Engrailed-1 and Netrin-1 regulate axon pathfinding by association interneurons that project to motor neurons

Harald Saueressig, John Burrill and Martyn Goulding*

Molecular Neurobiology Laboratory, The Salk Institute, 10010 North Torrey Pines Rd, La Jolla, CA 92037, USA

*Author for correspondence (e-mail: goulding@salk.edu)

Accepted 19 July; published on WWW 7 September 1999

SUMMARY

During early development, multiple classes of interneurons are generated in the spinal cord including association interneurons that synapse with motor neurons and regulate their activity. Very little is known about the molecular mechanisms that generate these interneuron cell types, nor is it known how axons from association interneurons are guided toward somatic motor neurons. By targeting the axonal reporter gene tau-lacZ to the En1 locus, we show the cell-type-specific transcription factor Engrailed-1 (EN1) defines a population of association neurons that project locally to somatic motor neurons. These EN1 interneurons are born early and their axons pioneer an ipsilateral longitudinal projection in the ventral spinal cord. The EN1 interneurons extend axons in a stereotypic manner, first ventrally, then rostrally for one to two segments where their axons terminate close to motor neurons. We show that the growth of EN1 axons along a ventrolateral pathway toward motor neurons is dependent on netrin-1 signaling. In addition, we demonstrate that En1 regulates pathfinding and fasciculation during the second phase of EN1 axon growth in the ventrolateral funiculus (VLF); however, En1 is not required for the early specification of ventral interneuron cell types in the embryonic spinal cord.

Key words: Engrailed-1, Netrin-1, Pathfinding, Association interneuron, Motor neuron, Mouse

INTRODUCTION

The development of the embryonic spinal cord is marked by the generation of multiple neuronal cell types that arise at different dorsoventral positions in the neural tube in response to inductive signals that pattern the neural tube along its dorsoventral (DV) axis (Tanabe and Jessell, 1996). Recent studies have revealed that the patterning of neuronal cell types by these inductive signals is regulated by transcription factors that are expressed in restricted populations of dividing neuronal precursors within the ventricular zone (Burrill et al., 1997; Ericson et al., 1997; Briscoe et al., 1999). These transcription factors are thought to function by controlling the expression of a second class of “cell-type-specific” transcription factors that are expressed in restricted populations of early postmitotic neurons. Three cell-type-specific transcription factors that are expressed in the embryonic spinal cord are the homeodomain proteins, EN1, CHX10 and EVX1, which mark three populations of ventral interneurons (Burrill et al., 1997; Ericson et al., 1997; Matise and Joyner, 1997). However, the role that these proteins play in the differentiation program of spinal interneurons has not been determined, primarily because the cell types in the embryonic spinal cord that express these transcription factors are not known. Instead, studies of these and other cell-type-specific transcription factors in the spinal cord have been largely restricted to using them as molecular markers to examine the patterning of early neuronal cell types in the embryonic neural tube (Pfaff et al., 1996; Burrill et al., 1997; Ericson et al., 1997; Matise and Joyner, 1997; Lee et al., 1998; Briscoe et al., 1999).

EN1 is a prototypic cell-type-specific transcription factor that is expressed in a restricted population of early postmitotic ventral neurons that are located in two bilateral columns dorsal to motor neurons (Davis et al., 1991; Burrill et al., 1997; Matise and Joyner, 1997). The expression of EN1 in these neurons is controlled by inductive signals that pattern the ventral neural tube (Pfaff et al., 1996; Ericson et al., 1997). EN1 expression in the ventral spinal cord is dependent on the activity of the PAX6 transcription factor which is expressed in ventral progenitors that give rise to EN1 interneurons, and EN1 is no longer expressed in the ventral spinal cord of Small eye (Pax6) mutant embryos (Burrill et al., 1997; Ericson et al., 1997). The restricted expression of EN1 in early postmitotic neurons, together with the specific loss of these cells in Pax6 mutant mice has led to the hypothesis that EN1 marks a subclass of ventral interneurons and that this interneuron subclass may be specified in part by EN1 (Burrill et al., 1997; Matise and Joyner, 1997). However, the interneurons in the embryonic spinal cord that express EN1 have not been characterized in detail, nor has the function of En1 in these neurons been determined.

In this study, we have used tau-lacZ as a neuronal tracer to characterize in detail the EN1-expressing neurons in the embryonic spinal cord and analyze the role En1 plays in their development. We find that EN1 is expressed specifically by ipsilaterally projecting association interneurons that extend...
locally toward motor neurons. Axons from the EN1 interneurons project ventrally then rostrally in the VLF, where they establish local ipsilateral projections to motor neurons in the embryonic spinal cord. The EN1 axons express DCC and require netrin-1 to extend axons ventrally toward motor neurons where they normally pioneer the formation of the VLF. Our studies demonstrate that En1 does not control early cell fate in ventral spinal interneurons, instead En1 controls late aspects of EN1 interneuron differentiation where it is necessary for the organized growth of EN1 axons in the VLF toward motor neurons.

