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RESEARCH ARTICLE

Draxin Is Involved in the Proper Development of the dI3 Interneuron in Chick Spinal Cord

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Generation of the appropriate types, numbers and distribution of neurons during the development of the nervous system requires the careful coordination of proliferation, differentiation, and patterning. In this work, we analyzed the roles of a repulsive axon guidance protein, draxin, on the development of chick spinal cord dI3 interneuron. *draxin* mRNA and/or protein were detected in the roof plate at first and then the boundary region between the ventricular and the mantle zones in chick spinal cord and dorsal basement membrane of the chick spinal cord. Overexpression of draxin caused the decreased and delayed migration of the dI3 interneuron, the reduction of progenitor cell proliferation, and abnormal localization of some ectopic progenitor-like cells in the mantle zone of the spinal cord. Our data reveal that draxin may be involved in the proper development of the dI3 interneuron in chick spinal cord. *Developmental Dynamics 239:1654–1663, 2010*. © **2010 Wiley-Liss, Inc.**

Key words: draxin; dI3 interneuron; chick; spinal cord; proliferation; migration

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INTRODUCTION

Neurogenesis in the embryonic spinal cord is marked by the emergence of distinct classes of neurons at sharply delineated positions along the dorsoventral (DV) axis of the neural tube (Jessell, 2000). Cell types arising from progenitor domains in the dorsal half of the spinal cord are dedicated to processing sensory signals between the periphery and the brain. The ventral progenitor populations give rise to motor neurons and interneurons essential for the control of posture and locomotion. Roof plate–derived signals establish regional identities in the dorsal neural progenitors of the ventricular zone by inducing the expression of the proneural basic helix loop helix (bHLH) factors Math1 (or its chick homolog Cash1), Ngn1/2 and Mash1 (or its chick homolog Cash1) (Gowan et al., 2001; Timmer et al., 2002). These progenitors differentiate into six classes of early dorsal interneurons (dI1–6). The six populations can be divided into two classes: class A and class B neurons. Class-A neurons, dI1–3, give rise to commissural and other interneurons of the deep dorsal horn (Müller et al., 2002), and their generation is largely roof plate–dependent (Lee et al., 2000; Millonig et al., 2000). The generation of class-B neurons, dI4–6, appears to be roof plate–independent (Gross et al., 2002; Müller et al., 2002).

Interkinetic nuclear migration (INM) is the proliferative cell behavior in which the nuclei of neuroepithelial cells migrate in an apical-to-basal and then basal-to-apical manner. INM has been documented in multiple systems, including the developing spinal cord, cerebral cortex, and retina. Inside these systems, the neural progenitors make cytoplasmic connections with both the apical and the basal sides of the neural epithelium, whereas the position of the nucleus along the apical-basal

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axis varies depending on the stage of the cell cycle (Frade, 2002; Buchman and Tsai, 2008). Following cell-cycle exit, progenitors migrate via nuclear translocation, a mode of cellular migration in which the soma of the neuroepithelial cell moves by way of nuclear migration while maintaining apically and basally directed processes (Ghashghaei et al., 2007). INM may serve as a mechanism for increasing the density of the proliferative pool while still maintaining adherence junctions during mitosis (Frade, 2002). Preservation of the apical and basal processes allows for the maintenance of localized protein signaling complexes, such as the adherence and tight junction-associated protein complexes at the apical end of neuroepithelial cells (Chenn et al., 1998), and distinct signaling complexes, including those associated with focal adhesions at the basal surface (Li and Sakaguchi, 2002; Wozniak et al., 2004). Therefore, the displacement of interphase nuclei throughout the epithelium allows apical-basal localized signaling complexes to be maintained throughout the cell cycle in the densely packed proliferative neuroepithelium (Baye and Link, 2008).

We recently identified a novel secreted repulsive axon guidance molecule that we named draxin, which stands for "dorsal repulsive axon guidance protein" (Islam et al., 2009). Draxin is required for the development of the spinal cord and forebrain commissures (Islam et al., 2009) and may also function as a regulator of chick neural crest migration (Su et al., 2009). In the present study, we localized the draxin protein and mRNA during development of the chick spinal cord dI3 interneuron. Ectopic overexpression of draxin caused the decreased and delayed migration of dI3 interneurons. Furthermore, we found some ectopic progenitor-like cells that localized to the mantle zone of the spinal cord abnormally after the ectopic overexpression of draxin. This might cause a reduction of the progenitor cell pool and cell proliferation. Our data reveal that draxin may be involved in the development of the dI3 interneuron in chick spinal cord.

RESULTS

Draxin Is Expressed in the Roof Plate, Basement Membrane, and Dorsal Part of the Chick Spinal Cord

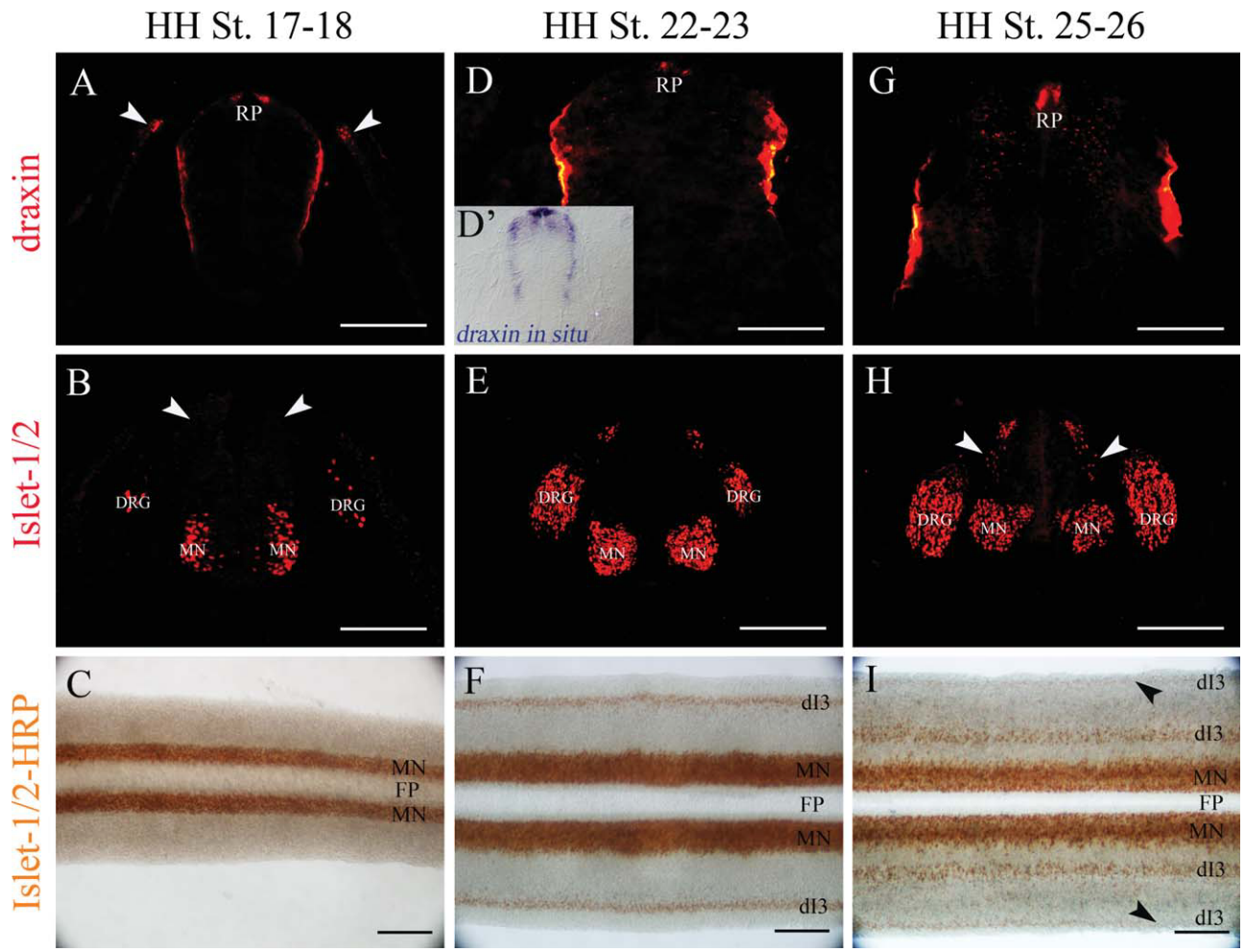
Anti-draxin immunohistochemistry was carried out by transverse section staining (Fig. 1A, D, and G) to examine the distribution pattern of draxin protein in the chick spinal cord. At HH (Hamburger and Hamilton, 1951) stage 17–18 of the chick spinal cord, draxin was detected in the roof plate, the most lateral part of the basement membrane, and the dorsal lip of the dermomyotome (Fig. 1A). At stage 22–23, draxin was similarly detected except for additional strong staining in the dorsolateral basement membrane and absence in the dorsal dermomyotome (Fig. 1D). At the same stage, we performed *draxin* in situ hybridization experiment. The *draxin* mRNA was detected strongly in the roof plate, the boundary region between the ventricular and mantle zones, and weakly in the dorsal part of spinal cord (Fig. 1D′). This result indicated that at this stage some cells in the dorsal central part of the spinal cord had already started to express draxin. At stage 25–26, many cells in the dorsal central part of the spinal cord expressed draxin in addition to the earlier expression (Fig. 1G).

