Developmental Biology **244,** 180–198 (2002)
doi:10.1006/dbio.2002.0580, available online at http://www.idealibrary.com on IDEAL®

# Evidence for a Cell-Specific Action of Reelin in the Spinal Cord

**Patricia E. Phelps,*[,1] Rachel Rich,* Shannon Dupuy-Davies,† Yesenia Ríos,* and Tina Wong***

**Department of Physiological Science, UCLA, Box 951527, Los Angeles, California 90095-1527; and †Department of Developmental Neurobiology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105*

**Reelin, the extracellular matrix protein missing in *reeler* mice, plays an important role in neuronal migration in the central nervous system. We examined the migratory pathways of phenotypically identified spinal cord neurons to determine whether their positions were altered in *reeler* mutants. Interneurons and projection neurons containing choline acetyltransferase and/or NADPH diaphorase were studied in E12.5–E17.5 *reeler* and wild-type embryos, and their final locations were assessed postnatally. While three groups of dorsal horn interneurons migrated and differentiated normally in *reeler* mice, the migrations of both sympathetic (SPNs) and parasympathetic preganglionic neurons (PPNs) were aberrant in the mutants. Initially *reeler* and wild-type SPNs were detected laterally near somatic motor neurons, but by E13.5, many *reeler* SPNs had mismigrated medially. Postnatally, 79% of wild-type SPNs were found laterally, whereas in *reeler,* 92% of these neurons were positioned medially. At E13.5, both *reeler* and wild-type PPNs were found laterally, but by E14.5, *reeler* PPNs were scattered across the intermediate spinal cord while wild-type neurons correctly maintained their lateral location. By postnatal day 16, 97% of PPNs were positioned laterally in wild-type mice; in contrast, only 62% of PPNs were found laterally in mutant mice. In E12.5–E14.5 wild-type mice, Reelin-secreting cells were localized along the dorsal and medial borders of both groups of preganglionic neurons, but did not form a solid barrier. In contrast, Dab1, the intracellular adaptor protein thought to function in Reelin signaling, was expressed in cells having positions consistent with their identification as SPNs and PPNs. In combination, these findings suggest that, in the absence of Reelin, both groups of autonomic motor neurons migrate medially past their normal locations, while somatic motor neurons and cholinergic interneurons in thoracic and sacral segments are positioned normally. These results suggest that Reelin acts in a cell-specific manner on the migration of cholinergic spinal cord neurons.** 

## INTRODUCTION

The mouse autosomal recessive mutation, *reeler,* is a well-studied, naturally occurring mutation known to be associated with a characteristic neurological phenotype that includes impaired motor coordination, tremors, and ataxia (Falconer, 1951). These deficits are thought to be produced by major migrational errors in the laminated structures of the cerebral and cerebellar cortices and the hippocampal formation (Caviness, 1982; Goffinet, 1983a, 1984b; Mariani *et al.,* 1977). In addition to errors in migration in laminated structures, the *reeler* mutation has been reported to perturb the migration and/or final location of neurons in various nonlaminated regions of the brainstem, such as the inferior olivary complex, the nucleus ambiguous, and the facial nerve nucleus (Fujimoto *et al.,* 1998; Goffinet, 1983b, 1984a).

The molecular basis of the *reeler* phenotype was revealed with the cloning of *reelin,* the gene mutated in *reeler* mice, and the subsequent identification of the protein missing in these mice (D'Arcangelo *et al.,* 1995). Reelin is a secreted glycoprotein containing eight EGF-like repeats, a cleavable signal peptide, and several potential glycosylation sites (D'Arcangelo *et al.,* 1995, 1997). The structural organization and amino acid composition of Reelin resemble those of other extracellular matrix-type glycoproteins that are known to be involved in cell adhesion and neuronal migration (Curran and D'Arcangelo, 1998). Subsequently, both spontaneous (*scrambler*) and targeted (*yotari*) mouse muta-

[1] To whom correspondence should be addressed. Fax: (310) 206-9184. E-mail: pphelps@physci.ucla.edu.

0012-1606/02 $35.00

tions in the *disabled-1* (*Dab1*) gene demonstrated phenotypes virtually identical to *reeler* (Howell *et al.,* 1997; Sheldon *et al.,* 1997; Yoneshima *et al.,* 1997). These mice expressed little or no Dab1 protein, a phosphoprotein thought to function as an adaptor molecule in the transduction of protein kinase pathways (Howell *et al.,* 1997; Rice and Curran, 2001; Rice *et al.,* 1998; Sheldon *et al.,* 1997). Most recently, mice lacking both the very low density lipoprotein (VLDLR) and apolipoprotein E2 (ApoER2) receptors also displayed a *reeler*-like phenotype. Subsequently, Reelin was shown to be a high affinity ligand for these lipoprotein receptors (D'Arcangelo *et al.,* 1999; Trommsdorff *et al.,* 1999). Increased levels of Dab1 were reported in both *reeler* and *Vldlr/ApoER2* mutants, additional evidence placing Reelin, VLDLR, ApoER2, and Dab1 in a common signaling pathway that functions to influence cell migration (Rice and Curran, 1999, 2001; Trommsdorff *et al.,* 1999).

While the *reeler* mutation induces well-known migrational errors in the cerebral cortex, cerebellum, and hippocampal formation (Caviness, 1982; Goffinet, 1984b; Mariani *et al.,* 1977), the spinal cords of *reeler* mice were thought to be normal until Yip *et al.* (2000) demonstrated that the sympathetic preganglionic neurons (SPNs) in *reeler* were located medially, in contrast to those in wild-type mice that were concentrated laterally in the intermediolateral horn of the thoracic spinal cord (Yip *et al.,* 2000). This report of a migrational error in a single population of spinal cord neurons led us to question whether the migrations of other phenotypically defined neurons throughout the spinal cord also might be affected by the *reeler* mutation.

In both rats and mice, several groups of spinal cord neurons are known to use acetylcholine as their neurotransmitter and to contain NADPH-diaphorase as well as nitric oxide synthase (Barber *et al.,* 1984; Blottner and Baumgarten, 1992; Brüning, 1992; Dun *et al.,* 1993; Valtschanoff *et al.,* 1992; Wetts *et al.,* 1995; Wetts and Vaughn, 1994). Developmental studies in rat spinal cord detected choline acetyltransferase (ChAT) immunoreactivity within several types of cholinergic interneurons as well as in SPNs during their migration to their final locations, thus providing information about their migratory pathways (Phelps *et al.,* 1990, 1991). Somatic motor neurons seemed to migrate radially, while other cholinergic neurons, the "U-shaped" group, migrated in a tangential direction, perpendicular to radial glia (Phelps *et al.,* 1996; Phelps and Vaughn, 1995). In contrast to these direct migratory patterns, SPNs have a complex pathway that consists of three distinct movements. Initially, they migrate radially from the ventral ventricular zone to the ventral intermediate zone together with somatic motor neurons. Then, SPNs move dorsally toward the intermediolateral region along a nonradial pathway. Finally, a subset of SPNs migrate medially, back toward the ventricular zone (Barber *et al.,* 1993; Phelps *et al.,* 1991, 1993).

The present study examines the effect of the *reeler* mutation on radially and tangentially directed migrations of phenotypically defined spinal cord neurons. We compared the locations of cholinergic and/or diaphorase-positive neurons in postnatal *reeler* with those of wild-type mice and then examined the migrations leading to the postnatal pattern. In addition, the relationships between both groups of preganglionic neurons and Reelin or Dab1-expressing cells were examined to determine whether cells demonstrating aberrant migrations might do so as a direct result of altered Reelin signaling.

## MATERIALS AND METHODS

*Reeler* (*Reln*$^{rl}$/*Reln*$^{rl}$) mice were obtained from breeding colonies maintained at St. Jude Children's Research Hospital and UCLA and were purchased from The Jackson Laboratory (B6C3Fe/J-*reeler* mice). Wild-type, heterozygous, and homozygous *reeler* animals were obtained from heterozygous intercrosses. The day a vaginal plug was observed was recorded as embryonic day 0.5 (E0.5) according to established conventions (Kaufman, 1992). Litters at the following ages were used for this investigation: E12.5–E14.5, E17.5, E18.5, and postnatal day 16 (P16). Pregnant animals were anesthetized with avertin (0.3 ml/10 g body weight) before the cesarean delivery of the embryos. Postnatal animals were anesthetized individually with the same concentration of avertin. Embryos were either perfused immediately or briefly stored in cold oxygenated Gey's solution (Freshney, 1987) until the perfusion. Animal protocols conformed to NIH and AAALAC guidelines and were approved by the St. Jude Children's Research Hospital Animal Care and Use Committee and the Chancellor's Animal Research Committee at UCLA. Spinal cords from animals E14.5 and younger were blocked and fixed by immersion in 4% paraformaldehyde in 0.12 M Millonig's phosphate buffer (pH 7.4) for 4 h or overnight at 4°C, while animals older than E14.5 were fixed by intracardial perfusion with the same fixative. Genotypes were determined by using polymerase chain reaction screening of yolk sac, brain, or tail genomic DNA (D'Arcangelo *et al.,* 1996).

Embryonic spinal cords were blocked within the vertebral canal, while P16 spinal cords were dissected before blocking. Spinal cords were washed in three rinses of Millonig's buffer and infiltrated with 30% sucrose. Specimens were frozen in M-1 embedding medium (Shandon-Lipshaw), and 40-$\mu$m transverse or horizontal sections were cut with a cryostat and stored in serial order. Sections were mounted on gelatin-coated slides and allowed to air dry prior to immunocytochemistry.

### *Immunocytochemical and Histochemical Procedures*

Immunocytochemical procedures to locate ChAT were adapted from previous studies (Houser *et al.,* 1983; Phelps *et al.,* 1990). Sections surrounded by silicone rings were washed in Tris-buffered saline (TBS; 0.1 M Tris, pH 7.4, containing 1.4% NaCl) and incubated in 0.3% hydrogen peroxide and 0.1% sodium azide for 30 min. After rinsing, sections were incubated in TBS containing 0.8% Triton X-100 for 15 min and in 3% normal horse serum diluted in TBS for 1 h with 0.1% bovine serum albumin and 0.1% Triton X-100. Sections were incubated in anti-ChAT (AB144P, dilution 1:250; Chemicon) overnight at room temperature. After washing, sections were incubated for 1 h in rat-adsorbed horse anti-mouse