MATERIALS AND METHODS

Generation and breeding of En1tau-lacZ “knock-in” mice
The En1tau-lacZ targeting vector is shown in Fig. 1. The vector was created by replacing sequences encoding the endogenous methionine initiation codon and first 72 amino acids of EN1 with a gene cassette encoding the tau-lacZ gene and the PGKneoA gene flanked by loxP sites (see Fig. 1) similar to the strategy described by Hanks et al. (1995). CCE ES cells were cultured on neomycin/gancyclovir-resistant LIF-secreting STO fibroblast cells. 2x10^5 ES cells were electroporated with 25 µg of KpnI-linearized DNA using standard settings (Biorad Gene Pulser) and recombinant clones were selected in G418 (200 µg/ml active drug) and FIAU (0.2 µM) containing media. ES cell clones were screened for correctly targeted integration of tau-lacZ using an external 3' genomic DNA probe (HindIII-digested genomic DNA probed with 700 bp EcoRI/HindIII DNA fragment). Positive clones were confirmed by Southern blot analysis using a 5' genomic DNA probe (Hanks et al., 1995). Two ES cell clones (C7 and C145) were microinjected into C57Bl6 blastocysts and reimplanted into pseudopregnant ICR foster. Chimeric mice were bred with C57Bl6 females to obtain germline transmission and the En1loxZ allele was maintained on a mixed 129Sv/C57Bl6 background. Three chimeric mice transmitted the En1loxZ allele through the germline and their offspring were used for these studies. Genotyping of En1loxZ/loxZ embryos was performed by Southern analysis as described above using yolk sac DNA. The En1loxZ/loxZ mutant embryos were also identified by the loss of midbrain structures and altered limb morphology. The axonal projection pattern of the EN1 interneurons was examined in netrin-1-deficient embryos by crossing En1loxZ/+ heterozygous mice with netrin-1+/− mice (Skarnes et al., 1995). En1loxZ/+; netrin-1−/− mutant embryos were obtained by crossing En1loxZ/+; netrin-1+/− mice with netrin-1−/− females. Genotyping was performed using PCR primers specific for each gene.

Whole-mount X-Gal staining
Mouse embryos were fixed in 0.2% glutaraldehyde in 100 mM phosphate buffer, pH 7.3, 5 mM EGTA for 30 minutes at 4°C, then rinsed in the same buffer without glutaraldehyde. X-gal staining was performed as previously described (Matise and Joyner, 1997).

Whole-mount antibody staining
Mouse embryos were immersion fixed in PBS containing 4% paraformaldehyde (PFA), rinsed twice in phosphate-buffered saline PBS containing 0.5% Triton X-100 (PBST), blocked with PBST/10% NGS and incubated in primary antibody for 1-3 days. The following antibodies were used in this study: anti-β-galactosidase (1:10,000; Organan Technikon-Cappel), anti-Engrailed (1:3, 4G11 monoclonal, Developmental Hybridoma Bank), anti-TAG1 (1:100; 4D7 monoclonal, Developmental Hybridoma Bank), anti-DCC (1:200; Calbiochem), anti-GAD65 (1:1000; monoclonal, Developmental Hybridoma Bank), anti-TUJ1 (1:500; BABoC), anti-Isl1 (1:10; monoclonal, Developmental Hybridoma Bank) and anti-LIM3 (1:3000, S. Pfaff). After several washes with PBST, specimens were incubated for several hours with HRP- or FITC/Cy3-conjugated secondary antibodies.

Fig. 1. Generation of En1loxZ knock-in mice. (A) Structure of the mouse En1 locus. The En1tau-lacZ knock-in targeting vector is shown below. Abbreviations: B, BamHII; C, ClaI; H, HindIII; R, EcoRI; X, XbaI; neo, PGKneoA G418 resistance cassette used for positive selection flanked by loxP recombination sites (triangles); T-lacZ, coding region of the tau-lacZ fusion gene; pA, SV40 polyadenylation signal. DNA probe: A 0.7 kb EcoRI-HindIII fragment from the 3' end of the En1 gene was used to screen ES cells for homologous recombinants by Southern blotting. The mutated allele of En1 gives a predicted band of 4.5 kb with this 3' DNA probe compared to the wild-type allele which gives a 7.5 kb band. (B) Southern blots of HindIII digested genomic DNA from 129Sv mouse DNA, the CCE ES cell line and two ES cell lines that have undergone homologous recombination at the En1 locus (C7 and C145). The 7.5 kb wild-type band is present in all four samples. A diagnostic 4.5 kb HindIII band is present in the two ES cell lines, C7 and C145, where the En1 gene is correctly targeted. (C) Southern analysis of genomic DNA from a litter of F2 embryos. (Both parents were En1loxZ/loxZ mice). (D) β-galactosidase (β-gal) expression in E12 En1loxZ knock-in mouse embryos. β-gal is expressed in the spinal cord (arrow) and in the midbrain (arrowhead). Note the loss of the midbrain En1 expression domain in En1loxZ/loxZ embryos.
secondary antibodies (Jackson ImmunoResearch) for DAB staining reactions or immunofluorescence, respectively. After final washes, specimens were mounted in PBS/glycerol (1:9) or glycerol/DABCO for microscopy.

**Retrograde tracing experiments**

Spinal cords from E11-E12 mouse embryos were dissected using sharpened tungsten needles and then pinned out straight in Sylgard-coated Petri dishes. Fluorescein-conjugated Dextran (Molecular Probes) was injected into the ventral spinal cord in the area of the VLF and allowed for transport for several hours (see Burrill et al., 1997). After fixation in 4% PFA, whole-mount antibody-labeling was performed as described above with antibodies to β-galactosidase. Spinal cords were then separated into two halves and cleared in glycerol/DABCO before being mounted on coverslips for confocal microscopy. Double-labeled cells were scored and their position relative to the injection site measured.

**Confocal microscopy**

Whole-mounted spinal cords and sections were analyzed using a Zeiss confocal microscope (LSM 510, Version 1.5). Confocal images were assembled with Adobe Photoshop 4.0.