Formation and Migration Pattern of the dI3 Interneuron in the Chick Spinal Cord

Immunostaining of Islet-1/2 at different stages of chick spinal cord development was carried out to show the formation and migration pattern of chick dI3 interneurons by transverse section staining (Fig. 1B, E, and H) and a lateral view of the whole-mount open-book preparation (Fig. 1C, F, and I). The floor plate is located in the middle and the roof plate is located in the upper and lower edge in whole-mount open-book preparation. At stage 17–18, the Islet-1/2-positive dI3 interneuron was not formed in the dorsal part of the spinal cord (white arrowheads in Fig. 1B and C). Many Islet-1/2-positive cells were detected in the DRG and motor neuron column (Fig. 1B and C). At stage 22–23, some dI3 interneurons were formed in the dorsal part of the spinal cord (Fig. 1E). Whole-mount open-book preparation and Islet-1/2-HRP immunostaining showed that the newly formed dI3 interneurons formed a single straight band near the roof plate (Fig. 1F). Many Islet-1/2-positive cells were also detected in the DRG and motor neuron column (Fig. 1E and F). At stage 25–26, many dI3 interneurons were formed in the dorsal part of the spinal cord (Fig. 1H). Some dI3 interneurons started to migrate ventrally (white arrowheads in Fig. 1H). Whole-mount open-book preparation and Islet-1/2-HRP immunostaining showed that the cells in the dorsal spinal cord form a straight band near the roof plate. The migrating dI3 interneurons formed another straight band near the motor neuron column (Fig. 1I). The draxin expression in the dorsal middle part of the spinal cord started at a slightly later stage (stage 22–23). At the same time, the draxin signal became stronger and concentrated to the dorsolateral region of the basement membrane. These changes happen together with the commencement of dI3 interneuron differentiation. From this correlation, we speculate that draxin, especially draxin expressed in the dorsal middle part of the spinal cord and the dorsolateral basement membrane, may be involved in the development of dI3 interneurons.

dI3 Interneurons Can Bind Draxin-AP Protein

To determine whether trunk dI3 interneurons express a draxin receptor, a draxin-AP-binding assay was performed. When sections from the trunk spinal cord region of stage 21–22 chick embryos were incubated with draxin-AP protein, positive signals were detected in the roof plate, dorsal basement membrane, and dorsolateral spinal cord (Fig. 2D). Islet-1/2 immunostaining in the same section showed that almost all the dI3 interneurons could bind draxin-AP protein (Fig. 2E and F). But when control-AP protein was incubated with spinal cord sections, no signal was detected inside the spinal cord (Fig. 2A–C). This result indicates that dI3 interneurons express a draxin receptor and have the ability to respond to draxin.


HH St. 17-18
HH St. 22-23
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draxin
Islet-1/2
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A
RP
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G
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Fig. 1.

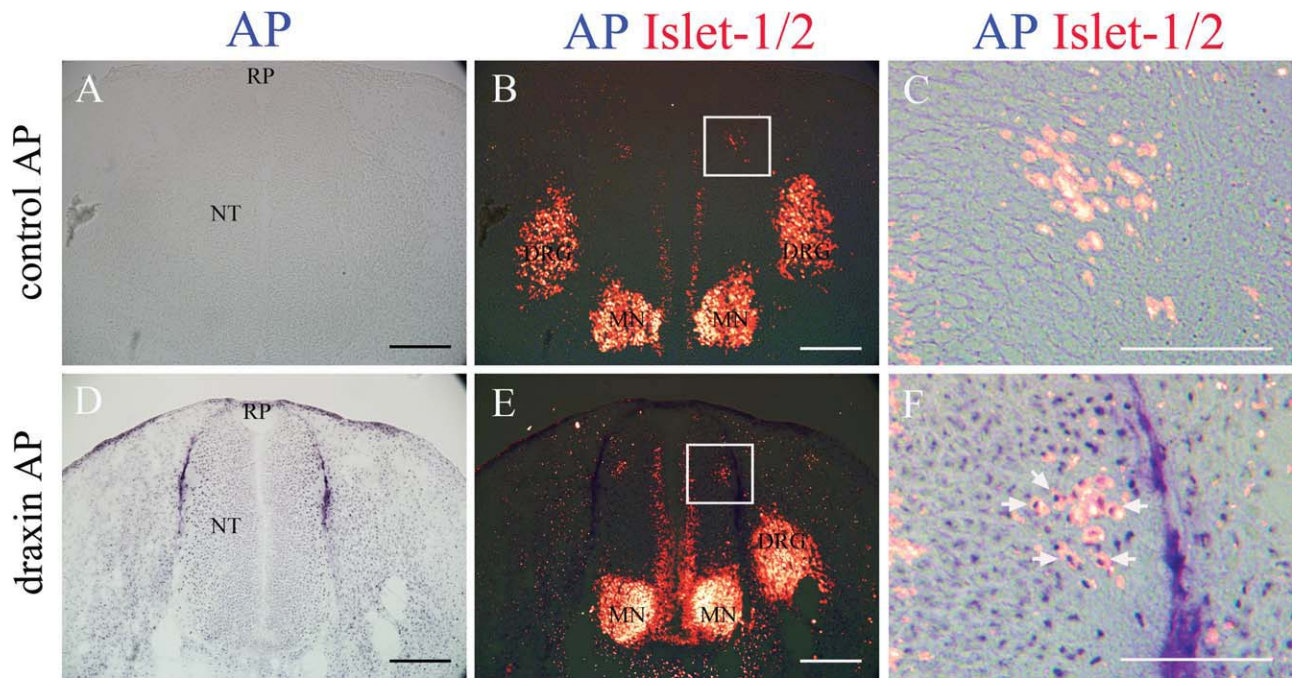
AP
AP Islet-1/2
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Fig. 2.