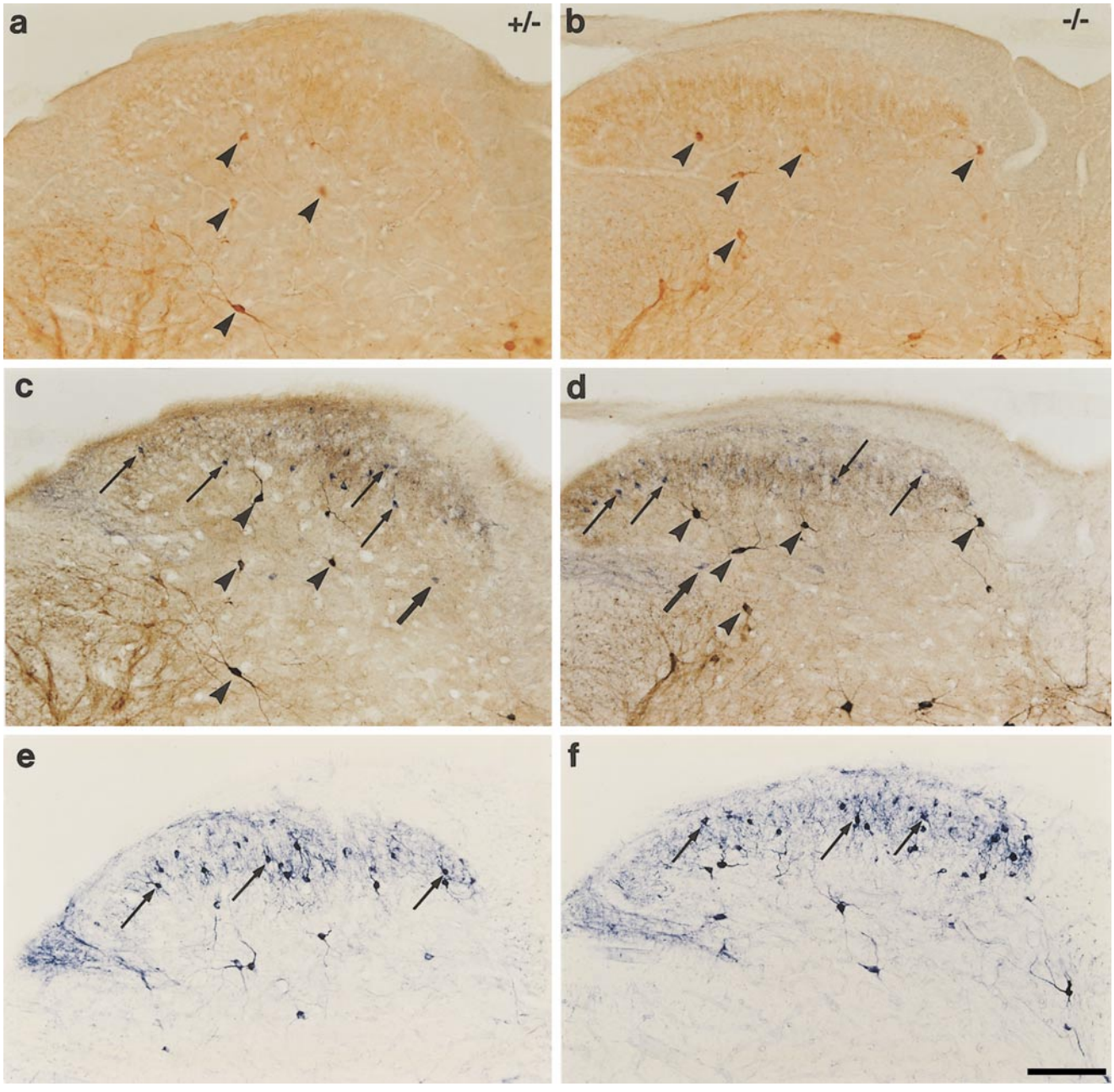



**FIG. 1.** Dorsal horn interneurons identified with ChAT immunocytochemistry (a–d) and/or NADPH-diaphorase histochemistry (c–f) are found in similar locations in P16 heterozygous (+/−, a, c, e) and *reeler* (−/−, b, d, f) mice. Photomicrographs are oriented with the midline to the right and dorsal toward the top. (a, b) ChAT-immunoreactive neurons (arrowheads) within the cervical spinal cord are scattered throughout the deep dorsal horn. (c, d) Subsequently, the sections illustrated in (a) and (b) were double-labeled for diaphorase histochemistry. Both ChAT-diaphorase-labeled neurons (brown-black, arrowheads in a–d) and diaphorase-positive cells (blue, thick arrows in c, d) are observed in the deep dorsal horn. Smaller diaphorase-only-labeled cells are found in the superficial dorsal horn (thin arrows). (e, f) Diaphorase-positive neurons are intensely labeled and are found in both superficial (thin arrows) and deep layers of the dorsal horn. Scale in a–f, 100 $\mu$m.

antibodies (1:200; Vector), rinsed, and incubated for 1 h in an avidin-biotin complex (1:100, Vector Elite Mouse IgG Kit). Specimens were rinsed in sodium acetate buffer (0.1 M, pH 7.4) for 10 min and then reacted for 8–13 min with 0.06% 3,3′-diaminobenzidine · 4 HCL (DAB) enhanced with NiCl and glucose oxidase (Shu *et al.*, 1988). After removing the silicone rings,

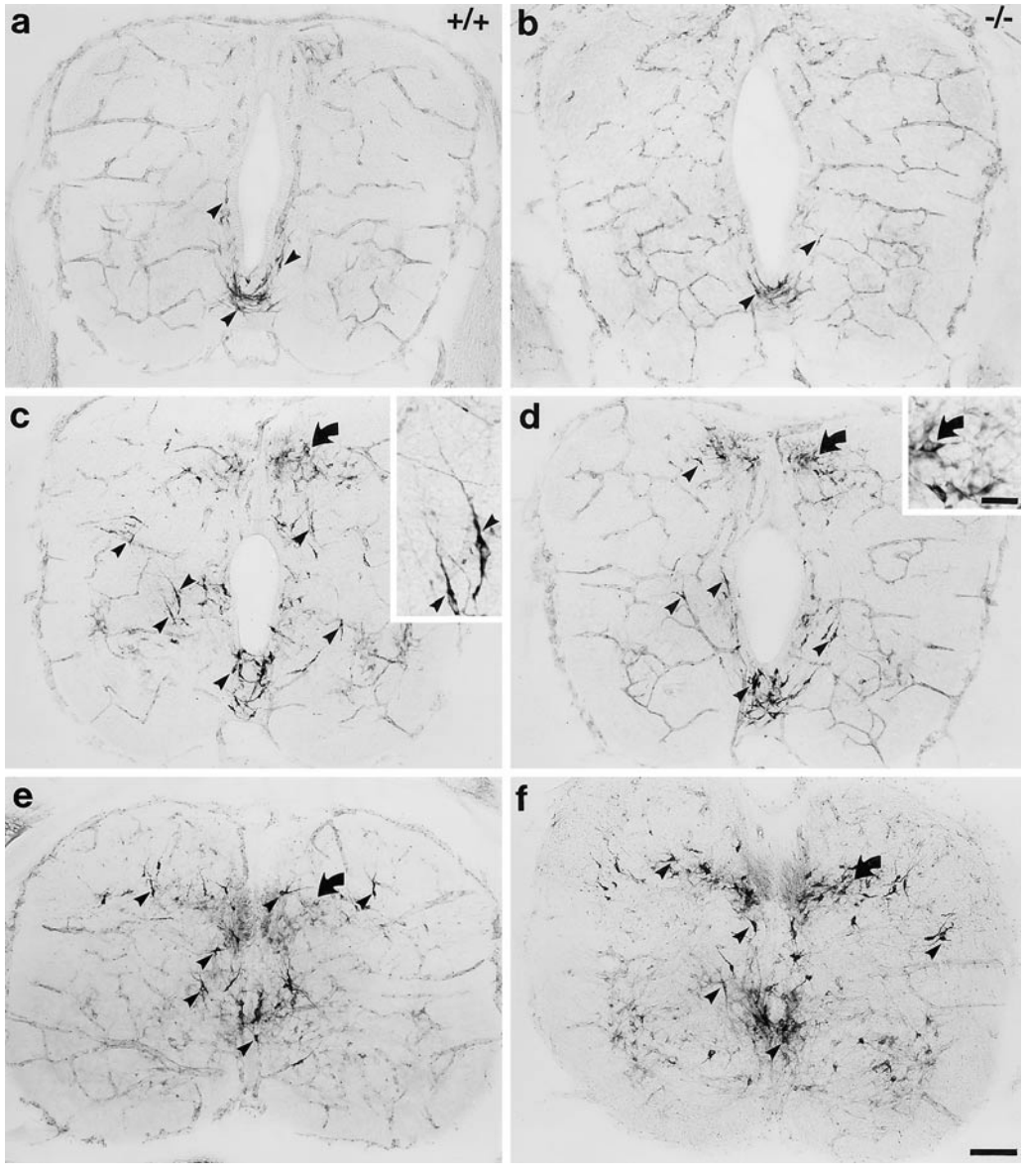



**FIG. 2.** Migrations of diaphorase-labeled dorsal horn interneurons are similar in wild-type (+/+, a, c, e) and *reeler* (−/−, b, d, f) embryos on E13.5 (a, b), E14.5 (c, d), and E17.5 (e, f). Endothelial cells of blood vessels are diaphorase-positive at all ages and visible in many of these and later figures. (a, b) On E13.5, a "U-shaped" group of diaphorase-positive neurons (arrowheads) surrounds the ventral ventricular zone, including the floor plate. (c, d). By E14.5, some cells from the U-shaped group (arrowheads) have migrated further dorsally, whereas others remain around the ventral ventricular zone. Many cells derived from the U-shaped group have the appearance of migrating neurons with leading processes as seen in the inset in (c). Dorsally originating diaphorase-only cells (curved arrows) are clustered near the midline of the dorsal horn. Typically, at this age, these cells are small and have mediolaterally oriented processes (inset in d, curved arrow). (e, f). On E17.5, neurons from the U-shaped group (arrowheads) are found around the central canal and scattered within the deep dorsal horn. The dorsally originating diaphorase-only cells (curved arrows) have smaller somata and shorter processes than those derived from the U-shaped group. Scales: a–f, 100 $\mu$m; insets c, d, 50 $\mu$m.

sections were dehydrated and coverslipped. Postnatal specimens to be subsequently processed for NADPH-diaphorase histochemistry were temporarily coverslipped in glycerin, photographed, and washed before further processing.

The diaphorase histochemical procedure was adapted from studies of rat spinal cord (Wetts *et al.*, 1995; Wetts and Vaughn, 1994). Slide-mounted sections were placed in Millonig's buffer with 0.8-1% Triton X-100 for 20 min, followed by a solution containing

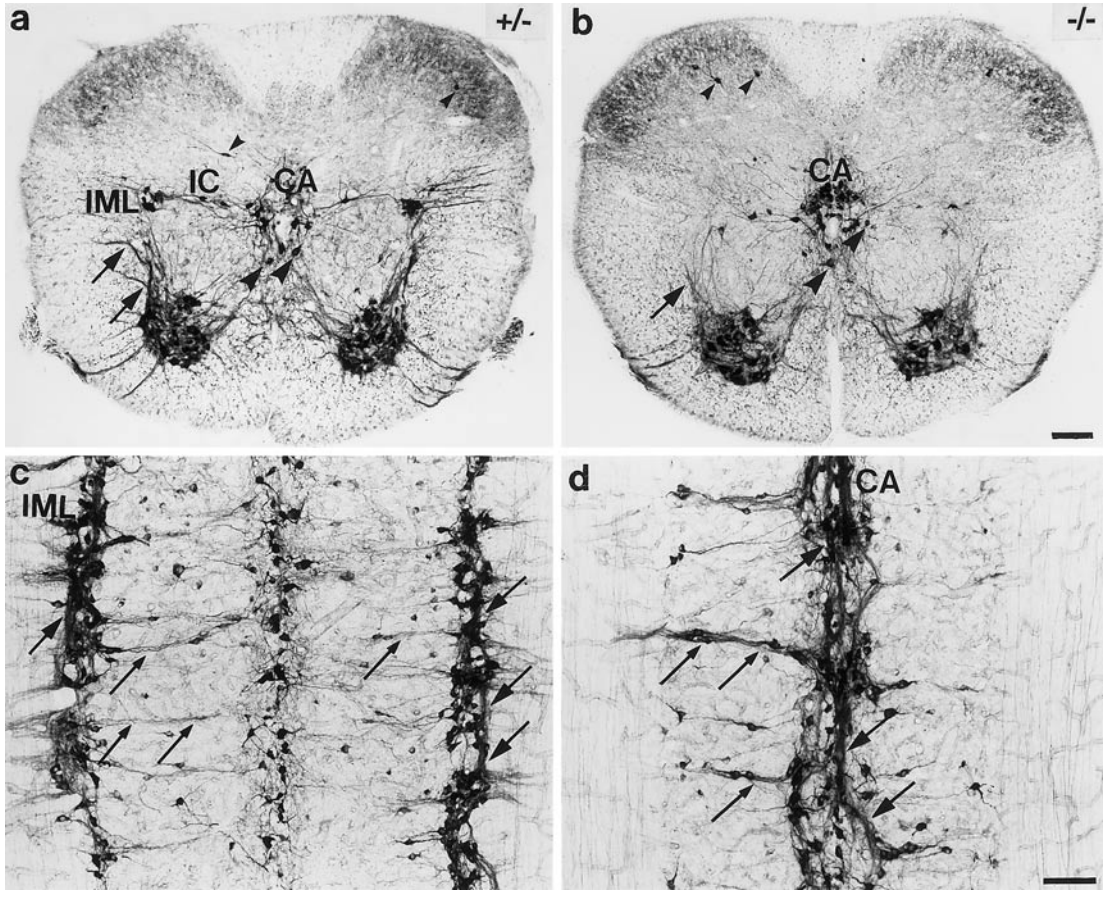



**FIG. 3.** Postnatal day 16 thoracic spinal cord labeled with either ChAT immunocytochemistry in transverse sections (a, b) or NADPH-diaphorase histochemistry in horizontal sections (c, d). (a) In heterozygous mice, cholinergic SPNs are concentrated in the intermediolateral nucleus (IML), but also are distributed in the intercalated (IC) and central autonomic (CA) nuclei. ChAT-positive somatic motor neurons are found ventrally and have prominent dendritic bundles (arrows). Additional populations of cholinergic interneurons can be observed around the central canal (large arrowheads) and in the deep dorsal horn (small arrowheads). (b) In *reeler,* ChAT-labeled SPNs are concentrated in the midline CA area, with a few labeled cells found in the IC region. ChAT-positive somatic motor neurons are ventrally located but have dendritic bundles (arrow) that appear to be reduced in size and length. Cholinergic interneurons are found around the central canal (large arrowheads) and in the deep dorsal horn (small arrowheads). (c) In heterozygous mice, SPNs identified with diaphorase histochemistry in this horizontal section are found primarily in the IML. The SPNs have prominent longitudinally and mediolaterally oriented dendritic bundles (large and small arrows, respectively), forming a ladder-like structure. (d) In *reeler,* the massive longitudinally projecting dendritic bundles (large arrows) are positioned incorrectly at the midline (CA), just dorsal to the central canal. In contrast, mediolaterally oriented dendrites (small arrows) remain correctly positioned but appear to be fewer in number than observed in heterozygous and wild-type mice. Scales: a, b, 100 $\mu$m; c, d, 100 $\mu$m.

0.2-1% Triton, nitro blue tetrazolium (NBT, 0.01-0.06 mg/ml; Sigma), and nicotinamide adenine dinucleotide phosphate (NADPH, 0.025–0.15 mg/ml; Sigma). Sections were left in the dark overnight at either room temperature or 37°C, depending on the necessary intensification. Specimens then were rinsed, dehydrated in acetone, and coverslipped.

