol. 332(2), 351-359.

2. Su Y, **Naser IB**, Islam SM, Zhang S, Ahmed G, Chen S, Shinmyo Y, Kawakami M, Yamamura K, Tanaka H, 2009. Draxin, an axon guidance protein, affects chick trunk neural crest migration. Dev. Growth. Diff. (In press).

3. 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. 2009. Draxin, a repulsive guidance protein for spinal cord and forebrain commissures. Science. 323(5912), 388-393.

Acknowledgements

All praises to Almighty God whose mercy and kindness enabled me to successfully finish this work.

I would like to express my sincere gratitude to my supervisor and mentor Professor Hideaki Tanaka for his kindness, guidance and constructive criticism of my research work and critical reading of my publication.

I would like thank Dr. Kunimasha Ohta and Dr. Yohei Shinmyo for their advice and support.

I would like to thank all lab members for their help and cooperation.

I am thankful to Ministry of Education, Science, Sports and Culture of Japan (MEXT), the 21st Century COE program, the Global COE program (Cell Fate Regulation Research and Education Unit), Japan and Kumamoto University for financial support.

Finally, I am glad to dedicate this work to my family members especially to my wife and daughter for their love and support.

1. Introduction

During nervous system development, pioneer axons are navigated to their correct targets by a variety of guidance signals in their surrounding environment. These signals include different kinds of molecules that are secreted or presented on the cell surface and the extracellular matrix by the intermediate or final cellular targets of the axons (Tessier-Lavigne, 1994; Tessier-Lavigne and Goodman, 1996; Dickson, 2002). These cues are classified into two major groups attractants and repellents, with four different modes of actions, chemoattraction, chemorepulsion, contact attraction and contact repulsion. Usually secreted guidance cues mediate their action through chemoattraction and chemorepulsion and membrane bound cues mediate by contact attraction or repulsion. Netrins, slits, semaphorins and ephrins are four conserved families of axon guidance molecules identified (Tessier-lavigne and Goodman, 1996; Dickson, 2002).

Netrin was identified by Tessier-Lavigne *et al*. in a ground-breaking study in which small explants of embryonic rat ventral neural tube (embryonic spinal cord) were shown to attract commissural axons from rat dorsal neural tube explants (Kennedy *et al*., 1994 and Serafini *et al*., 1994). Knock out mice study showed that Netrin is required for spinal commissural axon growth toward the floor plate and for the correct formation of

forebrain commissures: the corpus callosum, hippocampal commissures and anterior commissures (Serafini *et al*., 1996). Netrin is required for the final exit of retinal axons from the retina to the optic nerve and lack of Netrin function results in optic nerve hypoplasia (Deiner *et al*., 1997). Cortical axon projection (Métin *et al*., 1997) and thalamocortical axon projection (Braisted *et al*., 2000) are also defected in Netrin knock out mice. Trochlear motor axons were reported to be repulsed by Netrin (Colamarino and Tessier-lavigne, 1995). Several receptors like DCC (Keino-Masu *et al*., 1996), Unc5 (Hong *et al*., 1999), Neogenin (Rajagopalan *et al*., 2004), DSCAM (Ly *et al*., 2008) were reported to mediate Netrin function.

Slits function as chemorepellent in both vertebrates and invertebrates (Brose *et al*., 1999). This protein mediates the midline guidance of commissural axons of the central nervous system (Battye *et al*., 1999; Kidd *et al*., 1999; Zou *et al*., 2000; Shu *et al*., 2003). Retinal ganglion cell axon responsiveness to Slit molecules differ depending whether the axon is interacting with Slit molecules within the retina or at the optic chiasm. In the molecular environment of the retina, axon growth is supported by Slit (Jin *et al*., 2003). Slit 1 and Slit 2 help to properly position RGC axons within the optic chiasm by chemorepulsion (Plump *et al*., 2002). Roundabout (Robo) family receptors mediate intracellular signaling of Slits (review in Brose and Tessier-Lavigne, 2000).

Semaphorins are a large family of axon guidance molecule defined by the presence of a conserved 420–amino acid Sema domain at their amino termini. Eight classes of semaphorins are identified on the basis of their structure. Classes 1 and 2 are found in invertebrates, classes 3 to 7 are found in vertebrates, and class 5 semaphorins are encoded by viruses (Luo *et al*., 1993). Semaphorins function as short-range inhibitory cues that deflect axons (Hippocampal Axon) away from inappropriate regions, or guide them through repulsive corridors (Cheng *et al*., 2001). Semaphorins may also act as attractive cues for certain axons (developing peripheral neurons) (Wong *et al*., 1999).

Ephrin and Eph molecules are reported to be involved in a wide range of developmental events such as cell migration (Poliakov, A. *et al,* 2004) blood vessel formation (Klagsbrun, M. *et al* 2005) and axon pathfinding (Sakurai. *et al*, 2002., Kullander *et al*., 2002). Two different subfamilies of Ephrins are present. Ephrin-A proteins are GPI-linked molecules that bind EphA receptor proteins, while ephrin-Bs are transmembrane molecules that bind to EphB proteins. Ephrin-B2 can also bind to EphA4. Ephrins play most crucial role in topographic map formation of the retino-collicular projection system both as repulsive and attractive guidance cues (review in O'Leary and Wilkinson, 1999). Post crossing spinal cord commissural axons

were also been reported to be guided by ephrins (Imondi *et al*., 2000).

Several families of morphogenetic proteins, for example; BMP, Shh, Wnt, which are known to specify cell fates along major body axes, are now well-accepted guidance molecules for axonal pathfinding. BMP signaling regulates retinotopic map formation in the Lateral geniculate nucleus and Superior colliculas (Plas *et al*., 2008). Wnt were reported to modulate the medio-lateral patterning of retinocollicular map formation (Schmitt *et al*., 2004). Shh, emanated from floor plate, attract commissural axons to grow them towards the ventral midline (Charron *et al*., 2003). Shh also repel post crossing commissural axons toward anterior direction (Bourikas *et al*., 2005).

Axon guidance research made a great advance to identify several classes of axon guidance molecules but all these molecules could not explain the precise network formation of the brain. So, immense complexity of the brain makes it likely that more other classes of guidance molecules might exist. We recently identified another axon guidance molecule that might function as a repulsive guidance protein for commissural axons in developing spinal cord and forebrain (Islam *et al*., 2009). In the midbrain of chick and mouse, draxin is expressed from early embryonic stage in a dorsal high to ventral low gradient. Our data suggest that draxin might function as a repulsive guidance cue for the ventrally directed axon projection in chick early embryonic brain.

Continuous expression of *draxin* even after the midbrains ventrally directed axon projections completed led us to analyse the retinocollicular projection in *draxin* knock out mice. Miss projections of retinal axons in mouse midbrain/superior colliculas suggest that draxin might also involved in correct retinocollicular projection.

In the chick embryo at stage 14 (Hamburger and Hamilton, 1951) one of the first major axonal tract is constituted of the processes of numerous neurons located near the roof plate of the mesencephalon or midbrain on the both side of the dorsal midline (Chédotal *et al*., 1995; Hunter *et al*., 2001). This group of cells corresponds to the mesencephalic nucleus of the trigeminal nerve (nmes V). Their axons follow descending roots circumferentially from the dorsal midline of the mesencephalon towards the ventral side (Fig. 1A). The tectobulbar tract is another well characterized long-distance axon projection and starts from stage 18 where the axons of newly generated neurons extend away from the dorsal midline, taking a ventral and circumferential route through the dorsal tectum toward the ventral midline (Kröger and Schwarz, 1990, Henke-Fahle *et al*., 2001) (Fig. 1A). One axonal population, the ipsilateral tectobulbar tract, turns abruptly in a caudal direction before reaching the tegmentum, and follows the course of the medial longitudinal fasciculus. Another population crosses the ventral midline and subsequently turns posteriorly.

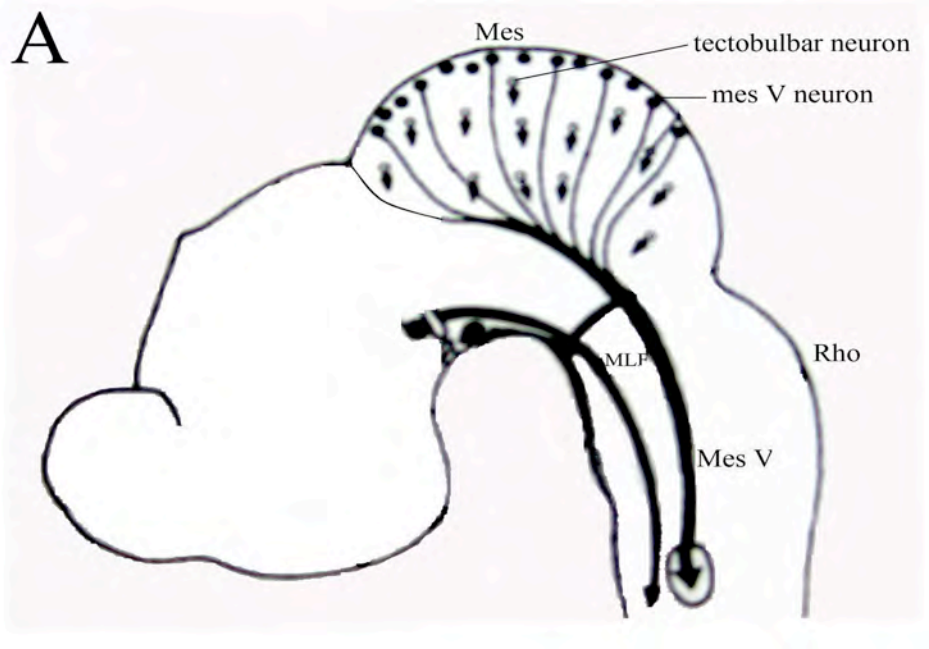


Fig 1: (A), Schematic representation of ventrally directed axonal tracts in chick midbrain (Chédotal *et al*., 1995). Mesencephalic V neurons (black circles) present near the dorsal midline. They start to project axons towards ventral direction from stage 14 and turn caudally in the ventral tectum. Tectobulbar neurons (open circles) also project axons toward ventral direction and turn caudally before reaching the tegmentum and follow the path of medial longitudinal fascicles. Mes, mesencephalon; Rho,rhombencephalon; Mes V, mesencephalic V neuron; MLF, medial longitudinal fasciculus.

Several reports have stated that the ventrally projecting axons along the rostro-caudal axis are attracted by the chemoattractant netrin-1, which is secreted by the floor plate (Kennedy *et al.*, 1994; Serafini *et al.*, 1994, 1996; MacLennan *et al.*, 1997; Shirasaki *et al.*, 1995). However the floor plate of mesencephalon does not attract axons of the dorsal tectum (Tamada *et al.*, 1995; Henke-Fahle *et al.*, 2001). The positive effect of netrin-1 may be neutralized by inhibitory molecule(s) secreted by the floor plate.

Therefore, the initial ventral course of tectobulbar and nmes V neuron axons seems not to be attracted by guidance molecules from the floor plate *in vivo*.

Chemorepellents from the floor plate repel the ipsilaterally projected tectobulbar axons and prevent them from crossing the midline. Semaphorin 3A is one of the repulsive molecules secreted by the medial longitudinal fasciculus, located parallel to the floor plate, that repels the ipsilaterally projected axons originating from the dorsal tectum (Henke-Fahle *et al.*, 2001). The mechanism and molecule(s) that determine the initial ventral course of these axons are largely unknown. One possibility is that chemorepellent molecule(s) from the roof plate may guide the pioneer axons of the tract towards the ventral course. BMPs were reported to mediate roof plate repulsion of commissural axons in the spinal cord (Augsburger *et al.*, 1999, Butler and Dodd, 2003). We hypothesized that draxin might be one of the unknown guidance cue that repels these axons to grow ventrally.

Topographic map forms in a precise and complex spatial order in the central nervous system where neighboring points in one structure project to neighboring points in a target structure. One extensively studied example of topographic map is retinocollicular projection where retinal axons project their axons to the midbrain target superior colliculas. Dorsal retinal axons project to the lateral colliculas and ventral

retinal axons project to the medial colliculas. Axons from the nasal retina find their destination in posterior colliculas and temporal retinal axons terminate at anterior colliculas. For topographic map formation, in 1963 Sperry proposed, molecules expressed in gradients across the projecting and target fields give a unique positional identity to each location that help the axons mapping to their correct location by matching up the positional value. In retinocollicular projection Eph receptors and their ligand ephrins are reported to play the key role. Several lines of evidence suggest that ephrin A ligands expressed in gradients along the antero-posterial (A-P) axis of the superior colliculas are repulsive to axons expressing EphA receptors, and elongation of retinal axons stops at proper locations on the superior colliculas according to the expression level of EphA receptors on the axons. The map formation along the anterior-posterior axis is largely mediated by EphA-ephrinA molecules.