Overexpression of Draxin Causes a Delay in Migration and a Decreased Number of dI3 Interneurons

To determine the in vivo function of draxin during the development of dI3 interneurons, we overexpressed secreted *draxin* or trans-membrane form *draxin* cDNA in the chick spinal cord by in ovo electroporation. The empty vector was electroporated as the control. Electroporation effectively induced ectopic expression of draxin by the co-induced EGFP (Islam et al., 2009). The trans-membrane form of *draxin* or vector was first overexpressed in chick spinal cord at stage 18–19 and then Islet-1/2 immunostaining was checked at stages 25–26 by transverse section (Fig. 3A, B, D, and E) and by the lateral view of the whole-mount open-book preparation (Fig. 3C and F). When the vector was overexpressed, dI3 interneuron formation and migration were normal in both the control and the electroporated side of the spinal cord (Fig. 3A–C). When the trans-membrane form of draxin was overexpressed, the migration of dI3 interneurons was delayed and the number of dI3 interneurons also seemed to be reduced in the electroporated side compared with the control side (Fig. 3D–F). Whole-mount open-book preparation and Islet-1/2-HRP immunostaining showed clearly that the migration was delayed, so the straight band formed by migrating dI3 interneurons was further toward the motor neuron column compared to the control side (black arrowhead in Fig. 3F). The black arrow (Fig. 3F) shows that some cells were located between the two bands because of the delay in migration. The cell density was also reduced compared with the control side so that the two dI3 interneuron bands were lighter than the two bands in the control side (Fig. 3F).

To get detailed information about the effects of ectopic draxin in the development of dI3 interneurons in the chick spinal cord, we overexpressed the secreted or trans-membrane forms of draxin at different stages of spinal cord development and counted the number of dI3 interneurons. We found that ectopic secreted draxin also caused a decrease of dI3 interneurons (Fig. 4A and B). The effect of secreted draxin was slightly weaker than that of the trans-membrane form of draxin. The relative dI3 interneuron number (the number of dI3 interneuron in the electroporated side of spinal cord/ the number of dI3 in the control side) was counted and compared (Fig. 4C). Overexpression of vector did not cause a change in the number of dI3 interneurons, so the relative dI3 interneuron number was 1.01 ± 0.05 (mean$\pm$SD, $n>12$). When secreted draxin was overexpressed from stage 12–13 to stage 24–25, the relative dI3 interneuron was reduced to 0.83 ± 0.08 (mean$\pm$SD, $n>12$). When the trans-membrane form of draxin was overexpressed from stage 12–13 to stage 24–25, the relative dI3 interneuron was reduced to 0.54 ± 0.12 (mean$\pm$SD, $n>12$). When secreted draxin was overexpressed from stage 18–19 to stage 24–25, the relative dI3 interneuron was reduced to 0.92 ± 0.12 (mean$\pm$SD, $n>12$). When the trans-membrane form of draxin was overexpressed from stage 18–19 to stage 24–25, the relative dI3 interneuron was reduced to 0.72 ± 0.08 (mean$\pm$SD, $n>12$). Compared with the control, overexpression of the secreted or trans-membrane forms of draxin both caused a decrease in the number of dI3 interneurons (Fig. 3C). Under the same experimental condition, the trans-membrane form of draxin caused a greater reduction in dI3 interneurons than the secreted draxin ($P<0.001$, *t*-test). When the overexpression of secreted draxin is performed at an earlier stage (stage 12–13), the dI3 interneuron is decreased more compared with overexpression performed at a later stage (stage 18–19) ($P<0.05$, *t*-test). The same is true for the trans-membrane form of draxin ($P<0.001$, *t*-test).

Fig. 1. Draxin distribution and migration pattern of dI3 interneurons. **A**: Draxin is detected in the roof plate (RP), lateral basement membrane of the spinal cord, and dorsal lip of the dermomyotome (arrowheads). **B**: dI3 interneurons are not formed in dorsal spinal cord (arrowheads). **C**: dI3 interneurons are not detected near RP. **D**: Draxin is detected in RP, thick bands of lateral basement membrane, some cells in dorsal spinal cord. **D′**: *draxin* mRNA is detected strongly in RP and dorsal central spinal cord at stage 23. **E**: Some dI3 interneurons are formed in dorsal spinal cord. **F**: dI3 interneurons are formed in a straight band near RP. **G**: Draxin is detected in RP, lateral basement membrane, many cells in the dorsal middle spinal cord. **H**: Many dI3 interneurons are formed dorsally. Some dI3 interneurons have started to migrate ventrally (arrowheads). **I**: Newly formed dI3 interneurons form a straight band near RP (arrowheads). Migrating dI3 interneurons form another straight band near MN. RP, roof plate; DRG, dorsal root ganglion; MN, motor neuron column; FP, floor plate; dI3, dI3 interneuron; St, stage. Scale bars = 100 μm in A, B, D, and G; 200 μm in C, E, F, H, and I.

Fig. 2. dI3 interneurons can bind draxin-AP protein. **A**: Control-AP binding assay on stage-21–22 chick trunk spinal cord. No AP signal can be detected inside of the spinal cord. **B**: Control-AP binding assay and anti-Islet-1/2 immunostaining in the same section with A. **C**: Higher magnification of the boxed region in B. At this stage, many dI3 interneurons were formed in the dorsal spinal cord. Among them, no cells bound control-AP protein. **D**: Draxin-AP binding assay on stage-21–22 chick trunk spinal cord. Many AP signals can be detected inside of the spinal cord, such as in the roof plate, dorsal basement membrane, and dorsolateral part of the spinal cord. **E**: Draxin-AP binding assay and anti-Islet-1/2 immunostaining in the same section with D. **F**: Higher magnification of the boxed region in E. At this stage, many dI3 interneurons were formed in the dorsal part of the spinal cord. Almost all of the dI3 interneurons can bind draxin-AP protein. Arrows mark some of the double-stained dI3 interneurons. NT, neural tube; RP, roof plate; DRG, dorsal root ganglion; MN, motor neuron column. Scale bars = 100 μm in A, B, D and E; 50 μm in C and F.

