

Embryonic GABAergic Spinal Commissural Neurons Project Rostrally to Mesencephalic Targets

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ABSTRACT

Although spinal commissural neurons serve as a model system for studying the mechanisms that underlie axonal pathfinding during development, little is known about their synaptic targets. Previously we identified a group of ventromedially located commissural neurons in rat spinal cord that are γ -aminobutyric acid (GABA)-ergic and express L1 CAM on their axons. In this study, serial sagittal sections of embryos (E12–15) were processed for glutamic acid decarboxylase (GAD)-65 and L1 immunocytochemistry and showed labeled commissural axons coursing rostrally within the ventral marginal zone. Both GAD65- and L1-positive axons extended rostrally out of the spinal cord into the central part of the medulla and then into the midbrain. GAD65-positive axons branched and ended abruptly within the lateral midbrain. To determine the targets of these ventral commissural neurons, embryos (E13–15) were injected with DiI into the ventromedial spinal cord. At all three ages, DiI-labeled axons projected rostrally in the contralateral ventral marginal zone, turned into the central medulla, and then traveled to the midbrain. DiI-labeled axons appeared to terminate in the lateral midbrain by branching into small, punctate structures. In reciprocal experiments, DiI injected into the lateral midbrain identified an axon pathway that coursed through the brainstem, into the spinal cord ventral marginal zone, and then filled cell bodies in the contralateral ventromedial spinal cord. A spatial and temporal coincidence was apparent between the GAD65/L1- and the DiI-labeled pathways. Together these findings suggest that some GABAergic commissural neurons are early projection neurons to midbrain targets and most likely represent a spinomesencephalic pathway to the midbrain reticular formation. *J. Comp. Neurol.* 475:327–339, 2004. © 2004 Wiley-Liss, Inc.

Indexing terms: GAD65; axonal pathfinding; spinal cord; L1 CAM; DiI

During development, growing axons navigate toward and subsequently innervate their intended targets. This feat of pathfinding requires that axons extend long distances and respond to multiple cues along their route. Spinal commissural neurons are a model system for studying axonal guidance cues and are known to originate along the dorsoventral extent of the medial intermediate zone. They extend their axons toward and into the ventral commissure before crossing and turning rostrally within the contralateral ventral marginal zone (Holley, 1982; Oppenheim et al., 1988; Silos-Santiago and Snider, 1992; Stoekli and Landmesser, 1995; Kaprielian et al., 2001). Commissural neurons were described by Ramón y Cajal (1933) as being among the earliest neurons born in the spinal cord and as being both numerous and diverse (Wentworth, 1984; Silos-Santiago and Snider, 1992). However, the syn-

aptic targets for most spinal commissural neurons are unknown.

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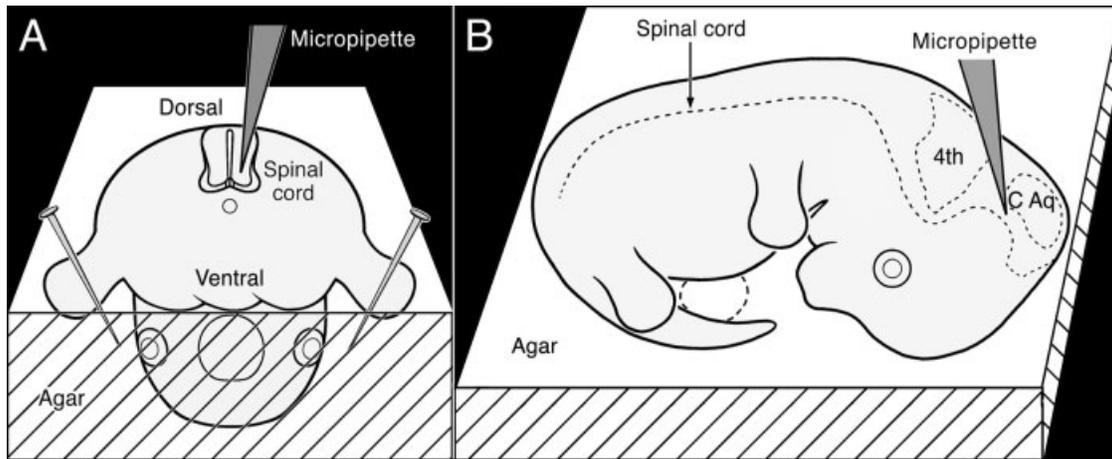


Fig. 1. Embryos oriented for spinal cord (A) or brainstem (B) DiI injections. **A:** After transection below the forelimbs, the embryo was turned upside down, with its head embedded in agar and the forelimbs pinned for support. DiI was injected unilaterally into the ven-

tromedial spinal cord. **B:** The embryo was placed on its side with the fore- and hindlimb buds embedded in the agar. After removal of the thin membrane overlying the fourth ventricle and cerebral aqueduct, DiI was injected unilaterally into the lateral midbrain.

Recent immunocytochemical studies of the two forms of glutamic acid decarboxylase (GAD65 and GAD67) and γ -aminobutyric acid (GABA) in the embryonic rat spinal cord established that a group of commissural neurons uses GABA as their neurotransmitter (Phelps et al., 1999; Tran et al., 2003). As early as E12, GAD65 was detected in commissural cell bodies, axons, and growth cones. By E13, large, ventromedial and small, dorsolateral groups of GAD65-positive commissural neurons were detected. In addition, some of these GABAergic commissural neurons expressed L1 CAM on both ipsi- and contralateral axonal surfaces (Tran and Phelps, 2000). L1 is found on axons and axonal growth cones and is thought to play an important role in axon outgrowth (Lagenaur and Lemmon, 1987; Lemmon et al., 1989), fasciculation (Stoeckli and Landmesser, 1995), and guidance (Lemmon et al., 1992; Cohen et al., 1997).

Most evidence to date suggests that spinal commissural neurons are interneurons (Imondi and Kaprielian, 2001;

Moran-Rivard et al., 2001; Butt and Kiehn, 2003). DiI tracing studies of dorsally located spinal commissural neurons in the mouse and chick have localized their axons in the contralateral ventral marginal zone for over 100 μ m (Imondi and Kaprielian, 2001). Additional studies of the expression pattern of the Engrailed (En1) homeodomain transcription factor have shown that some En1 neurons colocalized with GAD65 ventromedial spinal cord neurons. However, the axons of these En1 neurons did not appear to project beyond the spinal cord (Saueressig et al., 1999). Another transcription factor, Evx1, that defines a population of ventral commissural neurons has an axonal projection pathway that ascends in the contralateral ventral funiculus, but only for a few spinal segments (Moran-Rivard et al., 2001). Finally, electrophysiological studies of the coordination of neonatal rat hindlimbs have identified three classes of ventral commissural interneurons with axons that course over several segments of the lumbar spinal cord (Butt and Kiehn, 2003).

In this study, we tested the hypothesis that some of the GAD65- and L1-positive commissural neurons are projection neurons rather than interneurons. Thus, we set out to determine whether the earliest GAD65- and L1-positive commissural axons projected out of the spinal cord and, if so, where their targets might be. Subsequently, DiI injections in the ventral spinal cord, along with reciprocal injections in the brainstem, demonstrated that ventromedial spinal commissural axons projected out of the spinal cord to terminate in the mesencephalic tegmentum.