The medio-lateral patterning is believed to be mediated by B class of Eph, ephrin molecules. EphB2, EphB3, and EphB4 are each expressed in high-ventral to-low-dorsal gradient in the retina (Holash & Pasquale 1995, Henkemeyer *et al*. 1996, Connor *et al*. 1998, Birgbauer *et al*. 2000, Hindges *et al*. 2002), and ephrin-B1 is present in a high-medial-to-low-lateral gradient in the mouse Superior colliculas (Braisted *et al*. 1997, Hindges *et al*. 2002). These gradients mean that ventral retinal ganglion cells with

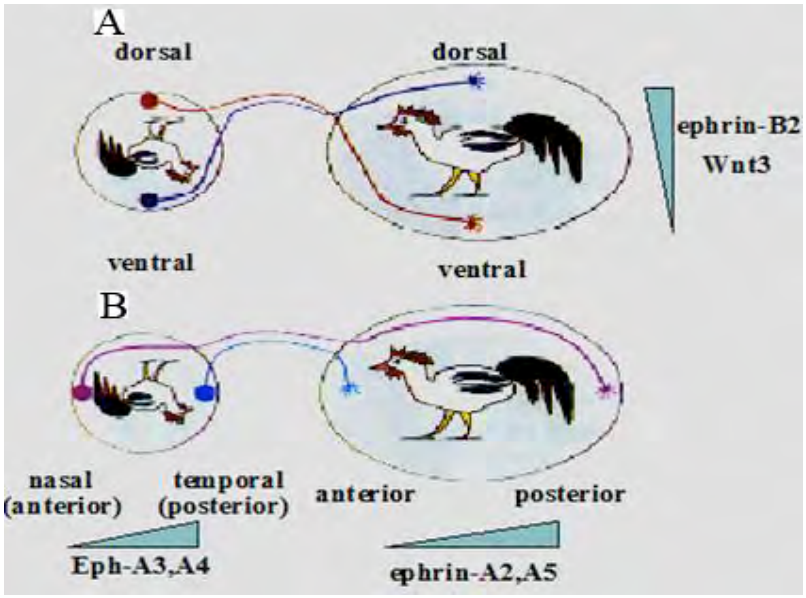


Fig2: Schematic representation of retinotectal map formation. (A) Dorsal retinal axons project to the ventral (lateral) tectum and ventral retinal axons project to the dorsal (medial) tectum. Ephrin B2 and Wnt3 expressed in dorsal high to ventral low gradient in the tectum. (B) Axons from the nasal retina find their destination in posterior tectum and temporal retinal axons terminate at anterior tectum. Along the nasal-temporal axes of the retina and tectum ephrin A and their receptors Eph A are expressed in high temporal to low nasal gradiant.

the most EphBs project to medial collicular targets where ephrin-B levels are highest, the exact opposite of the situation with the EphAs/ephrin-As on the anterior-posterior axes.

Along the medio-lateral axis Wnt family protein are recently reported to play a vital role (Schmilt *et. al.* 2005). Wnt3 is expressed in a medial high to ventral low gradient in chick optic tectum and mouse superior colliculas. Wnt3 repulses ventral retinal axons through Ryk receptor, which is expressed in ventral high to dorsal low gradient. And

dorsal retinal axons are attracted by low concentration of Wnt3 in the lateral superior colliculas. This attraction is mediated by Frizzled receptor. Another morphogene BMP (Plas *et.al.* 2008) is also reported to play a role in retinocollicular projections. In BMP transgenic mice medio-lateral patterning is defected in the superior colliculas and LGN. On the basis of gradient expression of *draxin* and phenotype analyses we hypothesized that draxin might also play important function in retinocollicular projection.

2. Experimental procedures

2-1. Chick embryos

Fertilized chick eggs were purchased from a local supplier and incubated at 38 °C in a humidified chamber. Chick embryos were staged according to Hamburger and Hamilton (1951).

2-2. In situ hybridization

Sense and antisense riboprobes labeled with digoxigenin (DIG) were synthesized from a pBluescript construct (SK^{-}) containing 1050-bp fragment of *draxin* cDNA using T7 and T3 RNA polymerases, respectively. Probes for section *in situ* hybridization were treated with alkali to trim them to approximately 200 bp.

Embryos were dissected in diethyl pyrocarbonate (DEPC) treated phosphate buffered saline (pH 7.0) and fixed in 4% Paraformaldehyde in PBS at 4°C overnight.

For section *in situ* hybridization, fixed embryos were cryoprotected by immersion in 30% sucrose in PBS and embedded in OCT compound (Sakura Fine Technical Co. Ltd, Tokyo, Japan). Twenty μm thick sections were cut on a cryostat and mounted on silanized glass slides (DAKO Cytomation). *In situ* hybridization was performed using the method of Schaeren-Wiemers and Gerfin-Moser (1993). Briefly, sections were treated with 10 μg/ml proteinase K and permeabilized with 1% Triton X-100 and then incubated with herring sperm DNA. Hybridization was performed overnight at 68°C with 400 ng/ml probe in prehybridization solution. After hybridization, sections were washed in 0.2X SSC at the hybridization temperature and blocked with 10% heat-inactivated sheep serum. For immunochemical detection, alkaline phosphatase (AP)-conjugated anti-DIG Fab fragment (Roche) diluted 1:5000 in 1% heat inactivated sheep serum (HISS) and buffer B1 was applied to the sections and incubated overnight at 4°C. Samples were washed and signals were detected using 337.5 μg/ml ρ-nitroblue tetrazolium (NBT) and 175 μ g/ml 5-bromo-4-chlro-3-indolylphosphate (BCIP) in NTMT solution [0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$, 0.24 mg/ml levamisole] for 6-72 hours at room temperature in the dark. Slides were dried overnight, dehydrated in a graded series of ethanol and xylene, and then mounted. Whole-mount *in situ* hybridization was performed following a procedure described previously

(Schaeren-Wiemeres and Gerfin-Moser, 1993). Briefly, after fixation with 4% PFA, embryos were dehydrated with graded series of methanol in PBT (0.1% tween -20 in PBS) and chilled at -20°C in absolute methanol. Embryos were then rehydrated in a reversed graded methanol series in PBT. Treatment with proteinase K (20 μg/ml) was performed for 10-20 minutes before refixation. Samples were pretreated in prehybridization solution for one hour in 70°C and hybridized with 0.1 μg/ml probe in prehybridization solution at 70°C overnight. Embryos were washed and blocked with 20% heat inactivated sheep serum (HISS), and incubated with anti-DIG-AP Fab fragment (Roche) diluted 1:2000 in 20% HISS in TBST [137 mM NaCl, 2.68 mM $MgCl_2$, 25 mM Tris-HCl (pH 7.5), 1% Tween-20] at 4°C overnight. After washing the samples, signals were detected in 337.5 μg/ml NBT and 175 μg/ml BCIP in NTMT solution [0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$, 1% Tween-20, and 0.48 mg/ml levamisole].

2-3. Immunohistochemistry

Chick embryos were fixed with 4% PFA/PBS for 2 hours at 4°C, immersed in 30% sucrose/PBS at 4°C, embedded in OCT compound and frozen at -80°C. Cryostat sections 10-15 μm thick were cut in a cryostat and mounted on gelatin-coated glass slides. Sections were washed with PBS for 5-10 minutes, fixed, blocked and then

stained with anti-draxin monoclonal antibody for one hour at RT. After several washes with PBS, the sections were incubated with anti-mouse IgG-Cy3 (1:300 in blocking solution (Jackson IR) for one hour at RT.

2-4. *In vitro* explant culture

Stage 25 chick embryonic midbrains were opened dorsally and a stripe of tissue was cut out along the dorso-ventral axis on either side from the middle part (excluding the floor plate). From these stripes, the dorsal one-third was taken and cut into small pieces to prepare the explants. The explants were embedded in rat-tail collagen (Sigma) as described (Islam *et al*., 2009) and incubated in *draxin* or mock DNA–transfected COS7 conditioned medium diluted 1:1 with fresh culture medium (DMEM/F12 supplemented with L-glutamine, 10% FCS) for 36 hours in a 5% CO incubator at 37°C. To visualize the neurites, explants were fixed in 4% PFA for 1 hour and then stained with a monoclonal antibody against neurofilament 82E10 (Go *et al*., 1989).

For co-culture experiments, *draxin* cDNA or control vector was transfected into COS7 cells using LipofectAmine 2000 (Gibco BRL). Forty-eight hours later, cell pellets containing 1000-2000 cells were generated by hanging drop cultures (Ohta *et al*., 1999). Explants and cell aggregates were embedded in rat-tail collagen and placed at a distance of 200-300 μ m from each other and cultured in DMEM/F12 supplemented with

L-glutamine, 10% FCS for 48 hours at 37°C. Neurites were visualized by immunostaining as described above.

2-5. *In ovo* electroporation and axon tracing by DiI

Fertilized chicken eggs were incubated at 38°C for 50-53 hours in a horizontal position to obtain embryos at stages 10-16. Eggs were windowed on the top. A solution of 10% pelican ink diluted in 1´ Tyrode was injected under the embryo to aid visualization. *draxin* expression plasmid at a concentration of 5 μg/μl was injected into the lumen of the midbrain. Control vector at the same concentration was used as a control. *In ovo* electroporation into the mesencephalon was achieved with a train of four square wave pulses at 25 V, with a pulse length of 20 ms and pulse intervals of 999 ms using an electroporator. Eggs were sealed with clear tape and incubated for desired hours. Embryos were harvested and fixed for 6 hours in 4% PFA in PBS at 4°C. Midbrains of the electroporated embryos were opened dorsally and placed on a nylon membrane with the ventricular side facing upward. DiI powder (Molecular Probe) was added with a fine tungsten needle approximately 300 μm dorsal to the electroporated site. Samples were incubated at 38°C for 2-3 days. Labeled axons were photographed with a Leica fluorescence stereomicroscope.

2-6. Lac Z staining

We designed a target vector to produce a null mutation of *draxin* by replacing the second exon containing the ATG start codon with a lacZ-neo selection cassette. The targeting vector was constructed and the *draxin* mutant mice (Acc. No. CDB0484K) were generated as described previously (Yagi *et al*., 1993). *draxin* hetero mice were used to know *draxin's* expression by Lac Z staining (Islam *et al*., 2009). Fixed cryostat sections were washed with PBS twice and then colour were developed by dipping the sections in colour reagents. After overnight incubation or depend on colour development sections were fixed, washed and dehydrated by ethanol before mounting.

2-7. DiI labelling

Axon projection from retina to the superior colliculas was traced by DiI labelling. P8 mice were first anesthetised and eyelid were cut open. Fine crystals of DiI were inserted in to the desired position of the eye by thin tungsten needle. After two days, at P10, mice were analyzed blind to genotype. Mice were first fixed by heart perfusion by 4% PFA and midbrains were photographed with fluorescence optics as whole mounts.

2-8. *In vitro* mouse retinal explant culture

P0 mice retina were dissected and cultured in Lysin-laminine coated dish in presence of draxin conditioned medium or control conditioned medium diluted 1:1 with fresh culture medium (DMEM/F12 supplemented with L-glutamine, 10% FCS) for 36

hours in a 5% CO incubator at 37°C. To visualize the neurites, explants were fixed in 4% PFA for 1 hour and then stained with a monoclonal antibody against neurofilament.

3. Results

3-1. Expression patterns of *draxin* mRNA in the chick optic tectum

To analyze the expression pattern of *draxin* mRNA in chick optic tectum, embryos were examined by whole mount *in situ* hybridization with DIG-labeled antisense RNA probes. *draxin* mRNA expression started from stage 10 in the chick midbrain and persisted up to stage 36 (data not shown). At stage 18, *draxin* mRNA was expressed strongly near the dorsal midline of the tectum (Figs. 3A, B). The expression seemed to have a spot-like pattern. At this stage, *draxin* was also found to be expressed in the areas of diencephalon. Later, at stage 25, expression expanded towards the ventral direction with a dorsal high to ventral low gradient (Figs. 3C, D). *draxin* was not expressed in the dorsal midline at this stage.

Next, we examined *draxin* expression in detailed by section in situ hybridization. At stage 16, *draxin* was expressed only at the dorsal midline of the tectum (Fig. 4A). At stage 22, the expression of *draxin* was observed near the dorsal midline and started to extend laterally where the staining pattern was punctate (Fig. 4B). From stage 22 to stage 25 (Figs. 4B, C) *draxin* expression became stronger and extended laterally