Draxin May Cause a Reduction of Progenitor Proliferation in the Chick Spinal Cord

To determine why draxin caused a reduction of dI3 interneurons, the proliferation of progenitor cells in the chick spinal cord was monitored by PH3 (phosphorylated form of histone H3, present in the G2/M phase of the cell cycle) immunostaining. Given that the overexpression of the trans-membrane form of draxin at younger stages had the strongest effects in the reduction of dI3 interneurons, we focused our analysis on the overexpression of the trans-membrane form

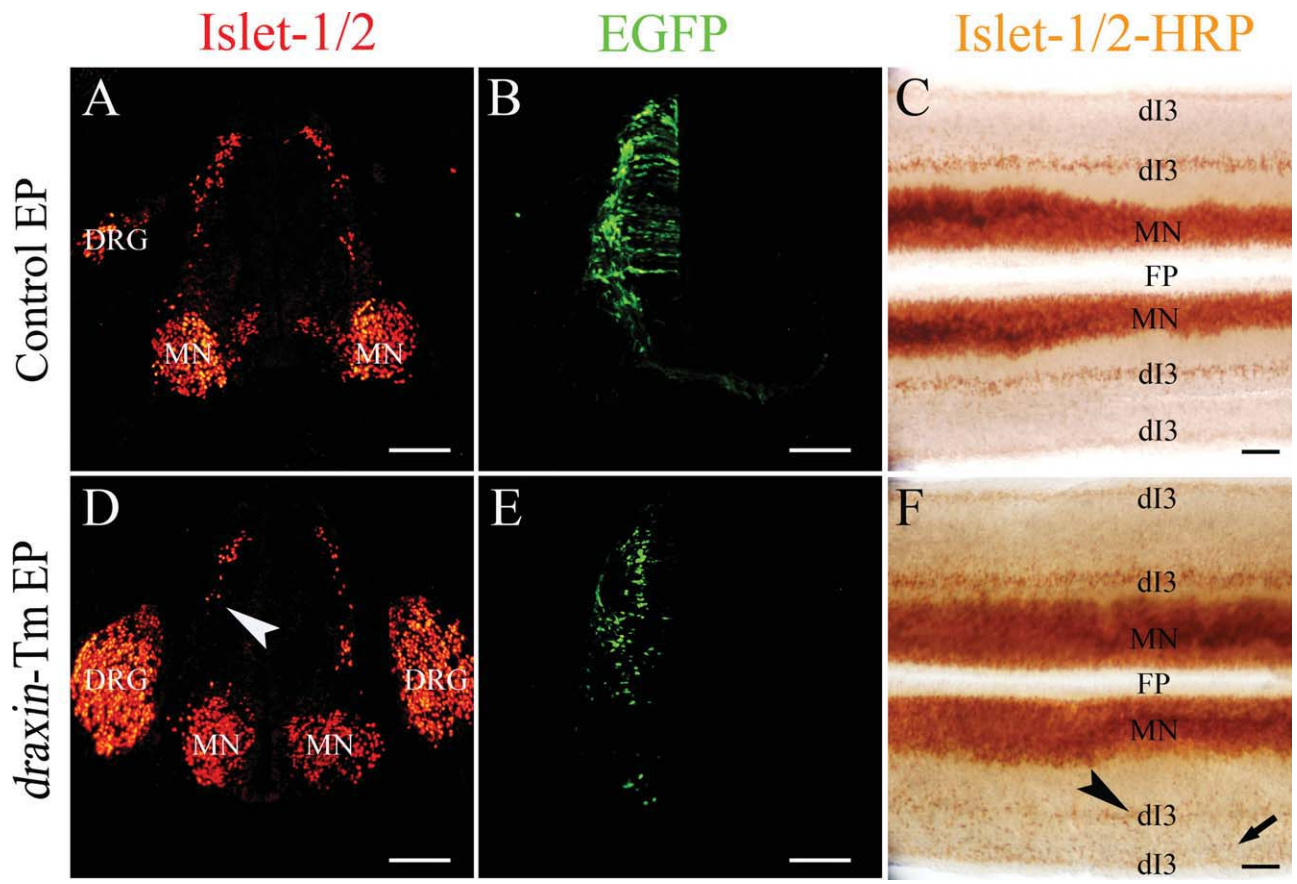

Fig. 3. Overexpression of trans-membrane draxin causes delayed migration and a decrease in the number of dI3 interneurons. **A**: Vector overexpression. **B**: EGFP expression in the same section with A shows the left side is overexpressed. **C**: Vector overexpression. EGFP-AP staining (data not shown) confirmed that the bottom side was overexpressed. dI3 interneuron formation and migration are normal in both sides of the spinal cord after vector overexpression. **D**: Trans-membrane draxin overexpression. **E**: EGFP expression in the same section with D shows that left side is overexpressed. The migration of dI3 interneurons is delayed (arrowhead), and the number of dI3 interneuron also seems to be decreased on the overexpressed side. **F**: Trans-membrane draxin overexpression. EGFP-AP staining (data not shown) confirmed that the bottom side was overexpressed. The straight band formed by migrating dI3 interneurons was further from the MN in the overexpressed side (arrowhead). Some cells are located between the two bands because of the delay of migration (black arrows). Therefore, the two dI3 interneuron bands are lighter than those on the control side. DRG, dorsal root ganglion; MN, motor neuron column; FP, floor plate; dI3, dI3 interneuron; EP, electroporation; Tm, transmembrane. Scale bar = 100 μm.

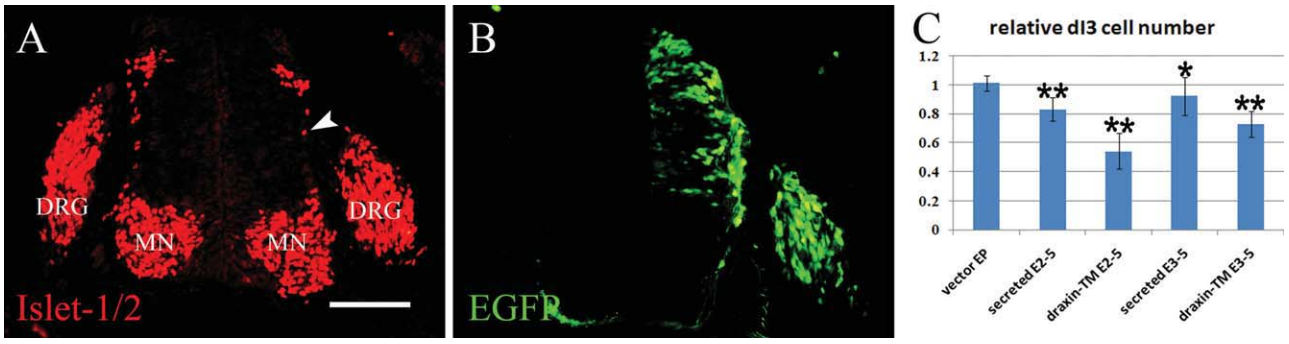

Fig. 4. The effect of secreted draxin is slightly weaker than that of trans-membrane draxin. **A**: Secreted draxin is overexpressed in stage-12–13, and Islet-1/2 immunostaining was examined around stage 24. **B**: EGFP signal in the same section with A shows that draxin is overexpressed on the right side. The migration of dI3 interneurons is slightly delayed (white arrowhead). **C**: Relative dI3 cell number (the number of dI3 interneurons in the electroporated side of the spinal cord/the number of dI3 in the control side) at different experimental conditions. Vector EP: EGFP vector was overexpressed from stage 12–13 to stage 25. Secreted E2–5: secreted draxin was overexpressed from stage 12–13 to stage 25. Draxin-TM E2–5: trans-membrane draxin was overexpressed from stage 12–13 to stage 25. Secreted E3–5: secreted draxin was overexpressed from stage 18–19 to stage 25. Draxin-TM E3-5: trans-membrane draxin was overexpressed from stage 18–19 to stage 25. Ectopic expression of draxin caused the decrease of dI3 interneurons. *$P<0.05$, **$P<0.001$. The decrease of dI3 interneurons was significantly enhanced by trans-membrane draxin and by overexpression at younger stages than older stages. DRG, dorsal root ganglion; MN, motor neuron column; EP, electroporation; dI3, dI3 interneuron. Scale bar = 100 μm.