MATERIALS AND METHODS

Animal and tissue preparation

All procedures were approved by the Chancellor's Animal Research Committee at the University of California, Los Angeles. Male and female Sprague-Dawley rats were caged together overnight for breeding, and the day of a positive smear was recorded as embryonic day 0 (E0). Pregnant rats were deeply anesthetized with intraperitoneal injections of ketamine (90 mg/kg) and xylazine

Abbreviations

C Aq	cerebral aqueduct
Cb	cerebellum
Cf	cephalic flexure
DRA	dorsal root afferents
Dt	dorsal thalamus
LV	lateral ventricle
Mb	midbrain/mesencephalon
Md	medulla oblongata
MGE	medial ganglionic eminence
Op	olfactory placode
Pc	posterior commissure
Pn	pons
Pt	pretegmentum
SC	spinal cord
vc	ventral commissure
vmz	ventral marginal zone
1st cn	first cranial nerve
3rd cn	third cranial nerve
4th cn	fourth cranial nerve
4th v	fourth ventricle
12th cn	twelfth cranial nerve

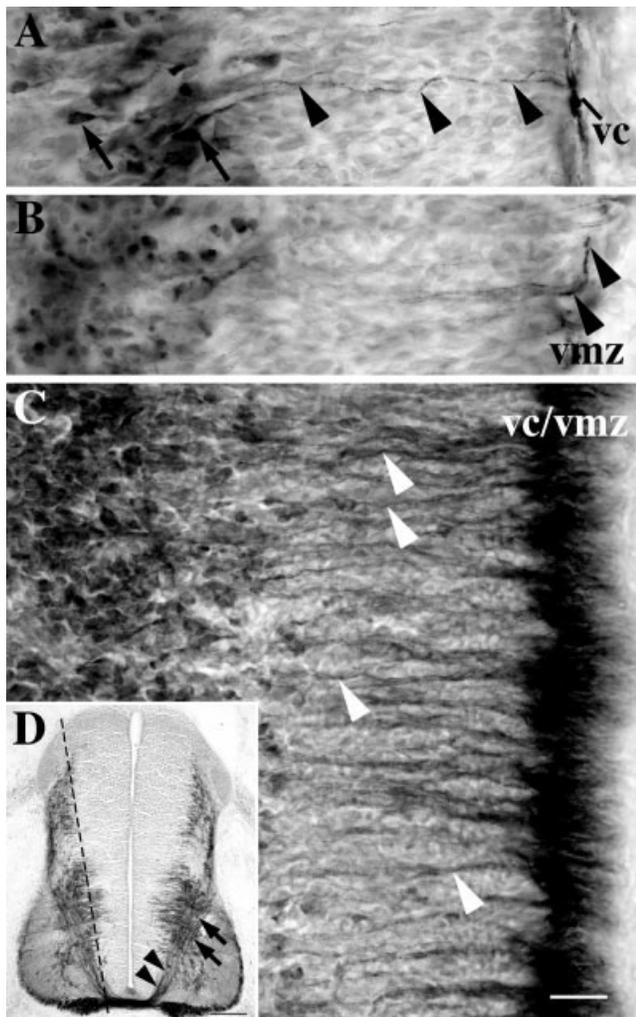


Fig. 2. GABAergic commissural neurons project their axons into the ventral commissure in sagittal (A–C) or coronal (D) sections labeled with GAD65 immunocytochemistry. Sagittal sections in this figure and subsequent figures are oriented with ventral to the right and rostral to the top. **A:** GAD65-positive neurons (arrows) were first detected on E12 at the cervical level, and a few of their axons (arrowheads) projected as far as the ventral commissure (vc). **B:** In a section adjacent and lateral to A, labeled commissural axons (arrowheads) turned rostrally in the ventral marginal zone (vmz). Relatively few GAD65-labeled commissural axons were detected on E12 compared with 1 day later (C,D). **C:** An enlargement of the E13 cervical spinal cord illustrated in Figure 3C. Numerous GAD65-positive commissural neurons projected axons (arrowheads) into the ventral commissure and adjacent ventral marginal zone (vc/vmz). **D:** Coronal section of an E13 embryo illustrates the location of ventromedial GAD65-positive neurons (arrows), with their axons (arrowheads) coursing into the ventral commissure. The dashed line represents the approximate plane of section for A–C. Scale bars = 25 μm in C (applies to A–C); 50 μm in D.

(10–20 mg/kg) before embryos (12–15 days old) were collected. E12–13 animals were fixed by immersion in 4% paraformaldehyde or 0.01 M periodate, 0.075 M lysine, and 2% paraformaldehyde (PLP; McLean and Nakane, 1974); E14–15 animals were fixed by vascular perfusion. All embryos were postfixed in the same fixative for 4–12 hours at 4°C. After being rinsed in Millonig's buffer, em-

bryos were infiltrated with 30% sucrose before being incubated in a mixture of 7.5% gelatin/30% sucrose for 1 hour at 37°C and embedded in plastic molds containing the warm gelatin/sucrose mixture (Phelps et al., 1996). Frozen embryos were cut into 40- μm -thick sections for immunocytochemistry.

GAD65 and L1 immunocytochemical procedures

Monoclonal antibodies GAD-6 (1:50 dilution; Chang and Gottlieb, 1988) and ASCS4 (1:25 dilution; Sweadner, 1983) supernatants (both from DSHB, Department of Biological Sciences, University of Iowa) were used to localize GAD65 and L1 CAM, respectively. Immunocytochemical methods were identical to those previously described (Phelps et al., 1999; Tran and Phelps, 2000; Tran et al., 2003).

DiI tracing procedures

After Cesarean section, embryos were placed in cool oxygenated Gey's solution (Freshney, 1987). The extraembryonic membranes were removed, and the embryo was supported in an agar-filled petri dish during the DiI injection. DiI is a lipophilic, vital dye that is known to incorporate into the plasma membrane of living neurons, including their processes and axon terminals (D-282; Molecular Probes, Inc., Eugene, OR; Honig and Hume, 1986, 1989; Cohen-Cory and Fraser, 1995; Cohen-Cory, 1999). For DiI spinal cord injections, embryos with beating hearts were completely transected just below the forelimbs and then flipped upside down, so that their heads were held in place by the agar (Fig. 1A). For brainstem injections, the embryos were placed on their side, and the thin membrane over the fourth ventricle was peeled away (Fig. 1B). In total, 17 litters of embryos ranging from E13 to E15 were used for both spinal cord and brainstem injections. Littermates at each age were fixed and processed for immunocytochemistry. Approximately half of these litters were used to determine the optimal injection parameters, such as the postinjection survival time and the time required for DiI to travel passively to the target sites. A 0.5% DiI solution made in 95% ethanol was loaded into a micropipette for unilateral pressure injections into the ventromedial spinal cord region or brainstem. After injections, embryos were maintained at 37°C for 15–24 hours in Gey's solution and regularly gassed with 95% O₂ and 5% CO₂ to facilitate active dye transport (Serbedzija et al., 1989, 1990).

On the following day, injected embryos were fixed with 4% paraformaldehyde and subsequently kept at 37°C for 1–4 months before being sectioned into 150–200- μm -thick sections with a vibratome. Serial sagittal sections were cut beginning from the side opposite of the injection. Sections were mounted consecutively on slides, coverslipped in gel/mount (Biomedica Corp.), and viewed immediately with a fluorescent microscope. The only evidence of non-specific dye transfer was found at the injection site.

