THE JOURNAL OF COMPARATIVE NEUROLOGY 468:165–178 (2004)

Developmental Distribution of Reelin-Positive Cells and Their Secreted Product in the Rodent Spinal Cord

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ABSTRACT

To date, only sympathetic and parasympathetic preganglionic neurons are known to migrate abnormally in *reeler* mutant spinal cord. Reelin, the large extracellular matrix protein absent in *reeler*, is found in wild-type neurons bordering both groups of preganglionic neurons. To understand better Reelin's function in the spinal cord, we studied its developmental expression in both mice and rats. A remarkable conservation was found in the spatiotemporal pattern of Reelin in both species. Numerous Reelin-expressing cells were found in the intermediate zone, except for regions containing somatic and autonomic motor neurons. A band of Reelin-positive cells filled the superficial dorsal horn, whereas only a few immunoreactive cells populated the deep dorsal horn and dorsal commissure. High levels of diffuse Reelin product were detected in the lateral marginal and ventral ventricular zones in both rodent species. This expression pattern was detected at all segmental spinal cord levels during embryonic development and remained detectable at lower levels throughout the first postnatal month. To discriminate between the cellular and secreted forms of Reelin, brefeldin A was used to block secretion in organotypic cultures. Such perturbations revealed that the high levels of secreted Reelin in the lateral marginal zone were derived from varicose axons of more medially located Reelin-positive cells. Thus, the laterally located secreted Reelin product may normally prevent the preganglionic neurons from migrating too far medially. Based on the strong evolutionary conservation of Reelin expression and its postnatal detection, Reelin may have other important functions in addition to its role in neuronal migration. J. Comp. Neurol. 468:165–178, 2004.

Indexing terms: ***reeler*; sympathetic preganglionic neurons; parasympathetic preganglionic neurons; cell migration; secretion**

Reelin, a large extracellular matrix type protein, has an important but not well understood role in neuronal migration and is mutated in the *reeler* mouse (D'Arcangelo et al., 1995, 1997). Currently, there are seven known mutations in mouse *reelin* (Andersen et al., 2002; Falconer, 1951; Flaherty et al., 1992; Miao et al., 1994; Royaux, 1997; Sweet, 1993; Takahara et al., 1996); Mutations mapped to the *reelin* gene also have been documented in rat and human (DeSilva et al., 1997; Hong et al., 2000; Kikkawa et al., 2003; Yokoi et al., 2000). Thus far, the *reelin* gene has been identified in all vertebrate species investigated, including zebrafish, hyla, turtle, lizard, cat, hedgehog, dolphin, chick, and human, but it has not yet been detected in invertebrate species (Bar et al., 2000; Bermier et al., 2000; Hong et al., 2000; Perez-Garcia et al., 2000). Reelin conservation appears in both the amino acid and the nucleotide sequences, with the mouse sequences being 94% and 87% identical, respectively, to that of human (DeSilva et

Grant sponsor: National Science Foundation; Grant number: IBN-9734550 (P.E.P.); Grant sponsor: Council on Research of the University of California, Los Angeles Academic Senate; Grant sponsor: Center for Academic and Research Excellence Scholars Summer Research Program (S.A.V.); Grant sponsor: Marc U Star Fellowship (S.A.V.).

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Received 25 June 2003; Revised 18 August 2003; Accepted 20 August 2003

DOI 10.1002/cne.10946

Published online the week of November 17, 2003 in Wiley InterScience (www.interscience.wiley.com).

al., 1997). In addition, expression patterns in the developing cerebral cortex seem well conserved, insofar as Reelin is found in marginal zone cells in a wide variety of species. Thus Reelin was proposed to play a key role in mammalian cortical evolution (Bar et al., 2000).

The mouse mutants *scrambler* and *yotari* both exhibit *reeler*-like phenotypes, as do *disabled-1* (*dab1*) knockouts and the very-low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2) double knockouts (D'Arcangelo et al., 1999; Howell et al., 1997; Trommsdorff et al., 1999). Together, these findings have elucidated the first part of the Reelin-signaling pathway. Reelin is known to bind to the extracellular domains of both VLDLR and ApoER2, thereby activating a tyrosine kinase and phosphorylating Dab1 (Rice and Curran, 2001; Trommsdorff et al., 1999).

The defects in the migration and final positioning of spinal cord neurons identified to date are moderate (Phelps et al., 2002) compared with the severe migratory errors found in the cerebral cortex (Caviness, 1982; Goffinet, 1984). The sympathetic and parasympathetic preganglionic neurons within the *reeler* spinal cord sustain migratory errors in only the last phase of their complex migration (Phelps et al., 2002; Yip et al., 2000, 2003). Their migratory errors differ from those of cerebral cortical cells in that the preganglionic neurons migrate past their normal positions in the spinal cord, and the majority of cortical cells are unable to complete their normal migration. In addition, many neurons in the *reeler* spinal cord do migrate correctly (Phelps et al., 2002) in contrast to the few cerebral cortical neurons that achieve their normal position (Caviness and Rakic, 1978; Lambert de Rouvrout and Goffinet, 1998; Rice and Curran, 1999; Terashima et al., 1983). Thus, the spinal cord may be a simpler model than the cerebral cortex for elucidating the different functions of Reelin (Phelps et al., 2002; Yip et al., 2000).

Taking advantage of the conservation of Reelin across species, we first examined the spatial and temporal expression of Reelin in embryonic and early postnatal mouse and rat spinal cords. The distribution of Reelin was remarkably similar between these two rodents. Specifically, novel Reelin-secreting cells were present in the developing superficial dorsal horn and the dorsal commissure of both species. In addition, diffuse Reelin was detected in the lateral marginal zone and ventral ventricular zone. Finally, to improve the cellular resolution of Reelin-labeled cells, we blocked protein secretion in slice cultures. We were able in these experiments to differentiate clearly the cellular and secreted forms of Reelin and to characterize many features of the Reelin-labeled neurons.