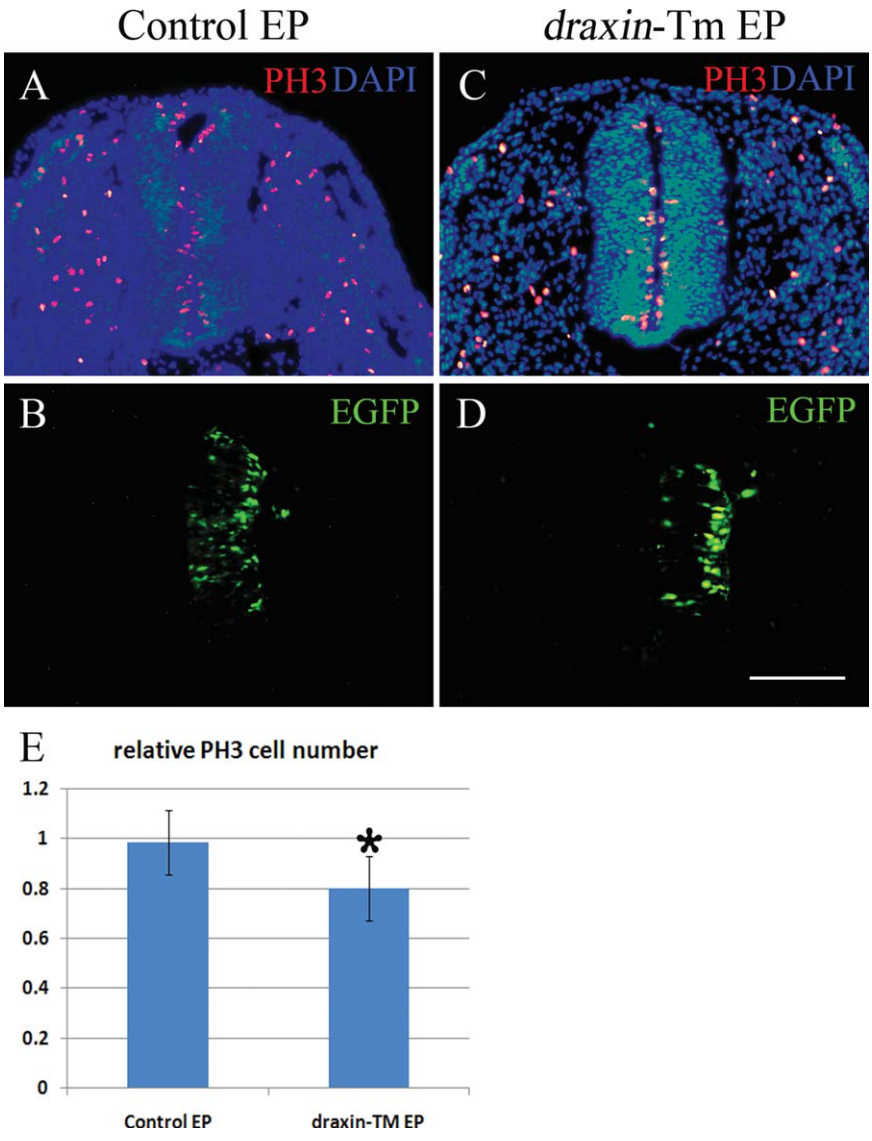

Fig. 5. Draxin causes the proliferation reduction of progenitor cells in chick embryonic spinal cord. Overexpression was performed at stage 12–13 and PH3 immunostaining was performed at stage 18–19. Nuclei were stained by DAPI. **A**: Vector is overexpressed. **B**: EGFP signal in the same section with A shows that overexpression is on the right side. The number of PH3-positive cells is not changed. **C**: Trans-membrane draxin is overexpressed. **D**: EGFP signal in the same section with C shows that overexpression is on the right side. The number of PH3-positive cells is reduced in the overexpressed side. **E**: Relative PH3 cell number (the number of PH3-positive cells in the electroporated side of the spinal cord/the number of PH3-positive cells in the control side) is reduced after overexpression of trans-membrane draxin (*$P<0.01$, *t*-test, n>12). Control EP: only EGFP vector was overexpressed. Draxin-TM EP: trans-membrane draxin was overexpressed. EP, electroporation; Tm, trans-membrane. Scale bar = 100 μm in A–D.

of draxin from stage 12–13 to stage 18–19. After overexpression of the vector, the cell number of PH3-positive cells was not changed (Fig. 5A and E). After overexpression of the trans-membrane form of draxin, the cell number of PH3-positive cells was reduced (Fig. 5C and E). After overexpression of the control vector, the relative PH3 cell number (the number of PH3-positive cells in the electroporated side of the spinal cord/the number of PH3-positive cells in the control side) was 0.984±0.132 (mean±SD, n>12). After overexpression of the trans-membrane form of draxin, the relative PH3 cell number was reduced to 0.797±0.128 (mean±SD, n>12, $P<0.01$, *t*-test). This result indicated that ectopic draxin could cause a reduction of progenitor cell proliferation in the chick spinal cord.

Overexpression of the Trans-Membrane Form of Draxin Causes Ectopic Progenitor-Like Cells in the Mantle Zone of the Spinal Cord

To trace the proliferation and migration of the progenitor cells in the ventricular zone of the spinal cord, BrdU incorporation was performed after trans-membrane draxin was overexpressed at stage 12–13 or stage 18–19. We first noted that when BrdU was incorporated for 3 hr, some BrdU-positive ectopic progenitor-like cells were located in the mantle zone of the spinal cord (arrows in Fig. 6A). Very few BrdU-positive cells were also found in the mantle zone of the control side (arrowhead in Fig. 6A). At the same time, some dI3 interneurons were already forming at the near region (arrowheads in Fig. 6B), so these ectopic BrdU-positive cells may be the differentiated cells that incorporated BrdU before exiting the cell cycle and leaving the ventricular zone or progenitor cells ectopically located in the mantle zone that continue to proliferate. To clarify the source of the ectopic BrdU-positive cells in the mantle zone, we performed 1-hr BrdU incorporation experiments after trans-membrane draxin was overexpressed from stage 18–19 to stage 24–25. In the electroporated side, some ectopic BrdU-positive cells were found (arrows in Fig. 6D) and the dI3 interneuron formation and migration were clearly delayed (arrowhead in Fig. 6E). No such changes were found in the control side (Fig. 6D and E). To determine whether the ectopic BrdU-positive progenitor-like cells can differentiate into dI3 interneurons, we also performed anti-BrdU and anti-Islet-1/2 double staining using 30 min of BrdU incorporation. There were no BrdU and Islet-1/2 double positive cells in the electroporated side after trans-membrane draxin was overexpressed from stage 12–13 to 18–19 (Fig. 6G) and from stage 12–13 to stage 24–25 (Fig. 6J). There were no ectopic BrdU-positive cells in the control side (Fig. 6G and J). BrdU incorporation during a short time