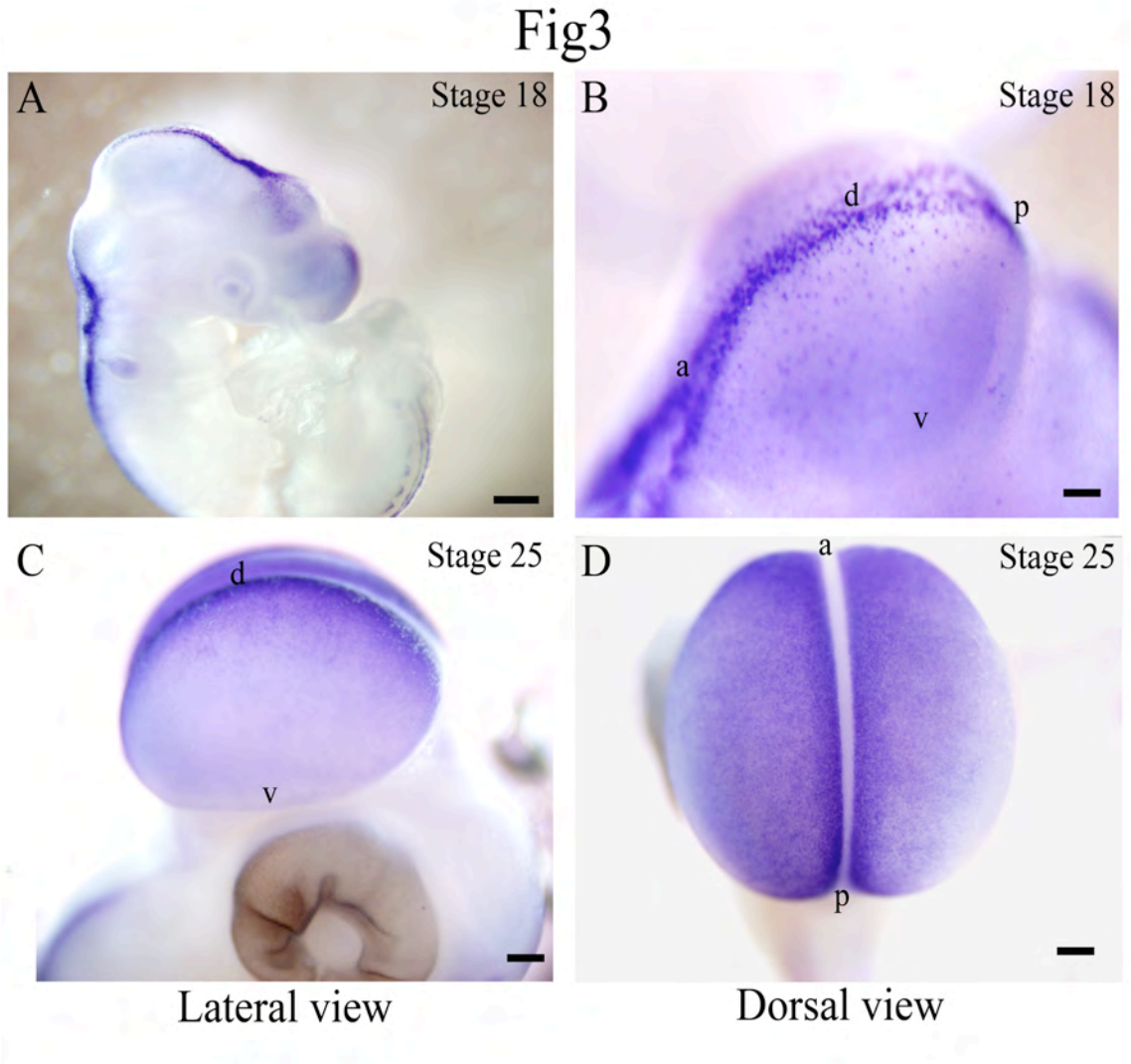


Fig. 3. *draxin* mRNA expression is graded in the midbrain (optic tectum) of stage 18 to 25 chick embryos. (A-D) whole mount *in situ* hybridization of *draxin* in chick embryo. (A, C) shows the lateral view, and (B, D) shows dorsal view at stage 18 (A, B) and stage 25 (C, D). Strong spot-like expression of *draxin* was detected near the dorsal midline of the tectum at stage 18 (B). Expression area expands at stage 25 (C, D) with a dorsal high to ventral low gradient. d, dorsal; v, ventral; a, anterior; p, posterior. Scale bars= 400 μm for A, B; 500 μm for C, D.

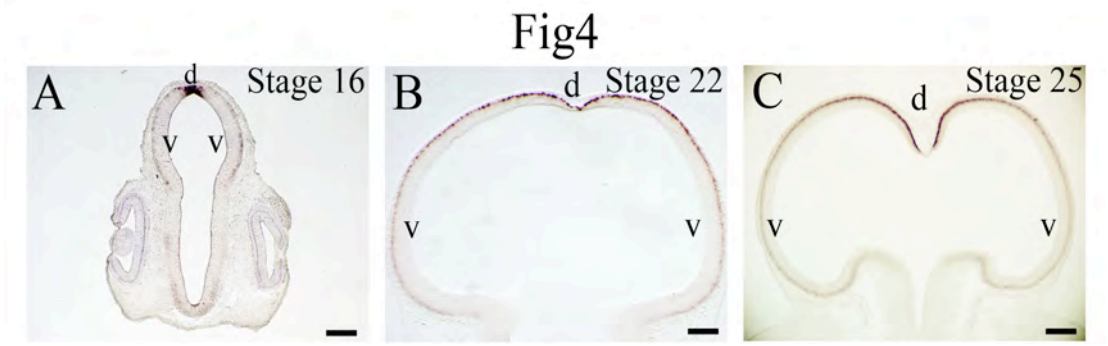


Fig. 4. Localization of *draxin* mRNA and protein in optic tectum of stage 16-25 chick embryos. (A-C) Coronal section *in situ* hybridization of stage 16 (A), stage 22 (B) and stage 25 (C). *draxin* mRNA was expressed in the dorsal midline and nearby cells at stage 16 (A). At stage 22 (B) and stage 25 (C) *draxin* expression expands from the dorsal to the ventral direction, and a dorsal high to ventral low gradient was observed.

towards the ventral portion of the tectum. A dorsal high to ventral low gradient was very clear at these stages. At stage 25, dorsal midline cells seemed to be negative for *draxin.*

3-2. Draxin protein expression

A monoclonal antibody against draxin was developed (Islam *et al*., 2009) and used to detect the expression of draxin protein at different stages of chick embryonic development. At stage 16, we could not detect the draxin protein (data not shown). This might be due to a very low level of expression of the protein. Draxin protein was detected at stage 18 (Figs. 5A). It was deposited on the most outer layer of the tectum. The intensity of expression was higher at stage 18 near the dorsal midline. We also double-labeled the tectum sections at stages 18, 20 and 22 with antibodies against

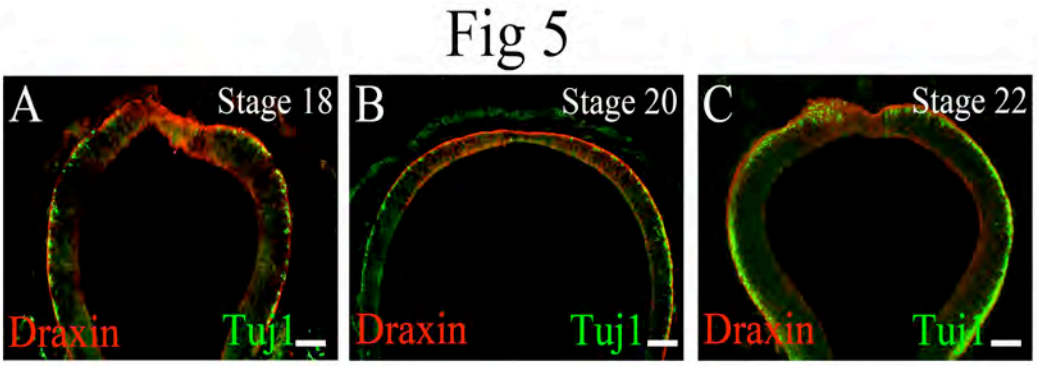


Fig. 5. Draxin protein expression. (A-C) Double staining of draxin and Tuj1 to show the expression of draxin and developing tectum axons at different developmental stages in series. At stage 18 (A), draxin expression is stronger near the dorsal midline and tectum axons are sporadic. In Stage 20 (B) and stage 22 (C) axons are increased beneath the draxin deposited layer. d, dorsal; v, ventral. Scale bars= 150 μm for A and 200 μm for B and C.

chicken draxin and β III-tubulin (Tuj1) (Figs. 5A-C) to show the relation of draxin expression and axon tract formation. At stage 18, dorsal midline and nearby area expressed draxin and sporadic tectum axons were observed (Fig. 5A). With the course of development more neuronal cells extended axons and thus tract size increased (Figs. 5B, C). At these stages deposited draxin did not overlap, but closely associated with axons (Figs. 5B, C).

3-3. *draxin* is not expressed by neuronal cells

draxin mRNA was expressed in the outermost layer of the tectum (Fig. 6A), whereas the neuronal cells, stained by the HuC/HuD antibody, were located in deeper layers of the tectum (Fig. 6B). These results indicated that *draxin* is not expressed by

Fig6

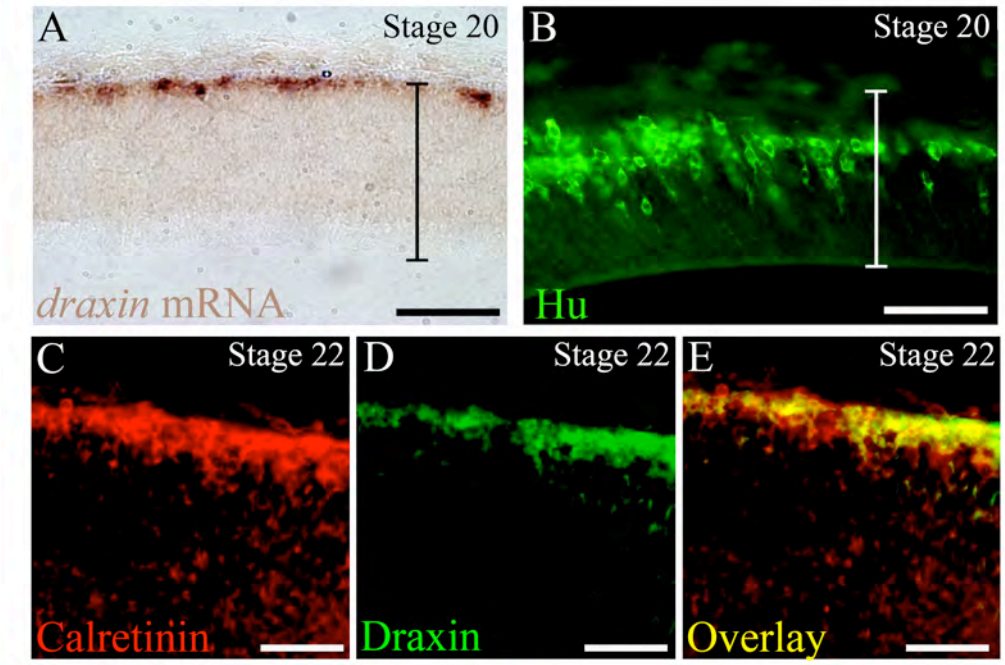


Fig. 6. Draxin-expressing cells. (A, B) Adjacent sections were stained for *draxin* mRNA (A) and neuronal cell marker Hu (B). (A) *draxin* is expressed by the outer most layer of dorsal tectum at stage 20. The bar represents the width of the tectum. (B) Anti-Hu antibody stains the cells of the middle layer of tectum. These results imply that draxin is not produced by the neuronal cells. (C-E) Same tectum section was stained for calretinin (C) and draxin (D). (E) Merge picture shows that certain population of calretinin positive Cajal-Retzius cells expresses draxin. Scale bars= 100 μm for A, B; 200 μm for C, D, E.

the neuronal cells. To know what type(s) of cell population express draxin we double-labeled the tectum sections at stage 22 with chicken draxin and calretinin antibodies (Figs. 6C, D). Since calretinin is a good marker of the Cajal-Retzius cells (Alcántara, *et al.* 1998), some Cajal-Retzius cells seemed to be positive for draxin (Fig. 6E).

3-4. Draxin affects the axon outgrowth from chick tectum explants

To perform functional analyses of draxin, we first cultured dorsal tectum explants from stage 22 chick embryos. To avoid contamination with neurons forming the posterior commissure in the anterior tectum, explants were prepared only from the medial portion with respect to the anterior-posterior axis of the stage 22 chick midbrain and cultured in collagen gel. After 36 hours of culture, neurite outgrowth from dorsal tectum explants was greatly inhibited in draxin-conditioned medium (Fig. 7B), whereas there was robust neurite outgrowth from the explants cultured in the control mock-transfected conditioned medium (Fig. 7A). After replacing draxin conditioned-medium with fresh culture media, neurite outgrowth was robust within 24 hours (data not shown). This data excluded the possibility of cell death in draxin conditioned-medium. Quantification of neurite outgrowth showed that dorsal tectum axons are sensitive to draxin in a dose dependent manner (Fig. 7C). To test whether

Fig7

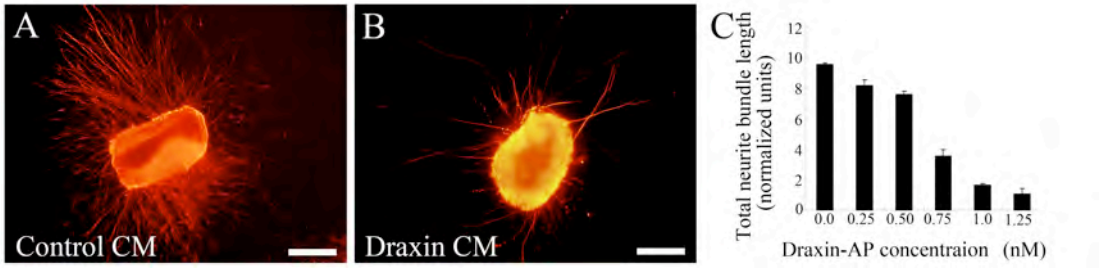


Fig. 7. Draxin inhibits axon outgrowth from the dorsal tectum explants. (A, B) draxin inhibits neurite outgrowth from dorsal tectum explants from stage 22 embryos. Dorsal tectum explants were cultured in collagen gel for 36 hours either with mock transfected COS conditioned medium (A) or *draxin* transfected COS conditioned medium (B). After culture, the explants were immunostained for the neurofilament marker 82E10. (A) Profuse outgrowth of neurites from explants cultured with mock transfected COS conditioned media are observed. (B) draxin strongly inhibits neurite outgrowth from dorsal tectum explants. The number of neurites is reduced significantly. (C) Quantification of neurite outgrowth inhibition (n=4 for each bar and difference compared with the control is significant statistically, $P \leq 0.001$). Scale bars= 200 μm for A-B.

draxin inhibited axon growth by directly binding to the neurites, we incubated the dorsal and ventral tectum neurites with a draxin-AP fusion protein. Draxin-AP bound to the dorsal tectum neurites (Fig. 8B) stronger than to those of the ventral tectum (Fig. 8C). AP protein alone was used as a negative control and did not bind to the tectum neurites (Fig. 8A). Unfixed tectum sections were incubated with draxin-AP protein to have an idea about the receptor distribution. Draxin-AP protein bound to the most outer layer of the tectum identically to the anti-draxin antibody staining (Fig. 5C) with dorsal high and ventral low gradient at stage 22 (Fig. 8E). Since draxin bound to the dorsolateral basement membrane in the spinal cord (Islam *et al.*, 2009), the tectum basement membrane might have some difference in draxin binding along the dorso-ventral axis. Control-AP protein did not bind to the section (Fig. 8D).