To determine the relationship between the Reelin-secreting cells and the preganglionic neurons, double-labeling experiments were conducted first with the diaphorase histochemical procedure followed by Reelin immunocytochemistry. Both monoclonal anti-Reelin antibodies, G10 (de Bergeydk *et al.*, 1998) and CR-50 (Ogawa *et al.*, 1995) (gifts of Drs. Susan Magdaleno and Tom Curran, St. Jude Children's Research Hospital), were localized with the Inno-Genex Mouse-on-Mouse kit and produced similar results. A control monoclonal antibody that recognizes a chick erythrocyte antigen (Ad2) (Miller *et al.*, 1982) was used to determine nonspecific background levels and help troubleshoot procedures. Avidin/biotin blocking (Vector) and the power blocking steps both preceded the primary antibody incubation. The G10 (1:1000) or control (Ad2, 1:5000) antibodies were combined with the biotin-labeling reagent for 30-60 min before the MouseBlock reagent was added to the mixture. Following a 4-h incubation in primary antibody, sections were washed with buffer (PBS containing 0.1% Tween 20) and then incubated in Streptavidin-HRP for 20 min.

**TABLE 1**
Analysis of Locations of Wild-Type and *Reeler* P16 Preganglionic Neurons

(a) Sympathetic preganglionic neurons in upper thoracic spinal cord

| Genotype | Medial spinal cord (%) | Lateral spinal cord (%) |
|---|---|---|
| (−/−) | 94 (164)[a] | 6 (11)[a] |
| (−/−) | 97 (100) | 3 (3) |
| (−/−)[b] | 85 (799) | 15 (143) |
| (−/−)[b] | 91 (797) | 9 (83) |
| (+/+) | 12 (35) | 88 (254) |
| (+/+) | 20 (89) | 80 (355) |
| (+/+)[b] | 23 (200) | 77 (656) |
| (+/+)[b] | 29 (130) | 71 (326) |

(b) Parasympathetic preganglionic neurons in sacral spinal cord

| Genotype | Medial Spinal Cord (%) | Lateral spinal cord (%) |
|---|---|---|
| (−/−) | 37 (125) | 63 (215) |
| (−/−) | 39 (124) | 61 (190) |
| (−/−)[b] | 37 (192) | 63 (326) |
| (−/−)[b] | 45 (365) | 55 (448) |
| (+/+) | 4 (15) | 96 (342) |
| (+/+) | 4 (12) | 96 (260) |
| (+/+)[b] | 4 (21) | 96 (547) |
| (+/+)[b] | 2 (14) | 98 (662) |

[a] Percentages of preganglionic neurons calculated per animal with absolute cell counts indicated in parentheses.
[b] Horizontal sections contained more preganglionic neurons than coronal sections.

After a buffer wash, sections were reacted with 0.06% DAB and 0.007% $H_2O_2$ for 2-8 min, yielding an amber-brown product.

To determine the distribution of Dab1 protein, thoracic and sacral sections from *reeler* mutants were examined with Dab1 immunocytochemistry. A polyclonal anti-Dab1 antiserum (CT38, 1:20,000; gift of Drs. Susan Magdaleno and Tom Curran) was used for these experiments with a protocol similar to that described above for ChAT. Adjacent sections were processed for diaphorase histochemistry to compare the localization of the preganglionic neurons with those that were Dab1-immunoreactive.

### *Mapping Analysis of Autonomic and Somatic Motor Neurons*

Photographs of nonadjacent sections processed with ChAT immunocytochemistry were used to analyze the location of SPNs and PPNs in eight P16 mice (4 each of *Reln*$^{rl}$/*Reln*$^{rl}$, *wt*/*wt*). Within each genotype, two spinal cords were sectioned coronally and two horizontally. The medial or lateral position of each neuron was determined by dividing the distance from the central canal to the lateral boundary of the gray matter in half. All ChAT-positive SPNs and PPNs that contained a nucleus within the section were mapped and their medial or lateral location was recorded. The total number of SPNs and PPNs mapped within each animal was counted, and the percentage of medially vs. laterally located neurons was calculated as a percentage of the total number of neurons. The statistical significance of these distributions was analyzed with the chi-square test. For comparison, the number and positions of the somatic motor neurons (SMNs) were mapped in the same coronal sections used to analyze the preganglionic neurons. Sacral sections containing sexually dimorphic SMNs were excluded from the analysis. The number of ChAT-positive SMNs containing a nucleus within 12 sections per genotype was counted and averaged within both thoracic and sacral spinal cord, and standard deviations were calculated.

## RESULTS

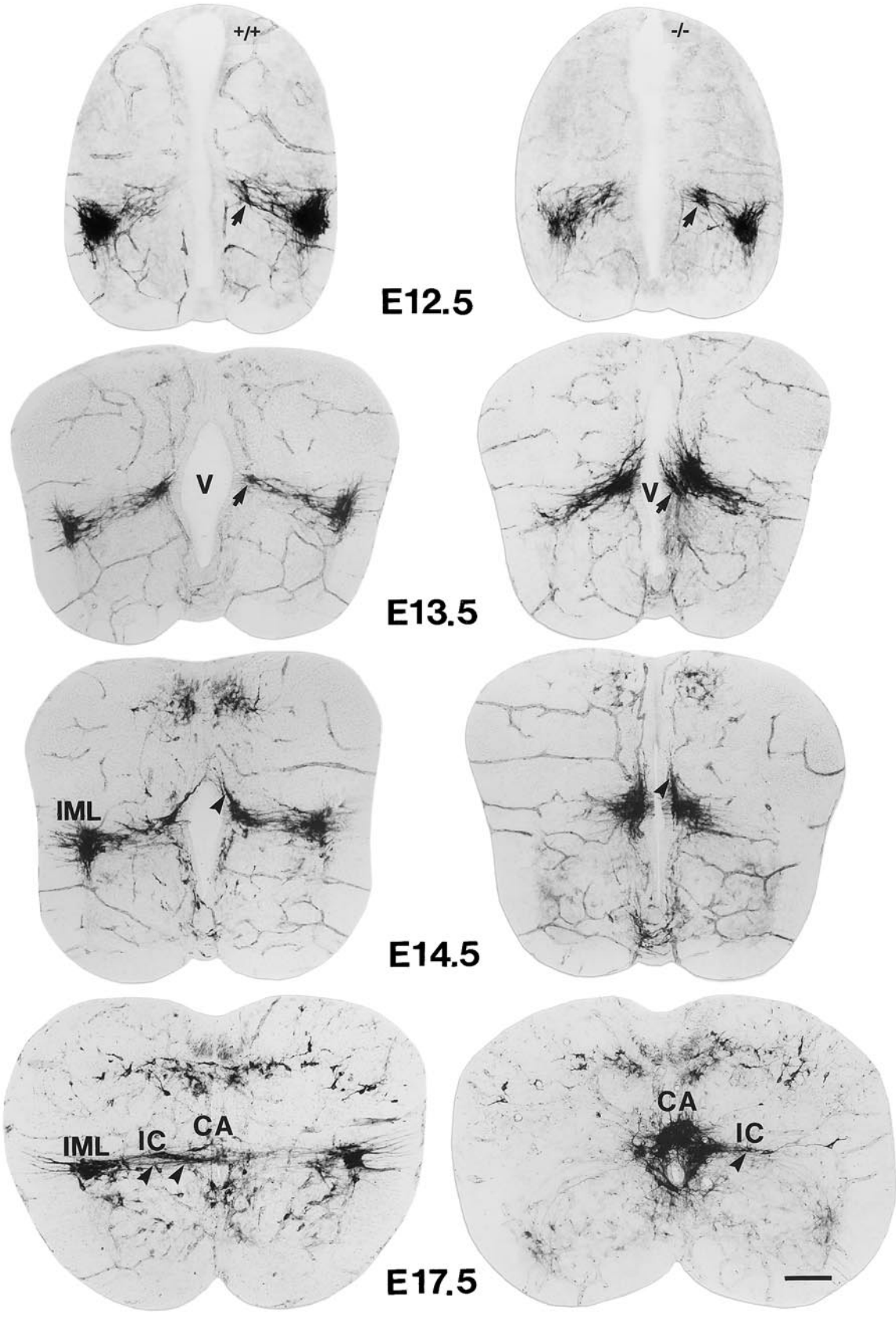
Several of the cholinergic neuronal populations in spinal cord coexpress NADPH diaphorase during their migration and thus can be labeled with either ChAT immunocytochemistry or diaphorase histochemistry (Phelps *et al.*, 1996; Wetts *et al.*, 1995). Both of these markers were used to determine whether the migrations of phenotypically defined spinal cord neurons were altered in postnatal and embryonic *reeler* mutants.

### *ChAT and Diaphorase-Positive Dorsal Horn Interneurons Migrate Normally in reeler*

Previous studies in rats report that there are two morphologically similar but chemically distinct groups of diaphorase-positive cells in the deep dorsal horn (laminae III–V); one type expresses both ChAT and diaphorase, and the other, only diaphorase (Spike *et al.*, 1993; Wetts *et al.*, 1995; Wetts and Vaughn, 1994). In addition to being chemically distinct, these two types of deep dorsal horn neurons have separate developmental histories. The ChAT/diaphorase neurons originate from the ventral ventricular zone as part of the "U-shaped" group of neurons, whereas the diaphorase-only neurons originate from the dorsal ventricular zone (Phelps *et al.*, 1996).

Adjacent sections of P16 cervical spinal cord were processed separately for ChAT immunocytochemistry (Figs. 1a and 1b) and diaphorase histochemistry (Figs. 1e and 1f). Then, direct comparisons were made in double-labeled material (Figs. 1c and 1d). ChAT-positive cells were scattered within the deep dorsal horn in *reeler* mutant, heterozygous, and wild-type littermates and typically had three to four primary dendrites (Figs. 1a and 1b; wild-type data not shown). When these sections subsequently were processed for diaphorase histochemistry, the blue reaction product filled the cholinergic interneurons, revealing their processes more distinctly (Figs. 1c and 1d). A few additional diaphorase-only cells were detected in the deep dorsal horn (Figs. 1c and 1d) and many small diaphorase-only cells were distributed throughout the superficial lamina (Figs. 1c–1f). Thus, the positioning of ChAT-diaphorase or diaphorase-only dorsal interneurons was similar in mutant, heterozygous, and wild-type mice.

Because these two types of deep dorsal horns neurons had not been previously identified in mice, we first determined



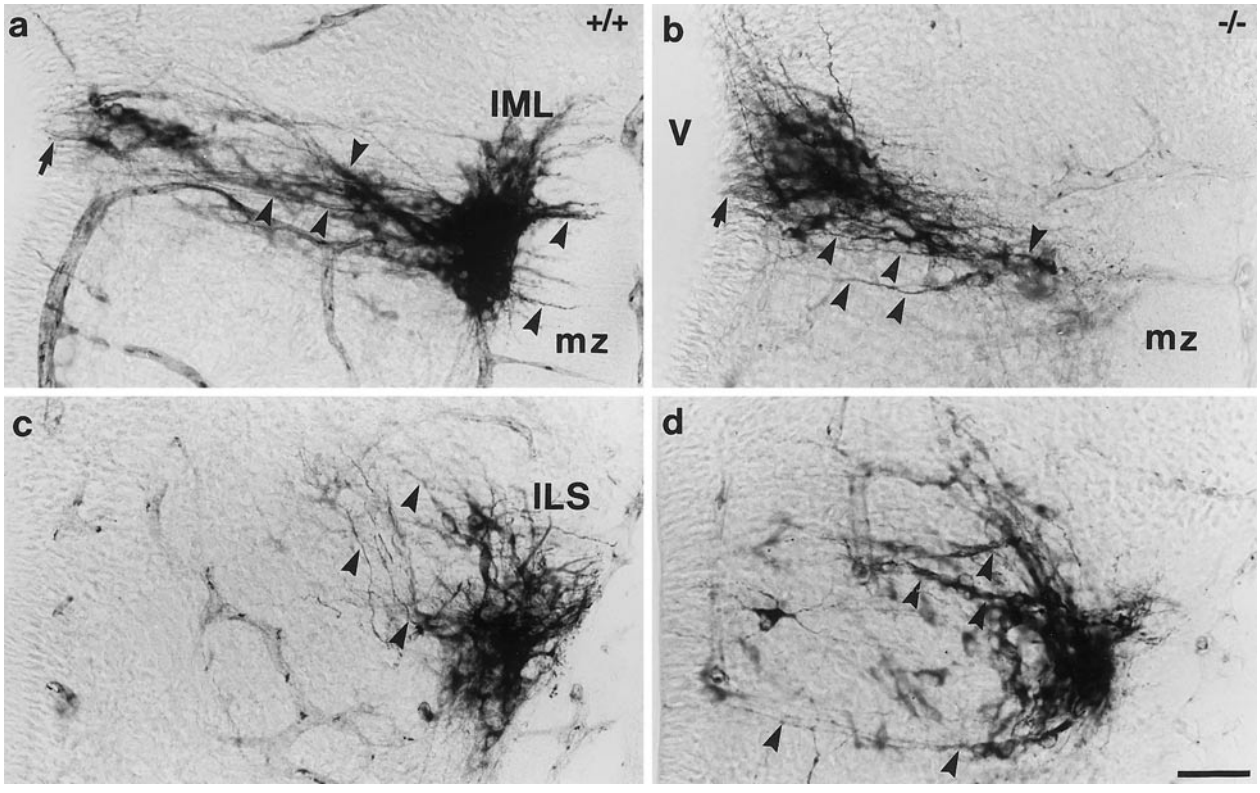



**FIG. 5.** Comparison of the processes of diaphorase-positive sympathetic (a, b) and parasympathetic (c, d) preganglionic neurons in wild-type (a, c) and mutant (b, d) mice. The ventricle (V) is oriented to the left and the lateral marginal zone (mz) to the right in each image. Endothelial cells within blood vessels are diaphorase-labeled in these enlargements. (a) In E13.5 wild-type thoracic spinal cord, the majority of the SPNs are found in the intermediolateral nucleus (IML) with processes (arrowheads) extending medially, deep into the ventricular zone (arrow) and laterally into the marginal zone (mz). (b) In *reeler,* most of the SPNs are found in more medial positions than observed in wild-type mice (a). Processes (arrowheads) of *reeler* SPNs project primarily medially through the ventricular zone (arrow) or dorsomedially. (c) In E14.5 wild-type sacral spinal cord, the PPNs are clustered laterally in the intermediolateral sacral nucleus (ILS) and have processes (arrowheads) projecting dorsomedially. (d) In *reeler,* many of the PPNs are clustered laterally but others form strings of cell bodies and processes (arrowheads) that project medially. Scale: a–d, 40 $\mu$m.