MATERIALS AND METHODS

Animals and tissue preparation

The *reeler* mice (B6C3Fe-*a/a-Reln*^{rl}; Jackson Laboratory, Bar Harbor, ME) and Sprague-Dawley rats (Charles River, Wilmington, MA) were obtained from breeding colonies maintained at UCLA. After overnight breeding, vaginal plugs or smears were used to confirm insemination; and this day was recorded as embryonic day 0.5 (E0.5) in mice and E0 in rats according to standard conventions. Reelin expression was studied during the period of sympathetic and parasympathetic preganglionic neuron migration (mice, E11.5–14.5; rats, E13–16), at late embryonic ages (mice, E17.5; rats, E19) and at postnatal ages from birth to P28. Pregnant and postnatal mice were anesthetized with avertin (0.3 ml/10 g body weight) and pregnant rats with ketamine (90 mg/kg) and xylazine (20 mg/ml) before cesarean delivery of the embryos. Postnatal rat pups were anesthetized with 18% chlorohydrate (0.01 ml/g). The youngest embryos were immersed in fixative; older embryonic and postnatal animals underwent intracardiac perfusion with 4% paraformaldehyde in Millonig's phosphate buffer (pH 7.4). Animals were postfixed for 4–12 hours, washed, cryoprotected in 30% sucrose, embedded, and stored at −80°C. Forty-micrometer-thick sections were cut with a cryostat and stored in buffer with azide. Sections from embryos were mounted on slides and surrounded with silicone caulking to create incubation wells; postnatal sections were processed freely floating.

Immunocytochemical and histochemical procedures

The diaphorase histochemical procedure used to identify sympathetic and parasympathetic preganglionic neurons was identical for both species. After mounting, sections were exposed to a presoak of 0.12 M Millonig's buffer (pH 7.4) with 0.8–1% Triton for 20 minutes. Then, sections were placed in a solution containing 0.2–1% Triton, β-nicotinamide adenine dinucleotide phosphate (NADPH; 0.025–0.15 mg/ml; Sigma, St. Louis, MO) and nitro blue tetrazolium (NBT; 0.01–0.06 mg/ml; Sigma), with NADPH and NBT used at a ratio of 5:2. Slides remained in the dark overnight at room temperature. After repeated washing, sections were doubly labeled for Reelin, with the detergent-presoak step omitted.

The procedures to localize Reelin in mouse sections were identical to those reported with use of the InnoGenex Mouse-on-Mouse kit (Phelps et al., 2002). To visualize Reelin in rat sections, standard avidin-biotin immunolabeling techniques were used. Initially, sections were washed with 0.1 M Tris buffer containing 1.4% NaCl and 0.1% bovine serum albumin (TBS; pH 7.4), followed by a 30-minute presoak in 0.3% H_2O_2 and 0.1% sodium azide. Sections were placed into a 0.8% Triton presoak for 15 minutes, followed by blocking in 1.5% normal horse serum and 0.1% Triton for 1 hour. Sections were incubated in sequential avidin and biotin blocking steps (diluted 1:1; Vector Laboratories, Burlingame, CA), before being placed into monoclonal anti-Reelin antibody overnight (G10 diluted 1:10,000; gift of Drs. Tom Curran and Susan Magdaleno; de Bergeyck et al., 1998). A control monoclonal antibody to a chick erythrocyte antigen (Miller et al., 1982) was substituted for Reelin in the rat protocol and resulted in the absence of labeling (data not shown).

After rinsing, sections were incubated with rat adsorbed biotinylated anti-mouse IgG (diluted 1:200 in TBS plus 0.1% Triton) for 1 hour. After a buffer rinse, sections were incubated an additional 1 hour in avidin-biotin complex (diluted 1:125), followed by a 10-minute rinse in 0.1 M acetate buffer before being developed with diaminobenzidine (DAB) intensified with Ni-glucose oxidase and rinsed in acetate buffer (Shu et al., 1988). For counterstaining, embryonic sections were air dried and then briefly immersed in buffered cresylecht violet (Cell Point Scientific) before dehydration. Free-floating sections were mounted on slides before dehydration and coverslipping. Sections

that contained diaphorase reaction product were dehydrated with acetone.

Culture procedures

Embryonic day 16 rat spinal columns were dissected, embedded in agarose (type IX ultralow gelling temperature; Sigma), and cooled on ice before sectioning with a Vibroslicer (Barber et al., 1993; Phelps et al., 1996). Slices 300 μm thick of cervical and thoracic spinal cord and surrounding tissues were cut, transferred to collagen-coated coverslips, and placed in flat-bottom culture tubes (Robertson et al., 1989). Cultures were grown in 0.5 ml of defined EOL culture medium (Annis et al., 1990) containing 10 ng nerve growth factor (NGF) and remained stationary for 6–7 hours before being placed on a roller drum in a 5% CO_2 incubator. On the following day, cultures were divided into control and brefeldin A-treated groups. Brefeldin A specifically and reversibly blocks protein transport from the endoplasmic reticulum to the Golgi apparatus in many cell types (Lippincott-Schwartz et al., 1989; Onci et al., 1991). Brefeldin A (5 μg/ml; Epicenter) was added to fresh media for 6 hours while the control group received media only. All cultures were fixed with 4% paraformaldehyde for 2 hr at 4°C and rinsed in buffer. Cultures were infiltrated with sucrose (5–20%), followed by a mixture of 7.5% gelatin/20% sucrose at 37°C, then removed from the coverslips, embedded in warm gelatin/sucrose mixture, and frozen in cold isopentane (Phelps et al., 1996; Stern, 1993). After sectioning, cultures were mounted on slides and processed for Reelin immunocytochemistry using either the G10 (de Bergeyck et al., 1998) or the CR-50 antibody (1:4,000; gift of Drs. Tom Curran and Susan Magdaleno; Ogawa et al., 1995) and the immunocytochemical procedure described above. No differences were detected in the pattern of Reelin distribution between the two monoclonal antibodies. Photomicrographs were taken with an Olympus AX 70 microscope and either an Olympus analog camera (Figs. 1–4, 6, 7) or a Zeiss AxioCam digital camera (Fig. 5).

RESULTS

Positioning of sympathetic and parasympathetic preganglionic neurons relative to the Reelin-secreting cells is similar in mice and rats

Sympathetic (SPN) and parasympathetic (PPN) preganglionic neurons are known to migrate incorrectly in *reeler* mice (Phelps et al., 2002; Yip et al., 2000). By E14.5, wild-type SPNs are correctly positioned, with the majority of cells found laterally in the intermediolateral (IML) nucleus and the remainder situated more medially in the central autonomic (CA) nucleus or between these regions (Fig. 1a). In contrast, most of the *reeler* SPNs reside medially (Fig. 1c). On E14.5, PPNs in wild-type mice are located laterally in the intermediolateral sacral (ILS) nucleus in a well-ordered group (Fig. 1b), whereas they are dispersed across the intermediate zone in *reeler* mutants (Fig. 1d).