Data were recorded with a Zeiss AxioCam digital camera and Openlab 3.1.1 software. Subsequently, all Openlab files were converted to Adobe Photoshop 7.0 files. In addition, selected sections were analyzed with a Zeiss 310 confocal microscope with a Texas red/rhodamine filter. Images were captured with the LSM 310/410 software and converted to TIF files. Montages were assembled in Adobe Photoshop, and minor modifications in color balance,

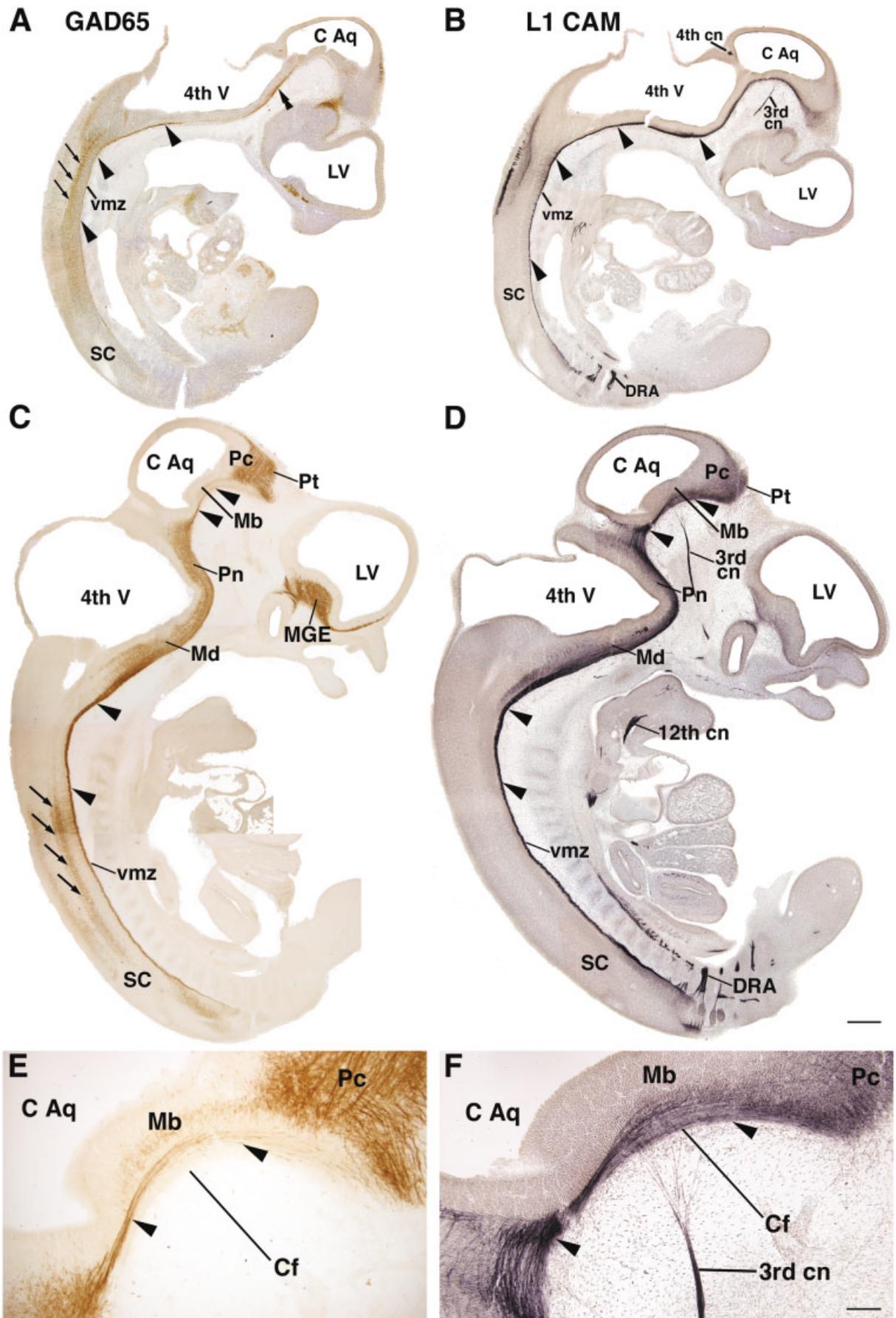


Figure 3

brightness, and contrast were made if necessary. Anatomical terminology for structural subdivisions is based on embryonic mouse and rat atlases (Paxinos et al., 1991, 1994; Puelles and Rubenstein, 1993; Alvarez-Bolado and Swanson, 1996).

RESULTS

Earliest axonal projections of GABAergic spinal commissural neurons

We previously identified a group of commissural neurons in the ventromedial spinal cord that were committed to the GABAergic phenotype as early as E12 (Phelps et al., 1999) and expressed L1 CAM on their axonal surfaces (Tran and Phelps, 2000). By using a monoclonal antibody that labels the GAD65 protein, GABAergic spinal commissural neurons first were identified in sagittal sections at E12 (Fig. 2A,B) and were more intensely labeled at E13 (Fig. 2C,D). The earliest GAD65-labeled axons projected toward and into the ventral commissure (Fig. 2A) and then turned rostrally into the ventral marginal zone (Fig. 2B).

The earliest continuous GAD65-positive axonal pathway was detected in serial sagittal sections of the ventral marginal zone in E12.5 animals (Fig. 3A). A column of GAD65-labeled commissural cell bodies was found in the ventromedial intermediate zone of the spinal cord (Fig. 3A, arrows). In addition, GAD65-labeled axons coursed in the ventral marginal zone, projected out of the spinal cord into the brainstem, and were followed as far rostrally as the medulla (Fig. 3A). A similar axonal pathway was seen in adjacent sections of the same embryo immunolabeled for L1 CAM (Fig. 3B). The L1 pathway appeared to extend farther rostrally than the GAD65-positive fibers (Fig. 3A,B). In fact, L1-immunoreactive fibers were traced as far rostrally as the midbrain (Fig. 3B). Additional structures labeled exclusively with L1 were the third and fourth cranial nerves and dorsal root afferents. The pathways of both GAD65- and L1-labeled axons appeared to

project along the ventral margin of the developing spinal cord and brainstem.

Spinal GABAergic and L1-positive axons project to the brainstem

By E13, numerous GAD65-positive commissural neurons were detected in sagittally (Fig. 2C, enlargement of Fig. 3C) and coronally (Fig. 2D) sectioned spinal cords. At this age, a dramatic increase in the number of GABAergic commissural neurons was found in the ventromedial spinal cord (Figs. 2D, 3C, arrows). Consequently, many more GABAergic commissural axons had joined the ventral marginal zone to form a continuous projection pathway from the spinal cord to as far rostrally as the midbrain (Fig. 3C). In an adjacent section from the same embryo, a similar L1-positive axonal projection pathway was observed (Fig. 3D). In addition, some GAD65- and L1-positive axon bundles appeared to end in the midbrain, beyond the level where the third cranial nerve exits near the cephalic flexure (Fig. 3E,F). Note that cranial nerves are L1 positive and GAD65 negative. The distal ends of both the GAD65 and the L1 axonal pathways were seen to defasciculate into small fiber bundles and were not followed beyond the posterior commissure at this or subsequent ages (Fig. 3E).

