Olfactory bulb axonal outgrowth is inhibited by draxin

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ABSTRACT

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. These results suggest that draxin functions as an inhibitory guidance cue for OB axons and indicate its contribution to the formation of the LOT.

Keywords: Draxin, olfactory bulb, axonal outgrowth, LOT, repulsive guidance cue

1. Introduction

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. The axons of ORNs then relay the information to the main olfactory bulb (MOB) where they synapse with the dendrites of mitral and tufted cells. The axons of these MOB neurons convey the olfactory information ipsilaterally to the higher order of olfactory structures in the brain [1, 2, 3, 4]. The OB axons form the lateral olfactory tract (LOT), which lies immediately beneath the pial surface. 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 [5, 6]. 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* [7, 8, 9]. 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 [10, 11]. This suggests that other molecules with redundant function may exist either in the cortex or in the septum of these mutants and may regulate the development of the LOT rostrocaudally.

We recently reported [12] a chemorepulsive axon guidance molecule, draxin, which is expressed strongly in various parts of the brain and spinal cord. Draxin inhibits or repels chicken spinal cord commissural and mouse cortical axons *in vitro*. *Draxin* knockout mice showed agenesis of all forebrain commissures. In another recent study, draxin inhibited chicken tectal axonal outgrowth *in vitro* and *in vivo* [13]. The expression of *draxin* in the mouse neocortex and septum from early developmental to postnatal stages suggests that draxin might have a role in the formation of the LOT. Here, we explored this possibility by checking the sensitivity of OB axons against draxin *in vitro*. We report here that the

outgrowth of axons emanating from mouse olfactory bulb explants was significantly inhibited by draxin-conditioned medium and by draxin expressing 293 cells. We have shown the presence of draxin receptors in the LOT axons *in vivo* by observing a bound draxin-AP signal in brain sections. Although the LOT in *draxin-/-* mice was able to reach the telencephalon, we observed very mild defasciculation within the OB axonal trajectory, especially at the rostral part.

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 [12]. Wild type mice were used for *in vitro* culture experiments. 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 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 [14]. Section immunohistochemistry was performed following a protocol previously described in detail [15]. The following primary antibodies were used for immunostaining: rabbit polyclonal anti-draxin (mouse protein), rat anti-L1 (Chemicon), and mouse anti-neurofilament (2H3) (Hybridoma Bank). For whole-mount immunostaining, embryonic mouse brains were dissected and staining was performed with rabbit polyclonal anti-neuropilin-1 [16], following a protocol that was previously described

in detail [6].

2.3. Preparation of Draxin and Control-alkaline phosphatase conditioned medium

The cDNAs encoding chick draxin-AP and empty AP tag vector [12] were transfected in 293T cells 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.

2.4. Explant Cultures and draxin-AP binding

Explants from E14.5 olfactory bulbs (OB) of wild type mice were dissected as described previously [10] 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.

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 [12]. 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 [17]. 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.

Draxin-AP binding to unfixed brain sections was performed following a protocol described previously [18] using the conditioned medium described above.

3. Results

3.1. Expression pattern of draxin in the cortex and septum

Earlier studies [5, 7, 8, 9, 10, 11] 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 [12]. 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 [19]. Strong *draxin* expression in terms of β-galactosidase staining was observed in the neocortex and septum of *draxin* heterozygous mouse embryos at E13 (Fig. 1A). However, at E17.5, the expression became weaker in the septum while it remained stronger in the neocortex (Fig. 1B). In the mouse neocortex, robust *draxin* expression persists throughout embryogenesis and in 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

[12]. Therefore, we wanted to examine whether draxin protein is also present in the LOT. Since L1 is abundantly expressed in LOT axons [11], we performed double immunostaining with antibodies against draxin and L1. Draxin protein expression was not merged with L1 in the LOT (Fig. 1C 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.

3.2. 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. 2A and C), whereas neurite outgrowth was significantly diminished when the explants were incubated with draxin-AP conditioned medium (Fig. 2B 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. 2D 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. 2E and F). The effect of draxin on the OB axon outgrowth demonstrated here was similar to that observed in our earlier studies with other type of axons [12, 13]. Diffusible axon guidance molecules function through their receptors [20, 21]. 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. 2G and H). 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*.

3.3. 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. 3A 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. These data suggest that although draxin inhibited axonal outgrowth *in vitro,* other molecules with similar functions might compensate for its function *in vivo*.