**RESULTS**

**Generation of En1tauαlacZ knock-in mice**

To determine the identity of the EN1 cells, we generated a “knock-in” mouse that expresses the axonal reporter protein tau-β-galactosidase (Callahan and Thomas, 1994) under the control of En1 regulatory sequences. A tau-lacZ reporter gene cassette was recombined into the first exon of En1 in ES cells and these cells were then injected into blastocysts to generate chimeric mice (see Methods for details, Fig. 1A,B). En1<sup>tlZ/+</sup> heterozygous mice were analyzed at a number of different developmental ages, revealing a pattern of tau-β-galactosidase (tau-β-gal) activity that was indistinguishable from the endogenous expression pattern of En1 (Figs 1D, 2A). Two stripes of tau-β-gal expression were observed in the ventral spinal cord from E9.5 until E16. Expression of tau-β-gal was highest from E9.5 to E13, declining in older embryos to undetectable levels at birth. The insertion of the tau-lacZ gene into the first exon of En1 was predicted to result in an En1 null allele and consistent with this, EN1 protein staining was absent in homozygous En1<sup>tlZtlZ</sup> embryos (data not shown). Homozygous En1<sup>tlZtlZ</sup> embryos also exhibited a truncation of the midbrain and limb defects (Fig. 1D), as had previously been described for the En1 knockout mice (Wurst et al., 1994; Loomis et al., 1996). No morphological defects were found in En1<sup>tlZ/+</sup> heterozygous embryos demonstrating that the En1 gene is haplosufficient.

**EN1 expression defines a population of locally projecting ipsilateral interneurons in the spinal cord**

En1<sup>tlZ/+</sup> heterozygous embryos of various ages were stained with an antibody to β-galactosidase to characterize the EN1 interneurons. Tau-β-gal was localized to the cell bodies and the axonal processes of the EN1 interneurons, enabling their morphology to be visualized in detail (Fig. 2). In E10 and E12 embryos, stained EN1 axons could be seen throughout the ventral horn at spinal cord and hindbrain levels. In E10 embryos, two prominent phases of early axon growth were...
discernible. Soon after they are born, the EN1 interneurons migrate laterally into the mantle zone where they extend axons along a ventrolateral trajectory toward newly born motor neurons in the ventral horn (Fig. 2B). Upon reaching the same dorsoventral level as motor neurons, the EN1 axons turn rostrally where they grow for varying distances in the developing ventrolateral funiculus, immediately lateral to motor neurons. Since the spinal cord at E10 already contains large numbers of EN1 interneurons making it difficult to visualize individual axons, chimeric En1tlZ embryos were generated that contained fewer marked neurons in the spinal cord. In E10 chimeric embryos, individual EN1 interneurons were seen extending axons along a ventrolateral route toward motor neurons in the ventral horn (Fig. 2C). At E10.5, individual EN1 interneurons could be seen extending axons in a rostral direction for distances of up to 200 μm, immediately adjacent to differentiating motor neurons (Fig. 2D). In spinal cords from E10.5 En1tlZ/+ heterozygous embryos, these EN1 axons formed a broad non-fasciculated tract immediately lateral to motor neurons and in older E12–E13 embryos the EN1 axons were distributed in an arc encompassing all somatic motor neurons (see Figs 7 and 8).

To examine whether axons from the EN1 interneurons project into the motor neuron column, fluorescein-conjugated dextran was injected into the forelimb muscles of E12.5 En1tlZ/+ embryos to retrogradely label motor neurons, and an antibody to β-galactosidase was then used to visualize EN1 axonal processes. Axons from EN1 interneurons were found intermingled with motor neurons throughout the ventral horn (Fig. 2E). To determine whether these EN1 axons contacted motor neurons, sections through E12.5 retrogradely labeled En1tlZ/+ embryos were then imaged in 2 μm optical slices with a confocal microscope. Throughout these sections, axonal processes from EN1 interneurons were seen closely abutting the soma of motor neurons (Fig. 2E). However, it should be noted that, using this approach, we were not able to determine whether the EN1 axons directly contact motor neurons. Furthermore, attempts to analyze synapse formation in older embryos by immuno-EM have been hindered by reduced En1tlZ expression at later times in development. Nevertheless, our observation that axons from EN1 interneurons terminate adjacent to motor neurons and that their processes closely abut motor neurons supports our hypothesis that they form connections with motor neurons. This conclusion is supported by studies in the embryonic avian spinal cord showing Renshaw cells and a number of last order interneurons express EN1 (Wenner et al., 1998). Interestingly, no axons from EN1 interneurons were seen projecting to preganglionic sympathetic neurons in the intermediolateral column, arguing the EN1 interneurons form connections specifically with somatic motor neurons.

EN1 interneurons establish early ipsilateral projections in the ventral spinal cord

The EN1 interneurons develop early when few other differentiated neurons are present in the embryonic spinal cord suggesting that the EN1 interneurons pioneer ipsilateral rostral projections in the ventral spinal cord. We were therefore interested in ascertaining whether the EN1 axons navigate independently toward motor neurons or use other axons to direct their growth. Previous studies in the mouse and chick have shown commissural neurons are among the first neurons that are born and differentiate in the embryonic spinal cord (Wentworth, 1984; Holley and Silver, 1987), suggesting commissural axons might provide a scaffold for the ventral growth of the EN1 axons. Since TAG1 is expressed on the axons of commissural neurons, an antibody to TAG1 (4D7) was used to compare the growth of commissural neurons with EN1 axon outgrowth. When the EN1 axons first begin projecting at E10, little or no TAG1 expression was seen in the dorsal spinal cord that could be attributed to commissural axons (Fig. 3A). Although, TAG1 expression was observed in the ventral horns in the vicinity of the EN1 axons, further analysis showed this expression to be restricted to motor neurons and their axons. These axons exit the ventral spinal cord and are therefore unlikely to function as a substratum for EN1 axon extension. In older E10.5 embryos, TAG1 commissural axons were seen projecting directly toward the floor plate along a characteristic medial pathway (Fig. 3C), whereas the EN1 interneurons extended axons along a more lateral route toward motor neurons (Fig. 3B,C). Furthermore, our analysis of TAG1 and β-gal staining in En1tlZ/+ embryos shows the first EN1 axons reach the ventral horn and turn rostrally before any commissural axons have crossed the floor plate (data not shown).