To elucidate the target of the spinal GAD65- and L1-positive commissural axons, we examined the projection pathways in older embryos. At E14, GAD65- and L1-labeled commissural axons within the ventral marginal zone were similar to those at earlier ages, except that the band of L1-labeled axons appeared to be wider than that of GAD65 (Fig. 4A,E), a finding consistent with previous results in the ventral marginal zone in coronal sections (Tran and Phelps, 2000). Differences in axon density were most apparent in the brainstem. Beginning in the medulla, both the GAD65- and the L1-labeled axons expanded into the deeper or central part of the medulla and remained in this position throughout the medulla and pons. A group of centrally located GAD65-positive axons defasciculated and appeared to terminate in the rostral midbrain (Fig. 4B), in an area similar to that seen in E13 embryos (Fig. 3C). Although L1-positive axons also were detected in the midbrain (Fig. 4E,F), the bundles were too massive to distinguish whether any axons terminated there. However, in both E13 and E14 sections, GAD65-immunoreactive axons appeared to course as far rostrally as the midbrain, just ventral to the posterior commissure, in an area identified in atlases as the mesencephalic tegmentum (Figs. 3E,F, 4B,F; Paxinos et al., 1994; Alvarez-Bolado and Swanson, 1996).

We initially suspected, based on the axonal projection patterns of both GAD65- and L1-positive fibers in the ventral spinal cord and brainstem at the earlier ages, that these commissural axons traveled close to the ventral border of the brainstem. However, by E14 it was apparent that both GAD65- and L1-labeled fiber tracts traveled in a wider pathway that spanned the ventral half of the brainstem and continued into the midbrain (Fig. 4A,E,F). When transverse sections of E14 midbrain were examined, numerous GAD65-immunoreactive terminal-like structures were concentrated in the mesencephalic tegmentum (Fig. 4C,D). By this age, many other GABAergic and L1-positive axons also were detected. For example, GAD65- and L1-positive axons were found in the posterior commissure and dorsal thalamus (Fig. 4A,E), and numerous

Fig. 3. Sagittal sections of embryos at E12.5 (A,B) and E13 (C–F) processed for GAD65 (A,C,E) and L1 (B,D,F) immunocytochemistry. **A:** GAD65-positive spinal cord neurons (arrows) were present at cervical levels. A small GAD65-positive axon bundle (single arrowheads) projected rostrally from the ventral marginal zone (vmz) into the brainstem. GAD65 immunoreactivity also was detected in the pons (double arrowhead). **B:** In a section adjacent to A, L1-labeled axons (arrowheads) were detected in the vmz and then coursed out of the spinal cord (SC) in a continuous pathway to the rostral midbrain. Structures labeled only by L1 were the dorsal root afferents (DRA) and parts of the third and fourth cranial nerves. **C:** Numerous GAD65-positive commissural axons (SC; arrowheads) were present in the vmz and could be followed as far rostrally as the midbrain (Mb, arrowheads). Axons from ventral GAD65-positive commissural somata (arrows) contributed to this pathway (enlargement in Fig. 2C). **D:** In a section adjacent to C, L1-positive axons (arrowheads) in the vmz projected into the brainstem. A few dorsal root afferents as well as the third and twelfth cranial nerves were identified with L1. **E:** Enlargement of the midbrain region in C shows defasciculated GAD65-positive axons (arrowheads) in the cephalic flexure (Cf). **F:** Enlargement of the midbrain region in D also shows defasciculated L1-positive axons just before reaching the posterior commissure. Note that the third cranial nerve exits the midbrain at the cephalic flexure. Scale bars = 400 μ m in D (applies to A–D); 100 μ m in F (applies to E,F).

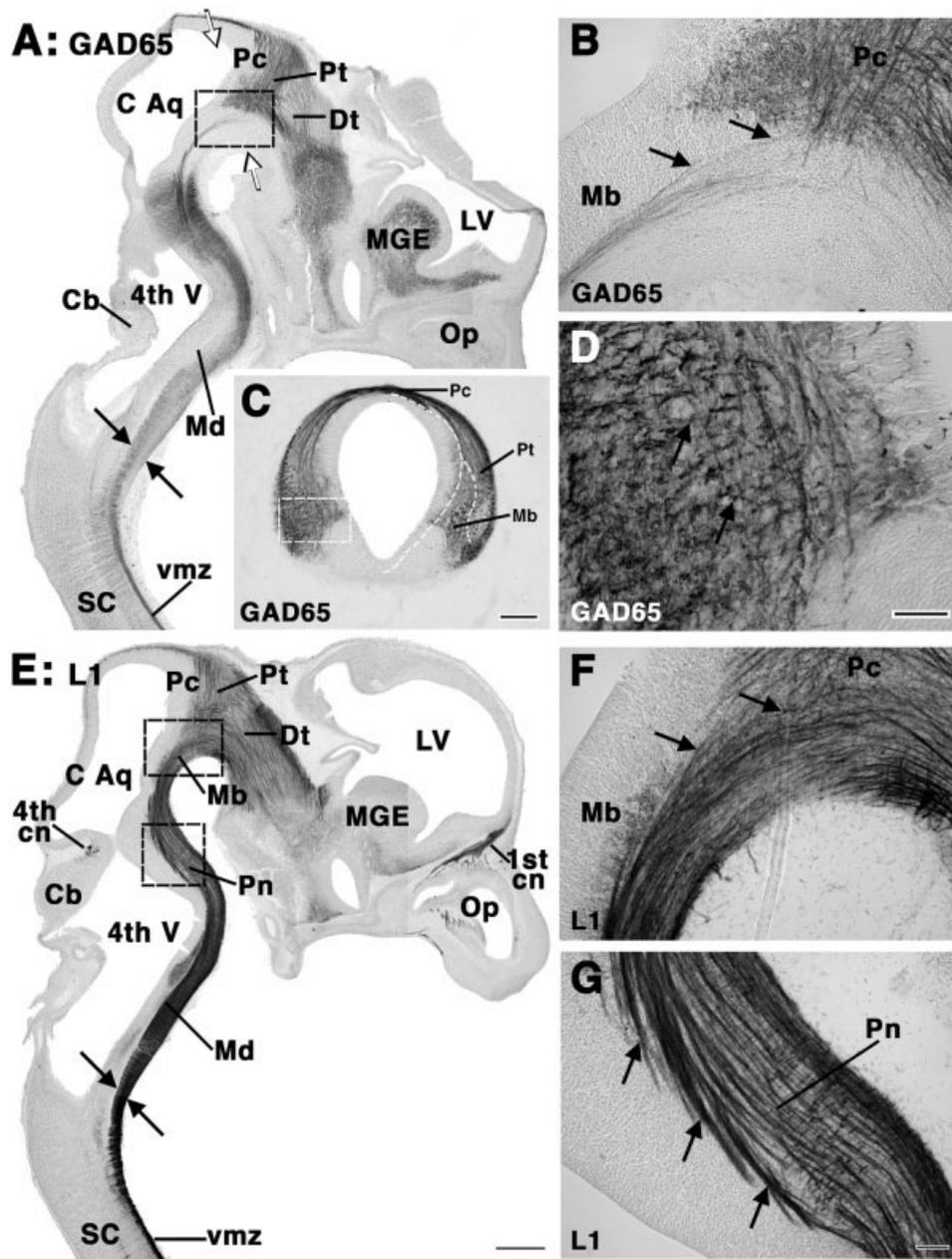


Fig. 4. Adjacent sagittal sections of the same E14 animal labeled with GAD65 (A,B) and L1 (E–G). A transverse section through an E14 midbrain was labeled with GAD65 (C,D). **A:** The tightly bundled GAD65-positive pathway within the ventral marginal zone (vmz) broadened upon entering the medulla (Md; black arrows). Other GABAergic elements were present in the medial ganglionic eminence (MGE) as well as in the dorsal thalamus (Dt). **B:** Enlargement of the boxed region in A illustrates the defasciculation of GAD65-positive axons (arrows) as well as GAD65-labeled fibers in the posterior commissure (Pc). **C:** The approximate level of this midbrain section is indicated by the white arrows in A. GAD65 immunoreactivity was detected in the midbrain, pretectum, and axons of the posterior commissure. Dashed lines at right indicate the regional separation adapted from Alvarez-Bolado and Swanson (1996). **D:** Enlargement of