4. Discussion

It was earlier reported [2] 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 [5]. 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 [8, 9] 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 [10]. 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 [11]. These data indicate that Slits, through their Robo receptors, regulate the precise formation of the LOT *in vivo* [11]. 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 [10, 11]. 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 [10, 11, 22, 23].

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 [6, 24, 25, 26] 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 [11]. Thus, multiple diffusible factors, together with a variety of short-range cues, work in concert to regulate the LOT development *in vivo*. Last, 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*.

In conclusion, we propose a scheme, modified from de Castro et al. [7], to show the expression pattern of diffusible cues that may act together to regulate the development of the LOT (Fig. 3E).

Acknowledgments

We are very grateful to Drs. Tatsumi Hirata and Takahiko Kawasaki for their generous cooperation and for providing the anti-neuropilin-1 antibody. This work was supported by grants-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan

(MEXT), by the 21st Century COE Program and by the Global COE Program (Cell Fate Regulation Research and Education Unit), MEXT, Japan.

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Figure Legends

Fig. 1. 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.

Fig. 2. 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). (G, H) 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 H). Control-AP proteins did not bind to the sections (G). 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, G, and H.

Fig. 3. 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. (E) 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. Scale bars = 500 μm for A-D.

Figure-1
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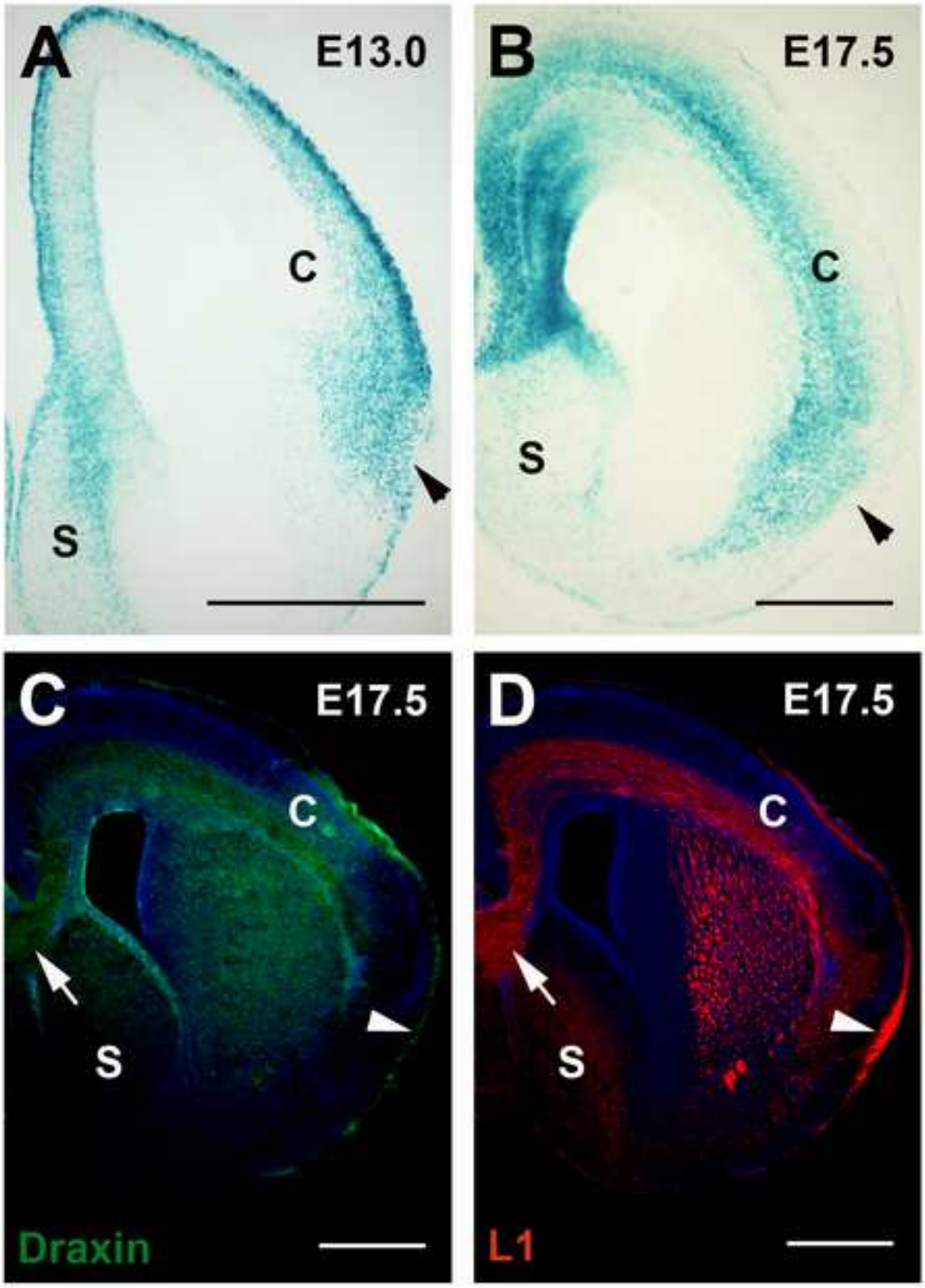
Figure-1