To investigate whether other axons provide a substratum for growing EN1 axons, we then compared the onset and timing of EN1 axon outgrowth with the development of other early-born neurons in the spinal cord using an antibody to neuron-specific β-tubulin (Yagamuna et al., 1990; Easter et al., 1993; Burrill et al., 1997). En1tlZ/+ embryos were stained with antibodies to β-gal and β-tubulin (TuJ1) and the trajectories of ventrally directed EN1 axons were then imaged by confocal microscopy and compared with other axons marked by β-tubulin expression. None of the individual EN1 axons examined (n=38) were seen fasciculating with axons from TuJ1+/EN1− interneurons or with other EN1 axons, arguing that the first EN1 axons that grow toward the ventral horn do so independently (Fig. 3D, also see Fig. 2B). In addition, we examined the early trajectory of isolated EN1 axons as they turned rostrally within the ventral spinal cord (Fig. 3E,F). Once again, no other longitudinally aligned axons were seen in the immediate vicinity of EN1 axons either during or after they had turned rostrally in the VLF (n=27). Consequently, when the EN1 axons enter the VLF, they do not grow together with other axons, nor do they join a pre-existing axon scaffold. Although a small number of EN1− longitudinal processes were seen within the VLF at E10, in most cases these were well separated from the EN1 axons. Our finding that relatively few longitudinally projecting EN1− axons are present in the ventral spinal cord when EN1 interneurons begin extending axons rostrally, leads us to conclude the EN1 interneurons help pioneer the formation of the VLF in the mammalian spinal cord.

Netrin-1 controls the ventral growth of ipsilateral interneurons in the spinal cord

The EN1 interneurons project axons ventrally albeit by a different pathway than commissural neurons that extend axons medial to motor neurons en route to the floor plate. While previous studies have demonstrated an essential role for netrin-1 in guiding commissural neurons toward the floor plate, its
role in diverting the growth of axons from association neurons has not been examined due to an inability to specifically mark these cells. We were therefore interested in determining whether ipsilaterally projecting association neurons also respond to netrin-1 to guide their axons ventrally toward motor neurons, since low levels of netrin-1 have been reported to be present throughout the ventral spinal cord (Kennedy et al., 1994; Serafini et al., 1996). Spinal cords from E10.5 En1+/−/+ heterozygous embryos were first examined to determine whether the netrin receptor Deleted in Colorectal Cancer (DCC) is expressed on the axons of the EN1 interneurons (Fig. 4). Both proteins were found co-localized in EN1 neurons and their axons (Fig. 4C, see arrow) at E10.5. Interestingly, DCC was not expressed by immature neuroepithelial-like EN1 cells that lacked a ventrally directed axon (Fig. 4C, arrowhead), nor was it expressed at E12.5, when EN1 interneurons no longer extend axons ventrally toward the ventral horn (data not shown).

To test directly whether netrin-1 regulates the ventral growth of ipsilaterally projecting axons, En1+/−/+ mice were crossed with netrin-1 (net-1+/−) heterozygous mice (Skarnes et al., 1995). Analysis of the axonal morphology of EN1 interneurons in second generation progeny from these crosses revealed a dramatic difference in the projection pattern of the EN1 interneurons in spinal cords from En1+/−/+; net-1−/− mutant embryos. In E11 wild-type and net-1 heterozygous embryos, all the EN1 neurons project ventrally to the level of the motor neurons before turning rostrally in the VLF (Fig. 5A). In contrast, the majority of these axons were either truncated or turned rostrally before reaching the VLF in homozygous net-1−/− embryos (Fig. 5B,C). While a few EN1 axons exhibited near normal projection patterns in net-1−/− embryos (Fig. 5C, arrowheads), the vast majority of the ventrally projecting EN1 axons possessed short ventral processes with an average axon length of 60-70 μm compared with 140-150 μm in wild-type embryos (Fig. 5D). A number of longitudinally directed axons were also seen at the same dorsoventral level as the EN1 cell bodies (see arrow in Fig. 5E) and among these, we were able to trace individual axons that projected in a rostral direction. This observation demonstrates netrin-1 is necessary for the first phase of EN1 axon growth and that it seems to play no role in the decision to project rostrally or caudally. Furthermore, this result suggests that rostrocaudal cues for association axons are present throughout the spinal cord and are not restricted to the VLF. We also observed small numbers of EN1 neurons with dorsally directed axons in netrin-1 mutant spinal cords (Fig. 5F), further supporting our conclusion that netrin-1 is required for the ventral growth of ipsilateral axons, since dorsally directed EN1 axons were never observed in wild-type embryos.