whether their migratory patterns would be similar to those in rat spinal cord (Phelps *et al.,* 1996; Wetts *et al.,* 1995). Both types of deep dorsal horn neurons could be distinguished in diaphorase preparations of embryonic mouse spinal cord. Initially, the neurons of the U-shaped group were detected around the ventral ventricular zone on E13.5 in cervical spinal cord of both *reeler* and wild-type mice (Figs. 2a and 2b), and by E14.5, some of these cells appeared

**FIG. 4.** Embryonic sympathetic preganglionic neurons (SPNs) are identified with diaphorase histochemistry in E12.5, E13.5, E14.5, and E17.5 wild-type (left column) and *reeler* (right column) mice. In both genotypes on E12.5, SPNs are found further ventrally than at later ages. A few preganglionic neurons also are seen projecting medially (arrows) in both wild-type and *reeler* littermates. On E13.5 (second row), wild-type SPNs are detected across the intermediate gray. Most cells are located laterally, but others are found at the midline (arrow), directly adjacent to the ventricle (V). In contrast, SPNs in *reeler* are concentrated medially with a few cells dispersed more laterally. On E14.5 (third row), most of the wild-type SPNs are found in the intermediolateral nucleus (IML). Those in more medial positions extend processes (arrowheads) into the dorsal commissure along the borders of the receding ventricle. In E14.5 *reeler,* SPNs are aligned along the medial surface of the ventricle with labeled processes (arrowhead) projecting dorsally. By E17.5 (bottom row), wild-type mice have the normal distribution of SPNs in the IML, intercalated (IC), and central autonomic (CA) regions as well as mediolaterally directed dendritic bundles (arrowheads). In *reeler,* the distribution appears reversed, with most cells in the CA area and just a few in the IC region. However, transversely oriented dendritic bundles (arrowhead) are still present. Scale, 100 $\mu$m.

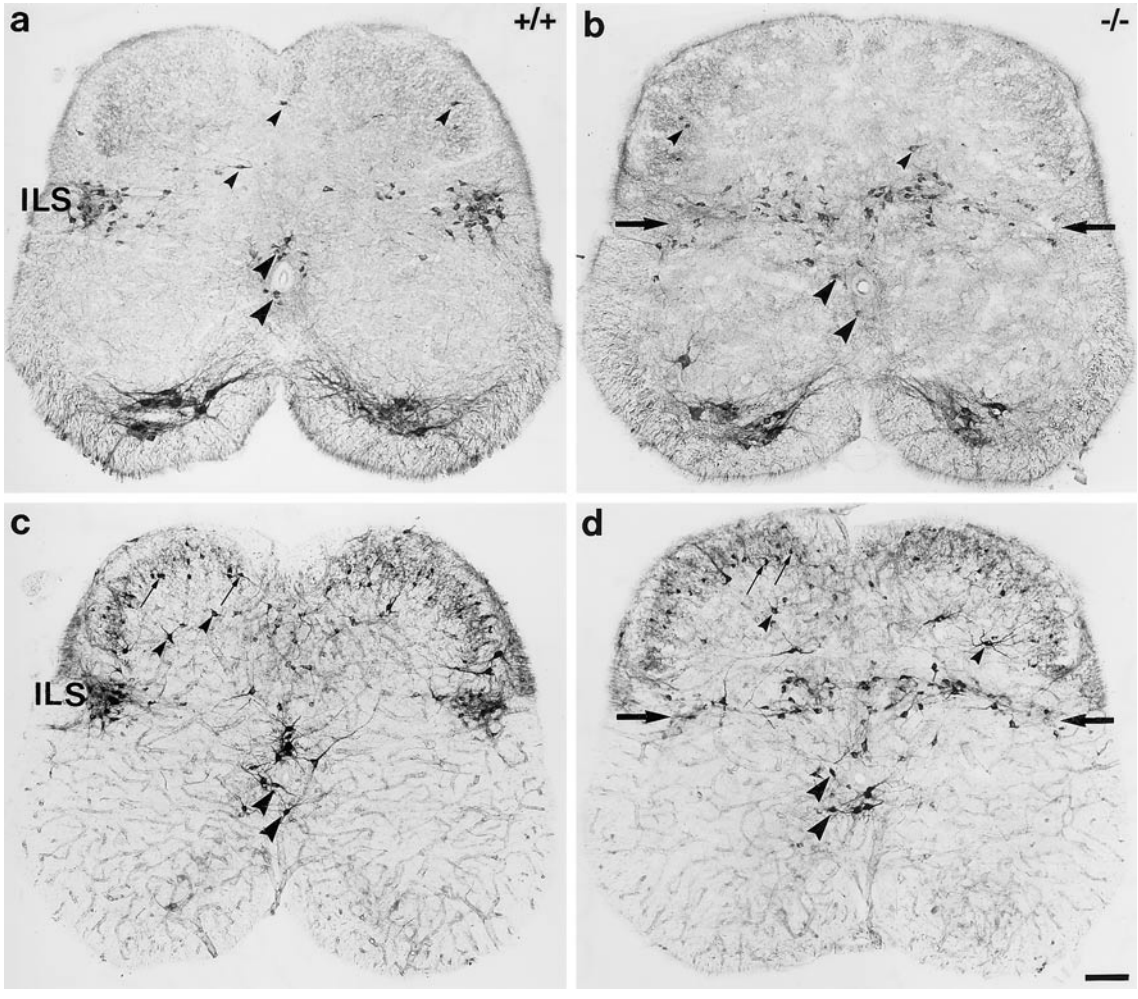



**FIG. 6.** Postnatal day 16 parasympathetic preganglionic neurons (PPNs) can be identified in sacral cord with either ChAT immunocytochemistry (a, b) or diaphorase histochemistry (c, d). (a) Wild-type cholinergic PPNs are found in the intermediolateral sacral nucleus (ILS), whereas ChAT-positive somatic motor neurons are located in the ventral horn. Cholinergic interneurons are found clustered around the central canal (large arrowheads) and scattered in the deep dorsal horn (small arrowheads). (b). In *reeler*, ChAT-labeled PPNs (between arrows) are dispersed between the intermediolateral region and the midline and have a disorganized appearance. Somatic motor neurons are correctly located in the ventral horn, and ChAT-positive interneurons are in their normal locations around the central canal (large arrowheads) and in the deep dorsal horn cells (small arrowheads). (c) Wild-type sacral spinal cord section adjacent to (a) was processed with diaphorase to label the PPNs (ILS). Additional diaphorase-labeled cells are distributed in the superficial (small arrows) and deep (small arrowheads) dorsal horn as well as around the central canal (large arrowheads). (d) Sacral section from the *reeler* mutant illustrated in (b) was labeled with diaphorase to identify the PPNs (between larger arrows). These neurons are widely dispersed across the intermediate spinal cord. Diaphorase-labeled interneurons are found in locations similar to those observed in wild-type mice (c). Scale: a–d, 100 $\mu$m.

to have migrated along a tangential pathway into the deep dorsal horn (Figs. 2c and 2d). At these early ages, many neurons of the U-shaped group had bipolar profiles with leading and trailing processes characteristic of migrating neurons (Fig. 2c, inset). Although the number and final positions of the neurons of the U-shaped group varied between individual sections (see Figs. 2c and 2d), no consistent differences were detected between mutant and wild-type mice. By E17.5, cells derived from the U-shaped group were found in the deep dorsal horn and around the central canal of both *reeler* and wild-type mice (Figs. 2e and 2f).

Diaphorase-only neurons of the deep dorsal horn were detected first on E14.5 in both mutant and wild-type mice and could be differentiated from those of the U-shaped group by location and morphology (Figs. 2c and 2d). Initially these small neurons were detected dorsomedially, contained less diaphorase reaction product than those of the U-shaped group, and had short processes projecting later-