Reelin-secreting cells were positioned along the dorsal and medial borders of wild-type mouse SPNs (Fig. 1a) and PPNs (Fig. 1b), a location consistent with the interpretation that Reelin might specifically inhibit the medial migration of these preganglionic neurons along radial glia (Phelps et al., 2002). Additionally, the superficial dorsal horn contained Reelin-secreting cells (Fig. 1a,b, thin arrows), and a portion of the ventral ventricular zone (vz) was immunolabeled. Specific Reelin immunoreactivity was not detected in sections from *reeler* mutants (Fig. 1c,d). However, both wild-type and *reeler* sections were surrounded by nonspecifically stained meninges and peripheral tissue despite the use of a mouse-on-mouse kit (Fig. 1a–d). Penetrating blood vessels also were detected in mutants (Fig. 1c).

To confirm that the relationship between mouse preganglionic neurons and Reelin-positive cells was conserved in the rat spinal cord, thoracic and sacral sections of an equivalently aged rat (E16) were doubly labeled for diaphorase and Reelin. Rat SPNs and PPNs (Fig. 2a,b) were found in positions similar to those in wild-type mouse tissue. In addition, Reelin-expressing cells in rat were detected along the dorsal and medial borders of the preganglionic neurons in a pattern similar to that seen in mouse (Figs. 1, 2). Absent in the rat sections, however, was the nonspecific staining associated with the meninges, dorsal root ganglia, and blood vessels observed in mouse spinal cord (Fig. 1a–d).

Reelin is detected in mouse and rat thoracic spinal cord from early embryonic ages through the first postnatal week

Reelin immunostaining was detected in mouse thoracic spinal cord as early as E11.5 (Fig. 3a) and in rat by E13 (Fig. 3d). The pattern of early Reelin expression was similar between the two species but not identical. Numerous Reelin-positive cells filled the intermediate zone from the level of the unstained dorsal root entry zone to the dorsal boundary of the primary motor column. In addition, several Reelin-positive cells were found medial to the immunonegative motor column (asterisks, Fig. 3a,d). The adjacent marginal zone contained immunoreactive processes that appeared to be derived from axons of more medially located somata (Fig. 3c). More ventrally, a circular group of Reelin-positive cells was detected, primarily in rat embryos (Fig. 3d, large arrowhead) with fewer such cells found in mice (Fig. 3a, large arrowhead). Several immunoreactive axons were detected in both mouse and rat ventral commissure but only at the earliest ages (Fig. 3a,c,d). Generally the ventricular zones were unlabeled, with two exceptions: 1) a group of immunoreactive neurons in the ventral ventricular zone (Fig. 3a,d, small arrowheads) and 2) immunoreactive cells that were associated with the E13 rat roof plate (Fig. 3d,f) but were not distinguishable in the mouse (Fig. 3a).

One day later, Reelin-labeled cells in both rodents had dispersed within the spinal cord. The areas occupied by unlabeled sympathetic preganglionic and somatic motor neurons were separated by immunoreactive cells that projected Reelin-labeled processes into the lateral marginal zone (Fig. 3b,e). The midline of the ventral ventricular zone expressed Reelin in E14 rat thoracic cord (Fig. 3e) and occasionally in E12.5 mouse sections. Again, a Reelin-positive area was associated with the E14 rat roof plate but was not distinguishable in the E12.5 mouse spinal cord (Fig. 3b,e,g).

Developmentally, Reelin expression appeared most intense between E14.5 and E17.5 in mouse (Figs. 1a, 4a) and between E16 and E19 in rat (Figs. 2a, 4c) thoracic spinal