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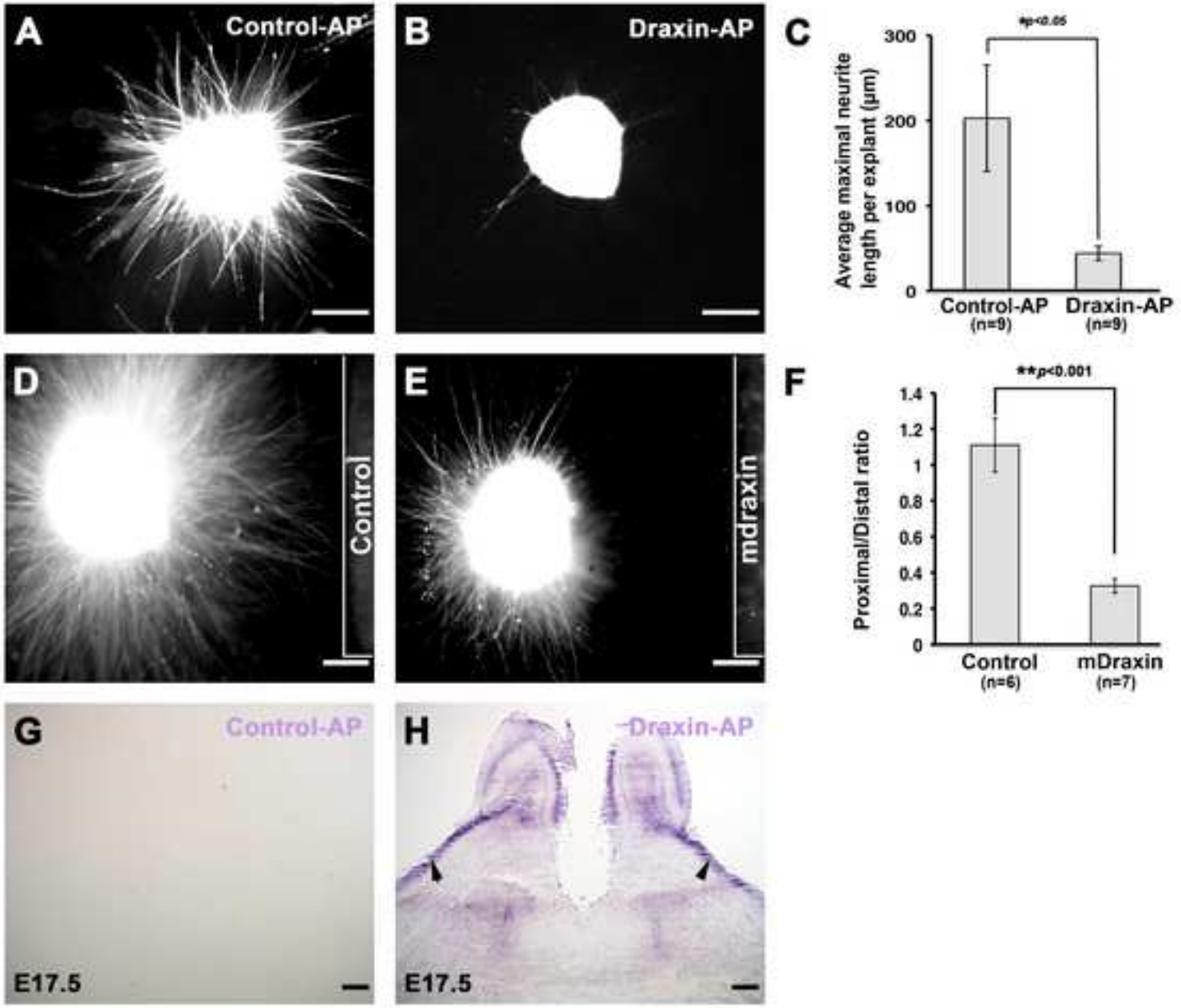

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