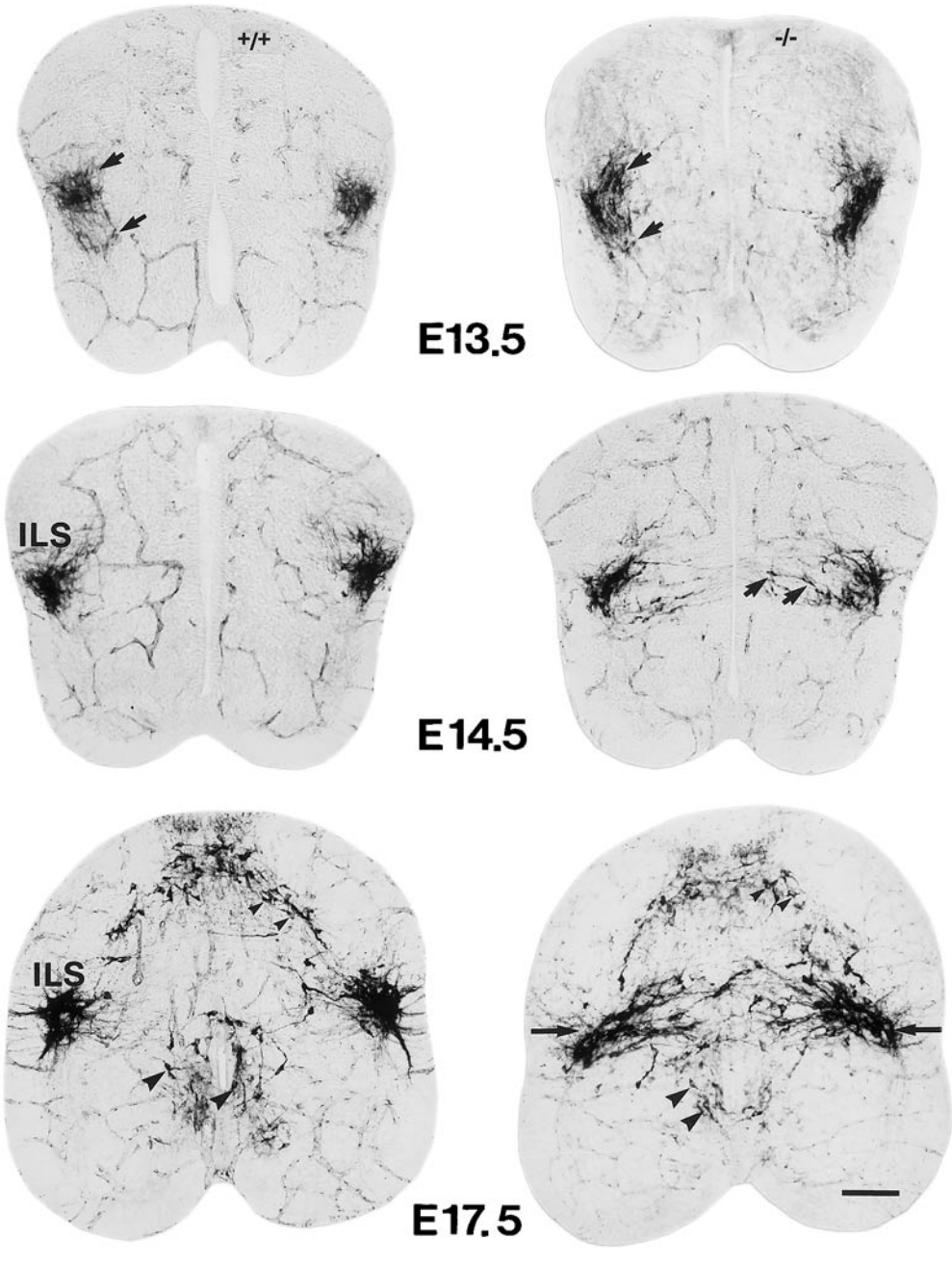
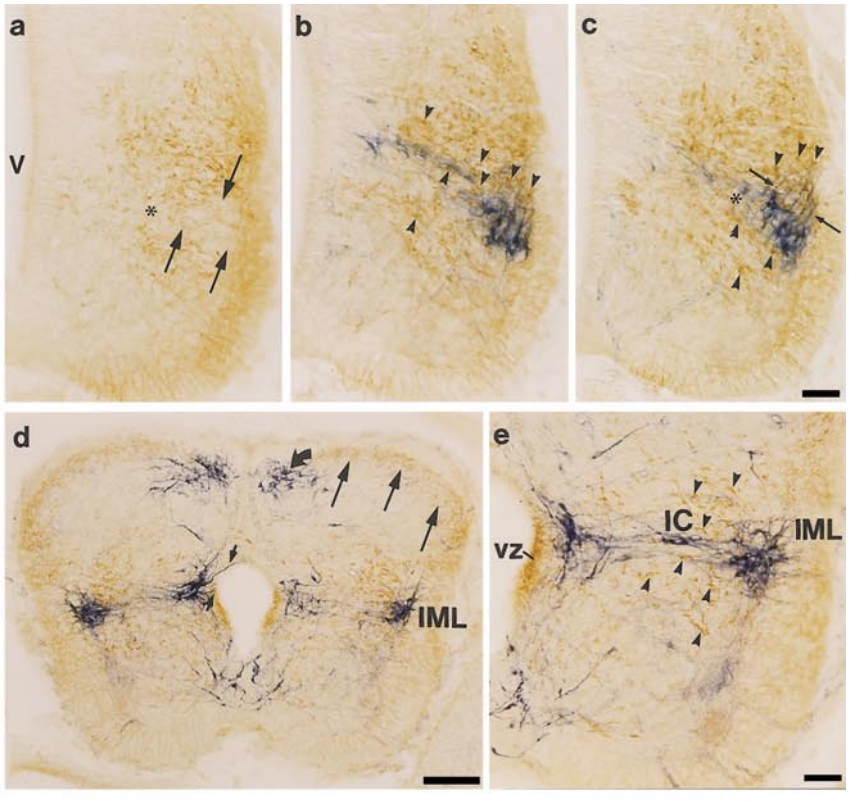
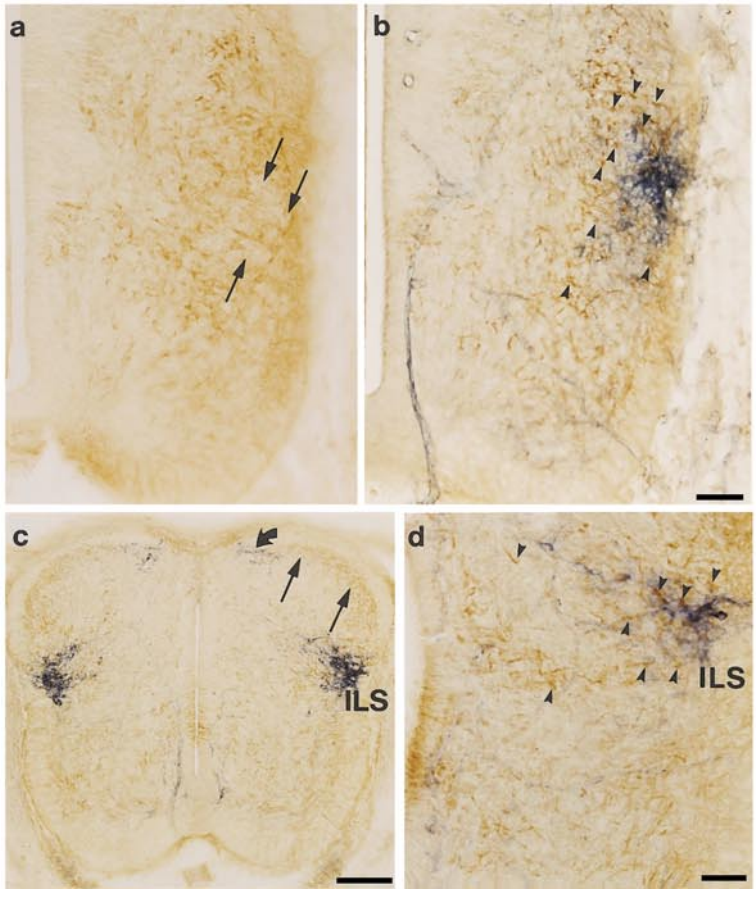



**FIG. 7.** Embryonic PPNs identified with diaphorase histochemistry in E13.5, E14.5, and E17.5 in wild-type (left column) and *reeler* (right column) mice. In E13.5 wild-type mice (top row), PPNs (arrows) are dispersed laterally across a more extensive dorsoventral area than seen at older ages, Similarly, *reeler* PPNs are distributed in a broad band along the lateral border of the spinal cord. By E14.5 (middle row), wild-type PPNs have migrated into the intermediolateral horn to form the sacral nucleus (ILS). In *reeler* medially directed, diaphorase-labeled PPNs (arrows) and their processes are detected on E14.5. On E17.5 (bottom row), wild-type mice have tightly clustered diaphorase-labeled cells in the ILS. In contrast, PPNs (between arrows) in *reeler* are found scattered across the intermediate spinal cord. Despite obvious differences in the PPNs, other populations of diaphorase cells in the dorsal horn (small arrowheads) and around the central canal (larger arrowheads) appear in similar positions in wild-type and *reeler* mice. Scale, 100 $\mu$m.




8




9

ally, in the direction of their apparent radial migration (Fig. 2d, inset) (Wetts *et al.*, 1995). By E17.5, many cells derived from the diaphorase-only group appeared to have dispersed laterally into the deep dorsal horn in both wild-type and mutant mice (Figs. 2e and 2f).

### *Migration of Thoracic and Sacral Somatic Motor Neurons Is Normal in reeler*

ChAT-immunoreactive SMNs were identified and analyzed in thoracic and sacral sections of P16 spinal cord that also contained autonomic motor neurons in abnormal locations as reported below. In the thoracic spinal cord, both *reeler* and wild-type mice had SMNs clustered into a single, ventrally located column (Figs. 3a and 3b). While their cell body locations were normal, the ChAT-containing dendritic arbors of thoracic SMNs in *reeler* appeared reduced in length and number compared with those of heterozygous and wild-type mice (Figs. 3a and 3b). To exclude the possibility that the aberrant dendritic bundles observed in mutants were due to fewer SMNs, their number per section was counted and averaged in wild-type (26 ± 6 SMNs, mean ± SD) and mutant (29 ± 6) thoracic spinal cord.

When SMNs were evaluated in sacral sections, their number per section (wild-type, 29 ± 9; *reeler* 28 ± 3) and their ventral location were similar in *reeler* and wild-type mice. In contrast to the thoracic SMNs, the dendritic arbors of the sacral SMNs appeared normal in mutants (see Figs. 6a and 6b).

### *Migration of Sympathetic Preganglionic Neurons Is Aberrant is reeler*

In the upper and middle thoracic regions of P16 heterozygous and wild-type mice, SPNs labeled with either ChAT immunocytochemistry or diaphorase histochemistry were located primarily in the intermediolateral nucleus (IML) with a few cells apparent in the adjacent funiculus. These neurons have dendritic bundles projecting medially in both ChAT- (Fig. 3a) and diaphorase-labeled (Fig. 3c) sections, and cell bodies within these bundles were located in the intercalated (IC) region (Fig. 3a). Additionally, a small number of SPNs were found in the central autonomic (CA) region, just dorsal to the central canal (Fig. 3a). In horizontal sections from wild-type and heterozygous mice, prominent bundles of dendrites were observed projecting rostrocaudally in the IML nucleus, and periodic dendritic bundles were seen extending medially, thus forming a ladder-like structure (Fig. 3c) similar to that described in the rat (Barber *et al.*, 1984; Markham *et al.*, 1991).

In P16 *reeler*, the SPNs were drastically rearranged. In all areas of thoracic spinal cord, ChAT-or diaphorase-labeled

**FIG. 8.** Single (a) or double-labeled (b–e) Reelin immunocytochemistry (amber-brown chromogen) combined with diaphorase histochemistry (purple-blue product) identify separate populations of neurons in wild-type thoracic spinal cord. The E12.5 images (a–c) are of nearby sections from the same embryo that are oriented with the ventricle (V) on the left and dorsal to the top. (a) In this E12.5 thoracic section, Reelin-positive cells are scattered around the dorsal, ventral, and medial sides of a vacant area (large arrows) occupied by unlabeled SPNs. Along the lateral border, the marginal zone contains a diffuse pattern of Reelin. Gaps between the Reelin-secreting cells (e.g., asterisk) may allow some PN neurons to escape the effects of Reelin and migrate medially. (b) Discrete Reelin-positive cells (arrowheads) are located both dorsal and medial to the SPNs, but do not form a solid barrier. A stream of diaphorase-labeled cells (purple-blue product) have migrated medially. (c) On E12.5, the SPNs have not yet reached their final locations in the intermediolateral region. Multiple dorsally oriented processes (between small arrows) are detected emanating from the SPNs in the direction of their migration into the intermediolateral area. One more medially located SPN (asterisk) resides in a Reelin-free area. Reelin-labeled cells (arrowheads) are concentrated dorsal, ventral, and medial to the SPNs. (d) By E14.5, most SPNs are located laterally in the intermediolateral nucleus (IML) or medially, near the receding ventricle. Processes (small arrows) of the medially located SPNs project into the dorsal commissure or the receding ventricular zone. Additional diaphorase-labeled cells detected on E14.5 are the dorsally originating diaphorase-only cells (curved arrow) and the U-derived cells found primarily in the ventral commissure. Reelin-positive cells still surround the IML, and at this age, form a band of cells in the superficial dorsal horn (large arrows). (e) High magnification of E14.5 wild-type spinal cord illustrates the relationship between Reelin-positive cells (arrowheads) and SPNs in three locations, the IML and IC nuclei and the centrally located cells adjacent to the receding ventricular zone. A portion of the ventral ventricular zone (vz) expresses Reelin at E14.5 (d, e) that was not observed in control sections (data not shown) or at E12.5 (a–c). Scales: a–c, 40 $\mu$m; d, 100 $\mu$m; e, 40 $\mu$m.

**FIG. 9.** Single (a) or double-labeled (b–d) Reelin localization (amber-brown chromogen) and diaphorase histochemistry (purple-blue product) identify separate populations of neurons in wild-type sacral spinal cord sections at E13.5 (a, b) and E14.5 (c, d). Sections are oriented with the ventricular zone to the left and dorsal to the top. (a) On E13.5 Reelin-labeled cells are found within the intermediate zone of the sacral spinal cord, but only a few are within the region occupied by unlabeled PPNs (outlined by arrows). (b) On E13.5, purple-blue diaphorase-positive PPNs are scattered along the lateral spinal cord, having not quite reached their final position in the intermediolateral spinal cord. Amber-brown Reelin-secreting cells (arrowheads) are dispersed in positions dorsal, ventral, and medial to the PPNs. (c) By E14.5, most PPNs are concentrated in the intermediolateral sacral nucleus (ILS) with a few cells positioned more medially. The dorsally derived diaphorase-only cells (curved arrow) are lightly labeled compared to the preganglionic neurons. Reelin-positive cells are detected around the ILS nucleus, in a small portion of the ventral ventricular zone, and in the superficial laminae of the dorsal horn (arrows). (d) Reelin-positive cells (arrowheads) are scattered within the intermediate zone and surround the ILS. A portion of the ventral ventricular zone in sacral E14.5 spinal cord contains Reelin immunoreactivity that was not observed in control sections (data not shown) or at earlier ages (a, b). Scales: a, b, 40 $\mu$m; c, 100 $\mu$m; d, 40 $\mu$m.

SPNs primarily were clustered in the CA nucleus (Figs. 3b and 3d), with a small number of cells detected in the IML or IC regions. Axons emanating from individual SPNs were observed projecting across the intermediate zone to reach the ventral root. In horizontal sections from mutants, rostrocaudally projecting dendrites of the SPNs were prominent; however, they were incorrectly positioned at the midline (compare Figs. 3c and 3d). In contrast, the periodic mediolaterally projecting dendrites were present in their correct locations (Figs. 3c and 3d). In rats, SPNs located in the IML, IC, and CA nuclei all contributed dendrites to these mediolateral bundles (Barber *et al.*, 1984; Markham *et al.*, 1991). In *reeler* mutants, most of these bundles were derived from medially placed cells.

Sympathetic preganglionic neurons were analyzed in the ChAT-immunoreactive sections in upper thoracic segments, levels in which Strack *et al.* (1988) reported that 95% of these cells in rat were found in the IML nucleus or adjacent lateral funiculus. The medial or lateral location of SPNs was mapped in wild-type and *reeler* mice and the results reported in Table 1a. An average of 79% of the SPNs were located laterally in wild-type mice in contrast to only 8% in *reeler* mutants. Thus, an average of 21% of SPNs were medially located in wild-type mice compared with 92% in *reeler* mutants. Differences in these proportions between genotypes were highly significant ($\chi^2 = 8669$; df = 1; $P > 0.0001$). These results are similar to findings reported by Yip *et al.* (2000) when Fluorogold-retrograde labeled SPNs were analyzed in P30 mice.