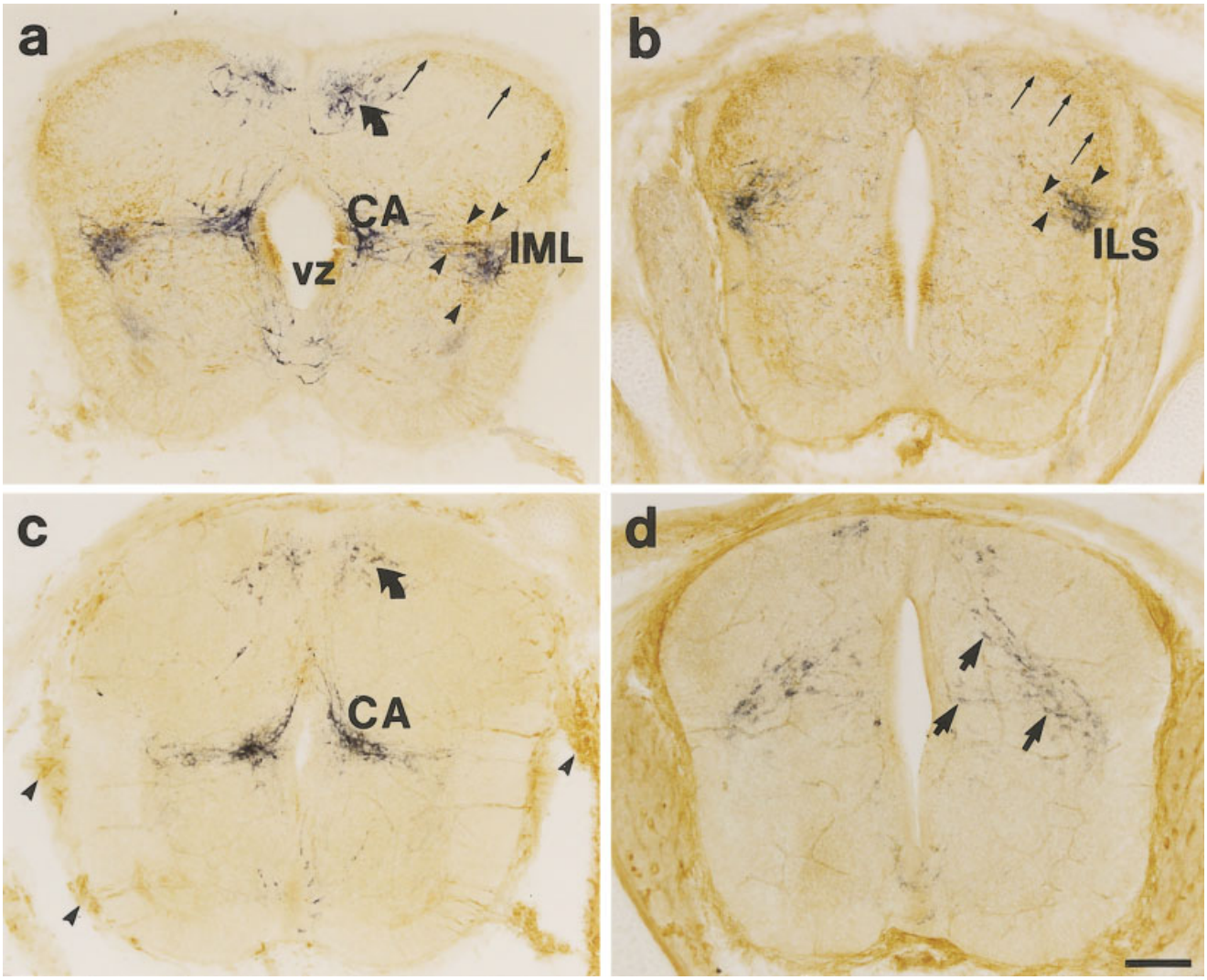

Fig. 1. NADPH-diaphorase histochemistry (blue-purple product) identifies sympathetic (a,c) and parasympathetic (b,d) preganglionic neurons in sections of E14.5 wild-type (a,b) and *reeler* (c,d) mouse spinal cord. Reelin-immunoreactive cells (amber-brown product) also were identified. **a:** Sympathetic preganglionic neurons are found between the intermediolateral (IML) and the central autonomic (CA) nuclei in thoracic spinal cord. Reelin-positive cells are located dorsal and medial to the IML neurons, in the superficial lamina of the dorsal horn (thin arrows) and in the ventral ventricular zone (vz). Diaphorase-positive interneurons are detected in the ventral midline and in the dorsal horn (thick arrow). **b:** In the sacral spinal cord, parasympathetic preganglionic neurons are positioned in the intermediolateral sacral (ILS) nucleus with Reelin-positive cells (arrowheads) located nearby. Reelin-secreting cells are found in the dorsal horn (thin arrows) and the ventral ventricular zone. **c:** The *reeler* sympathetic preganglionic neurons are concentrated medially in the central autonomic nucleus (CA). Bilateral dorsal clusters of diaphorase-labeled cells (arrow) are detected. As expected, no Reelin is detected in *reeler* spinal cord, but there is nonspecific staining in surrounding tissues (arrowheads). **d:** The *reeler* parasympathetic preganglionic neurons (arrows) are dispersed across the mediolateral extent of the spinal cord. Nonspecific staining is present in the peripheral tissues. Scale bar = 100 μm.

cord. At these ages, numerous Reelin-secreting cells were detected in the intermediate gray and superficial dorsal horn, whereas smaller groups of cells populated the dorsal commissure and the ventromedial grey matter. In addition, part of the inner edge of the ventral ventricular zone was strongly immunoreactive in E14.5 mouse and E16 rat sections (Figs. 1a, 2a). Diffuse Reelin was detected in some of the embryonic marginal zones, areas known to contain primarily axons rather than cell bodies. Strong Reelin labeling overlaid the lateral and sometimes the ventral marginal zones, but the dorsal marginal zone remained Reelin negative (Fig. 4a,b). During the first postnatal month, this diffuse Reelin product was maintained in the ventral and lateral funiculi (Fig. 4c,d). At a higher magnification, Reelin-immunoreactive processes emerged from Reelin-labeled somata and extended toward the lateral marginal zone (Fig. 4e,f, insets).

To determine the laminar location of the Reelin-positive dorsal horn cells, both mouse and rat sections first were labeled with Reelin and then were counterstained. In Reelin-only sections, prominent Reelin-labeled cells were detected along the outer border of the dorsal horn (Fig.

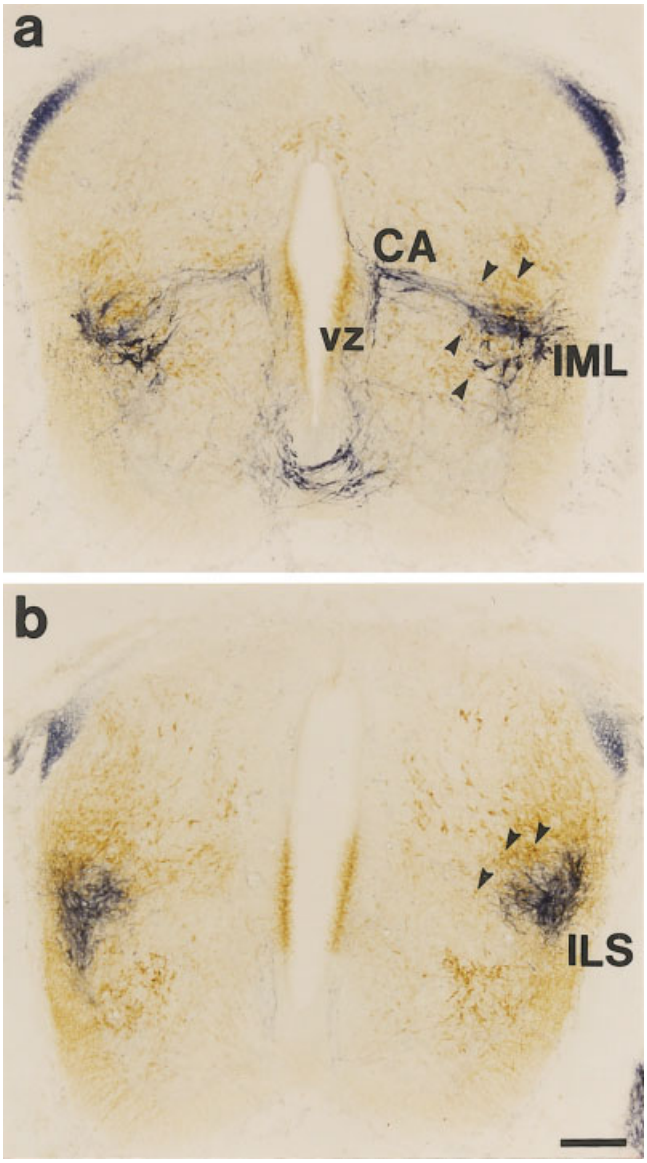

Fig. 2. Thoracic (a) and sacral (b) spinal cord sections from E16 wild-type rat were processed successively for diaphorase histochemistry (blue-purple product) and Reelin immunocytochemistry (amber-brown product). Reelin is found within part of the ventral ventricular zone (vz) at both levels. **a:** Most sympathetic preganglionic neurons in thoracic spinal cord are located in the intermediolateral nucleus (IML), with a small number found medially in the central autonomic nucleus (CA). Reelin-positive cells (arrowheads) are detected dorsal and medial to the IML. **b:** Diaphorase-positive parasympathetic preganglionic neurons are located in the intermediolateral sacral nucleus (ILS) and are bordered medially and dorsally by Reelin-positive cells (arrowheads). Diffuse Reelin product is found in the lateral marginal zone, near the ILS. Scale bar = 100 μm.