**En1 does not control early aspects of ventral interneuron cell fate**

Previous functional studies of engrailed (en) in invertebrates have revealed a role for en in controlling neuronal cell fate and neurotransmitter phenotype. In the grasshopper embryo, en controls the differentiation of glia and neurons that arise from the midline neuroblast (Condron et al., 1994) and en is also required for the development of serotonergic neurons from the NB 7-3 neuroblast in Drosophila (Lundell et al., 1996). The expression of EN1 in a discrete population of early postmitotic interneurons prompted us to ask whether En1 specifies early interneuron cell fate or neurotransmitter phenotype in the embryonic spinal cord. E12 En1 mutant embryos were examined to determine whether loss of the En1 gene causes the EN1 interneurons to assume an alternative cell fate, as evidenced by a change in their early axonal morphology. The axon trajectories of EN1 interneurons were examined by retrogradely labeling axons in the ventral spinal cord with a fluorescein-conjugated dextran tracer. In En1+/−/+ heterozygous embryos, retrogradely labeled EN1 interneurons were found with axons extending up to 500-600 μm or two spinal cord segments in a rostral direction (Fig. 6A), and many of these appeared to project intrasegmentally (<250 μm). While the vast majority of EN1 interneurons labeled in this manner possessed a rostrally directed axon, a few caudally projecting EN1 spinal interneurons (n=3/178) were also labeled. A careful comparison of retrogradely labeled EN1 interneurons in heterozygote (n=178) and En1 mutant spinal cords (n=179) did not reveal any dramatic change in the projections of the EN1 interneurons that would indicate a change in cell fate (Fig. 6A). Unlike the CHX10/LIM3 interneurons, the EN1 interneurons did not project over several spinal cord segments, nor did they project contralaterally like the EVX1 interneurons. A small difference, however, was seen in the axon length of retrogradely labeled cells in En1 mutant spinal cords, and this primarily involved an increase in the number of neurons that project 200-300 μm together with an associated loss of EN1 cells with short-projecting axons (<100 μm). Whether this difference is due to the pathfinding defects observed in En1 mutant embryos or instead involves the loss of a population of short-projecting EN1 interneurons remains to be determined.

The above retrograde analysis of the EN1 projections confirms our hypothesis that the EN1 interneurons project locally to motor neurons and suggests En1 does not control early aspects of cell fate in these cells. To further examine this, we asked whether En1 controls the expression of neurotransmitters such as GABA in these interneurons. Previous physiological studies in the spinal cord indicate that many of the locally projecting interneurons present in the ventral spinal cord are GABAergic. To first ascertain whether the EN1 interneurons synthesize GABA, we examined the expression of GAD65 in the embryonic spinal cord. Transverse sections through the spinal cord of E11 En1+/−/+ heterozygous embryo, when stained with antibodies to GAD65 and β-galactosidase, showed coexpression of GAD65 in all of the EN1 neurons as well as in a population of commissural neurons immediately adjacent to the EN1 interneurons (Fig. 6B). To ascertain whether EN1 is necessary for these interneurons to differentiate into GABAergic neurons, GAD65 expression was also analyzed in spinal cords from E11 En1+/− homozygous embryos. No difference was observed in the overall pattern of GAD65 staining at E11 in the En1−/− mutants and, in particular, expression of GAD65 was unchanged in the EN1 interneurons (Fig. 6C). Consequently, En1 function is not required for establishing the early GABAergic phenotype of the EN1 interneurons.

**EN1 interneurons exhibit fasciculation and pathfinding defects in En1 mutants**

Our morphological analysis of the EN1 interneurons shows they project axons locally to the ventral horn where they innervate motor neurons within 1-2 segments of their cell
bodies. Consistent with this, the EN1 axons upon entering the VLF do not fasciculate and are instead distributed throughout the funiculus in a parallel array of single axons that extend rostrally for short distances (Fig. 7). As early as E10.5, EN1 interneurons with a typical inverted L-shape morphology were seen at all levels of the spinal cord in En1 tlZ/+ embryos. While axons with roughly similar trajectories were also observed in En1tlZ/-/- embryos, clear differences could be seen between their trajectories and those present in heterozygous En1 tlZ/+ embryos. In E10.5 En1tlZ/-/- embryos, EN1 axons were often seen to turn dorsally soon after entering the VLF (Fig. 7B, inset). Furthermore, even at these early stages, axon bundles containing three or more EN1 axons were found throughout the VLF in E10.5 En1tlZ/-/- mutant spinal cords (see arrows in Fig. 7B), in sharp contrast to heterozygous En1tlZ/+ embryos where EN1 axons do not fasciculate with each other (Fig. 7A). When spinal cords from older E12 heterozygous embryos were scanned by confocal microscopy, sagittal sections through the VLF revealed individual EN1 axons aligned longitudinally in a parallel array at the lateral edge of the ventral horn (Fig. 7C). In contrast, many EN1 axons were bundled together into fascicles containing 10 or more axons in En1tlZ/-/- homozygous embryos (see arrows in Fig. 7F, c.f. 7E). In addition to these fasciculation defects, the EN1 axons also exhibited markedly disorganized trajectories within the VLF of E12 En1 mutants, giving them a matted appearance when viewed in sagittal sections (see Fig. 7D).

The abnormal morphology of the EN1 axons in homozygous En1 mutant embryos led us to ask whether these defects affect the normal development of EN1 projections to somatic motor neurons. Motor neurons in E12.5 En1tlZ/+ and En1tlZ/-/- embryos were visualized using an antibody to ISL1 and the distribution of EN1 axons relative to motor neurons was analyzed in sections stained with an antibody to β-gal. In transverse sections through heterozygous En1tlZ/+ embryos, axons from EN1 interneurons were distributed throughout the

**Fig. 3.** Analysis of early axon outgrowth by EN1 interneurons. (A-C) Spinal cords from E10-10.5 En1tlZ/+ heterozygous embryos stained with antibodies to β-gal (red) and TAG1 (green). (A) Dorsal and (B) ventral view of cross section through an E10 spinal cord. At the time that EN1 axons first begin projecting toward the ventral horn only a few weakly stained TAG1+ commissural axons are observed in the dorsal spinal cord. (C) Cross section through an E10.5 spinal cord. The EN1 axons (red, arrows) and commissural axons (green, arrowheads) project along different pathways in the ventral spinal cord. (D-F) Spinal cords from E10 En1tlZ/+ heterozygous embryos stained with antibodies to β-gal (red) and β-tubulin (green). (D,E) Sagittal confocal section through an E10 spinal cord. The arrowheads in D and E mark isolated EN1 axons (orange) that are growing ventrally toward the ventral horn. Note the absence of closely associated EN1+ (green) axons. A more medially located commissural axon is marked with an arrow. (F) A 20 μm collapsed confocal Z-series showing EN1 axons turning in the ventral horn. The large arrowhead in F indicates an EN1 axon that is turning and beginning to grow rostrally in the VLF. Very few longitudinally projecting EN1- axons are present in the VLF at this time (small arrowhead).