In developing rodents, the SPNs are generated synchronously with somatic motor neurons and migrate into the ventral intermediate zone as a single common group (Barber *et al.*, 1991; Phelps *et al.*, 1991). Then SPNs move dorsally along a tangential pathway into the intermediolateral region, and finally some migrate back medially, to reside in IC and CA regions (Phelps *et al.*, 1991, 1993). In E12.5 wild-type and mutant mice, most SPNs were detected along the lateral border of the ventral spinal cord, near the somatic motor neurons (Fig. 4). However, small numbers of diaphorase-positive cells already were located medially in mice of both genotypes (Fig. 4, arrows). By E13.5, the SPNs have migrated further dorsally in both wild-type and *reeler*, and substantial differences in the extent of their lateral-to-medial migration were apparent (Fig. 4). Most wild-type SPNs remained laterally, whereas the majority of *reeler* neurons were found near the midline. Prominent medially directed processes emanated from both wild-type and *reeler* SPNs, and frequently projected deep into the ventricular zone, whereas laterally projecting processes were associated only with wild-type neurons (Figs. 5a and 5b). On E14.5, SPNs in wild-type mice remained concentrated in the IML, while those in *reeler* occupied positions near the ventricular surface (Fig. 4). At this age, bundles of processes from both wild-type and *reeler* SPNs extended dorsally toward the roof plate (Fig. 4, arrowheads) and also medially projecting processes penetrated through the width of the ventricular zone (Fig. 4). By E17.5, most wild-type SPNs were positioned laterally in the IML nucleus (Fig. 4). In contrast, most SPNs in E17.5 mutants were found medially in the CA region, directly dorsal to or along the lateral edge of the central canal (Fig. 4).

### *Migration of the Parasympathetic Preganglionic Neurons Is Aberrant in reeler*

The parasympathetic preganglionic neurons (PPNs) are the second group of autonomic motor neurons that reside in the spinal cord and, like the SPNs, express both ChAT and NADPH-diaphorase (Barber *et al.*, 1984; Brüning, 1992; Vizzard *et al.*, 1994). The PPNs in P16 wild-type and heterozygous mice formed the intermediolateral sacral nucleus (ILS; Figs. 6a and 6c) and, unlike the SPNs, usually were not found in medial locations (Barber *et al.*, 1991; Nadelhaft and Booth, 1984; Nadelhaft *et al.*, 1986). In P16 mutants, some PPNs were located laterally but did not form a discrete group (Figs. 6b and 6d). Instead, *reeler* PPNs were scattered along the entire mediolateral extent of the intermediate region of sacral spinal cord (Figs. 6b and 6d).

The medial or lateral position of sacral PPNs was analyzed and compared in wild-type and *reeler* animals. In wild-type mice, an average of 97% of the PPNs were found in the lateral half of the spinal cord, compared with only 62% in *reeler* (Table 1b). Thus, a mean of 38% of *reeler* PPNs were located medially in contrast to only 3% in wild-type mice. Differences in the location of PPNs between wild-type and *reeler* mice were highly significant ($\chi^2 = 1026$; df = 1; $P > 0.0001$).

Sacral PPNs and somatic motor neurons are generated synchronously in rats and are born at least 1 day later than SPNs in thoracic spinal cord (Barber *et al.*, 1991). Because neither the development nor the normal migratory pattern of the PPNs has been examined previously, we first determined the ages these neurons were detectable in wild-type mice and their apparent sequence of migration. PPNs were inconsistently detected with diaphorase histochemistry in mice before E13.5, but by this age, they were found along the lateral border of the ventral spinal cord in both wild-type and *reeler* (Fig. 7). By E14.5, the PPNs in both genotypes were located further dorsally in a distinct intermediolateral sacral group (ILS; Fig. 7). Processes emanated from wild-type PPNs in a dorsomedial direction, whereas those from mutants projected medially as well as dorsomedially (Figs. 5c and 5d). In E14.5 mutants, the cell bodies of many PPNs were arranged loosely in the lateral portion of the spinal cord. Some PPNs, however, appeared to form strings of cell bodies with processes projecting toward the ventricle (Figs. 5d and 7).

In E17.5 wild-type mice, PPNs were clustered in the ILS with dendrites projecting laterally and medially (Fig. 7). Diaphorase staining of E17.5 mutant mice showed that, although some PPNs were found laterally, the majority of labeled cells were scattered in positions between the ILS and the midline (Fig. 7, between arrows). Dendritic bundles formed in the mutant but appeared disorganized and incor-

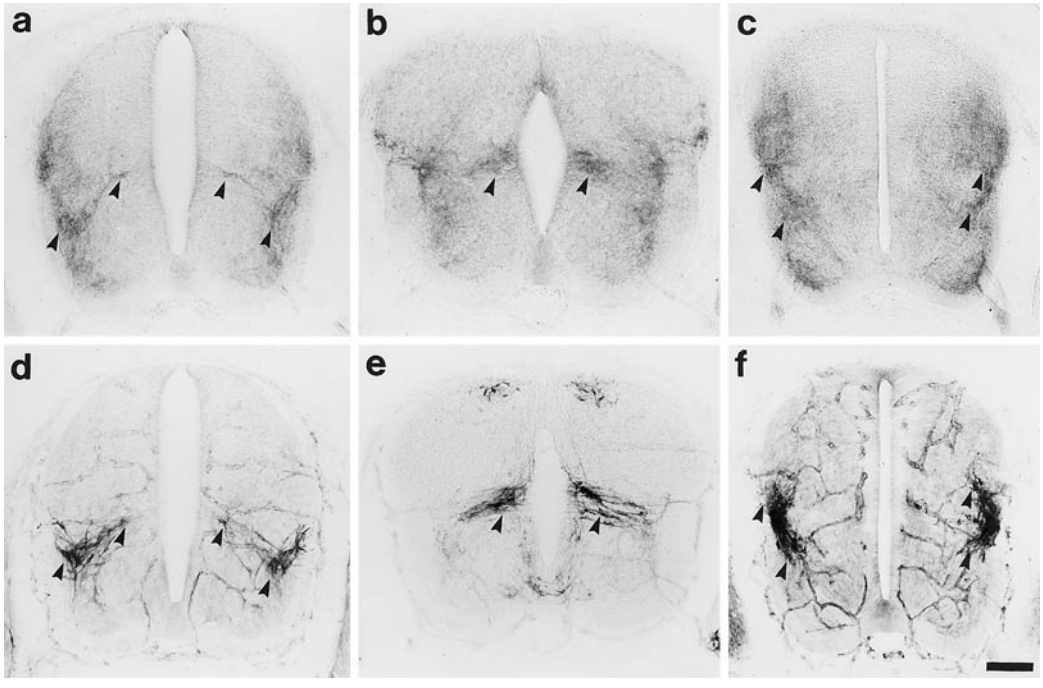



**FIG. 10.** SPNs (a, b, d, e) and PPNs (c, f) identified with anti-Dab1 antiserum (a–c) or diaphorase histochemistry (d–f) in pairs of adjacent sections from *reeler* mutants. (a, d) On E12.5, Dab1-labeled cells and processes (a, arrowheads) extend across the intermediate zone of thoracic spinal cord in the same position occupied by diaphorase-labeled SPNs (d, arrowheads). At this early stage, the majority of *reeler* SPNs are located laterally. (b, e) On E13.5, many SPNs (arrowheads) in *reeler* thoracic spinal cord are concentrated along the ventricle, in the midline. At E13.5, these SPNs are localized with Dab1 immunoreactivity (b) and diaphorase histochemistry (e). Additional neuronal populations also express high levels of Dab1 in mutants. (c, f) On E13.5, *reeler* sacral spinal cord contains a number of Dab1-positive cells, including PPNs (arrowheads) positioned in the intermediolateral sacral region (c). Adjacent section contains diaphorase-labeled PPNs (f, arrowheads) in an equivalent locations as those labeled with anti-Dab1 (c, arrowheads). Scale: a–f, 100 $\mu$m.

rectly oriented. During embryonic development, there appeared to be a progressive shift in the distribution of mutant PPNs to more medial locations, suggesting that these neurons fail to stop in their normal position in the ILS nucleus and instead migrate back toward the midline between E14.5 and E17.5 (Fig. 7).

### *Expression of Reelin in Thoracic and Sacral Spinal Cord*

Although Reelin was detected as early as E11.5 (data not shown), the pattern of Reelin expression was compared in wild-type thoracic and sacral spinal cord between E12.5 and E14.5, the period when the aberrant migration of *reeler* preganglionic neurons occurred. Reelin was not detected in the SPNs (Fig. 8a) (Yip *et al.*, 2000) or the PPNs (Fig. 9a) themselves, but rather was expressed in cells that bordered these groups of autonomic motor neurons (Figs. 8a–8c and 9a and 9b). In addition to the cellular localization, a pattern of diffuse Reelin labeling was observed in the lateral marginal zone near the SPNs and PPNs (Figs. 8a and 9a). It is likely that this diffuse Reelin represents the secreted extracellular form of the molecule. At older ages, a number of Reelin-secreting cells were detected in the superficial dorsal horn and a few were dispersed in the ventral horn (Figs. 8d and 9c). Reelin localization throughout the spinal cord is the focus of an additional study (Kubasak *et al.*, 2001).

In wild-type mice, many more SPNs than PPNs migrate back toward the midline, and thus we reasoned that the pattern of Reelin-containing cells would reflect these differences with more Reelin cells surrounding PPNs than SPNs. In E12.5 wild-type thoracic segments, Reelin-labeled cells located medial to the SPNs did not form a solid barrier, but instead had gaps between them through which medially directed neurons could migrate (Fig. 8a). The three-dimensional relationship of the Reelin-secreting cells and the medially migrating SPNs was complex. Sometimes the Reelin-free spaces appeared to border the migratory pathway used by the medially directed SPNs (Fig. 8c), while at other times, the diaphorase- and Reelin-labeled cells seemed directly adjacent to each other (Fig. 8b). However, once a single diaphorase-labeled neuron successfully navigated through the Reelin cells, others appeared to migrate along the same pathway, forming a cohort of cells migrating toward the midline (Fig. 8b). The dorsally directed processes of the SPNs projected individually (Fig. 8c) and these lateral

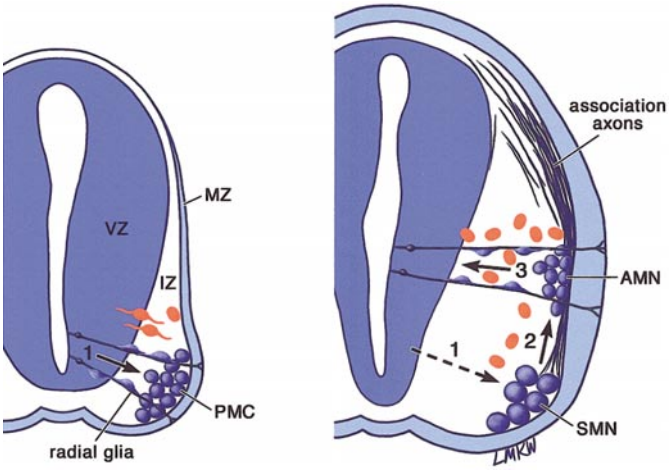



**FIG. 11.** Summary diagram of how Reelin may inhibit the migration of spinal autonomic motor neurons (AMN). Solid or dotted arrows indicate the period of active or completed migration, respectively. Somatic and autonomic motor neurons (blue cells) are generated synchronously in the ventricular zone (VZ) and then migrate radially to form the primary motor column (PMC). During the first step of their migration (1), only a few Reelin-secreting cells (red cells) are present in the spinal cord and they occupy positions dorsal to the developing PMC. Subsequently, the somatic motor neurons (SMN) remain in the ventral spinal cord, while the AMNs migrate both dorsally along a tangential pathway parallel to the association axons (2) and medially along the radial glia (3). Reelin-expressing cells positioned around the AMNs may inhibit the majority of these neurons from migrating medially (3). Cells that for some reason escape the effects of Reelin may migrate to the midline, while those that are inhibited would reside in intermediate locations between centrally and laterally located AMNs.

cells migrated dorsally into their correct locations by E13.5. Between E13.5 and E14.5, SPNs were found distributed across the intermediate zone with Reelin-positive cells surrounding them dorsally, ventrally, and medially (Figs. 8d and 8e). The centrally located SPNs projected long processes toward the dorsal commissure and shorter processes into the receding ventricular zone (Fig. 8d). In general, the processes of the SPNs that penetrated the ventricular zone did so just dorsal to the Reelin-labeled portion of the ventral ventricular zone (Figs. 8d and 8e).