5a,c). Large Reelin-positive cells with mediolaterally oriented processes were observed primarily within lamina I or occasionally in the adjacent white matter (Fig. 5a,b) and bore a strong morphological resemblance to Waldeyer's cells (Scheibel and Scheibel, 1968). Smaller Reelin-positive cells and overlying diffuse Reelin product were found in both lamina I and lamina II (Fig. 5a–d). At early ages, more Reelin-labeled cells were apparent in the lateral than in the medial dorsal horn, but, at older embryonic and postnatal ages, Reelin-positive neurons appeared evenly distributed across the superficial dorsal horn (Figs. 4a–d, 5a–d). Small numbers of Reelin-positive cells were scattered in the deep dorsal horn, an area that otherwise was Reelin negative (Figs. 4, 5a–d). Immunoreactive dorsal horn cells were maintained in wild-type mice and rats (Fig. 4c–f) and were detected as late as P21–28 (data not shown).

Reelin is expressed at all levels of the developing spinal cord

Sections from cervical, thoracic, lumbar, and sacral rat spinal cord were processed together for Reelin immunoreactivity to determine whether Reelin was distributed at all segmental levels or just in areas containing autonomic motor neurons. A dense pattern of Reelin-positive cells was present within the intermediate gray matter and ventricular zone at all E16 spinal cord levels (Fig. 6a–d). Other Reelin-positive cells, such as those in the dorsal horn or dorsal commissure, were present in a rostral-to-caudal developmental gradient. When incubated in the same immunoreagents, cervical and thoracic sections showed robustly stained Reelin-containing cells in the superficial dorsal horn (Fig. 6a,b), whereas Reelin expression was barely detected in the lateral dorsal horn of the lumbar sections (Fig. 6c) and was not yet apparent at sacral levels (Fig. 6d). In addition, distinct Reelin-positive cells were present within the dorsal commissure near the roof plate of all spinal levels, except for the sacral (Fig. 6a–d). Contrary to the abundance of Reelin-positive cells in the dorsal commissure, the ventral commissure near the floor plate was devoid of Reelin staining by E16 (Fig. 6e,f).

Histotypic expression of Reelin in slice cultures

To identify and manipulate Reelin-secreting cells better in the developing spinal cord, we utilized organotypic slice cultures (Barber et al., 1993; Phelps et al., 1996). Initially, we sought to determine whether the pattern of Reelin expression in slice cultures would mimic the patterns from similarly aged embryos. Slices from E16 rat were cultured for 1 day in vitro and then examined with Reelin immunocytochemistry (Fig. 7a). Several Reelin-positive cells in slices were found in the intermediate gray matter and in the dorsal commissure, but they were generally excluded from areas containing somatic and autonomic motor neurons. Distinct Reelin-labeled cells were difficult to detect in the superficial dorsal horn, but a laminated pattern of reaction product was present. Diffuse Reelin product was concentrated over the lateral marginal zone but was not detected in the dorsal marginal zone (Fig. 7a). Thus, the pattern of Reelin localization in vitro closely resembled that observed in embryos.

Blocking secretion enhances Reelin-positive cells and abolishes diffuse Reelin product

To visualize better the cellular origin of Reelin in the spinal cord, protein secretion was blocked in slice cultures. Brefeldin A is known to cause protein accumulation in the endoplasmic reticulum (Lippincott-Schwartz et al., 1990; Onci et al., 1991) and in our model greatly enhanced the