**Fig. 4.** DCC is expressed in EN1 interneurons. A transverse section through ventral half of an E10.5 mouse spinal cord stained with antibodies to DCC and β-gal. (A) DCC expression; (B) β-gal expression; (C) merged image showing DCC expression in EN1 axons in the VLF (arrow). DCC is not expressed by undifferentiated EN1 cells that have not extended an axon (arrowhead).
VLF in a punctate manner, forming a crescent-shaped axon tract encompassing both the medial motor column (MMC, Fig. 8A) and the lateral motor column (LMC, data not shown). When spinal cords from En1<sup>±/±</sup> homozygous embryos were examined, we observed a significant difference in the distribution of EN1 axons within the VLF. Processes from EN1 interneurons were markedly absent from ventromedial regions of the VLF (Fig. 8B,D). In particular, few if any EN1 axons were seen adjacent to the medial-most motor neurons in the MMC<sub>m</sub>. This loss of EN1 axons immediately adjacent to the medial MMC<sub>m</sub> was observed at all axial levels. In addition to reduced numbers of longitudinally projecting EN1<sup>+</sup> axons adjacent to the medial MMC<sub>m</sub> (Fig. 8D), spinal cords from En1 mutant embryos also contained far fewer EN1 axons within the MMC<sub>m</sub> when compared to age-matched controls (Fig. 8E). Although fewer EN1 axons were seen in the medial half of the MMC<sub>m</sub>, an increase was seen in the number of EN1 axons in the lateral half of the MMC<sub>m</sub> suggesting the EN1 axons that normally grow toward the medial MMC<sub>m</sub> instead enter the lateral MMC<sub>m</sub> in the En1<sup>−/−</sup> mutants.

### DISCUSSION

**EN1 axons pioneer local ipsilateral projections to motor neurons and are guided by netrin-1**

The EN1 interneurons are among the first postmitotic interneurons to develop in the embryonic spinal cord where they begin differentiating shortly after the first motor neurons are born (Pfaff et al., 1986 and Fig. 3). We observe that the earliest EN1 axons grow independently of other neighboring...
axons toward motor neurons (Figs 2, 3) indicating they respond autonomously to guidance cues in the embryonic spinal cord to establish an ipsilateral projection pathway in the ventral spinal cord. The CHX10/LIM3 interneurons also differentiate at the same time as the EN1 interneurons and have axons that project longitudinally in the VLF; however, our results indicate these cells are not required for EN1 axon guidance, since EN1 axons do not grow together with other axons at E10, including...
axons from CHX10/LIM3 interneurons (Fig. 3, data not shown). It is also unlikely that commissural neurons pioneer the formation of the VLF, since the first EN1 axons that turn rostrally in the ventral spinal cord do so before any commissural axons have crossed the floor plate (Fig. 3). Our findings do not support the presence of an early born population of ventral interneurons that help guide the EN1 axons that are like the primitive longitudinal (PL) cells, which have been proposed to pioneer the formation of the VLF in the chick (Yaganuma et al., 1990), and such cells have not been found in the spinal cords of rodents (H. S. and M. G., unpublished observations). In addition, our observation that the EN1 axons still project rostrally in netrin-1 mutant embryos (Fig. 5), albeit at more dorsal locations in the spinal cord, further argues against the presence of localized guidepost cells that direct the rostral growth of the EN1 axons. While the EN1 interneurons are among the first cells to extend axons longitudinally in the VLF, it is not clear whether they serve as pioneers for later developing interneurons as has been described elsewhere (Caudy and Bentley, 1986; Bastiani and Goodman, 1986; Kuwada, 1986).

This study provides the first evidence that netrin-1 is essential for guiding axons from ipsilaterally projecting association neurons along a circumferential pathway toward motor neurons. Netrin-1 was first identified as a chemoattractant for commissural neurons in vertebrates (Kennedy et al., 1994; Serafini et al., 1994) and, in both vertebrates and in *Drosophila*, netrin-1 signaling is required for axons to cross the ventral midline and establish the ventral commissure (Serafini et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996). Our results, together with other studies that have analyzed pathfinding by commissural neurons (Shirasaki et al., 1995; Serafini et al., 1996; Fazeli et al., 1997), demonstrate netrin-1 functions as a chemoattractant for multiple interneuron cell types in the vertebrate spinal cord. This is analogous to the described activity of the *C. elegans* netrin-homologue UNC6, which together with its receptors UNC5 and UNC40 controls the circumferential migration of multiple pioneer axons in *C. elegans* embryos (Hedgecock et al., 1990). We also find that commissural neurons and ipsilateral interneurons that are located close to each other in the ventral spinal cord extend axons along different pathways in the ventral spinal cord in response to netrin-signaling (T. Kagawa and M.G., unpublished observations). Consequently, while both cell types are capable of responding to similar levels of netrin-1 by extending axons ventrally, the pathways that are taken by these netrin-responsive axons differ significantly. Interestingly, EN1 expression also appears to overlap with that of Unc5H1, a mammalian homologue of the UNC-5 receptor that mediates chemorepulsion by UNC-6 in *C. elegans* (Leonardo et al., 1997). However, signaling via the netrin/DCC/UNC5 pathway alone appears unlikely to account fully for the circumferential growth of the EN1 axons and their avoidance of the ventral midline, since 2-3% of the EN1 axons exhibit near normal trajectories in netrin-1 mutant embryos (Fig. 5) and no EN1 axons are observed crossing the floor plate, even though commissural axons are still able to do so in netrin-1 and DCC mutant embryos (Serafini et al., 1996; Fazeli et al., 1997).