Although we expected to find a higher concentration of Reelin-secreting cells around the PPNs than the SPNs, the pattern of Reelin expression in sacral segments (Fig. 9a) was similar to that observed in thoracic levels (Fig. 8a). On E13.5, the region containing the PPNs contained little Reelin, while the surrounding area was populated by a number of Reelin-secreting cells. On E14.5, the wild-type PPNs had achieved their position in the ILS with occasional cells found more medially (Figs. 9c and 9d). Reelin was detected in an appropriate time and location to play a role in both the dorsal and medial migrations of both the SPNs and PPNs, and its pattern of distribution in thoracic and sacral spinal cord was similar.

### *Dab1 Is Expressed in Sympathetic and Parasympathetic Preganglionic Neurons*

Because Dab1 is a critical part of the Reelin signaling pathway, we asked whether both the SPNs and PPNs contained the Dab1 protein and thus would be capable of responding to Reelin. Dab1 was localized in *reeler* mutants rather than wild-type mice, due to the substantial increase in Dab1 protein levels reported in mutants (Rice *et al.*, 1998). In E12.5 *reeler* thoracic spinal cord, Dab1-labeled cell bodies and processes (Fig. 10a) were found in several regions, including positions identical to the diaphorase-positive SPNs in adjacent sections (Fig. 10d). By E13.5, most of the *reeler* SPNs (Fig. 10e) had migrated medially, and a similar shift to a midline location was detected in an adjacent section processed for Dab1 immunoreactivity (Fig. 10b). The coincident location of Dab1-immunoreactive cells (Figs. 10a and 10b) and SPNs identified with diaphorase (Figs. 10d and 10e) strongly supports the interpretation that *reeler* SPNs express relatively high levels of the cytoplasmic adaptor protein, Dab1. Similar comparisons were made in sacral spinal cord to determine whether *reeler* PPNs also contained high levels of Dab1. On E13.5, the age when PPNs are migrating dorsally, Dab1-labeled cells (arrowheads, Fig. 10c) were found in the same position as the diaphorase-labeled PPNs. These findings suggest that both populations of autonomic motor neurons contain substantial levels of the Dab1 protein during their aberrant migrations in *reeler* mutants. Because neurons in other areas of thoracic and sacral spinal cord also express high levels of Dab1, additional populations of spinal cord neurons may respond to Reelin signaling.

## DISCUSSION

In the present study, neurochemically identified populations of spinal cord neurons were shown to display both normal and abnormal migration patterns within *reeler*, suggesting that Reelin is critical to the proper migration of only specific neuronal phenotypes in the spinal cord. Interestingly, both neuronal populations with aberrant migrations are similar in their phenotype: visceral efferent neurons that project their axons to postganglionic neuronal targets within the peripheral nervous system. In addition to migratory errors, we found that the pattern of dendritic bundles was dramatically altered in both groups of *reeler* autonomic motor neurons and that dendrites of somatic motor neurons in the thoracic spinal cord were stunted. Other novel findings of this study include strong correlative evidence that both populations of autonomic motor neurons express Dab1 during their migration and are located adjacent to Reelin-secreting cells, features required for the preganglionic neurons to use Reelin as a short-range cue guiding their cell migration.

### *Many reeler Sympathetic and Parasympathetic Preganglionic Neurons Fail to Stop*

Based on sequential observations, mouse PPNs appear to follow the first two steps of the complex migratory pattern used by SPNs in rats (Barber *et al.*, 1993; Markham and Vaughn, 1991; Phelps *et al.*, 1991, 1993), mice (Yip *et al.*, 2000), and humans (Foster and Phelps, 2000). Initially, both SPNs and PPNs migrate laterally from the ventricular zone to the ventral intermediate zone, in combination with the somatic motor neurons (Fig. 11, step 1). At this point, the migration of somatic motor neurons is complete, whereas both populations of preganglionic neurons subsequently migrate dorsally (Fig. 11, step 2). Both SPNs and PPNs in *reeler* appear to sustain a normal lateral and dorsal migration, but then a number of these neurons proceed medially, rather than remaining in the intermediolateral gray matter (Fig. 11, step 3). Although both populations of preganglionic neurons have similar migratory defects, the final positioning of these two groups of neurons differed.

If the differential distribution of medially located preganglionic neurons was solely dependent on Reelin, higher levels of expression or more Reelin-secreting cells should reside in sacral than in thoracic wild-type spinal cord. To date, such differences were not detected, and thus, additional factors probably contribute to the differential migratory patterns of the wild-type SPNs and PPNs. One difference between these phenotypically similar neurons is that SPNs normally have a medially directed, third component of their migratory pathway, whereas PPNs do not. This variation in normal migratory patterns may explain why, in *reeler*, a much larger percentage of SPNs than PPNs resides at the midline. A second related difference between the spinal preganglionic neurons is that many of the leading processes of wild-type SPNs are oriented medially, parallel to the radial glia, whereas those of the PPNs project further dorsally. If the final lateral to medial segment of the SPN migration is guided by radial glia as previously proposed (Phelps *et al.*, 1993), the differences in the alignment of their leading processes with radial glia may play a role in the extent of their normal medial migration in wild-type mice and their mismigration in mutants. Other factors that would explain the variations in the normal SPN and PPN migrations would be if some of these neurons did not express Dab1, if they expressed only one of the lipoprotein receptors (VLDLR and ApoER2), and/or if there were other components required in the Reelin signaling pathway that were missing. Additional studies are necessary to further define the variation in migratory behavior of the spinal preganglionic neurons based on differences in expression of components of the Reelin signaling pathway.

### *Misplaced Sympathetic and Parasympathetic Preganglionic Neurons Differentiate Relatively Normally*

In general, *reeler* SPNs and PPNs seem to differentiate relatively normally, with prominent dendritic bundles forming early in development. The rostrocaudal or mediolateral orientation of these dendritic bundles was correctly maintained in *reeler*, although many bundles were incorrectly positioned. While it is likely that the afferent input to the dendrites and cell bodies of *reeler* autonomic motor neurons differs from those of wild-type mice, apparently these visceral efferent systems function well enough in *reeler* mutants that many animals survive and are able to breed and produce offspring (Falconer, 1951; Mariani *et al.*, 1977). Where examined in cerebral and cerebellar cortices, misplaced *reeler* neurons still maintain the specificity of synaptic connections (see reviews in Caviness and Rakic, 1978; Lambert de Rouvrout and Goffinet, 1998). For example, during development, thalamic axons in mutants were able to find their correct targets, layer IV cortical neurons, despite the incorrect location of the subplate cells that presumably guide them (Caviness, 1976; Molnár *et al.*, 1998; Steindler and Colwell, 1976). Findings of intact synaptic connectivity in *reeler* cortical areas, together with the maintenance of functions controlled by both the sympathetic and parasympathetic components of the autonomic nervous system, suggest that at least some appropriate afferents are able to find their targets on misplaced preganglionic neurons in *reeler* mice.

### *Role of Reelin in Spinal Cord*

Expression of Reelin mRNA was demonstrated previously in the ventral and intermediate spinal cord (Bernier *et al.*, 2000; Ikeda and Terashima, 1997; Schiffmann *et al.*, 1997), and just recently, Reelin protein was localized between the SPNs and the ventricular zone (Yip *et al.*, 2000). Our study is the first to demonstrate distinct cellular localization of Reelin-secreting cells along the dorsal, ventral, and medial borders of the preganglionic neurons in both thoracic and sacral spinal cord. In addition, we found the intracellular adaptor protein Dab1 within both populations of *reeler* preganglionic neurons. Together, these findings suggest that many spinal preganglionic neurons normally respond to Reelin signaling to accurately regulate their positional information during their complex migratory pattern.

It has been suggested that Reelin functions as an inhibitory molecule or "stop signal" for neuronal migration, as Reelin frequently is produced at the destination site of migrating neurons (D'Arcangelo *et al.*, 1995; Rice and Curran, 1999; Yip *et al.*, 2000). Based solely on the position of Reelin-secreting cells in spinal cord, Reelin could inhibit both the dorsal (Fig. 11, step 2) and the medial (Fig. 11, step 3) migrations of the preganglionic neurons. However, mutant SPNs and PPNs always stopped during their tangentially directed dorsal migration, but failed to stop along their radially directed medial migration. Thus, Reelin in spinal cord appears to regulate only radial migration, as the tangential migrations of both the U-shaped group and the dorsally directed component of the preganglionic neurons were normal in *reeler* mutants.

Some investigators have proposed that Reelin may act to signal radially directed neurons to disengage from the glial fiber, possibly by changing the adhesive interactions or surface properties between the migrating cells and their guidance substrates (Dulabon *et al.,* 2000; Lambert de Rouvrout and Goffinet, 1998; Ogawa *et al.,* 1995; Rice and Curran, 1999). Defects in forebrain radial glia have been reported in *reeler* mutants, including fewer branched processes and endfeet at the limiting membrane and a premature differentiation of radial glia into astrocytes (Hunter-Schaedle, 1997; Pinto-Lord *et al.,* 1982). Recent *in vitro* studies have directly demonstrated that Reelin can arrest cell migration by causing detachment of neurons from radial glia (Dulabon *et al.,* 2000). Previously, we proposed that there are two radially guided components in the migration pattern of the SPNs—the initial, medial to lateral migration carried out synchronously with somatic motor neurons and the later, lateral to medial migration (Phelps *et al.,* 1993). In *reeler* mutants, only errors in this later migration were detected, suggesting there is a differential effect on radial migration controlled by the timing and location of Reelin expression in spinal cord. As illustrated in Fig. 11, Reelin was not present ventrally along the medial to lateral migratory pathway used by somatic and autonomic motor neurons to move from the ventricular zone to form primary motor column (step 1; data not shown). However, a number of Reelin-secreting cells were found in the intermediate spinal cord during the later lateral to medial migration (step 3) and thus preferentially could inhibit the late component of their migration. Thus, autonomic motor neurons that completely escape the effects of Reelin would reside in the midline, those that were completely inhibited would remain in the intermediolateral position, and those that interacted with Reelin during the migration would reside within the processes connecting these medial and lateral locations. Our findings are consistent with previous suggestions that Reelin acts during the final stages of neuronal migration and may signal the detachment of the neurons from their migratory guides (Dulabon *et al.,* 2000; Rice and Curran, 1999, 2001). Clearly, additional migratory cues must be used to guide tangentially directed neuronal migration in spinal cord.

Alternately, it may be that neurons with complex radial and tangential migratory patterns are most susceptible to migrational errors in *reeler*. For example, the inferior olivary neurons that migrate from the 4th ventricle to the ventromedial rhombencephalon also are associated with abnormal positioning in *reeler* (Goffinet, 1983b). Specifically, their earlier nonradial migration appeared normal in *reeler,* while the later, radial component was associated with malpositioning (Goffinet, 1983b). The migratory errors in the *reeler* preganglionic neurons are similar in that only their later, radially directed migration was abnormal. The spinal preganglionic neurons in *reeler* are unique, however, in that they overmigrate or pass by their normal locations.

Our results showing that both populations of preganglionic neurons in spinal cord are mispositioned while other groups of cholinergic neurons are correctly positioned suggest that Reelin has a cell-specific action in the spinal cord. While additional populations of spinal cord neurons overexpress Dab1 in *reeler* mutants, and thus also may utilize the Reelin signaling pathway, the overall structure of the *reeler* spinal cord seems relatively normal in size and appearance. This is in contrast to cortical brain regions, where the defects in *reeler* are more severe. In developing *reeler* cerebral cortex, the entire inside-out pattern of cell migration is disrupted and in the *reeler* cerebellum the normal lamination and foliation is missing, Purkinje cells are incorrectly aligned, and the size is severely reduced (see reviews in Caviness and Rakic, 1978; Lambert de Rouvrout and Goffinet, 1998). Although currently Reelin appears to play a more limited role in the development of the spinal cord compared with its action in cortical regions, Reelin also is expressed in nonautonomic levels of the spinal cord during the period of neuronal migration (Kubasak *et al.,* 2001). Thus, it is possible that additional *reeler* spinal cord defects may be elucidated with studies in which neuronal populations are identified by functional markers such as their transmitter phenotypes.

## ACKNOWLEDGMENTS

We thank Dr. Thomas Curran at St. Jude Children's Hospital for his generous support and advice throughout this project and Dr. Ellen Carpenter for her guidance with the genotyping and helpful suggestions on the manuscript. Supported in part by the National Science Foundation Grant IBN-9734550 (P.E.P.), the Council on Research of the UCLA Academic Senate (P.E.P.), the CARE Scholars Summer Research Program (Y.R.), the NIH Cancer Center Support Grant P30 Ca 21765 (Dr. Thomas Curran), and the American Lebanese Syrian Associated Charities (S.D-D. and T. Curran).

## REFERENCES

Barber, R. P., Phelps, P. E., Houser, C. R., Crawford, G. D., Salvaterra, P. M., and Vaughn, J. E. (1984). The morphology and distribution of neurons containing choline acetyltransferase in the adult rat spinal cord: An immunocytochemical study. *J. Comp. Neurol.* **229,** 329–346.

Barber, R. P., Phelps, P. E., and Vaughn, J. E. (1991). Generation patterns of immunocytochemically identified cholinergic neurons at autonomic levels of the rat spinal cord. *J. Comp. Neurol.* **311,** 509–519.

Barber, R. P., Phelps, P. E., and Vaughn, J. E. (1993). Preganglionic autonomic motor neurons display normal translocation patterns in slice cultures of embryonic rat spinal cord. *J. Neurosci.* **13,** 4898–4907.