We also performed co-culture experiments. Stage 22 tectum explants from the dorsal and ventral portions were dissected as described earlier, and co-cultured with COS cell aggregates expressing draxin in three dimensional collagen gels (Fig. 9). After 36 hours in culture, neurites extended radially from explants in control experiments (Figs. 9A, C). In contrast, the number of neurites was significantly reduced on the side of the dorsal tectum explants facing the COS cell aggregates expressing draxin (Fig. 9B). Ventral tectum axons were not repelled by the draxin point source (Fig. 9D). These

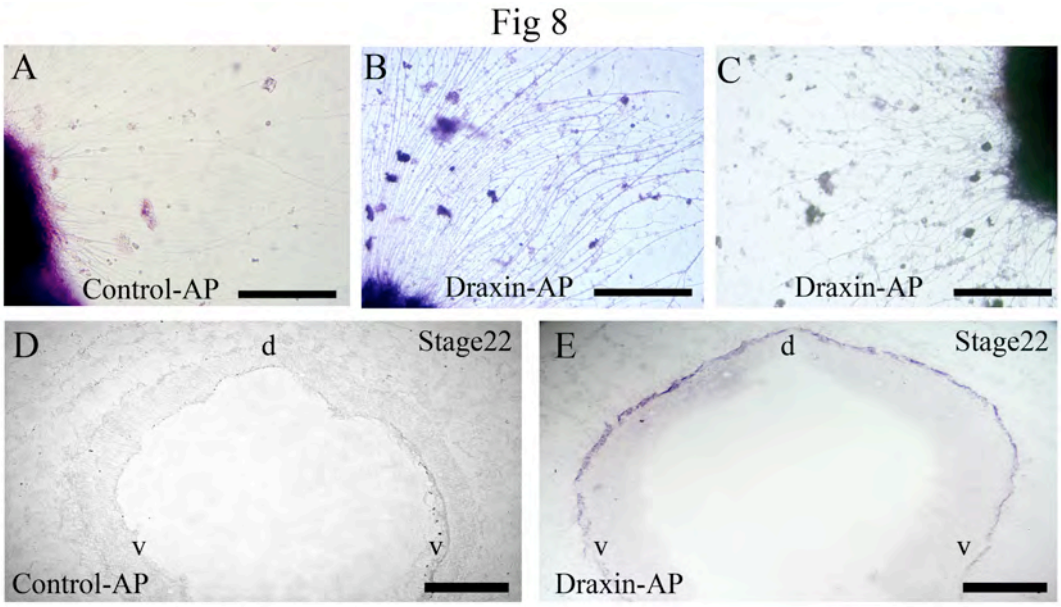


Fig. 8. Draxin bind to the tectum neurites.To test whether draxin binds directly to tectum neurite and inhibits their growth we performed a binding experiment between tectum neurites and alkaline phosphatase tagged draxin protein (A, B, and C). (B) draxin-AP protein bound to the dorsal tectum neurites. (A) Control AP protein did not bind to the dorsal tectum neurites. (C) draxin-AP protein bound to the ventral tectum neurites comparatively weaker than the dorsal tectum neurites. (D, E) Coronal sections of stage 22 chick midbrain. (E) draxin-AP protein seems to be bound to the basement membrane. (D) Control-AP Proteins did not bind to the sections. d, dorsal; v, ventral. Scale bars= 200 μm for A-E.

differences are quantified in Figure 5F. The P/D ratio was determined by dividing the number of neurites in the proximal quadrants by that of distal quadrants of the explants (Fig. 9E).

3-5. Overexpression of draxin causes misrouting and inhibiting of tectum axons

We have shown previously that draxin is a repulsive guidance protein for commissural axons in the forebrain and spinal cord (Islam *et al*., 2009). Our *in vitro* results shown here also imply that draxin may function as a repulsive guidance molecule for dorsal tectum axons. We overexpressed draxin by electroporating *draxin* cDNA-IRES-EGFP into the ventral part of the tectum of stage 16 chick embryos. After 40 hours of incubation, electroporated embryos were harvested and fixed with 4% PFA in PBS. To observe the response of pioneer tectum axons when they encountered the sites expressing ectopic draxin at the ventral tectum, we added DiI to the dorsal part of the tectum (Fig. 10A). We electroporated two different forms of *draxin*. The first was cDNA encoding the native secreted form of draxin. The second was a membrane-bound form of draxin, prepared by fusing the transmembrane domain of SC1 (Tanaka *et al.*, 1991) to a myc epitope at the C-terminus of the *draxin* cDNA sequence. As a control experiment we electroporated empty vector into embryos at the same stage. Strong EGFP signals confirmed the successful electroporation (Figs. 10B, E and H).

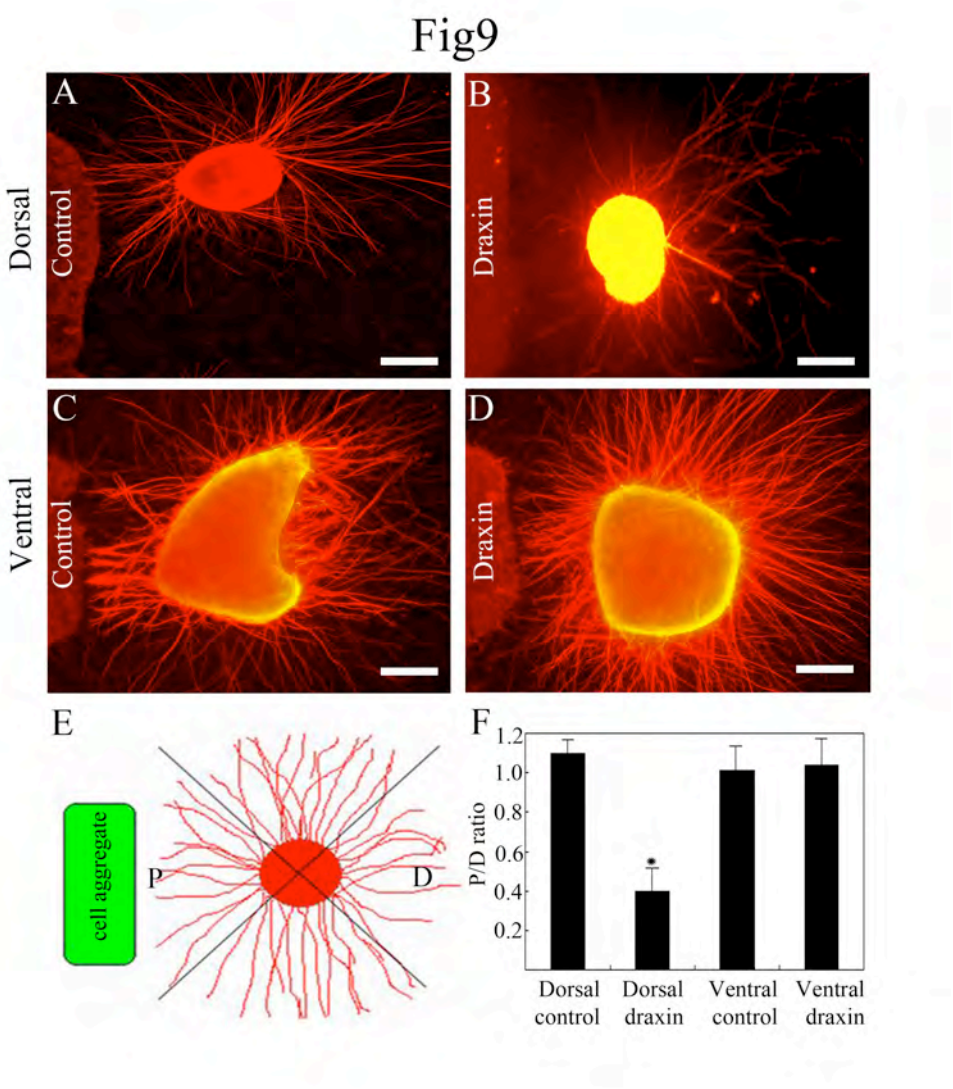


Fig. 9. Axons of dorsal tectum explants are repulsed by draxin. Dorsal and ventral tectum explants from stage 22 embryos were co-cultured with mock transfected COS cell aggregate (A, C) and *draxin* transfected COS cell aggregate (B, D). Axons from dorsal (A) and ventral (C) tectum explants grew radially when co-cultured with mock transfected COS cell aggregates. When co-cultured with *draxin* transfected cell aggregates, axons from the dorsal explants (B) were inhibited. Axons of ventral explants were not inhibited (D). (E) Scoring scheme used to test the effect of draxin on dorsal and ventral tectum axons**.** Explants were subdivided into four equal sectors. The two sectors used for quantification were designated as proximal (P) and distal (D) in relation to the COS cell aggregate. The number of axons in each sector was counted in every explant. Then the total number of axons in sector P was divided by that of D to get the P/D ratio. (F) Quantification of the axonal growth of dorsal and ventral tectum explants

cultured in collagen with mock-transfected COS cells (dorsal *n*= 10, ventral *n*= 8) or *draxin* transfected COS cells (dorsal *n*= 12, ventral *n*= 9) show a repulsive activity of draxin toward the dorsal tectum axons. Significant differences were observed among proximal sectors in the case of dorsal tectum explants co-cultured with draxin compared with control cases. *$p < 0.001$. Scale bar= 200 μm.

Axon growth was straight and long through the site where the control EGFP vector was overexpressed (arrows, Figs. 10B, C and D). Expression of ectopic membrane bound *draxin* caused highly disorganized tectum axon growth (Figs. 10E, F and G). Overlay images show that axon growth was stalled and then diverted upon encountering the ectopic membrane-bound form draxin (arrow, Fig. 10G). When the secreted form of *draxin* was overexpressed, we also observed disorganized axon growth (Figs. 10H, I and J). Many axons clearly avoided the site of draxin overexpression (arrow in Fig. 10J). Some tectum axons grew through the overexpressed sites (arrowheads in Figs. 10F, I). The reason might be due to the delay of expression of significant amount of draxin, which takes time, and some pioneer axons already grew through the overexpression sites. Alternatively, they might be draxin insensitive tectal neurons.

We also overexpressed *draxin* in the dorsal part of the tectum from where axons start to project. Electroporation were done at stage 16 and incubated until stage 22 then fixed and sectioned. Sections were stained with Tuj1 to observe the axon growth. EGFP

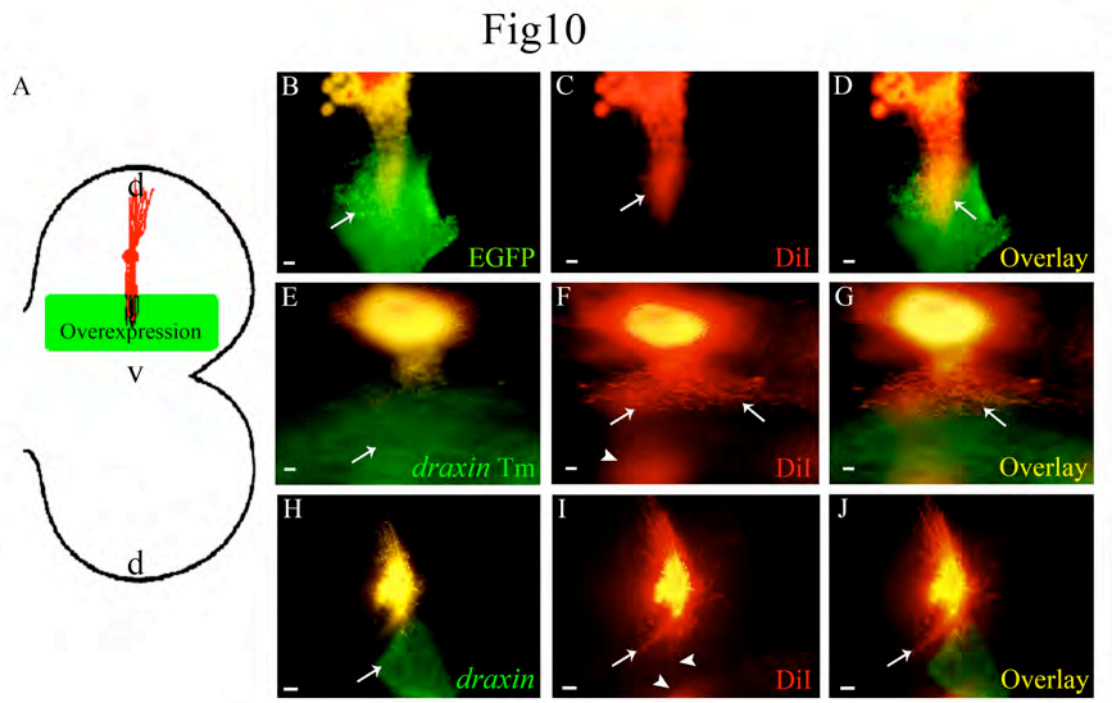


Fig. 10. Inhibition and misrouting of tectum axon projection by ectopic overexpression of *draxin in vivo*. (A) Schematic diagram shows the experimental design. DNA was electroporated into the ventral portion of the tectum of stage 16 chick embryos, and samples were isolated and fixed. The tectum was cut along the dorsal midline at stage 23. DiI was introduced at the dorsal part of the overexpressing site to trace the axons. Results are shown with the dorsal side at the top and the ventral side at the bottom. (B) Vector EGFP DNA was overexpressed in the ventral side of the tectum (arrow) (*n*= 15). (C) DiI labeling of the axons for two days. (D) Overlay image shows that growth of axons was long and straight through the EGFP overexpressed site (arrow). (E) To achieve a high concentration of ectopic draxin in one local area, membrane bound *draxin* cDNA was overexpressed in the ventral side of the tectum (*n*= 18). This construct contains a membrane anchored region and an IRES element driving co-expression of EGFP. EGFP shows the strong expression of *draxin* (arrow). (F) DiI was added to the dorsal side of the overexpressed site. As the pioneer axons proceeded towards the ectopic draxin, severe misrouting of axons was clearly observed (arrows). A part of the labeled axons are visible beyond the EGFP positive area (F, arrowhead). (G) Overlay picture shows that axon migration stopped and then proceeded in various directions in response to the site of *draxin* overexpression (arrow). (H) The secreted form of *draxin* was overexpressed in the ventral tectum (arrow) (*n*= 21) to determine the

effect of ectopic expression of the native form of draxin on tectum axons that have yet to enter the ventral tectum. (I) DiI labeling shows misrouted axons (arrow). Some labelled axons passed through the overexpressed area (I, arrowheads). (J) Overlay picture shows that the tectum axons were partially misrouted by the ectopic draxin. d, dorsal; v, ventral. *draxin* Tm, membrane-bound draxin. Scale bars= 100 μm for B-G; 200 μm for H-J.