The mechanisms that control this rostral turn and inhibit the EN1 axons from crossing the midline are not known. Recently, it has been demonstrated that the *Drosophila* protein, roundabout, and the *C. elegans* homologue, SAX3, are components of a guidance system that regulates midline crossing of axons (Kidd et al., 1998; Zallen et al., 1998). Based on the expression patterns of the mammalian roundabout homologues, robo-1 and robo-2, and their ligands, slit-1 and slit-2 (Brose et al., 1999), it appears that this guidance mechanism is conserved in higher vertebrates and may repel EN1 axons from the midline. The presence of a parallel repulsive signaling pathway during the initial ventral phase of EN1 axon growth would also explain why EN1 interneurons take a more lateral route before turning rostrally some distance from the floor plate. In particular, slit-2 is expressed at E10 in the ventral horn and may prevent the EN1 axons from projecting directly toward the floor plate like commissural neurons. None of the above mechanisms can account for the stereotypical rostral turning of EN1 interneurons suggesting additional signals must control this decision. Several genes involved in rostral/caudal axon elongation have been described in *C. elegans* and a similar independent guidance mechanism may account for the rostral turning behavior of the EN1 axons (McIntire et al., 1992; Wightman et al., 1997). Our observation that EN1 axons that fail to project ventrally in netrin-1 mutant embryos still turn rostrally (Fig. 5), argues that these cues for rostrocaudal guidance are distributed throughout the DV axis of the embryonic spinal cord.

**EN1 expression defines a class of locally projecting spinal interneurons in the embryonic spinal cord**

Our studies showing EN1 identifies a population of interneurons with a unique axonal projection pattern and is one of only four early ventral interneuron cell types in the ventral spinal cord, argues for an early limited diversification of neurons during development. Whereas neurons that co-express CHX10 and LIM3 also extend axons ipsilaterally in the VLF, their axons project for long distances (>3-5 segments) in both caudal and rostral directions, in contrast to the EN1 interneurons that have short axon trajectories (J. B. and M. G., unpublished results; K. Sharma and S. Pfaff, pers. comm.). This limited early diversification of ventral interneurons in the embryonic spinal cord appears to be a simple mechanism that generates a primitive interneuron axonal scaffold that can then be used to “wire up” the spinal cord. Rather than initially
generating many different neuronal cell types, neurons in the spinal cord would be initially segregated into groups of cells with similar axonal projections that then differentiate further to form the many physiologically distinct interneuron cell types that populate the adult spinal cord. According to this model, the CHX10/LIM3 interneurons would give rise to interneuron subtypes that innervate motor neurons over multiple spinal cord segments and function in propriospinal reflexes pathways. In contrast, neurons derived from the EN1 interneurons would differentiate into neurons that form local connections with motor neurons, either intrasegmentally or between adjacent segments, thereby generating local reflex circuits that coordinate motor neurons within a few spinal cord segments.

Although the mature morphologies and functional characteristics of the EN1 interneurons have not been described, studies in the E10 chick spinal cord indicate Renshaw cells and other last order inhibitory interneurons express EN1 (Wenner et al., 1998). Renshaw cells are located close to motor neurons in the ventral horn and synapse with them, forming a circuit that mediates the recurrent inhibition of motor neurons (Renshaw, 1946; Eccles et al., 1954). The adult phenotype of Renshaw cells is consistent with the expression of GAD65 in the EN1 interneurons, and with the distribution and morphology of the EN1 interneurons (see Figs 2, 6). Embryonic EN1 interneurons also share a number of morphological similarities with Ia inhibitory interneurons in the adult cat spinal cord (Jankowska and Lindstrom, 1972). Ia inhibitory interneurons are located dorsomedial to motor neurons, have short axonal processes in the VLF and make local projections to motor neurons, raising the possibility that Ia inhibitory interneurons also develop from embryonic interneurons that express EN1.

**En1 regulates late aspects of ventral interneuron phenotype**

In contrast to previous studies showing engrafted is required for the differentiation of specific neural cell types in invertebrates (Condron et al., 1994; Lundell et al., 1996), we find no evidence that En1 controls early interneuron cell fate in the embryonic spinal cord. Previous studies show that the EN1 interneurons are still present in the spinal cords of En1−/− mutant mice (Matise and Joyner, 1997); however, these studies were unable to provide any detailed analysis of changes to cell fate. We observe that, in homozygous En1+/- null embryos, the EN1 interneurons do not adopt the morphological characteristics of other ventral interneurons, nor do they ectopically express markers found in other interneuron cell types (data not shown). Furthermore, we find no evidence of a change in their neurotransmitter phenotype. GAD65 expression is normal in the spinal cords of En1 null embryos (Fig. 6) and other neurotransmitter-specific enzymes, e.g. ChAT and TH, are not ectopically expressed in the EN1 interneurons (data not shown). The lack of early cell fate changes in the En1 mutant embryos contrasts with the marked alterations to cell fate that occur when members of the LIM-homeodomain gene family are inactivated in mice. In mice lacking Lhx3/4, ventral motor neuron subtypes are lost or converted to dorsal motor neuron subtypes (Sharma et al., 1998), while loss of Isl1 function causes a complete failure of somatic motor neuron differentiation (Pfaff et al., 1996). The EN1 interneurons also fail to develop in Isl1 mutant mice; however, this is most likely due to the extensive cell death that occurs throughout the ventral spinal cord in these embryos. Our finding that En1 does not determine cell fate differs markedly from the functions that have been described for transcription factors that are expressed in the ventricular zone such as Pax6 and Nkx2.2, which have essential roles in determining the fate of the EN1 and SIM-1 ventral interneurons, respectively (Ericson et al., 1997; Briscoe et al., 1999). MNR2, which is also transiently expressed in dividing motor neuron precursors, may also function as a motor neuron determination factor (Tanabe et al., 1998). Our study shows that, although EN1 is expressed early in undifferentiated postmitotic neurons with a neuroepithelial morphology, it does not control an early step in the differentiation program of these cells.