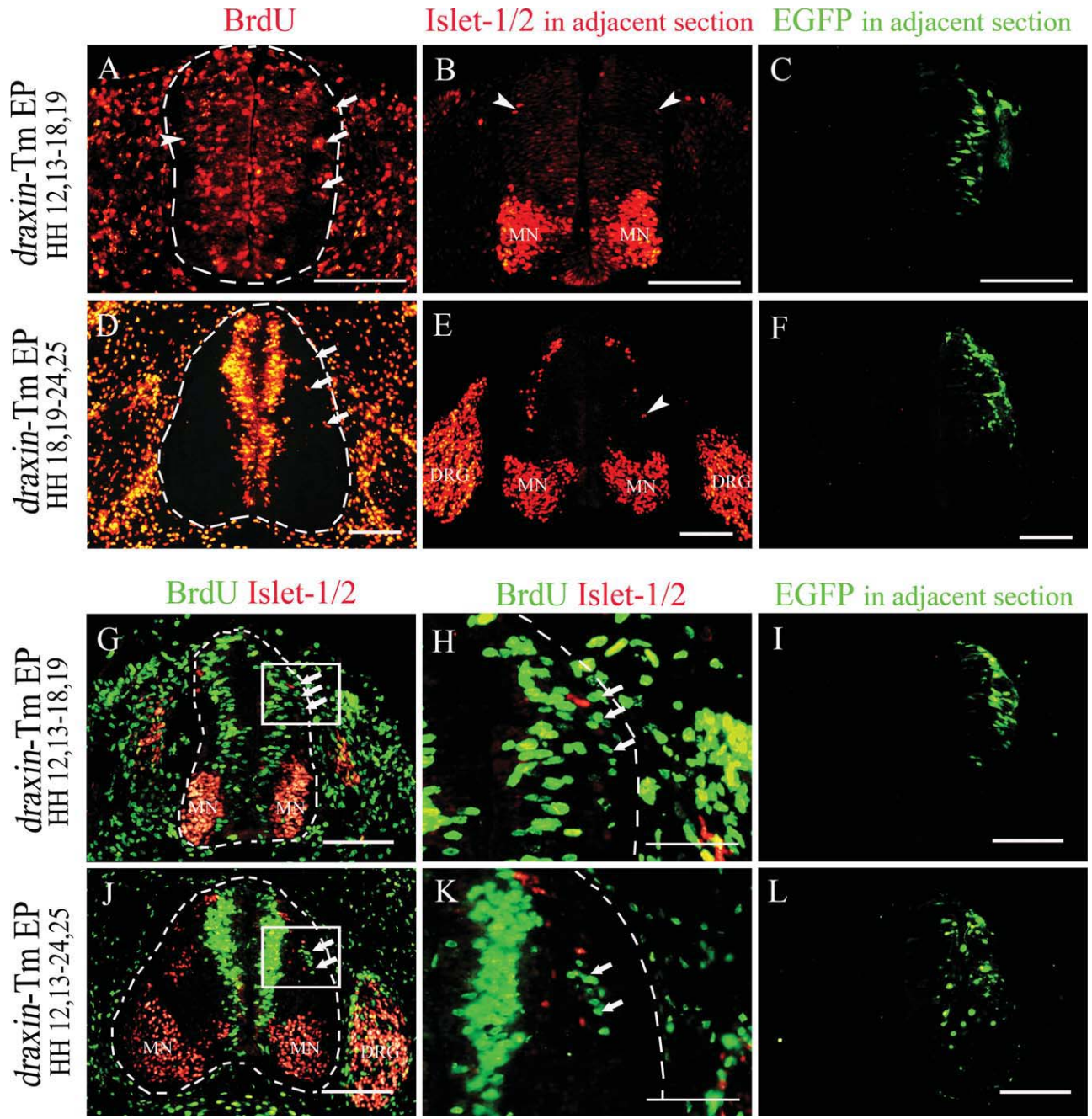

Fig. 6. Overexpression of trans-membrane draxin causes ectopic progenitor-like cells in the mantle zone of the spinal cord. Trans-membrane draxin was overexpressed in stage 12–13 (**A–C, G–L**) or stage 18–19 (**D–F**). The white dashed line outlines the boundary of spinal cord. A–C: Three hours of BrdU incorporation at stage 18–19 and (D–F) 1 hr at stage 24–25. Ectopic progenitor-like cells were localized in the mantle zone of the overexpressed side (arrows in A and D) and in the control side (arrowhead in A). B and E: Islet-1/2 immunostaining in the adjacent section of A and D, respectively. C and F: EGFP expression in the same section with B and E, respectively, shows the overexpression side. G–L: Thirty minutes of BrdU incorporation at stage 18–19 (G–I) and at stage 24–25 (J–L). H and K: Higher magnification of the boxed region in G and J, respectively. I and L: EGFP expression in the adjacent section with G and J, respectively. Ectopic BrdU-positive progenitor-like cells are not Islet-1/2-positive (arrows). DRG, dorsal root ganglion; MN, motor neuron column; EP, electroporation; Tm, trans-membrane. Scale bar = 50 μm in H and K, 100 μm in A–G, I, J, and L.

exposure, such as 30 min or 1 hr, always showed cell proliferation in the mantle zone. We concluded that after overexpression of trans-membrane draxin, some progenitor cells were ectopically located in the mantle zone, and they could not differentiate into dI3 interneurons but continued to proliferate at this abnormal position. The few ectopic BrdU-positive cells in the control side after 3 hr of BrdU incorporation might be the differentiated cells that incorporated BrdU before exiting the cell cycle and leaving the ventricular zone because such cells were not observed after a short period of BrdU uptake.

DISCUSSION

We examined the role of draxin during the development of dI3 interneurons in the chick spinal cord. Generation of the appropriate types and numbers of mature neurons during the development of the spinal cord requires the careful coordination of patterning, proliferation, and differentiation. Thus, first we checked the temporal correlation between the distribution pattern of draxin and the development of dI3 interneurons in the chick spinal cord (Fig. 1). During the development of dI3 interneurons, draxin protein was detected in the roof plate and dorsal basement membrane. Before the differentiation of dI3 interneurons, draxin was also detected in the dorsal dermomyotome. This distribution may be involved in the development of chick neural crest cells, such as the migration of early born neural crest cells (Su et al., 2009). When the differentiation of dI3 interneurons had already started, draxin protein and *draxin* mRNA were detected in the dorsal part of the spinal cord. *draxin* mRNA was just in the junction zone between the ventricular and mantle zones. Draxin-AP protein can bind to the entire surface of cultured living neural crest cells (Su et al., 2009). At the same developmental stage with the onset of dI3 interneuron differentiation and migration, we performed draxin-AP protein binding assay to the unfixed frozen sections. The result showed that almost all of the dI3 interneurons had draxin-binding activity (Fig. 2). When numerous dI3 interneurons were formed, the expression of draxin became very strong in all localizations. This temporal correlation suggested that draxin, especially the draxin expressed in the junction zone between the ventricular zone and the mantle zone, might be involved in the migration of progenitor cells, which undergo their final mitosis, from the ventricular zone to the mantle zone.

To check the probable function of draxin during the development of dI3 interneurons, we performed in vivo experiments and confirmed further that draxin plays an important role in dI3 interneuron development. First we found that the overexpression of both secreted and trans-membrane draxin caused the reduction and the delayed migration of dI3 interneurons (Figs. 3 and 4). We found that trans-membrane draxin had a stronger function than secreted draxin. This may be due to the difference of effective time. After secreted draxin is overexpressed, it will be distributed to the surrounding area, especially to the dorsal basement membrane, because of the high affinity of draxin to the dorsal basement membrane (Islam et al., 2009). But when trans-membrane draxin is overexpressed, it will move together with the overexpressing cells. Thus, the effective time of trans-membrane draxin is much longer than that of secreted draxin; as a result, trans-membrane draxin has a stronger effect than secreted draxin.