vector DNA electroporation (Fig. 11A) had no effect on the axon growth. Both in control side and electroporated side axon growth were unchanged (Fig. 11B). When membrane bound form of *draxin* was electroporated (Fig. 11C) axon growth were almost completely inhibited from the electroporated area (arrow in Fig. 11D). Secreted form of draxin also inhibits axon growth from the electroporated neurons (arrow in Fig. 11E) but the effect was weaker than the inhibition caused by membrane bound form. This result suggests that overexpressed draxin inhibited axonogenesis in the same way as bath application of draxin in the context of explant culture.

Mesencephalic V neurons are located near the dorsal midline. From stage 14 they project axons circumferentially towards the ventral direction (Chédotal *et al.*, 1995; Hunter *et al.*, 2001). We checked whether these neurons were sensitive to draxin by electroporating membrane bound form of *draxin* at stage 10 chick mesencephalon. After incubation up to stage 17 embryos were fixed and stained with Tuj1 antibody. Ectopic draxin strongly inhibited axon growth from the dorsally located neurons in the

Fig 11

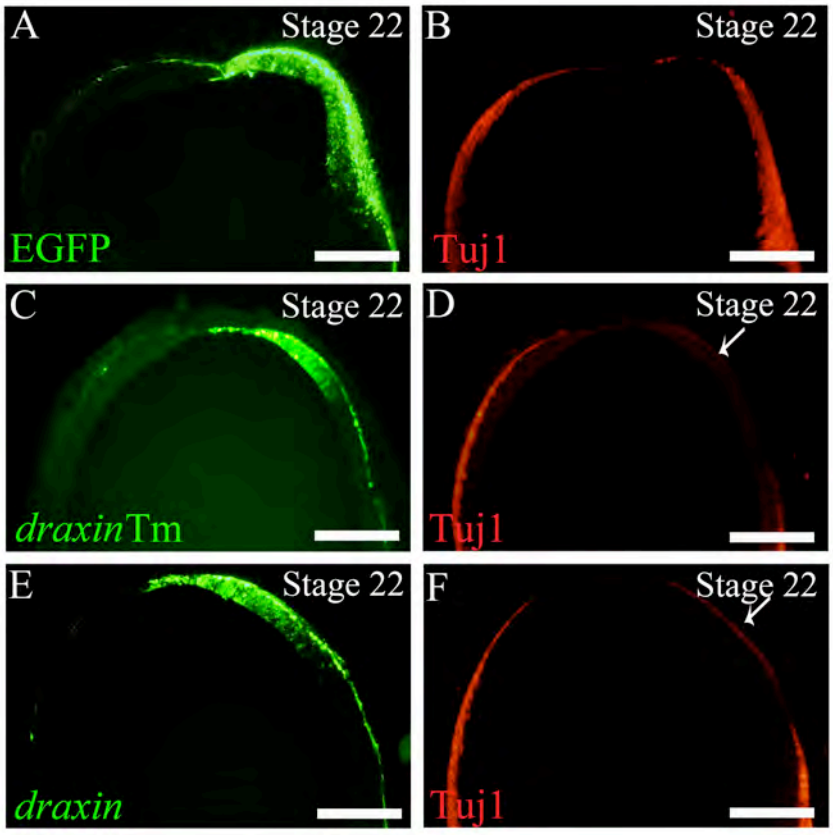


Fig.11. Overexpression of *draxin* caused inhibition of axon growth from dorsal tectum neurons. Control EGFP (A), *draxin* Tm (C) and secreted *draxin* (E) were electroporated in the dorsal tectum at stage 16. Embryos were incubated up to stage 22, fixed and stained with β-tubulin marker Tuj1 (B, D, and F). (B) With EGFP (*n*= 17) overexpression axon growth is normal in both electroporated side and control side. (D) *draxin* Tm expression greatly reduces the axon growth in the electroporated side (arrow) (*n*= 13). In the control side axon growth was normal. This effect mimics the effect of draxin on tectum explants in bath culture *in vitro*. We also electroporated secreted form of *draxin* (E). Secreted form of draxin also inhibits axon growth (F) (arrow) (*n*= 12) but the effect is weaker than that of the membrane bound form of draxin (compare arrows in D and F). Scale bar= 200 μm. *draxin* Tm, membrane-bound draxin.

electroporated side (Arrowheads in Figs. 12A-C). On the control side many neurons extended axon like processes (arrows in Fig. 12B). EGFP vector DNA electroporation did not affect axon growth from those neurons (Figs. 12D-F). As tectobulbar neurons do not start axon projection before stage 18, this result implies that mesencephalic V neurons might also be directed by draxin to their initial ventral course.

3-6. Expression patterns of *draxin* mRNA in the mouse

To analyze the expression pattern of *draxin* mRNA in mouse superior colliculus, embryos were examined by section Lac Z staining. At P0 coronal sections showed that *draxin* was expressed in medial high to lateral low gradient (Fig 13A). By sagittal sections expression along the anterior posterior axes were analyzed and no considerable gradient was observed (Fig 13B). In eye *draxin* expression was also checked at P0. *draxin* was expressed in eye but without any considerable gradient along both nasal-temporal and dorso-ventral axis (Figs 13C, D). Retinal ganglion cell layer and inner plexiform layer seem to express *draxin*.

3-7. Draxin affects the axon outgrowth from mouse retinal explants

To perform functional analyses of draxin, we first cultured mouse retinal explants from P0 mouse embryos. Explants were prepared from the different portion of the eye (dorsal, ventral, nasal, temporal) and cultured in lysin-laminine coated dish. After 36

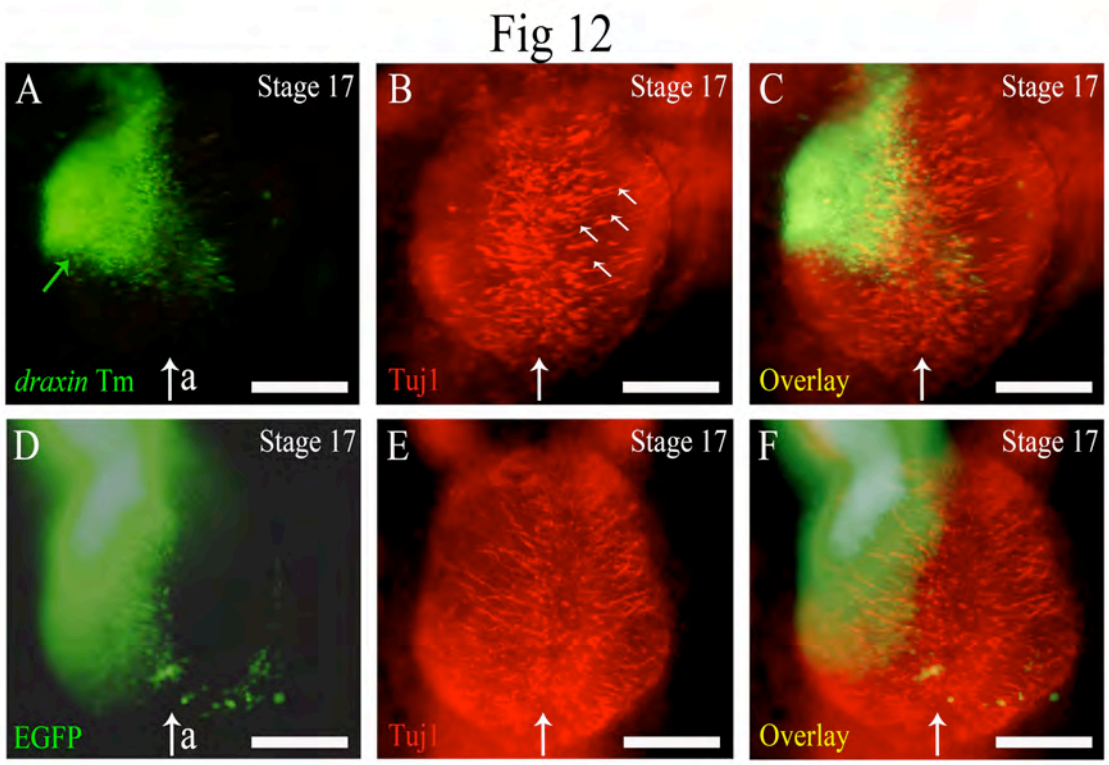


Fig. 12. Descending axons of the mesencephalic nucleus of the trigeminal nerve **(**mes V neurons) were also inhibited by overexpressed draxin. Stage 10 embryos were electroporated with membrane bound form *draxin* (A) (*n*= 30) or EGFP vector DNA (D) (*n*= 23). Embryos were incubated until stage 17, fixed and immunohistochemically stained with Tuj1 (B, E), and observed as whole mount from dorsal side. EGFP shows the strong expression of *draxin* (A, green arrow). (B, C) draxin stops the axon growth from the mesencephalic V neurons of the electroporated side. In the control side many V neurons project axons towards the ventral side of the mesencephalon (arrows). Large vertical arrows in all figures show the dorsal midline. (D, E) EGFP has no effect on the growth of the mesencephalic V neuron axons. Both in electroporated and control side axon growth are unchanged. *draxin* Tm, membrane-bound draxin. a, anterior. Scale bar= 200 μm.

hours of culture, neurite outgrowth from retinal explants was greatly inhibited in draxin-conditioned medium (Fig. 14B), whereas there was robust neurite outgrowth from the explants cultured in the control mock-transfected conditioned medium (Fig. 14A). The retinal explants derived from the all quadrants of eye did not show any difference in sensitivity towards draxin even with high concentration of draxin.

3-8. Phenotype analyses of *draxin* knock out mice

To assess the effects of *draxin* gene disruption on retinocollicular mapping, a focal injection of DiI was made in one retina, followed by examination of the contralateral midbrain. When retinal axons near the nasal extreme of wild-type mice were labeled, a characteristic arborization was seen at the posterior extreme of the Superior colliculas (Fig. 15A). When nasal axons of *draxin* knock out mice were labeled, an apparently normal arborization was always seen and additional more medial arborization were seen in approximately 80% of the animals (16 of 20 mice) (Fig. 15B, C, D).

Ventral retinal axon labelling in wild type mice always showed a single compact arborisation near the medial end of the superior colliculas (Fig. 16A). *draxin* knock out mice showed an apparently normal arborization near the medial end of the superior colliculas, and in approximately 76% of the mutants there were additional arborization

Fig 13

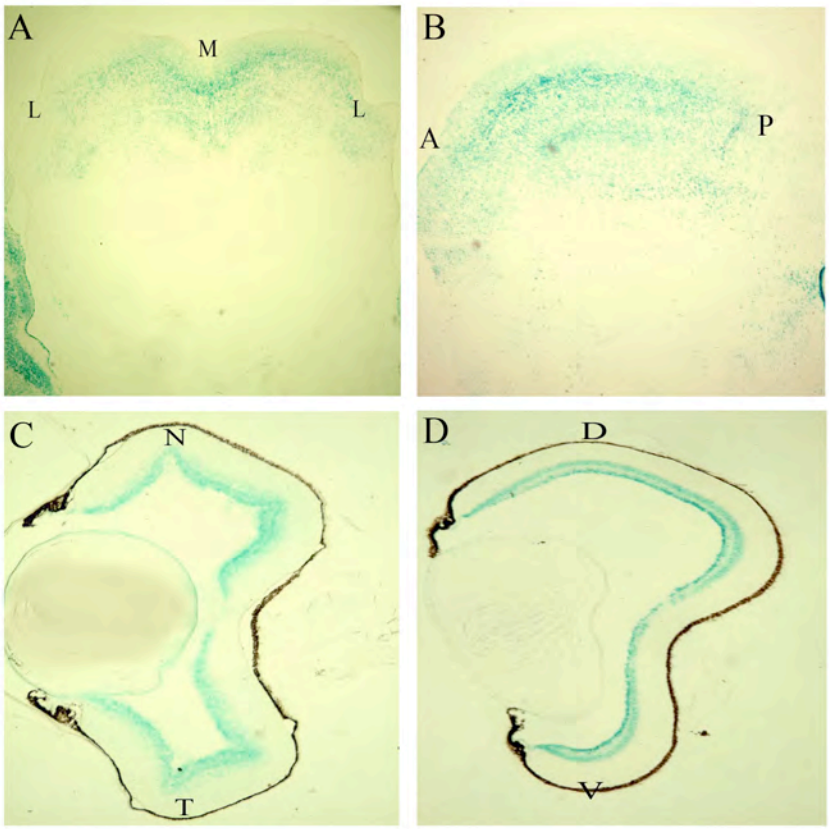


Fig. 13. *draxin* expression is graded in the superior colliculas of mouse. At P0 *draxin* was expressed in medial-high-to-lateral-low gradient (Fig 13A). Along the anterior-posterior axis *draxin* expression has no gradient (Fig 13B). In P0 retina *draxin* expression was strong with no obvious gradient (Figs 13C, D).