An important finding of this paper is that En1 controls fasciculation and axon pathfinding in association interneurons that project to somatic motor neurons. Normally these EN1 axons do not fasciculate with one another, a behavior that is
observed in regions of the developing CNS where axons are actively establishing connections with other neurons. Evidence that axon defasciculation is important for axon branching and connectivity has come from studies in both invertebrates and vertebrates (Stoeckli and Landmesser, 1995; Daston et al., 1996; Fambrough and Goodman, 1996; Tuttle et al., 1998). Association spinal interneurons, which include the EN1 interneurons, establish synapses with multiple motor neurons “en passant” over the length of their axons (Vaughn and Greishaber, 1973). In both mouse and rat embryos, synaptogenesis involves the extensive invasion of motor neuron dendrites into the VLF, and these dendrites intercalate with and form synapses with the axons of association neurons (Vaughn et al., 1977). Consequently, the defasciculated behavior of the EN1 axons is likely to be important for promoting contacts between axons and motor neuron dendrites, thereby facilitating synapse formation. The EN1 axons are abnormally bundled in En1−/− mutant embryos forming fascicles that contain 10 or more axons (Fig. 7 and 8). As a result, synapse formation between the EN1 association interneurons and motor neurons may be significantly impaired in En1−/− mutant mice.

In addition to the fasciculation phenotype, the EN1 axons fail to pathfind correctly toward somatic motor neurons in En1−/− mutant embryos (Fig. 8) and, as a result, two differences in the trajectories of the EN1 axons are seen. Firstly, the EN1 axons meander dorsoven-trally within the VLF and, secondly, there is a marked loss of EN1 axons projecting to motor neurons located in the medial MMCm. While the effects of these pathfinding defects on connectivity have not yet been determined, the aberrant trajectories of the EN1 axons in the VLF may have profound effects on the specificity of motor neuron innervation. During embryonic development, somatic motor neurons are organized into pools of cells that occupy discrete domains in the ventral horn (Landmesser, 1978; Smith and Hollyday, 1983; Gutman et al., 1993; Lin et al., 1998) and many of the inhibitory local circuit interneurons that innervate specific motor neuron pools do so in a topologically ordered manner (Eccles et al., 1957; Jankowska and Roberts, 1972). The majority of the early synapses between association neurons and motor neurons are either axiosomatic or are located on the proximal dendrites (Vaughn et al., 1977), indicating initial synapse formation is largely constrained by the axonal trajectories of the association interneurons. The EN1 axons project longitudinally in the VLF and, since motor neurons are also organized into columns that run longitudinally in the spinal cord, this indicates that individual EN1 neurons may preferentially form synapses with pools of motor neurons that innervate the same or synergistic muscles. In view of the dorsoventral meandering of the EN1 axons that occurs in En1 mutant spinal cords, this early organization of synapses between the EN1 interneurons and pools of motor neurons is likely to be altered in En1−/− mutant embryos.

Our finding that EN1 axons no longer project to motor neurons in the medial MMCm in the En1−/− mutant spinal cord demonstrates En1 function is essential for EN1 interneurons to pathfind their way to a subset of somatic motor neurons. These motor neurons are located in the medial half of the MMCm and specifically innervate the deep muscles of the back (Smith and Hollyday, 1983). Furthermore, dendrites from these motor neurons extend into the ventromedial region of the VLF where EN1 axons are no longer present in the En1−/− mutant embryos.

This suggests that EN1 may be part of a combinatorial transcription factor code that specifies connectivity between locally projecting interneurons and pools of somatic motor neurons. Alternatively, En1 may control the later emergence of a subclass of interneurons that innervate motor neurons located in the medial half of the MMCm and, in En1 mutants, these are transformed to interneurons that innervate more laterally located motor neurons.

We would like to thank Marc Tessier-Lavigne for the netrin-1 mutant mice, Chris Callahan and John Thomas for the tau-lacZ plasmid, Alexandra Joyner for the En1 targeting vector, Steve O’Gorman for ES cells, Yelena Marchuk for blastocyst injections and Marc Olivier and Shad Schidel for technical assistance. We would also like to thank Alexandra Joyner, Chris Kintner, Tom Jessell, Elise Lamar, Greg Lemke, Horst Simon, Chuck Stevens, John Thomas, Stefan Thor, Detlef Weigel and Todd Zorrick for their advice and comments on the manuscript. This work was supported by grants from NIH (M. G.), the Pew Charitable Trusts (M. G.) and the Fritz Burns Foundation (M. G.). Harald Saueressig was supported by a grant from the Deutsche Forschungsgemeinschaft (SA 652/2-1) and John Burrill was supported by a NRSA Postdoctoral Fellowship.

REFERENCES