The result that overexpression of draxin at younger stages caused a greater reduction of dI3 interneurons than that overexpressed at a little later stage suggested that overexpressed draxin might function in progenitor cells at a younger stage to cause reduced and/or delayed migration of dI3 interneurons. To demonstrate this hypothesis, we performed PH3 staining (progenitor cell proliferating marker) and BrdU incorporation experiments in younger stage chick embryos after trans-membrane draxin was overexpressed. The PH3-positive cell number was reduced, suggesting that dividing progenitor cells in the ventricular zone were decreased after overexpression of trans-membrane draxin (Fig. 5). Three hours of BrdU incorporation at the same stage showed some BrdU-positive cells ectopically located in the mantle zone of the spinal cord. These ectopic BrdU-positive cells may be the differentiated cells that incorporated BrdU before exiting the cell cycle and leaving the ventricular zone or progenitor cells ectopically located in the mantle zone that continue to proliferate. One-hour BrdU incorporation experiments at later stages showed some ectopic BrdU-positive cells located in the mantle zone (Fig. 6). We also found that no ectopic BrdU-positive cells were also Islet-1/2-positive (Fig. 6). Combining these results, we conclude that after overexpression of trans-membrane draxin, some progenitor cells ectopically localize to the mantle zone and continue to proliferate at this abnormal position. These results suggest that overexpression of draxin at younger stages can cause some progenitor cells to leave the ventricular zone prematurely.

During nervous system development, neurons are generated from progenitors located in the ventricles. The size of the progenitor pool is a key determinant of the total number of neurons produced during the entire neurogenesis period (Caviness et al., 1995; Bond and Woods, 2006; Rakic, 2006). Neurons arise during development from a pseudostratified columnar epithelium composed of mitotically active neuroepithelial cells. These cells have an elongated shape, with cytoplasmic connections to both the apical (ventricular) and the basal surfaces (Del Bene et al., 2008). The neural progenitors make cytoplasmic connections with both the apical and the basal sides of the neural epithelium. At the onset of neurogenesis, neuroepithelial cells differentiate into radial glial cells that exhibit reduced tight junctions and a reduced extent of interkinetic nuclear migration (Balslev et al., 1997; Götz and Huttner, 2005). Radial glial cells divide asymmetrically to produce two different types of daughter cells, progenitor cells, and postmitotic neural precursor cells (Fishell and Kriegstein, 2003). Following cell-cycle exit, postmitotic neural precursors migrate out and differentiate into mature neurons. There are two distinct migration modes: somal translocation or locomotion. The somal translocation cells first extend a long, basal process from the ventricular zone up to the pial surface, which is followed by rapid nucleokinesis and shortening of the basal process. Locomoting cells have a free leading process and move in a saltatory manner (Ghashghaei et al., 2007). Thus, the apically and basally directed processes and connections play an important role during progenitor cell proliferation and differentiation.

A small proportion of precursors were ectopically located in the cortical parenchyma of laminin γ1III4$^{-/-}$ mice, possibly because of the loosening of both the basal and the apical anchoring of some progenitor cells at earlier developmental stages (Haubst et al., 2006).

Overexpression of draxin caused some progenitor-like cells to ectopically

localize to the mantle zone of the chick spinal cord. This may also be caused by the disturbed connections between the progenitor cells and the basal and/or apical surface at early developmental stages. As a result, the progenitor pool inside of the ventricular zone was reduced to some extent. Immunostaining of ssDNA (single stranded DNA) was also performed to check the cell death. After overexpression of draxin, no significant increase of cell death was detected. This result excluded the possibility that reduction of progenitor pool was caused by cell death. On the other hand, draxin is identical to Neucrin, which is a secreted antagonist to canonical Wnt signaling (Miyake et al., 2009). Therefore, we could not exclude the possibility for the reduction in the progenitor pool, which is that draxin can reduce the proliferation of progenitor cells as an antagonist to canonical Wnt signaling. As a result, the reduction of the progenitor pool caused the reduction of progenitor cells that are undergoing cell division. Finally, the number of differentiated dI3 interneurons was reduced, and the development of dI3 interneurons was also delayed. This may happen not only to the dI3 interneuron progenitor cells but also to other subtype interneuron progenitors. Therefore, the examination of changes in the other subtypes of interneurons after draxin overexpression will be a next interesting step in the understanding of the function of draxin during dorsal spinal cord interneuron development.

EXPERIMENTAL PROCEDURES

Chick Embryos

Fertilized White Leghorn chick eggs were obtained from local commercial sources. All eggs were incubated at 38°C until embryos reached the desired HH (Hamburger and Hamilton, 1951) stages. Embryos were removed from the eggs, stripped of membranes, and fixed in 4% paraformaldehyde (PFA) at 4°C for 2 hr.

In Situ Hybridization and Immunohistochemistry

Transverse section in situ hybridization of *draxin* at stage 22–23 chick spinal cord was performed using digoxygenin (DIG)-labeled RNA antisense probes as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993; Okafuji and Tanaka, 2005). For immunohistochemistry staining, fixed embryos or sections were treated with blocking solution for 2 hr before being incubated in primary antibody (anti-Islet-1/2, 39.4D5, Developmental Studies Hybridoma Bank; anti-chick draxin monoclonal antibody; phosphorylated form Histone H3, rabbit, upstate, 1:400; BrdU, mouse IgG1, mAb G3G4, Developmental Studies Hybridoma Bank) overnight at 4°C or for 2 hr at RT. To check the cell death, anti-ssDNA antibody was used as previously described (Naruse et al., 1994; Zhang et al., 2010). Mouse anti-chick draxin monoclonal antibody was produced as previously described (Islam et al., 2009). After being washed extensively, embryos were detected with HRP-conjugated anti-mouse IgG (1:100 diluted in blocking solution), Cy3-conjugated anti-rabbit IgG (1:500 diluted in blocking solution), or Cy3-conjugated anti-mouse IgG (1:500 diluted in blocking solution). For the cell number comparison, the relative cell number was used for a standard *t*-test. First, three or four serial sections from one embryo were counted, and the average value was the absolute cell number of this embryo. The relative cell number is defined as the absolute cell number in the experimental side of the spinal cord divided by the absolute cell number in the control side of the spinal cord. Using this kind of treatment, the difference between the anterior and the posterior parts of the spinal cord or between the embryos at slightly different stages in the same time experiment was reduced.

In Ovo Electroporation

Electroporation of expression vectors into the chick neural tube was performed as previously described (Funahashi et al., 1999; Odani et al., 2008). *draxin* cDNA tagged with myc and His was cloned into the pMES-IRES-EGFP vector. The following cDNAs were used: pMES-IRES-EGFP, pMES-*draxin*-IRES-EGFP, and pMES-*draxin-Tm*-IRES-EGFP. A membrane-bound form of *draxin* was constructed by fusing the trans-membrane domain of *SC1* (Tanaka et al., 1991) at the C-terminal end. Plasmid DNA was concentrated to 4 μg/μl and supplemented with 0.1% fast green.