Fig 14

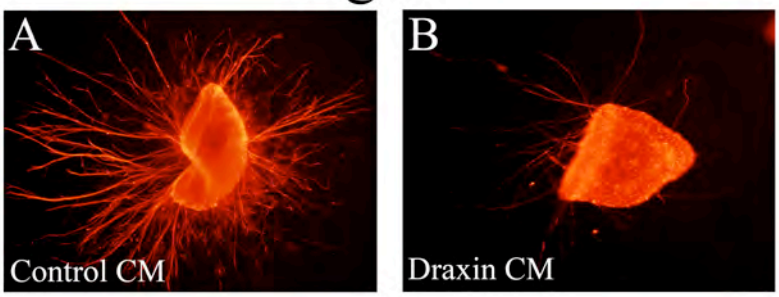


Fig. 14. Draxin inhibits axon outgrowth from the retinal explants (A, B). Retinal explants from all four quadrants were cultured in lysine-laminin coated dish for 36 hours either with mock transfected COS conditioned medium (A) or *draxin* transfected COS conditioned medium (B). (A) Profuse outgrowth of neurites from explants cultured with mock transfected COS conditioned media wee observed. (B) draxin strongly inhibits neurite outgrowth from explants of all retinal quadrants..

in anterior location of the correct termination zone (Figs. 16B- D) . The ectopic termination zones in this case were not in the lateral part of the correct termination zone.

When axons near the ventro-temporal region of the retina were labeled, wild-type mice always showed a single arborization near the anterior-medial end of the Superior colliculas (Fig. 17B). *draxin* knock out mice showed an apparently normal arborization near the anterior end of the Superior colliculas, and in approximately two third of the mutants there were multiple additional arborization in posterior locations of the correct termination zone (Fig. 17B) (12 of 20 mice). No comparable phenotypes of either temporal or nasal axons could be detected in *draxin* heterozygous (0 of 20 mice), although we cannot rule out more subtle defects that would not be seen by the methods used here.

When axons near the dorsal extreme of the retina were labeled, wild-type and *draxin* knock out mice always showed a single arborization near the lateral end of the superior colliculas (Figs. 18A, B). 35 *draxin* knock out mice were checked and no mice showed any abnormal projections, although we cannot rule out more subtle defects that would not be seen by the methods used here.

4. Discussion

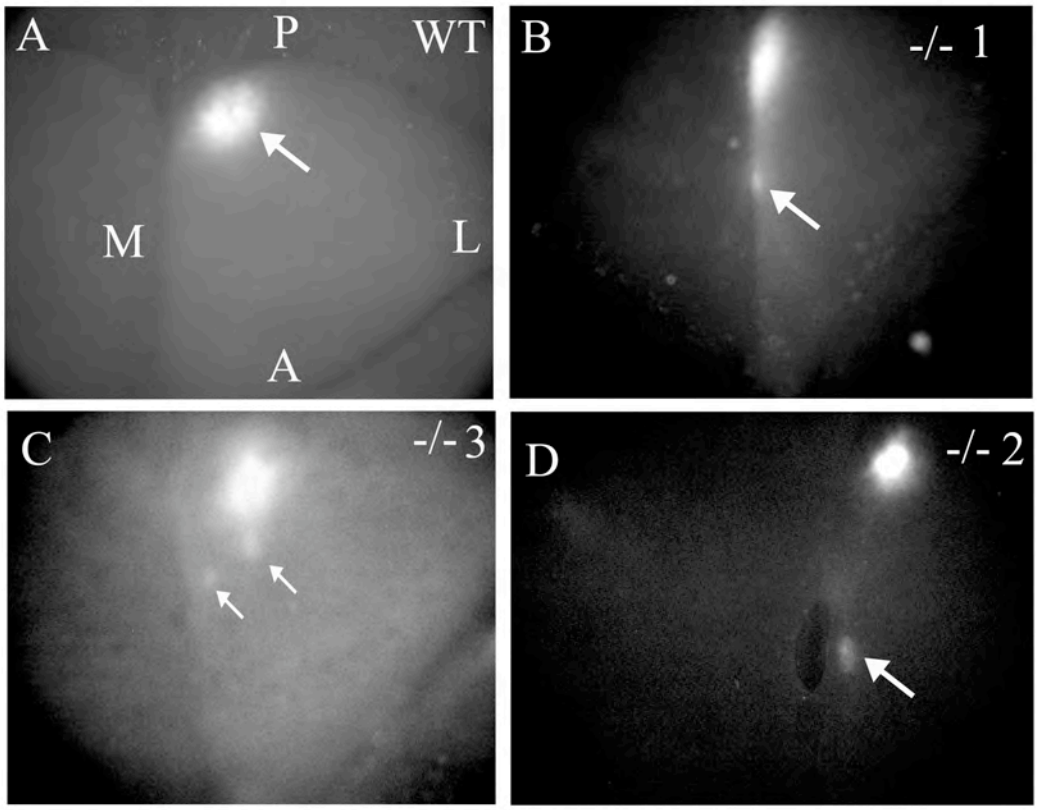
Correct wiring of the nervous system depends on the ability of axons to recognize

their appropriate targets. To help them find their way in the developing embryo, axons are tipped with the growth cones. Extracellular guidance cues can either attract or repel growth cones, and can operate either at close range or over a distance. In this study, we report the possible function of draxin, a repulsive axon guidance protein, in ventrally directed tectum axon projection in embryonic chick and mouse retinocollicular projection. Draxin is a chemorepulsive axon guidance molecule required for the development of spinal cord and forebrain commissures (Islam *et al*., 2009). Draxin inhibited neurite outgrowth from dorsal tectum explants *in vitro*. *In vivo* ectopic expression of draxin also caused misrouting of tectum axons. Draxin also inhibited axon outgrowth from mouse retinal explants. *draxin* knock out mice shows mapping defect along the medio-lateral and anterior-posterior axis.

***draxin* is expressed in chick optic tectum**

Roof-plate-mediated repulsion of ventrally projecting axons is reported in spinal cord commissural axons (Butler and Dodd, 2003). Repulsive guidance proteins from the roof plate prevent the axons from crossing the dorsal midline and guide them in the ventral direction. The chick mesencephalic V neuron axon tract and tectobulbar tract start to project ventrally beginning at stage 14 and stage 18 respectively and this ventral course might be guided by repulsive molecules from the roof plate. During the early

Fig 15



Nasal retinal labelling

Fig. 15. Nasal retinal axons are misprojected in *draxin* knock out mice. DiI were added to the nasal retina of wild type and *draxin* knock out mice and after two days superior colliculas were labelled (A, B, C, D). Wild type mice shows single termination zone (A). Ectopic termination zone along with normal one were observed in several *draxin* knock out mice (B, C, D).

development of the embryonic tectum, *draxin* mRNA begins to be expressed from stage 10 before the onset of mesencephalic V neuron and tectobulbar axon projection. Through the course of development this expression expands in the ventral direction with a decreasing gradient. Section *in situ* hybridization revealed that *draxin* is expressed by the dorsal midline from stage 16 to 22, when the initial ventral trajectory of the tectobulbar axons begins, and when it reaches the floor plate. *draxin* expression in the dorsal midline disappears at stage 25, when the tectobulbar and mesencephalic V neuron tract have already been formed. However, the dorsal high to ventral low gradient of *draxin* expression in the tectum persists until embryonic day 11. Repellent signal(s) from the roof plate might act to direct pioneer tectum axons ventrally. Expression of *draxin* near the roof plate from stage 10 and its repulsive activity on dorsal tectum axons make it a potential candidate as a repellent molecule from roof plate of chick tectum.

Although *draxin* mRNA was expressed in the tectum earlier than axonal growth of mesencephalic V neurons, we could first detect the protein deposited in the most outer layer of the tectum at stage 18. However, since draxin is secreted after synthesis and diffuses away from the expressing cells in the dorsal midline region (Islam *et al.,* 2009), it seems to be possible to regulate the axonal growth of mesencephalic V and

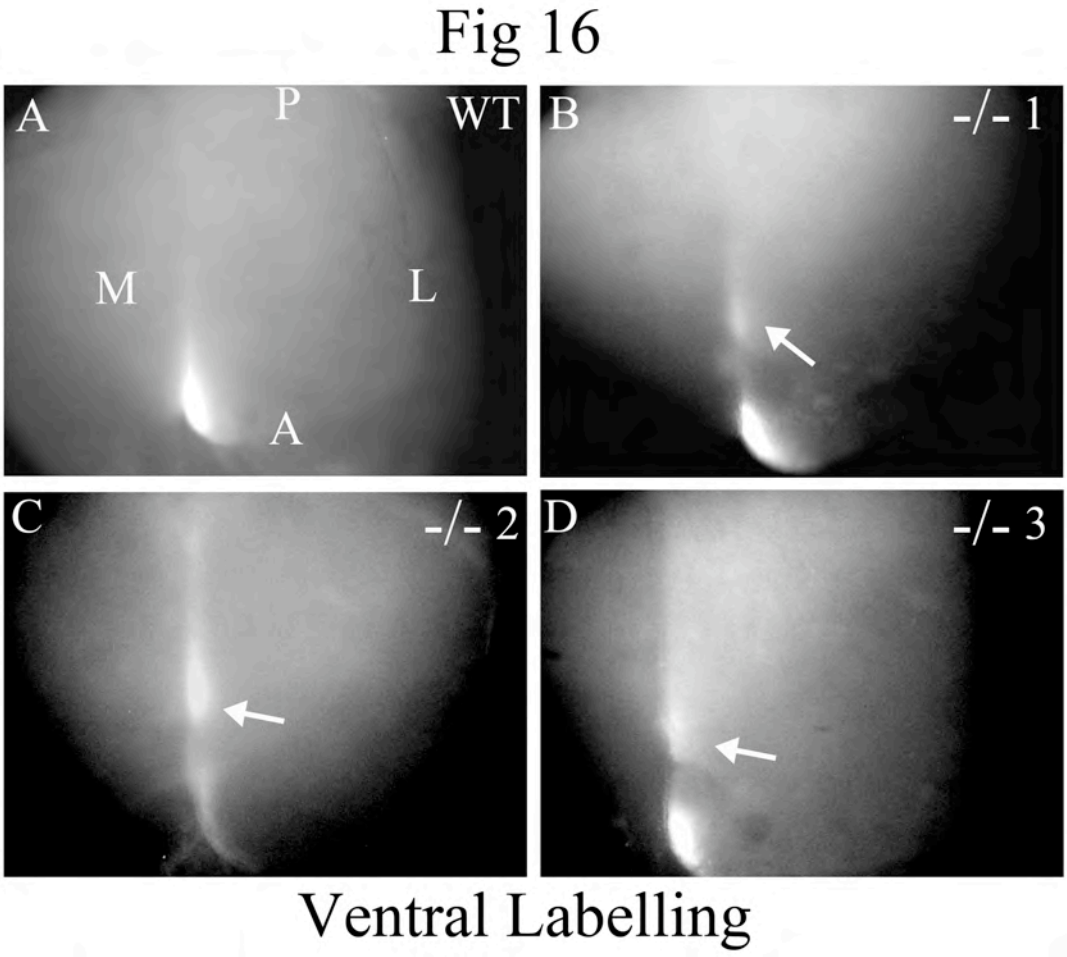


Fig. 16. Ventral retinal axons also misprojected in superior colliculas. When ventral retinal axons were labelled with DiI correct termination zone in the medial part of the superior colliculas always formed in wild type mouse (A). An ectopic termination zone along with the correct one were observed in case of draxin knock out mouse (B, C, D).

tectobulbar neurons toward the ventral directions.

One important question is whether draxin is synthesized by the neuronal cells that extend the tectum axons. Localization of *draxin* mRNA by *in situ* hybridization and staining for the neuronal cell marker Hu in the chick tectum at stage 20 shows that draxin is not synthesized by neuronal cells. Furthermore draxin protein expression does not overlap with the β -tubulin marker Tuj1 in the tectum. Some populations of Cajal-Retzius cells are positive for draxin protein. In mouse, spinal cord, corpus callosum, hippocampal, anterior commissural axons expressed draxin (Islam *et al*., 2009). We could not detect draxin protein in the tectum axons until stage 22.