the boxed area in C. Numerous GAD65 punctate structures (arrows) were detected in the developing midbrain. **E:** L1-positive axons were followed from the vmz of the spinal cord to the midbrain. The pathway widened as it entered the medulla (arrows) and then coursed through the pons and into the midbrain. Other L1-labeled axons were detected in the posterior commissure, the pretectum, the dorsal thalamus, the olfactory placode, and the first and fourth cranial nerves. **F:** Enlargement of the upper boxed region in E illustrates numerous L1-positive axons in the midbrain, just ventral to the posterior commissure. **G:** Enlargement of the lower boxed region in E shows numerous L1-positive fiber bundles (arrows) within the pons. Scale bars = 400 μ m in E (applies to A,E); 50 μ m in G (applies to B,F,G), D; 200 μ m in C.

GABAergic neurons were present in the medial ganglionic eminence, as previously reported (Anderson et al., 1997, 2001).

Finally, we studied the GABAergic and L1-positive projection pathways in E15 embryos, but, because of the enormous increase in complexity, we were unable to distinguish the pathways identified at earlier ages. Therefore, we turned to DiI tracing techniques to determine the rostral targets of the spinal commissural axons.

DiI-injected spinal commissural axons project to the contralateral mesencephalic tegmentum

Thirteen-day-old embryos were unilaterally injected with DiI in the ventromedial spinal cord (Figs. 1A, 5A), the site where many GABAergic commissural cell bodies reside (Fig. 2D; Phelps et al., 1999; Tran et al., 2003). A DiI-labeled axon tract within the contralateral ventral marginal zone was detected (Fig. 5B). Labeled axons from the spinal cord coursed rostrally into the brainstem, past the medulla and pons, and appeared to end abruptly at the level of the cephalic flexure in the midbrain (Fig. 5C).

At E14, a similar DiI-labeled axon pathway from the contralateral ventral marginal zone also projected into the brainstem (Fig. 5D). However, at the border between the spinal cord and the medulla, the DiI-labeled pathway appeared to turn gradually from the ventral marginal zone into the central region of the medulla (Fig. 5D, arrows). The broad DiI-labeled axon fascicle traversed through the medulla and continued into the pons and midbrain. DiI-labeled axons in the midbrain branched and displayed profiles similar to those of axons before their termination (Fig. 5E).

To identify the likely terminal fields of the ventral spinal commissural axons, DiI was injected unilaterally into the ventromedial spinal cord (Fig. 6A) in 15-day-old embryos. DiI-labeled axons within the contralateral ventral marginal zone were detected (Fig. 6B). However, as these axons entered the caudal brainstem, they gradually turned and spread out broadly in the central part of the medulla. A smaller fiber bundle continued into the pons and then rostrally to the midbrain (Fig. 6B). The axonal pathway appeared to terminate in the midbrain, between the cerebral aqueduct and the ventral part of the cephalic flexure (Fig. 6B). In the next two adjacent sections, likely terminal fields were detected in the rostral midbrain (Fig. 6C–F). Numerous punctate-like structures were detected branching from thin DiI-labeled axons (Fig. 6D,F).

At all three ages examined, a DiI-labeled axonal projection pathway ascended in a tight bundle in the ventral marginal zone and then spread and coursed into the central region of the medulla. These axons remained in this central location throughout the pons and ended in the midbrain. Finally, as these DiI-labeled axons approached their presumptive termination sites, they executed a lateral turn and appeared to terminate in the lateral midbrain.

Midbrain injections label cell bodies in the contralateral ventromedial spinal cord

To confirm that the DiI-labeled axons projecting through the brainstem originated from the contralateral spinal cord, DiI injections were made in the lateral midbrain, between the cerebral aqueduct and the cephalic

flexure (Fig. 1B). In E13 animals, serial sagittal sections on the ipsilateral side of the injection site were examined to locate the DiI-labeled pathway through the brainstem and spinal cord. As expected, a DiI-labeled axon tract was seen in the caudal medulla and entered the ventral marginal zone of the spinal cord (data not shown).

DiI injections into the lateral midbrain (Fig. 7A) of a 14-day-old embryo were examined in a series of five adjacent sagittal sections (Fig. 7). Two sections medial to the injection site contained DiI-labeled axons descending in a broad pathway through the brainstem (Fig. 7B) and then in a more tightly bundled fascicle in the central part of the pons (Fig. 7C). In the next two sections of the series, the DiI-labeled axon pathway reached the ventral marginal zone of the spinal cord (Fig. 7D), and small patches of what appeared to be cellular labeling were observed on the contralateral side (Fig. 7E). Confocal microscopy confirmed that the cellular profiles seen in Figure 7E were ventromedially located DiI-labeled cell bodies (Fig. 7F, arrowheads). Ventrally projecting axons that emanated from several of the DiI-labeled cell bodies (Fig. 7F, arrows) confirmed that commissural neurons were retrogradely labeled from the midbrain injection. These DiI-labeled cell bodies were found in a location similar to the location of those of the ventromedial GAD65-positive commissural neurons discussed above. No dorsally located DiI-labeled cell bodies were detected, but a few longitudinally directed DiI-labeled fibers were observed just ventral to the DiI-labeled cell bodies.

DISCUSSION

Spinal commissural neurons serve as a model system for studying the molecular cues that guide axonal pathfinding, yet to date little is known about the neurotransmitter phenotype of these neurons or their final synaptic targets. In the present study, we have shown that ventral GABAergic neurons extend their axons through the commissure before turning rostrally within the contralateral ventral marginal zone and coursing into the brainstem. In support of these findings, presumptive termination sites were labeled in the mesencephalon after contralateral DiI injections in the ventral spinal cord. In addition, cell bodies were detected in the ventromedial spinal cord from reciprocal DiI injections into the presumptive midbrain targets. Thus, we have shown that some commissural axons are projection neurons, and these are among the earliest axons to leave the spinal cord. Taken together, our data suggest that a group of GABAergic ventral commissural neurons represents an early-forming part of the spinomesencephalic tract.