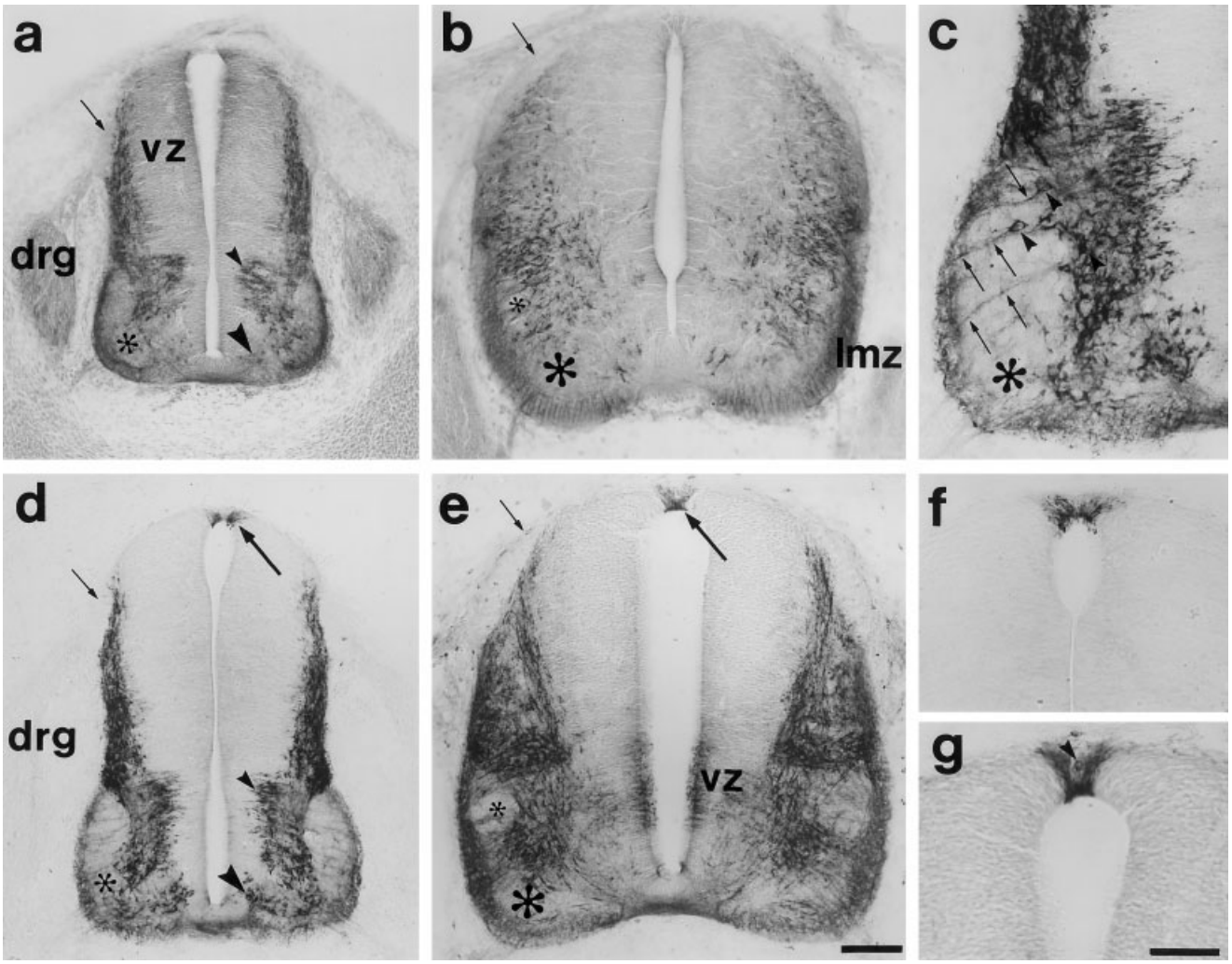

Fig. 3. Early Reelin expression in wild-type mouse (a,b) and the equivalently aged rat (c–g) thoracic spinal cords. Although the pattern of Reelin staining in mouse (a,b) is similar to that of the rat (d,e), background levels in the ventricular zone (vz), dorsal root ganglion (drg), and dorsal root entry zone (small arrow) are consistently higher in the mouse sections. **a:** An E11.5 mouse section illustrates immunoreactive Reelin-positive cells along the border of the ventricular zone (vz) from the dorsal root entry zone (small arrow) to the ventral part of the unlabeled primary motor column (asterisk). Some Reelin-labeled cells still occupy the ventricular zone (small arrowhead) and a few further ventral cells (large arrowhead) are detected. **b:** An E12.5 mouse section shows numerous Reelin-immunoreactive cells in the intermediate and ventral spinal cord and dense Reelin product in the lateral marginal zones (lmz). Immunonegative areas are detected in the intermediolateral region (small asterisk) and in the pools of somatic motor neurons (large asterisk). **c:** Enlargement of the ventral spinal cord from an E13 rat illustrates the Reelin-positive cells (arrowheads) that lie ventral and medial to the primary motor pool (asterisk). A few bundles of immunoreactive axons (arrows) project into the lateral marginal zone. **d:** An E13 rat section has a pattern of Reelin labeling similar to the E11.5 mouse (a). In addition, the area next to the roof plate (large arrow) shows Reelin expression in the rat sections. A small group of Reelin-positive cells is located ventrally (large arrowhead). **e:** An E14 rat section has numerous Reelin-positive cells in the intermediate and ventral spinal cord in a pattern similar to that found in the E12.5 mouse (b). Additionally, a distinct Reelin-positive region of the ventral ventricular zone (vz) and the area adjacent to the roof plate (large arrow) are detected. **f:** Enlargement of an E13 rat roof plate shows a densely Reelin-positive region on both sides of the midline. **g:** Enlargement of an E14 rat roof plate shows a cellular profile (arrowhead) that may be migrating away from the immunoreactive area. Scale bar = 100 μm in e (applies to a,b,d,e); 60 μm in g (applies to c,f,g).

Reelin accumulation in cell bodies and their processes (Fig. 7b–e). Control slices were exposed to fresh media only and processed along with brefeldin-treated slices for comparison. The most striking difference between the control and brefeldin-treated slices was that the diffuse Reelin product in the ventral and lateral marginal zones of control slices (Fig. 7a) was greatly reduced or absent in the brefeldin-treated slices (Fig. 7b,c). A second difference was that processes originating from Reelin-positive cells were found in the lateral and ventral marginal zones (Fig. 7c). Because brefeldin treatment caused the concomitant loss of diffuse Reelin product and the appearance of Reelin-filled processes, we concluded that the Reelin overlying the ventral and lateral marginal zones was most likely secreted by the immunoreactive processes found in these regions.