Sensitivity of tectal neurons to draxin is different along the dorso-ventral axis

The expression of *draxin* mRNA in the tectum of chick embryos suggests that this molecule may be a likely candidate for the signal(s) that determine the initial ventral trajectory of the tectum axon tract. Our results demonstrate that draxin effectively inhibits neurite outgrowth from tectum explants in culture. The strong inhibition of neurite outgrowth that we observed may be due to a higher concentration of draxin in the conditioned medium than would be present *in vivo*. A co-culture experiment was performed to determine whether a point source of minimal concentration of draxin can repel tectum neurites. In these co-culture experiments, we have shown that only neurites

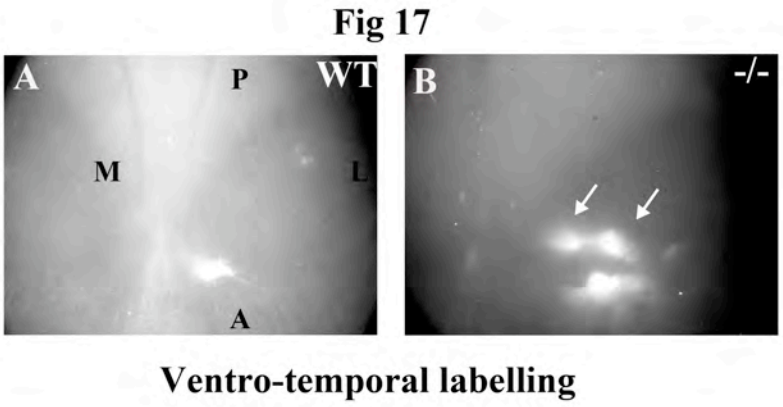


Fig. 17. Ventro-temporal retinal axons were labelled with DiI. A compact termination zone were observed in case of wild type mice (A), but multiple ectopic termination zone posterior to the correct one were observed in *draxin* knock out mice (B).

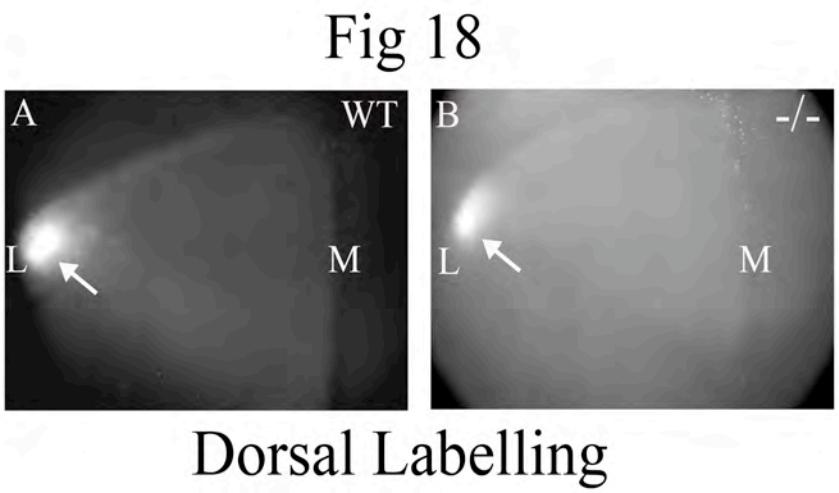


Fig. 18. Dorsal retinal labelling did not show any defect in *draxin* knock out mice. Both wild type (A) and *draxin* knock out (B) mice showed a compact termination zone at the lateral side of the superior colliculas.

from dorsal but not from ventral tectum were repelled by the source of draxin protein. Pioneer mesencephalic V neuron and tectobulbar axons began to project from the dorsal part of the tectum at stage 14 and stage 18 respectively. *In vivo* these dorsal neurons might be influenced by the presence of draxin protein in the dorsal midline and stop growth in the dorsal direction. Ventral neurons project axons later than dorsal neurons, and they may follow the dorsal neuronal axons, which are already oriented to grow in the ventral direction. Draxin binds directly to the neurites in order to exert its effect. This was confirmed by the binding of draxin-AP protein to the dorsal tectum neurites *in vitro,* indicating that draxin binding sites are present on these neurites. The difference in responsiveness of dorsal and ventral tectum neurites to draxin is paralleled by the preferential binding of draxin-AP to the neurites exiting from the dorsal tectum. *In vitro* experiments suggest that draxin repels tectum axons and thus might be involved in tectum axon patterning.

Tectum axons are sensitive to draxin *in vivo*

Our gain-of-function study demonstrates that tectum axons are repelled and misrouted by ectopic draxin expression. Mesencephalic V neurons project their axons ventrally at stage 14. Overexpression of membrane bound form draxin at stage 10 almost completely stopped axonogenesis at stage 17 before the tectobulbar axon

projection begins. This result implies that mesencephalic V neurons are sensitive to draxin. Overexpression of draxin at stage 16, also inhibited axon growth from the tectum axons indicated that mesencephalic V neurons as well as tectobulbar neurons were inhibited to grow their axons by ectopic draxin. The mechanism of axon growth inhibition by draxin is not clear. Overexpression cause abundant amount of draxin in the surrounding environment of the neurons might be resulting in an unfavorable condition to grow their axons in any direction.

Another experiment was designed to examine the effect of excess draxin on the pioneer tectum axons. *draxin* were overexpressed by electroporation in the ventral tectum at stage 16. Ectopic draxin protein was assumed to be synthesized before the first tectum axon reached the ventral tectum. When tectum axons encountered the ectopic draxin, they either were stopped or misrouted. This effect was stronger with the membrane-bound form of *draxin* because draxin was anchored on the cells, ensuring a high local concentration. The secreted and native form of draxin caused partial defects in axon projection, presumably because a large amount of ectopic draxin diffused away from the electroporated site. Although many axons were either stopped or misrouted by ectopic draxin, some axons passed through the overexpressed site might be before significant amount of draxin was produced or these axons were not sensitive to draxin.

Upon encountering ectopic draxin, the stopping and misrouting behavior of pioneer tectum axons strongly suggest the physiological role of draxin in the dorsal midline to direct the initial ventral course of tectum axons by repulsion.

For loss-of-function analyses we used siRNA approach. We had tried with two different constructs of siRNA for several times but were unsuccessful to find any difference in the ventrally directed growth of tectum axons. This might be due to the presence of some other molecular mechanism to play the same function with draxin or siRNA could not reduce the expression to a certain state to show any phenotype *in vivo*. These siRNA were tested on cultured cell lines and were found to be effective.

Floor-plate-mediated attraction of ventrally projecting axons is a major contributor to patterning of these axons. However, proteins that mediate this attraction appear to exert their influence either at short distances or in direct contact (Tessier-Lavigne and Goodman, 1996; Stoeckli and Landmesser, 1995). Therefore it is unlikely that tectum axons are guided by these proteins at a considerable distance from and parallel to the midline cells. Roof-plate-mediated repulsion of these axons can explain the precise trajectory they form without the attractive signals from the floor plate. Because *draxin* mRNA is expressed in the roof plate, and because *in vitro* and *in vivo* experiments suggest that draxin is repulsive for tectum axons, it may play an

important role in the formation of the ventrally directed tectum axon tract.

Draxin was involved in the retinotectal projection.

***draxin* is expressed in the superior colliculas and retina of the mouse**

Molecules that are involved in retinocollicular topographic map formation are expressed in the superior colliculas in a gradient manner. This gradient is important to explain the topographic nature of the map by differential responsiveness of the specific population of retinal ganglion cells to specific concentration of ligand in the superior colliculas. Ephrin A2 and ephrin A5 are expressed in a low-anterrior-to-high-posterior gradient in chick and mouse midbrains (Cheng *et al*. 1995, Zhang *et al*. 1996, Brennan *et al*. 1997, Marin *et al*. 2001 Drescher *et al*. 1995, Monschau *et al*. 1997).Their receptor Eph A is also expressed in gradient in retina. In the medio-lateral axis ephrin B1, is expressed in a high-medial-to-low-lateral gradient in the mouse superior colliculas (Braisted *et al* 1997, and Hindges *et al*. 2002) and their receptors Eph B2, B3, B4 are each expressed in high-ventral-to-low-dorsal gradient in eye (Holash & Pasquale 1995, Henkemeyer *et al*. 1996, Connor *et al*. 1998, Birgbauer *et al*. 2000, Hindges *et al*. 2002). Another molecule Wnt 3 is expressed in medial-high-to-lateral-low gradient in mouse superior colliculas (Schmitt *et al* 2005). *draxin* is expressed in medial-high-to-lateral-low gradient in mouse superior colliculas. At P0 we have

checked the expression. Detail expression analyses should be carried out to know the expression in different developmental stage. In eye *draxin* was also expressed but no obvious gradient was observed. Gradient expression of *draxin* induces us to study retinocollicular projection.

As draxin is a secreted molecule it must exert its effect through receptor molecule(s). We found draxin receptor and gives it alias name, as Receptor E. I will analyze the expression pattern of the receptor in mice.

Several molecules are characterized as modulators of retinocollicular projection like Eph receptors, ephrin ligand, Wnt, Ryk. Expression of These molecules in *draxin* knock out mice is important concern. I plan to analyze the expression of these known molecules in *draxin* knock out mice.

Draxin inhibits axon outgrowth from retinal explants

The dominant model for explaining retinocollicular map development has been the chemoaffinty hypothesis proposed by sperry. He postulated that retinal cells carry stable positional chemical labels, or chemoaffinity tags, that determine their proper tectal termination. Retinal ganglion cell axons from a specific position of the eye project to a specific location in the superior colliculas by the help of the molecules that either repel or attract them to their correct target. Ephrin A ligands repulse temporal retinal

axons that express high level of Eph A receptors (Feldheim *et. al.* 2000). This repulsion determines the position of termination zone of a RGC axon expressing certain level of Eph A receptors along the anterior-posterior axis. On the other hand, along the medio-lateral axis high concentration of ephrin B ligands in the medial superior colliculas attract Eph B expressing ventral retinal axons and that of Wnt 3 repels Ryk expressing dorsal retinal axons (Schmitt *et al* 2005). Draxin conditioned medium inhibited axon outgrowth from mouse retinal explants. Explants from all quadrants are inhibited by draxin conditioned medium might be due to the high concentration of draxin in the medium. Stripe assay should be done to find out positional specificity of draxin sensitivity. Retinal explants from different locations of the knock out mouse of the draxin receptor will also be checked for their sensitivity to mouse draxin.

Loss-of-function analysis shows endogenous draxin is required for proper topographic guidance *in vivo*

Loss-of-function analyses in different known molecules showed different phenotypes. Ephrin A2 and ephrin A5 knock out mice showed defect along the anterior-posterior axis of the superior colliculas (Feldheim *et. al* 2000). Due to loss of the repulsion activity of ephrin A2 and ephrin A5 in the posterior superior colliculas temporal retinal axons showed several ectopic termination zone in the posterior superior

colliculas (Feldheim *et. al* 2000). In the medio-lateral axes loss of attractive function of ephrin B2 and ephrin B3 led to formation of ectopic termination zone in the lateral superior colliculas when ventral retinal axons were labelled (Hindges *et al* 2002). The expression of *draxin* in high medial to low lateral gradient initially led to the idea that this graded draxin protein may act as topographically specific labels in the superior colliculas. Here, we demonstrated that gene targeting of the *draxin* in mouse results in topographically specific mapping errors in nasal, ventral and ventro-temporal axons. Since draxin inhibited axon outgrowth from retinal explant, loss of draxin inhibition/repulsion function in the medial superior colliculas led to formation of ectopic termination zone in the medial part of the superior colliculas when nasal and ventral retina were labelled. In case of ventro-temporal retinal labelling multiple ectopic termination zones were formed in the posterior side of the correct termination zone in the superior colliculas. Dorsal retinal labelling showed no defect in *draxin* knock out mice. As lateral superior colliculas express low level of draxin, dorsal retinal axons project to the lateral superior colliculas correctly. All these speculations are very preliminary about draxin function; extensive study should be carried out to find the possible mechanism of draxin function in retinocollicular projection. Major focus would be on the identification of functional draxin receptor. Projection pattern in optic chiasm,

LGN and branching of retinal axons in the superior colliculas should be checked. Expression of other guidance molecules in *draxin* knock out mice should be checked carefully. Positional specificity of retina towards draxin repulsion *in vitro* is a major concern to be answered.

5. Conclusion

Ventrally directed tectum axons are guided in parts by draxin in chick embryo and retinocollicular projection is disrupted in *draxin* knock out mice. Analyses of *draxin* knock out mice hopefully will reveal the function of draxin in the formation of retinocollicular projections. Draxin is a new axon guidance molecule that plays critical role in the early development of chick and mammalian brain.

6. References

Alcántara, S., Ruiz, M., D'Arcangelo, G., Ezan, F., Lecea, L., Curran, T., Sotelo, C., and Soriano, E., 1998. Regional and Cellular Patterns of *reelin* mRNA Expression in the Forebrain of the Developing and Adult Mouse. J. Neurosci 18, 7779–7799.

Augsburger, A., Schuchardt, A., Hoskins, S., Dodd, J., Butler, S., 1999. BMPs as mediators of roof plate repulsion of commissural neurons. Neuron 24, 127–141.

Battye, R., Stevens, A., Jacobs, J. R., 1999. Axon repulsion from the midline of the Drosophila CNS requires slit function. Development. 126, 2475-24781.

Birgbauer, E., Cowan, C. A., Sretavan, D. W., Henkemeyer, M., 2000. Kinase independent function of EphB receptors in retinal axon pathfinding to the optic disc from dorsal but not ventral retina. Development. 127, 1231-1241.