Some GABAergic commissural neurons are projection neurons

Although many reports have suggested that commissural neurons in the spinal cord are interneurons (Windle and Baxter, 1936; Holley, 1982; Yaginuma et al., 1991), it is likely that they project to a number of different targets and mediate a variety of functions based on their anatomical diversity (Wentworth, 1984; Silos-Santiago and Snider, 1992; Ramón y Cajal, 1995). For example, one group of commissural neurons was reported to project their axons into the contralateral motor neuron pools, whereas another group extended their axons into the con-

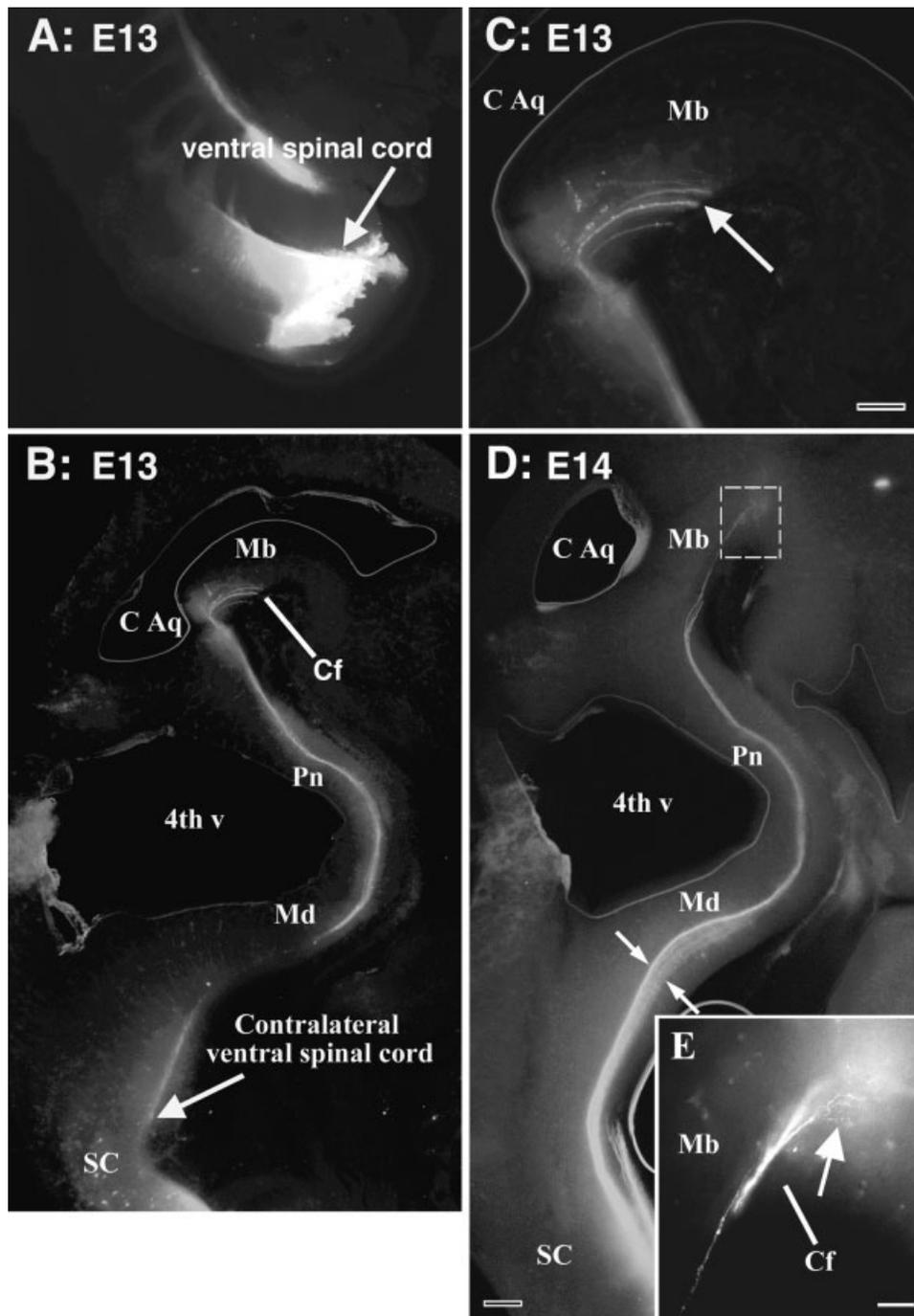


Fig. 5. Sagittal sections of E13 (A–C) and E14 (D,E) rat embryos were injected unilaterally with DiI in the ventral spinal cord. **A:** At E13, DiI was injected (arrow) into the ventral spinal cord. A small amount of dye spread to the sympathetic trunk. **B:** Approximately 400 μm medial to the injection site, DiI-labeled axons were detected in the contralateral ventral marginal zone, medulla (Md), and pons (Pn) and appeared to end in the midbrain (Mb) around the cephalic flexure (Cf). **C:** Enlargement of the midbrain area in B shows the presumptive

distal endings of the DiI-labeled axons (arrow). **D:** At E14, a similar DiI-labeled axon pathway projected from the ventral spinal cord to the rostral brainstem and appeared to terminate in the midbrain (boxed area). **E:** In the enlargement of the boxed area in D, DiI-labeled fibers appeared to end just above the cephalic flexure. The distal ends of the DiI-labeled axons arborized in a terminal-like field (arrow). Scale bars = 200 μm in D (applies to A,B,D); 100 μm in C; 50 μm in E.

tralateral marginal zone (Windle and Baxter, 1936; Ramón y Cajal, 1995). Additional studies indicated that the axons of commissural neurons crossed to the con-

tralateral ventral marginal zone, turned rostrally, and then extended their axons for only a few spinal segments (Oppenheim et al., 1988; Imondi and Kaprielian, 2001;