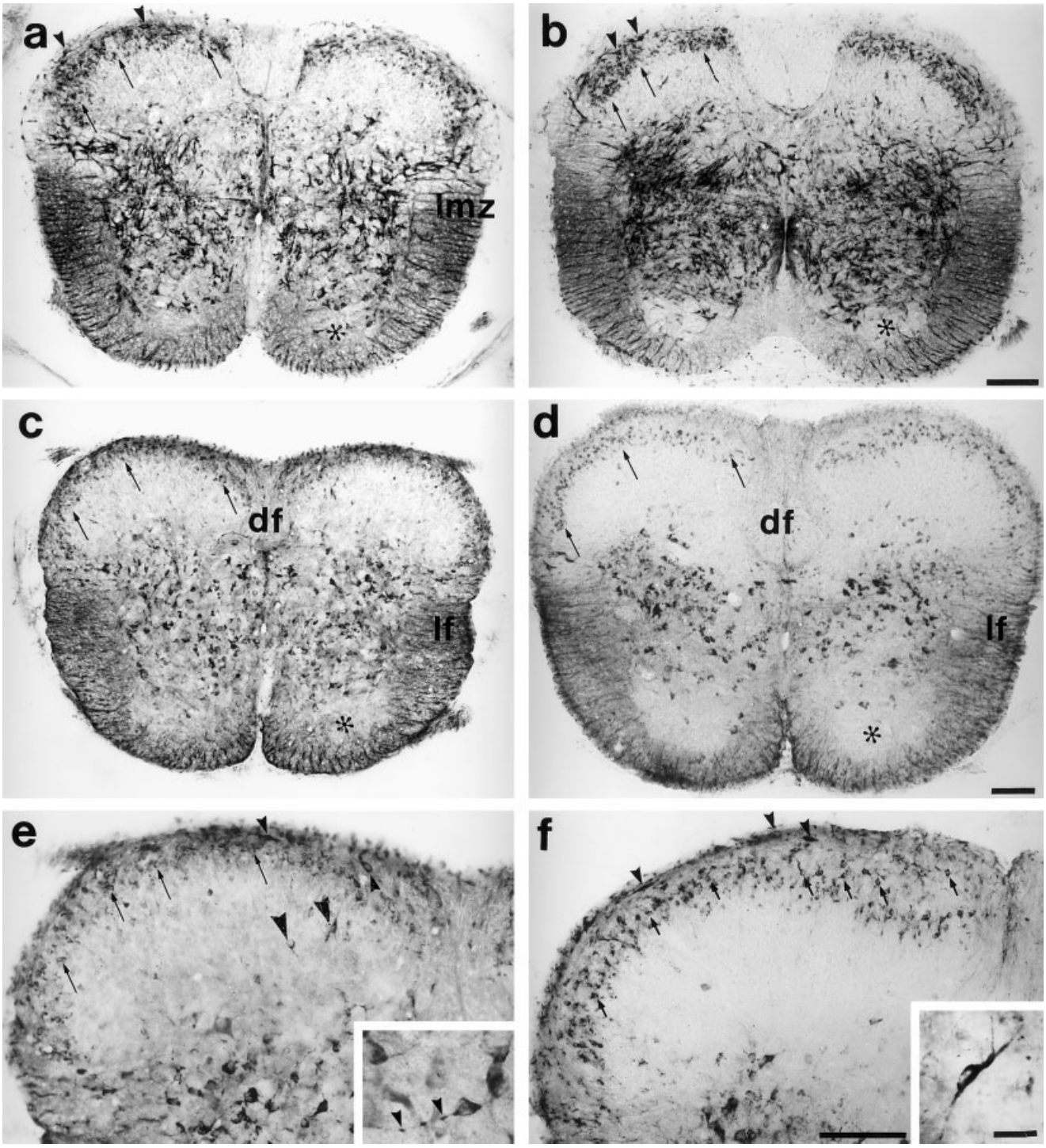

Fig. 4. Comparisons of mouse (a,c,e) and rat (b,d,f) Reelin expression in thoracic spinal cord at late embryonic (a,b) and early postnatal (c–f) ages. Somatic motor neuron pools (asterisks in a–d) are free of Reelin. **a,b:** E17.5 mouse and E19 rat sections have numerous Reelin-positive cells in the intermediate gray matter accompanied by dense immunoreactivity in the adjacent lateral marginal zone (lmz). The superficial dorsal horn (arrows) contains numerous small Reelin cells as well as a few larger cells (arrowheads) in lamina I. **c,d:** The overall Reelin immunoreactivity in P7 mouse and rat sections is less than that detected in embryonic ages. Reelin-positive cells remain most numerous in the intermediate gray and are detected in the superficial dorsal horn (arrows). Reelin product is concentrated in the lateral (lf) but not the dorsal (df) funiculus. **e,f:** Enlargements of P7 mouse and rat dorsal horns illustrate that most Reelin-positive cells have small somata (arrows), except for a few larger cells (small arrowheads) in lamina I. A few immunoreactive cells (large arrowheads) are detected in the deep dorsal horn. **Inset** in e: Enlargement of several Reelin cells from the intermediate gray matter. One cell has a varicose process (arrowheads) projecting laterally. **Inset in f:** An immunoreactive neuron in the intermediate gray has labeled processes. Scale bar = 100 μm in b (applies to a,b), d (applies to c,d), f (applies to e,f); 20 μm in insets.

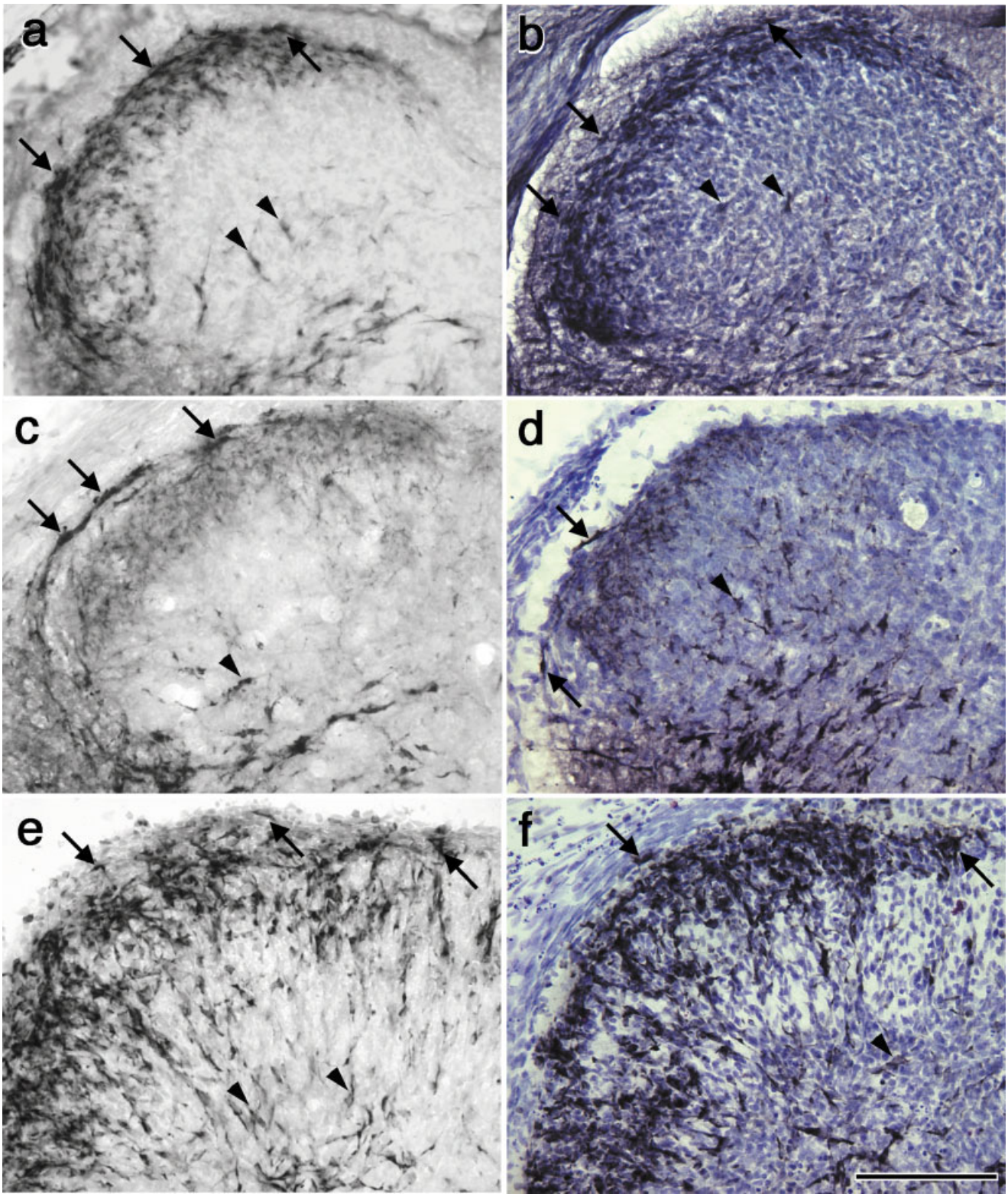

Fig. 5. Comparison of Reelin expression in the dorsal horn of an E14.5 wild-type mouse (a,b), an E16 rat (c,d), and an E16 rat slice culture grown 1 day in vitro (e,f). Pairs of sections were prepared with Reelin immunocytochemistry only (a,c,e) or were subsequently counterstained (b,d,f). All images are oriented with dorsal toward the top and medial to the right. **a,b:** Reelin-labeled cells outline the border of the E14.5 mouse superficial dorsal horn, with more cells found laterally than medially. Large Reelin-positive cells with long dendrites (arrows) are evident along the border of lamina I. Additional smaller immunoreactive cells populate lamina II as well. Only a few scattered Reelin-labeled cells are found within the deep dorsal horn (arrowheads). **c,d:** Large Reelin-labeled cells with thick projecting dendrites (arrows) are found at the border between the future white matter and lamina I of the E16 rat dorsal horn. Small immunoreactive cells reside in lamina II and occasionally cells are found in the deep dorsal horn (arrowheads). **e,f:** After Reelin secretion is blocked with brefeldin A, the diffuse product normally present in the superficial dorsal horn (c) is eliminated, and Reelin-positive cells are clearly visible. Large Reelin-labeled cells are present at the border of lamina I (arrows) and numerous smaller round Reelin-labeled cells are found within lamina II. A few Reelin-expressing cells also are found in the deep dorsal horn (arrowheads). Scale bar = 25 μm.