Bourikas, D., Pekarik, V., Baeriswyl, T., Grunditz, A., Sadhu, R., Nardó, M., Stoeckli, E. T., 2005. Sonic hedgehog guides commissural axons along the longitudinal axis of the spinal cord. Nat Neurosci. 8, 297-304.

Braisted, J. E., McLaughlin, T., Wang, H. U., Friedman, G. C., Anderson, D. J., O'leary, D. D., 1997. Graded and lamina-specific distributions of ligands of EphB receptor tyrosine kinases in the developing retinotectal system. Dev Biol. 191, 14-28.

Braisted, J. E., Catalano, S. M., Stimac, R., Kennedy, T. E., Tessier-Lavigne, M., Shatz, C. J., O'Leary, D. D., 2000. Netrin-1 promotes thalamic axon growth and is required for proper development of the thalamocortical projection. J Neurosci. 20, 5792-5801.

Brennan, C., Monschau, B., Lindberg, R., Guthrie, B., Drescher, U., Bonhoeffer, F., Holder, N., 1997. Two Eph receptor tyrosine kinase ligands control axon growth and may be involved in the creation of the retinotectal map in the zebrafish. Development. 124, 655–664.

Brose, K., Bland, K. S., Wang, K. H., Arnott, D., Henzel, W., Goodman, C. S., Tessier-Lavigne, M., Kidd, T., 1999. Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. Cell. 96, 795-806.

Brose, K., Tessier-Lavigne, M., 2000. Slit proteins: key regulators of axon guidance, axonal branching, and cell migration. Curr Opin Neurobiol. 10, 95-102.

Butler, S., Dodd, J., 2003. A Role for BMP Heterodimers in Roof Plate-Mediated Repulsion of Commissural Axons. Neuron 38, 389–402.

Charron, F., Stein, E., Jeong, J., McMahon, A. P., Tessier-Lavigne, M., 2003. The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. Cell. 113, 11-23.

Chédotal, A., Pourquié, O., Sotelo, C., 1995. Initial Tract Formation in the Brain of the Chick Embryo: Selective Expression of the BEN/SC1/DM-GRASP Cell Adhesion Molecule. Euro. J. Neurosci 7, 198-212.

Cheng, H. J., Nakamoto, M., Bergemann, A. D., Flanagan, J. G., 1995. Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. Cell. 82, 371-381.

Cheng, H. J., Bagri, A., Yaron, A., Stein, E., Pleasure, S. J., Tessier-Lavigne, M., 2001. Plexin-A3 mediates semaphorin signaling and regulates the development of hippocampal axonal projections. Neuron. 32, 249-263.

Colamarino, S. A., Tessier-Lavigne, M., 1995. The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. Cell. 81, 621-629.

Connor, R. J., Menzel, P., Pasquale, E. B., 1998. Expression and tyrosine phosphorylation of Eph receptors suggest multiple mechanisms in patterning of the visual system. Dev Biol. 193, 21-35.

Deiner, M. S., Kennedy, T. E., Fazeli, A., Serafini, T., Tessier-Lavigne, M., Sretavan, D. W., 1997. Netrin-1 and DCC mediate axon guidance locally at the optic disc: loss of function leads to optic nerve hypoplasia. Neuron. 19, 575-589.

Dickson, B. J., 2002. Molecular mechanism of axon guidance. Science 298, 1959-1964.

Drescher, U., Kremoser, C., Handwerker, C., Loschinger, J., Noda, M., Bonhoeffer F. 1995. In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. Cell. 82, 359–370.

Feldheim, D. A., Kim, Y. I., Bergemann, A. D., Frisen, J., Barbacid, M., Flanagan, J. G., 2000. Genetic analysis of ephrin-A2 and ephrin-A5 shows their requirement in multiple aspects of retinocollicular mapping. Neuron. 25, 563–574.

Go, M. J., Tanaka, H., Obata, K., Fujita, S. C., 1989. Developmental changes in phosphorylation state of neurofilament proteins in the chick embryonic optic nerve. Dev. Biol. 134, 85-102.

Hamburger, V., Hamilton, H. L., 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88, 49-92.

Henke-Fahle, S., Beck, K. W., Püschel, A. W., 2001. Differential responsiveness to the chemorepellent semaphorin 3A distinguishes Ipsi- and contralaterally projecting axons in the chick midbrain. Dev. Biol. 237, 381-397.

Henkemeyer, M., Orioli, D., Henderson. J. T., Saxton, T. M., Roder, J., Pawson, T., Klein, R., 1996. Nuk controls pathfinding of commissural axons in the mammalian central nervous system. Cell. 86, 35-46.

Hindges, R., McLaughlin, T., Genoud, N., Henkemeyer, M., O'Leary, D. D., 2002. EphB forward signaling controls directional branch extension and arborization required for dorsal-ventral retinotopic mapping. Neuron. 35, 475-487.

Holash, J. A., Pasquale, E. B., 1995. Polarized expression of the receptor protein tyrosine kinase Cek5 in the developing avian visual system. Dev Biol. 172, 683-693.

Hong, K., Hinck, L., Nishiyama, M., Poo, M. M., Tessier-Lavigne, M., Stein, E., 1999. A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. Cell. 97, 927-941.

Hunter, E., Begbie, J., Mason, I., Graham, A., 2001. Early development of the mesencephalic trigeminal nucleus. Dev. Dyn. 222, 484-493.

Imondi, R., Wideman, C., Kaprielian, Z., 2000. Complementary expression of transmembrane ephrins and their receptors in the mouse spinal cord: a possible role in constraining the orientation of longitudinally projecting axons. Development. 127, 1397-1410.

Islam, S. M., Shinmyo, Y., Okafuji, T., Su, Y., Naser, I. B., Ahmed, G., Zhang, S., Chen, S., Ohta, K., Kiyonari, H., Abe, T., Tanaka, S., Nishinakamura, R., Terashima, T., Kitamura, T., Tanaka, H., 2009. Draxin, a repulsive guidance protein for spinal cord and forebrain commissures. Science. 323, 388-393.

Jin, Z., Zhang, J., Klar, A., Chédotal, A., Rao, Y., Cepko, C. L., Bao, Z. Z., 2003. Irx4-mediated regulation of Slit1 expression contributes to the definition of early axonal paths inside the retina. Development. 130, 1037-1048.

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E. D., Chan, S. S., Culotti, J. G., Tessier-Lavigne, M., 1996. Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. Cell. 87, 175-185.

Kennedy, T. E., Serafini, T., de la Torre J. R., Tessier-Lavigne M., 1994. Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell. 78, 425-435.

Kidd, T., Bland, K. S., Goodman, C. S., 1999. Slit is the midline repellent for the robo receptor in Drosophila. Cell. 96, 785-794.

Klagsbrun, M., Eichmann, A., 2005. A role for axon guidance receptors and ligands in blood vessel development and tumor angiogenesis. Cytokine Growth Factor Rev. 16, 535-548.

Kröger, S., Schwarz, U., 1990. The avian tectobulbar tract: Development, explant culture, and effects of antibodies on the pattern of neurite outgrowth. J. Neurosci 10, 3118–3134.

Kullander, K., Klein, R., 2002. Mechanisms and functions of Eph and ephrin signalling. Nat Rev Mol Cell Biol. 3, 475-486.

Luo, Y., Raible, D., Raper, J. A., 1993. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell. 75, 217-227.

Ly, A., Nikolaev, A., Suresh, G., Zheng, Y., Tessier-Lavigne, M., Stein, E., 2008. DSCAM is a netrin receptor that collaborates with DCC in mediating turning responses to netrin-1. Cell. 133, 1241-1254.

MacLennan, A. J., McLaurin, D. L., Marks, L., Vinson, E. N., Pfeifer, M., Szulc, S. V., Heaton, M. B., Lee, N., 1997. Immunohistochemical localization of netrin-1 in the embryonic chick nervous system. J. Neurosci 17, 5466–5479.

Marin, O., Blanco, M. J., Nieto, M. A., 2001. Differential expression of Eph receptors and ephrins correlates with the formation of topographic projections in primary and secondary visual circuits of the embryonic chick forebrain. Dev. Biol. 234, 289–303.

Métin, C., Deléglise, D., Serafini, T., Kennedy, T. E., Tessier-Lavigne, M., 1997. A role for netrin-1 in the guidance of cortical efferents. Development. 124, 5063-5074.

O'Leary, D. D., Wilkinson, D. G., 1999. Eph receptors and ephrins in neural development. Curr Opin Neurobiol. 9, 65-73.

Monschau, B., Kremoser, C., Ohta, K., Tanaka, H., Kaneko, T., Yamada, T., Handwerker, C., Hornberger, M. R., Löschinger, J., Pasquale, E. B., Siever, D. A., Verderame, M. F., Müller, B. K., Bonhoeffer, F., Drescher, U., 1997. Shared and distinct functions of RAGS and ELF-1 in guiding retinal axons. EMBO J. 16, 1258-1267.

Ohta, K., Tannahill, D., Yoshida, K., Johnson, A. R., Cook, G. M., Keynes, R. J., 1999. Embryonic lens repels retinal ganglion cell axons. Dev. Biol. 211, 124–132.

Plas, D. T., Dhande, O. S., Lopez, J. E., Murali, D., Thaller, C., Henkemeyer, M., Furuta, Y., Overbeek, P., Crair, M. C., 2008. Bone morphogenetic proteins, eye patterning, and retinocollicular map formation in the mouse. J Neurosci. 28, 7057-7067.

Plump, A. S., Erskine, L., Sabatier, C., Brose, K., Epstein, C. J., Goodman, C. S., Mason,

C. A., Tessier-Lavigne, M., 2002. Slit1 and Slit2 cooperate to prevent premature midline crossing of retinal axons in the mouse visual system. Neuron. 33, 219-232.

Poliakov, A., Cotrina, M., Wilkinson, D. G., 2004. Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. Dev Cell. 7, 465-480.

Rajagopalan, S., Deitinghoff, L., Davis, D., Conrad, S., Skutella, T., Chedotal, A., Mueller, B. K., Strittmatter, S. M., 2004. Neogenin mediates the action of repulsive guidance molecule. Nat Cell Biol. 6, 756-762.

Sakurai, T., Wong, E., Drescher, U., Tanaka, H., Jay, D. G., 2002. Ephrin-A5 restricts topographically specific arborization in the chick retinotectal projection in vivo. Proc Natl Acad Sci U S A. 99, 10795-10800.

Schaeren-Wiemers, N., Gerfin-Moser, A., 1993. A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. Histochemistry 100, 431–440.

Schmitt, A. M., Shi, J., Wolf, A. M., Lu, C. C., King, L. A., Zou, Y., 2006. Wnt-Ryk signalling mediates medial-lateral retinotectal topographic mapping. Nature. 439, 31-37.

Serafini, T., Kennedy, T. E., Galko, M. J., Mirzayan, C., Jessell T. M., Tessier-Lavigne M., 1994. The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. Cell. 78, 409-424.

Serafini, T., Colamarino, S. A., Leonardo, E. D., Wang, H., Beddington, R., Skarnes, W. C., Tessier-Lavigne M., 1996. Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell. 87, 1001-1014.

Shirasaki, R., Tamada, A., Katsumata, R., Murakami, F., 1995. Guidance of cerebellofugal axons in the rat embryo: directed growth toward the floor plate and subsequent elongation along the longitudinal axis. Neuron 14, 961–972.

Shu, T., Sundaresan, V., McCarthy, M. M., Richards, L. J., 2003. Slit2 guides both precrossing and postcrossing callosal axons at the midline in vivo. J Neurosci. 23, 8176-8184.

Stoeckli, E. T., and Landmesser, L. T., 1995. Axonin-1, Nr-CAM, and Ng-CAM play different roles in the *in vivo* guidance of chick commissural neurons. Neuron 14, 1165–1179.

Tamada, A., Shirasaki, R., Murakami, F., 1995. Floor plate attracts crossed axons and chemorepels uncrossed axons in the vertebrate brain. Neuron 14, 1083–1093.

Tanaka, H., Matsui, T., Agata, A., Tomura, M., Kubota, I., McFarland, K. C., Kohr, B., Lee, A., Phillips, H. S., Shelton, D. L., 1991. Molecular cloning and expression of a novel adhesion molecule, SC1. Neuron 7, 535-545.

Tessier-Lavigne, M., 1994. Axon guidance by diffusible repellants and attractants. Curr. Opin. Genet. Dev. 4, 596–601.

Tessier-Lavigne, M., and Goodman, C. S. (1996). The molecular biology of axon guidance. Science 274, 1123-1133.

Wong, J. T., Wong, S. T., O'Connor, T. P., 1999. Ectopic semaphorin-1a functions as an attractive guidance cue for developing peripheral neurons. Nat Neurosci. 2, 798-803.

Yagi, T., Tokunaga, T., Furuta, Y., Nada, S., Yoshida, M., Tsukada, T., Saga, Y., Takeda, N., Ikawa, Y., Aizawa, S. 1993. A novel ES cell line, TT2, with high germline-differentiating potency. Anal Biochem. 214(1), 70-76.

Zhang, J. H., Cerretti, D. P., Yu, T., Flanagan, J. G., Zhou, R., 1996. Detection of ligands in regions anatomically connected to neurons expressing the Eph receptor Bsk: potential roles in neuron-target interaction. J Neurosci. 16, 7182-7192.

Zou, Y., Stoeckli, E., Chen, H., Tessier-Lavigne, M., 2000. Squeezing axons out of the gray matter: a role for slit and semaphorin proteins from midline and ventral spinal cord. Cell. 102, 363-375.