BrdU (Bromodeoxyuridine) Incorporation Experiment

The BrdU-labeling experiment was performed to estimate proliferation in the chick spinal cord. One hole was made in the blood vessels surrounding the embryo and a 20-μM BrdU solution (20 μl) was added near this hole for different times. The BrdU detection was performed by a modification of published methods (Dominov et al., 1998). Briefly, sections were blocked for 1 hr at RT, incubated in 2 N HCL for 10 min on ice, 10 min at RT, and 10 min at 37°C continuously and then neutralized in 0.1 M sodium borate buffer (PH 8.5) at RT for 15 min. Then sections were incubated with anti-BrdU (IgG1) and/or anti-Islet-1/2 (IgG2b) at 4°C overnight. After being washed, sections were incubated with anti-mouse IgG-cy3 for 1 hr and observed. Al546-conjucated anti-mouse IgG2b (1:300 diluted in blocking solution) and Al488-conjucated anti-mouse IgG1 (1:200 diluted in blocking solution) were used for BrdU and anti-Islet-1/2 double staining.

Draxin-AP Binding Assay to Spinal Cord Section

AP-tagged draxin protein was produced as previously described (Islam et al., 2009). For the binding assay, forelimb region spinal cord sections from stage 21–22 chick embryos were used. The same protocol was used as previously described (Su et al., 2009). After the binding assay, anti-Islet-1/2 immunostaining was performed in the same sections.

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REFERENCES

Balslev Y, Saunders NR, Møllgard K. 1997. Ontogenetic development of

diffusional restriction to protein at the pial surface of the rat brain: an electron microscopical study. J Neurocytol 26: 133–148.
Baye LM, Link BA. 2008. Nuclear migration during retinal development. Brain Res 1192:29–36.
Bond J, Woods CG. 2006. Cytoskeletal genes regulating brain size. Curr Opin Cell Biol 18:95–101.
Buchman JJ, Tsai LH. 2008. Putting a notch in our understanding of nuclear migration. Cell 134:912–914.
Caviness VS Jr, Takahashi T, Nowakowski RS. 1995. Numbers, time and neocortical neuronogenesis: a general developmental and evolutionary model. Trends Neurosci 18:379–383.
Chenn A, Zhang YA, Chang BT, McConnell SK. 1998. Intrinsic polarity of mammalian neuroepithelial cells. Mol Cell Neurosci 11:183–193.
Del Bene F, Wehman AM, Link BA, Baier H. 2008. Regulation of neurogenesis by interkinetic nuclear migration through an apical-basal notch gradient. Cell 134:1055–1065.
Dominov JA, Dunn JJ, Miller JB. 1998. Bcl-2 expression identifies an early stage of myogenesis and promotes clonal expansion of muscle cells. J Cell Biol 142:537–544.
Fishell G, Kriegstein AR. 2003. Neurons from radial glia: the consequences of asymmetric inheritance. Curr Opin Neurobiol 13:34–41.
Frade JM. 2002. Interkinetic nuclear movement in the vertebrate neuroepithelium: encounters with an old acquaintance. Prog Brain Res 136:67–71.
Funahashi J, Okafuji T, Ohuchi H, Noji S, Tanaka H, Nakamura H. 1999. Role of Pax-5 in the regulation of a mid-hindbrain organizer's activity. Dev Growth Differ 41:59–72.
Ghashghaei HT, Lai C, Anton ES. 2007. Neuronal migration in the adult brain: are we there yet? Nat Rev Neurosci 8: 141–151.
Götz M., Huttner WB. 2005. The cell biology of neurogenesis. Nat Rev Mol Cell Biol 6:777–788.
Gowan K, Helms AW, Hunsaker TL, Collisson T, Ebert PJ, Odom R, Johnson JE. 2001. Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. Neuron 31:219–232.
Gross MK, Dottori M, Goulding M. 2002. Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. Neuron 34:535–549.
Hamburger V, Hamilton HL. 1951. A series of normal stages in the development of the chick embryo. J Morph 88: 49–92.
Haubst N, Georges-Labouesse E, De Arcangelis A, Mayer U, Götz M. 2006. Basement membrane attachment is dispensable for radial glial cell fate and for proliferation, but affects positioning of neuronal subtypes. Development 133: 3245–3254.
Islam MS, Shinmyo Y, Okafuji T, Su Y, Naser IB, Ahmed G, Zhang S, Chen S, Kiyonari H, Takaya A, Tanaka S, Nishinakamura R, Kitamura T, Tanaka H. 2009. Draxin, a repulsive axon guidance protein for spinal cord and forebrain commissures. Science 323: 388–393.
Jessell TM. 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet 1: 20–29.
Lee KJ, Dietrich P, Jessell TM. 2000. Genetic ablation reveals that the roof plate is essential for dorsal interneuron specification. Nature 403:734–740.
Li M, Sakaguchi DS. 2002. Expression patterns of focal adhesion associated proteins in the developing retina. Dev Dyn 225:544–553.
Millonig JH, Millen KJ, Hatten ME. 2000. The mouse Dreher gene Lmx1a controls formation of the roof plate in the vertebrate CNS. Nature 403: 764–769.
Miyake A, Takahashi Y, Miwa H, Shimada A, Konishi M, Itoh N. 2009. Neurcin is a novel neural-specific secreted antagonist to canonical Wnt signaling. Biochem Biophys Res Commun 390: 1051–1055.
Müller T, Brohmman H, Pierani A, Heppenstall PA, Lewin GR, Jessell TM, Birchmeier C. 2002. The homeodomain factor lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. Neuron 34:551–562.
Naruse I, Keino H, Kawarada Y. 1994. Antibody against single-stranded DNA detects both programmed cell death and drug-induced apoptosis. Histochemistry 101:73–78.
Odani N, Ito K, Nakamura H. 2008. Electroporation as an efficient method of gene transfer. Dev Growth Differ 50: 443–448.
Okafuji T, Tanaka H. 2005. Expression pattern of *LINGO-1* in the developing nervous system of the chick embryo. Gene Exp Patterns 6:57–62.
Rakic P. 2006. A century of progress in corticoneurogenesis: from silver impregnation to genetic engineering. Cereb Cortex 16(Suppl 1):i3–17.
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.
Su Y, Naser IB, Islam SM, Zhang S, Ahmed G, Chen S, Shinmyo Y, Kawakami M, Yamamura KI, Tanaka H. 2009. Draxin, an axon guidance protein, affects chick trunk neural crest migration. Dev Growth Differ 51:787–796.
Tanaka H, Matsui T, Agata A, Tomura M, Kubota I, McFarland KC, Kohr B, Lee A, Phillips HS, Shelton DL. 1991. Molecular cloning and expression of a novel adhesion molecule, SC1. Neuron 7:535–545.
Timmer JR, Wang C, Niswander L. 2002. BMP signaling patterns the dorsal and intermediate neural tube via regulation of homeobox and helix-loop-helix transcription factors. Development 129: 2459–2472.
Wozniak MA, Modzelewska K, Kwong L, Keely PJ. 2004. Focal adhesion regulation of cell behavior. Biochim Biophys Acta 1692:103–119.
Zhang S, Su Y, Shinmyo Y, Islam SM, Naser IB, Ahmed G, Tamamaki N, Tanaka H. Draxin, a repulsive axon guidance protein, is involved in hippocampal development. Neurosci Res 66: 53–61.

Developmental Dynamics