Bernier, B., Bar, I., D'Arcangelo, G., Curran, T., and Goffinet, A. M. (2000). Reelin mRNA expression during embryonic brain development in the chick. *J. Comp. Neurol.* **422,** 448–463.

Blottner, D., and Baumgarten, H. G. (1992). Nitric oxide synthetase (NOS)-containing sympathoadrenal cholinergic neurons of the rat IML-cell column: Evidence from histochemistry, immunohistochemistry, and retrograde labeling. *J. Comp. Neurol.* **316,** 45–55.

Brüning, G. (1992). Localization of NADPH diaphorase, a histochemical marker for nitric oxide synthase, in the mouse spinal cord. *Acta Histochem.* **93,** 397–401.

Caviness, V. S., Jr. (1976). Patterns of cell and fiber distribution in the neocortex of the reeler mutant mouse. *J. Comp. Neurol.* **170,** 435–448.

Caviness, V. S., Jr. (1982). Neocortical histogenesis in normal and *reeler* mice: A developmental study based upon [$^3$H]Thymidine autoradiography. *Dev. Brain Res.* **4,** 293–302.

Caviness, V. S., Jr., and Rakic, P. (1978). Mechanisms of cortical development: A view from mutations in mice. *Annu. Rev. Neurosci.* **1,** 297–326.

Curran, T., and D'Arcangelo, G. (1998). Role of reelin in the control of brain development. *Brain Res. Rev.* **26,** 285–294.

D'Arcangelo, G., Homayouni, R., Keshvara, L., Rice, D. S., Sheldon, M., and Curran, T. (1999). Reelin is a ligand for lipoprotein receptors. *Neuron* **24,** 471–479.

D'Arcangelo, G., Miao, G. G., Chen, S. C., Soares, H. D., Morgan, J. I., and Curran, T. (1995). A protein related to extracellular matrix proteins deleted in the mouse mutant *reeler*. *Nature* **374,** 719–723.

D'Arcangelo, G., Miao, G. G., and Curran, T. (1996). Detection of the reelin breakpoint in *reeler* mice. *Mol. Brain Res.* **39,** 234–236.

D'Arcangelo, G. D., Nakajima, K., Miyata, T., Ogawa, M., Mikoshiba, K., and Curran, T. (1997). Reelin is a secreted glycoprotein recognized by the CR-50 monoclonal antibody. *J. Neurosci.* **17,** 23–31.

de Bergeydk, V., Naerhuyxen, B., Goffinet, A. M., and Lambert de Rouvroit, C. (1998). A panel of monoclonal antibodies against reelin, the extracellular matrix protein defective in *reeler* mutant mice. *J. Neurosci. Methods* **82,** 17–24.

Dulabon, L., Olson, E. C., Taglienti, M. G., Eisenhuth, S., McGrath, B., Walsh, C. A., Kreidberg, J. A., and Anton, E. S. (2000) Reelin binds $\alpha 3\beta 1$ integrin and inhibits neuronal migration. *Neuron* **27,** 33–44.

Dun, N. J., Dun, S. L., Wu, S. Y., Forstermann, U., Schmidt, H. H., and Tseng, L. F. (1993). Nitric oxide synthase immunoreactivity in the rat, mouse, cat and squirrel monkey spinal cord. *Neuroscience* **54,** 845–857.

Falconer, D. S. (1951). Two new mutants, *trembler* and *reeler,* with neurological actions in the house mouse. *J. Genet.* **50,** 192–201.

Foster, J. A., and Phelps, P. E. (2000). Neurons expressing NADPH-diaphorase in the developing human spinal cord. *J. Comp. Neurol.* **427,** 417–427.

Freshney, R. I. (1987). "Culture of Animal Cells: A Manual of Basic Technique." Wiley-Liss, New York.

Fujimoto, Y., Setsu, T., Ikeda, Y., Miwa, A., Okado, H., and Terashima, T. (1998). Ambiguus nucleus neurons innervating the abdominal esophagus are malpositioned in the *reeler* mouse. *Brain Res.* **811,** 156–160.

Goffinet, A. M. (1983a). The embryonic development of the cerebellum in normal and *reeler* mutant mice. *Anat. Embryol.* **168,** 73–86.

Goffinet, A. M. (1983b). The embryonic development of the interior olivary complex in normal and *reeler* ($rl^{ORL}$) mutant mice. *J. Comp. Neurol.* **219,** 10–24.

Goffinet, A. M. (1984a). Abnormal development of the facial nerve nucleus in *reeler* mutant mice. *J. Anat.* **138,** 207–215.

Goffinet, A. M. (1984b). Events governing organization of postmigratory neurons: Studies on brain development in normal and *reeler* mice. *Brain Res. Rev.* **7,** 261–296.

Houser, C. R., Crawford, G. D., Barber, R. P., Salvaterra, P. M., and Vaughn, J. E. (1983). Organization and morphological characteristics of cholinergic neurons: An immunocytochemical study with a monoclonal antibody to choline acetyltransferase. *Brain Res.* **266,** 97–119.

Howell, B. W., Gertler, F. B., and Cooper, J. A. (1997). Mouse disabled (mDab1): A Src binding protein implicated in neuronal development. *EMBO J.* **16,** 121–132.

Hunter-Schaedle, K. E. (1997). Radial glial cell development and transformation are disturbed in *reeler* forebrain. *J. Neurobiol.* **33,** 459–472.

Ikeda, Y., and Terashima, T. (1997). Expression of *reelin,* the gene responsible for the reeler mutation, in embryonic development and adulthood in the mouse. *Dev. Dyn.* **210,** 157–172.

Kaufman, M. H. (1992). "The Atlas of Mouse Development." Academic Press: Harcourt Brace & Company, London.

Kubasak, M. D., Brooks, R., and Phelps, P. E. (2001). Reelin expressing cells are found throughout the developing spinal cord. *Soc. Neurosci. Abstr.* **27,** 469.10.

Lambert de Rouvrout, C., and Goffinet, A. M. (1998). The *reeler* mouse as a model of brain development. *Adv. Anat. Embryol. Cell Biol.* **150,** 1–106.

Mariani, J., Crepel, F., Mikoshiba, K., Changeux, J. P., and Sotelo, C. (1977). Anatomical, physiological and biochemical studies of the cerebellum from *reeler* mutant mouse. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **281,** 1–28.

Markham, J. A., Phelps, P. E., and Vaughn, J. E. (1991). Development of rostrocaudal dendritic bundles in rat thoracic spinal cord: Analysis of cholinergic sympathetic preganglionic neurons. *Dev. Brain Res.* **61,** 229–236.

Markham, J. A., and Vaughn, J. E. (1991). Migration patterns of sympathetic preganglionic neurons in embryonic rat spinal cord. *J. Neurobiol.* **22,** 811–822.

Miller, M. M., Goto, R., and Clark, S. D. (1982). Structural characterization of developmentally expressed antigenic markers on chicken erythrocytes using monoclonal antibodies. *Dev. Biol.* **94,** 400–414.

Molnár, Z., Adams, R., Goffinet, A. M., and Blakemore, C. (1998). The role of the first postmitotic cortical cells in the development of thalamocortical innervation in the *reeler* mouse. *J. Neurosci.* **18,** 5746–5765.

Nadelhaft, I., and Booth, A. M. (1984). The location and morphology of preganglionic neurons and the distribution of visceral afferents from the rat pelvic nerve: A horseradish peroxidase study. *J. Comp. Neurol.* **226,** 238–245.

Nadelhaft, I., de Groat, W. C., and Morran, C. (1986). The distribution and morphology of parasympathetic preganglionic neurons in the cat sacral spinal cord as revealed by horseradish peroxidase applied to the sacral ventral roots. *J. Comp. Neurol.* **249,** 48–56.

Ogawa, M., Miyata, T., Nakajima, K., Yagyu, K., Seike, M., Ikenaka, K., Yamamoto, H., and Mikoshiba, K. (1995). The *reeler* gene-associated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. *Neuron* **14,** 899–912.

Phelps, P. E., Barber, R. P., Brennan, L. A., Maines, V. M., Salvaterra, P. M., and Vaughn, J. E. (1990). Embryonic development of four different subsets of cholinergic neurons in rat cervical spinal cord. *J. Comp. Neurol.* **291,** 9–26.

Phelps, P. E., Barber, R. P., and Vaughn, J. E. (1991). Embryonic development of choline acetyltransferase in thoracic spinal motor neurons: Somatic and autonomic neurons may be derived from a common cellular group. *J. Comp. Neurol.* **307,** 77–86.

Phelps, P. E., Barber, R. P., and Vaughn, J. E. (1993). Embryonic development of rat sympathetic preganglionic neurons: Possible migratory substrates. *J. Comp. Neurol.* **330,** 1–14.

Phelps, P. E., Barber, R. P., and Vaughn, J. E. (1996). Nonradial migration of interneurons can be experimentally altered in spinal cord slice cultures. *Development* **122,** 2013–2022.

Phelps, P. E., and Vaughn, J. E. (1995). Commissural fibers may guide cholinergic neuronal migration in developing rat cervical spinal cord. *J. Comp. Neurol.* **355,** 38–50.

Pinto-Lord, M. C., Evrard, P., and Caviness, Jr., V. S. (1982). Obstructed neuronal migration along radial glial fibers in the neocortex of the *reeler* mouse: A Golgi-EM analysis. *Brain Res.* **4,** 379–393.

Rice, D. S., and Curran, T. (1999). Mutant mice with scrambled brains: Understanding the signaling pathways that control cell positioning in the CNS. *Genes Dev.* **13,** 2758–2773.

Rice, D. S., and Curran, T. (2001). Role of the Reelin signaling pathway in central nervous system development. *Annu. Rev. Neurosci.* **24,** 1005–1039.

Rice, D. S., Sheldon, M., D'Arcangelo, G., Nakajima, K., Goldowitz, D., and Curran, T. (1998). *Disabled-1* acts downstream of *Reelin* in a signaling pathway that controls laminar organization in the mammalian brain. *Development* **125,** 3719–3729.

Schiffmann, S. N., Bernier, B., and Goffinet, A. M. (1997). Reelin mRNA expression during mouse brain development. *Eur. J. Neurosci.* **9,** 1055–1071.

Sheldon, M., Rice, D. S., D'Arcangelo, G., Yoneshima, H., Nakajima, K., Mikoshiba, K., Howell, B. W., Cooper, J. A., Goldowitz, D., and Curran, T. (1997). *Scrambler* and *yotari* disrupt the *disabled* gene and produce a *reeler*-like phenotype in mice. *Nature* **389,** 730–733.

Shu, S., Ju, G., and Fan, L. (1988). The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. *Neurosci. Lett.* **85,** 169–171.

Spike, R. C., Todd, A. J., and Johnston, H. M. (1993). Coexistence of NADPH diaphorase with GABA, glycine, and acetylcholine in rat spinal cord. *J. Comp. Neurol.* **335,** 320–333.

Steindler, D. A., and Colwell, S. A. (1976). Reeler mutant mouse: Maintenance of appropriate and reciprocal connections in the cerebral cortex and thalamus. *Brain Res.* **105,** 386–393.

Strack, A. M., Sawyer, W. B., Marubio, L. M., and Loewy, A. D. (1988). Spinal origin of sympathetic preganglionic neurons in the rat. *Brain Res.* **455,** 187–191.

Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimpf, J., Hammer, R. E., Richardson, J. A., and Herz, J. (1999). Reeler/disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. *Cell* **97,** 689–701.

Valtschanoff, J. G., Weinberg, R. J., and Rustioni, A. (1992). NADPH-diaphorase in the spinal cord of rats. *J. Comp. Neurol.* **321,** 209–222.

Vizzard, M. A., Erdman, S. L., Erickson, V. L., Stewart, R. J., Roppolo, J. R., and de Groat, W. C. (1994). Localization of NADPH diaphorase in the lumbosacral spinal cord and dorsal root ganglia of the cat. *J. Comp. Neurol.* **339,** 62–75.

Wetts, R., Phelps, P. E., and Vaughn, J. E. (1995). Transient and continuous expression of NADPH diaphorase in different neuronal populations of developing rat spinal cord. *Dev. Dyn.* **202,** 215–228.

Wetts, R., and Vaughn, J. E. (1994). Choline acetyltransferase and NADPH diaphorase are co-expressed in rat spinal cord neurons. *Neuroscience* **63,** 1117–1124.

Yip, J. W., Yip, Y. P., Nakajima, K., and Capriotti, C. (2000). Reelin controls position of autonomic neurons in the spinal cord. *Proc. Natl. Acad. Sci. USA* **97,** 8612–8616.

Yoneshima, H., Nagata, E., Matsumoto, M., Yamada, M., Nakajima, K., Miyata, T., Ogawa, M., and Mikoshiba, K. (1997). A novel neurological mutant mouse, *yotari*, which exhibits *reeler*-like phenotype but expresses CR-50 antigen/Reelin. *Neurosci. Res.* **29,** 217–223.

Received for publication July 3, 2001
Revised November 30, 2001
Accepted December 27, 2001
Published online February 25, 2002