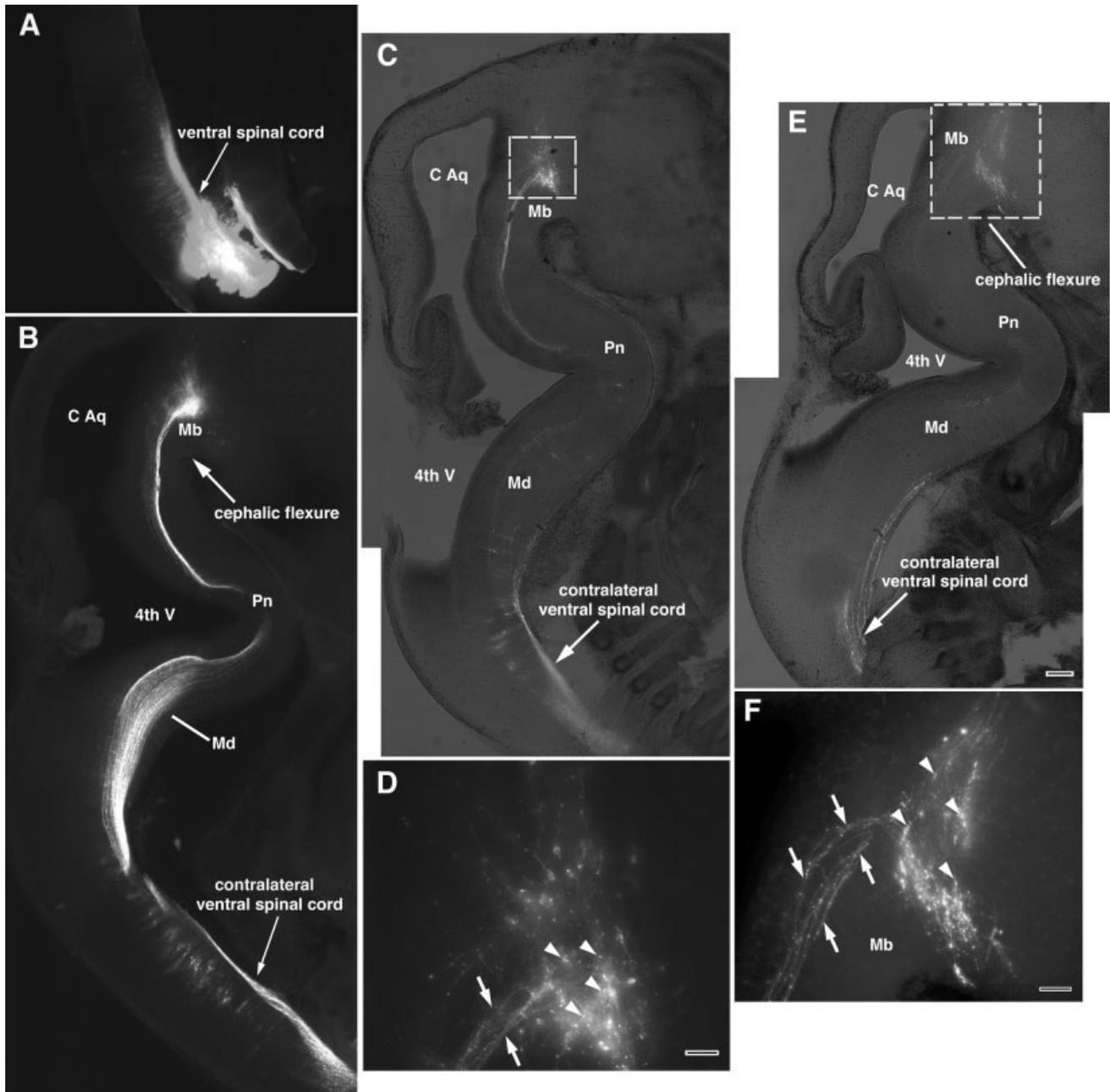


Fig. 6. Serial sections from a single 15-day-old embryo are illustrated in the sagittal plane. **A:** The ventral unilateral injection site has a short ascending pathway. **B:** Approximately 600 μm from the injection site in A, DiI-labeled axons were followed from the contralateral ventral marginal zone into the brainstem. The axon pathway widened as it traversed the medulla, then narrowed as it projected through the central part of the pons. A potential termination site was found in the midbrain, at the level of the cephalic flexure (arrow). **C:** In a section adjacent to B, DiI-labeled axons were detected in the

rostral pons that continued into the midbrain and formed an apparent termination site. **D:** Enlargement of the boxed region in C illustrates numerous punctate DiI-labeled structures (arrowheads) emanating from the ascending axons (arrows). **E:** In a section directly adjacent to C, another likely DiI-labeled termination site (boxed area) was detected in the midbrain. **F:** Enlargement of the boxed area in E shows DiI-labeled axons (arrows) ending in a crescent-shaped, punctate area (arrowheads) in the lateral midbrain. Scale bars = 200 μm in E (applies to A–C,E); 50 μm in D; 100 μm in F.

Moran-Rivard et al., 2001). This diversity in axonal projections suggests that distinct groups of commissural neurons have different synaptic targets and thus are likely to

have different functions as well. Such diversity in commissural targets has been reported for fish. One group of goldfish commissural neurons was associated with con-

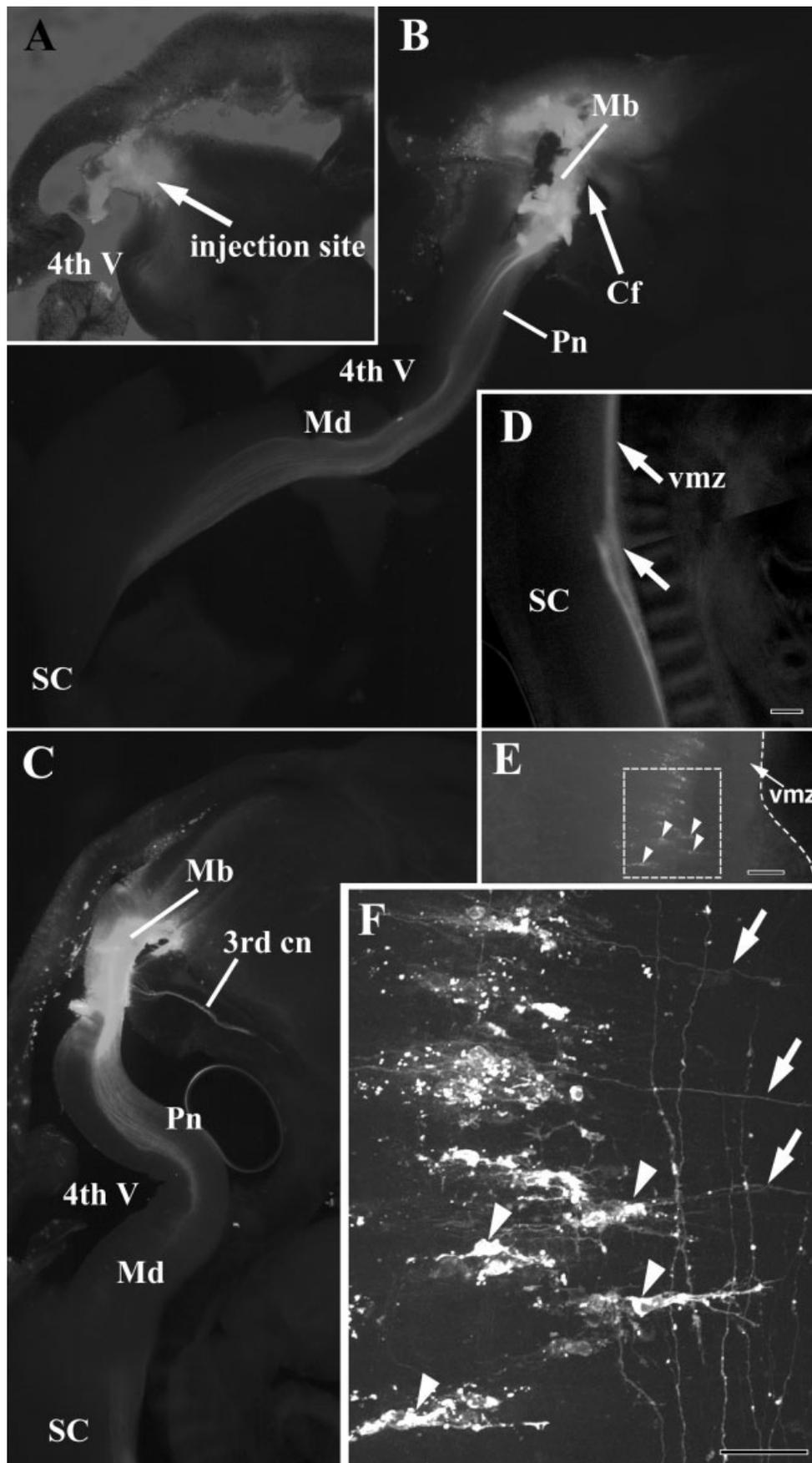


Figure 7

tralateral spinal interneuron reflexes (Fetcho, 1990), and three other groups of GABAergic commissural neurons projected rostrally into the brainstem of zebrafish (Bernhardt et al., 1992). Our findings are consistent with reports on zebrafish and are the first to demonstrate that some vertebrate GABAergic commissural neurons are projection neurons.

For years, the GABAergic neurons in the spinal cord also were thought to be spinal interneurons, most likely because of the abundance of GABAergic terminals within the gray matter, especially in the dorsal horn (Barber and Saito, 1976; Todd and Lochhead, 1990; Todd and Sullivan, 1990). In addition, little GAD immunoreactivity was detected in the surrounding white matter in the adult spinal cord (McLaughlin et al., 1975; Mugnaini and Oertel, 1985), another observation that might have led to the assumption that GABAergic spinal cord neurons are interneurons. The present experiments took advantage of the unexpectedly high levels of GAD65 expression within embryonic cell bodies and axons to provide the initial insight into the projection pathway of GABAergic commissural axons (Phelps et al., 1999; Tran et al., 2003). In addition, by blocking axonal transport in organotypic cultures, we changed the subcellular localization of GAD65 and GAD67 and thus explained the normal developmental shift in the GAD proteins from somata and axons at early ages to terminal-like structures at older ages (Tran et al., 2003).

GAD65-positive axons are a subset of those labeled by L1

Our present data illustrate a significant number of L1-positive axons coursing within the ventral marginal zone, which is consistent with previous reports (Stoeckli and Landmesser, 1995; Imondi and Kaprielian, 2001). In addition, our previous and present results show that both GAD65- and L1-positive commissural axons travel along the same pathway (Tran and Phelps, 2000). As early as E12.5, both GAD65- and L1-labeled pathways course in a tight fascicle in the ventral marginal zone and along the ventral edge of the brainstem. Subsequently, both GAD65- and L1-labeled fiber bundles broaden as they coursed from the ventral edge to the central core of the medulla, where they continue rostrally. However, many more L1- than GAD65-labeled axons were detected within the ventral marginal zone pathway. This finding indicates that multiple sources

contribute to the L1-labeled axonal pathway, including the ascending GAD65-positive axons.

Correlation of DiI and GABAergic axonal projections from the spinal cord to the midbrain

There are close spatial and temporal correlations between the axonal pathways detected with GAD65 immunoreactivity and the DiI injections into the ventral spinal cord. Both DiI- and GAD65-labeled axons crossed the ventral commissure and coursed rostrally within the contralateral marginal zone and then projected deep into the central medulla. Both pathways continued within the central pons until they reached the mesencephalic tegmentum. In addition, transverse midbrain sections immunolabeled with GAD65 displayed numerous GABAergic terminal-like structures at this stage, which is consistent with the previously reported pattern for the mouse (Katarova et al., 2000). Finally, the detection of GABAergic punctate structures in the mesencephalic tegmentum correlates well with the synaptic terminal location of the ascending GAD65-positive pathway.

Anterograde and retrograde DiI-labeled axons follow a reciprocal pathway

The pathway labeled by injections into the ventral spinal cord produced terminal-like structures in the contralateral mesencephalic tegmentum as identified in atlases (Paxinos et al., 1991, 1994; Alvarez-Bolado and Swanson, 1996). These findings suggest that DiI was actively and passively transported anterogradely. In contrast, DiI injections into the mesencephalic tegmentum labeled cell bodies within the contralateral ventromedial intermediate zone of the spinal cord, findings consistent with DiI moving in a retrograde direction. Furthermore, both pathways coursed through the central region of the brainstem to and from the midbrain. These findings suggest that both DiI-labeled axonal pathways identify a long projection pathway originating in the spinal cord and terminating in the midbrain.

Some commissural axons project in the spinomesencephalic tract to the midbrain reticular formation

Axon pathways labeled with GAD65, L1, and DiI all appear to originate in the ventromedial spinal cord and terminate in the lateral midbrain, suggesting that they compose part of the spinomesencephalic tract (Paxinos, 1995). Studies of the adult spinomesencephalic tract showed their cell bodies of origin in laminae I, III–V, VII, and X (Wiberg and Blomqvist, 1984; Paxinos, 1995). The ventromedial GAD65 commissural cell bodies found during development are most likely to be located in lamina VII of the adult spinal cord. To date, most axons in the spinomesencephalic tract are known to cross in the ventral commissure and then project rostrally in the ventrolateral funiculus along with other tracts of the anterolateral system (Heimer, 1995; Paxinos, 1995). The axons of GAD65- and DiI-labeled commissural neurons course in the ventral marginal zone and appear to terminate in the midbrain. All of these findings are consistent with previous reports of the spinomesencephalic pathway in adults.

The synaptic targets of the adult spinomesencephalic axons have been identified as the periaqueductal gray, the

Fig. 7. Serial sagittal sections of retrograde DiI tracing in a single 14-day-old embryo (A–F). **A:** DiI injection site was in the midbrain (arrow). **B:** A section adjacent to A illustrates DiI-labeled axons descending from the midbrain as far as the medulla. **C:** The section adjacent to B shows a DiI-labeled axonal pathway coursing through the center of the brainstem and the third cranial nerve exiting the midbrain in the cephalic flexure. **D:** In the section adjacent to C, DiI-labeled fiber bundles coursed in the ventral marginal zone (arrows) of the spinal cord. **E:** In the section adjacent to D, DiI was detected in the ventral one-third of the contralateral spinal cord. The edge of the ventral marginal zone is indicated by the dashed line. **F:** A confocal enlargement of the boxed area in E illustrates numerous DiI-labeled neurons (arrowheads). Several DiI-labeled axons (arrows) coursed between the midline of the spinal cord and the DiI-labeled cells, the expected axonal pathway of spinal commissural neurons. Additional longitudinally oriented DiI-labeled axons were detected. Scale bars = 200 μm in D (applies to A–D); 100 μm in E; 50 μm in F.

mesencephalic reticular formation, the ventral tegmental area, the interpeduncular nucleus, and the tectum (Wiberg and Blomqvist, 1984; Heimer, 1995; Paxinos, 1995). Among these different sites, our DiI-labeled axons appeared to terminate in a midbrain location too far ventrally to project to the tectum or the periaqueductal gray. The DiI-labeled terminal fields also were located too far laterally for them to be in midline structures such as the ventral tegmental area or the interpeduncular nucleus. Rather, the termination site of the DiI-labeled axons was most consistent with a target located in the mesencephalic reticular formation (Paxinos et al., 1994; Alvarez-Bolado and Swanson, 1996).

In conclusion, the cell body locations, axonal projections, and terminal fields of the GAD65-, L1-, and DiI-labeled pathways are all consistent with previous reports of the spinomesencephalic tract that projects to the midbrain reticular formation (Menétreay et al., 1982, 1983; Paxinos, 1995). In addition, numerous GAD65-immunoreactive terminals were found in the mesencephalic reticular area in E14–15 embryos, which is consistent with reports for adults (Mugnaini and Oertel, 1985; Paxinos, 1995). The consistency of these findings with three different labeling methods strengthens our conclusion that early-forming GABAergic ventral commissural neurons compose part of the spinomesencephalic pathway. Finally, the observation that GABAergic axons project rostrally at such an early developmental stage suggests that these pathways mediate important early connections between the spinal cord and the brainstem.

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