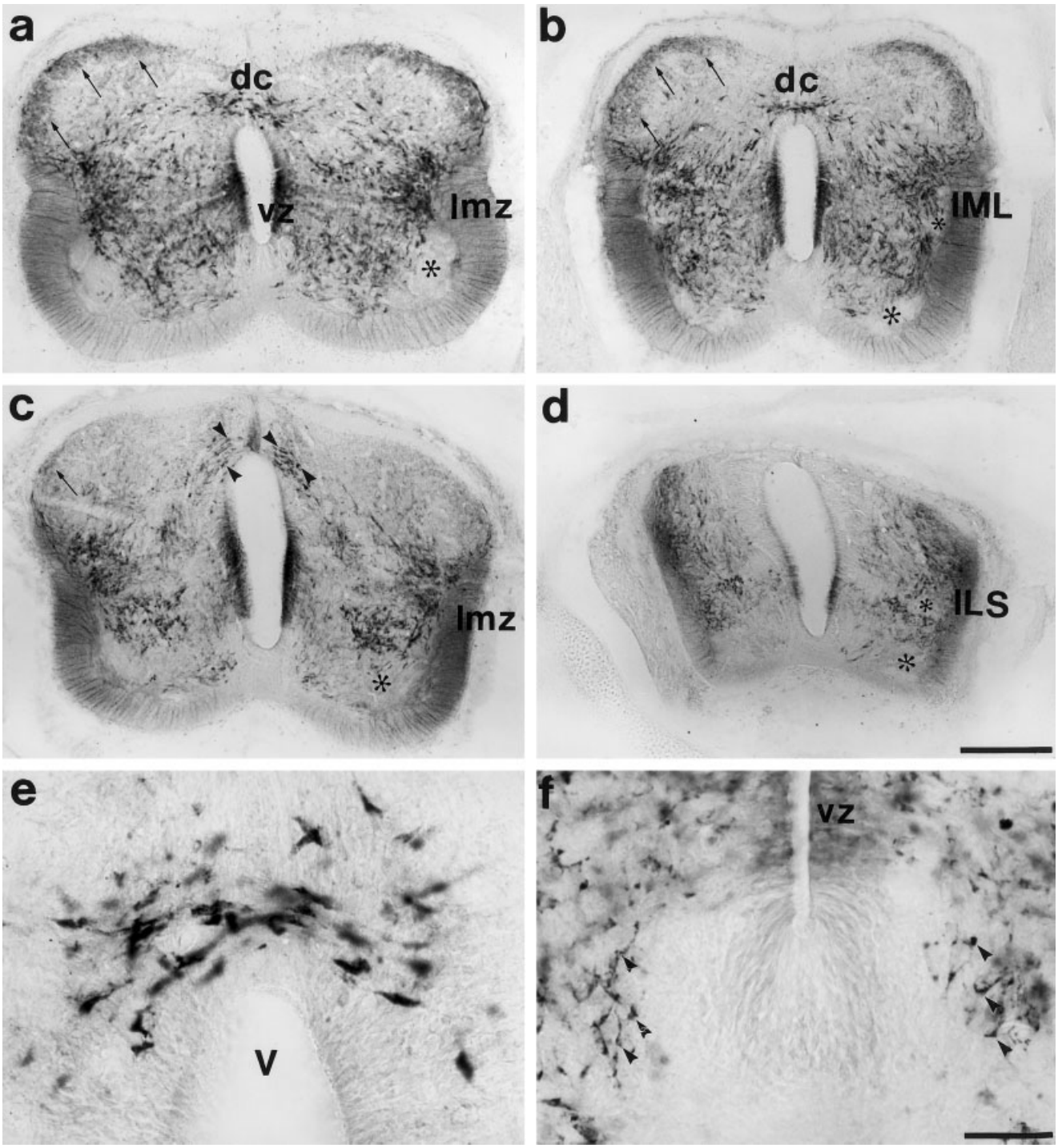

Fig. 6. Reelin is expressed in all segmental levels of a single E16 rat spinal cord. Reelin immunocytochemistry illustrated at cervical (a), thoracic (b), lumbar (c), and sacral (d) levels. The enlargement of the roof plate (e) and the floor plate (f) are from a single cervical section. Common to all levels are the numerous Reelin-positive cells in the intermediate gray, a discrete immunoreactive area of the ventricular zone (vz), and the diffuse Reelin product in the lateral marginal zone (lmz). Reelin displays a rostral-to-caudal developmental gradient. **a:** In this cervical section, Reelin-positive cells are excluded from the somatic motor neuron pools (asterisk), the ventral midline, and the deep layers of the dorsal horn. Reelin-positive cells are numerous in the ventromedial and intermediate gray matter. A band of Reelin immunoreactivity is detected in the superficial dorsal horn (arrows) and a group of large cells occupies the dorsal commissure (dc). **b:** The pattern of Reelin-positive cells in thoracic cord resembles that in cervical cord (a), with Reelin-labeled cells in the superficial dorsal horn (arrows) and dc. Areas containing immunonegative neurons are detected in the intermediolateral region (asterisk near IML) and in the pools of somatic motor neurons (asterisk). **c:** The band of Reelin in the superficial horn is not yet apparent at the lumbar level, except for some immunoreactivity (arrow) in the lateral region. Large Reelin cells (arrowheads) are not yet present in the dorsal commissure but are present on both sides of the midline. Somatic motor neuron areas (asterisk) are immunonegative. **d:** The pattern of Reelin expression in sacral spinal cord is the least mature of the levels illustrated. Very little Reelin is localized in the ventral ventricular zone or in the dorsal horn. The areas containing parasympathetic preganglionic (small asterisk near ILS) and somatic motor neurons (large asterisk) are immunonegative. **e:** Large Reelin-positive cells congregate in the dorsal commissural region, just dorsal to the midline of the ventricle (V). **f:** In this enlargement of the floor plate and ventral commissure, both regions are immunonegative. Reelin-positive processes are detected in the adjacent ventral spinal cord (arrowheads) and in the ventral vz. Scale bar = 200 μm in d (applies to a–d); 40 μm in f (applies to e,f).

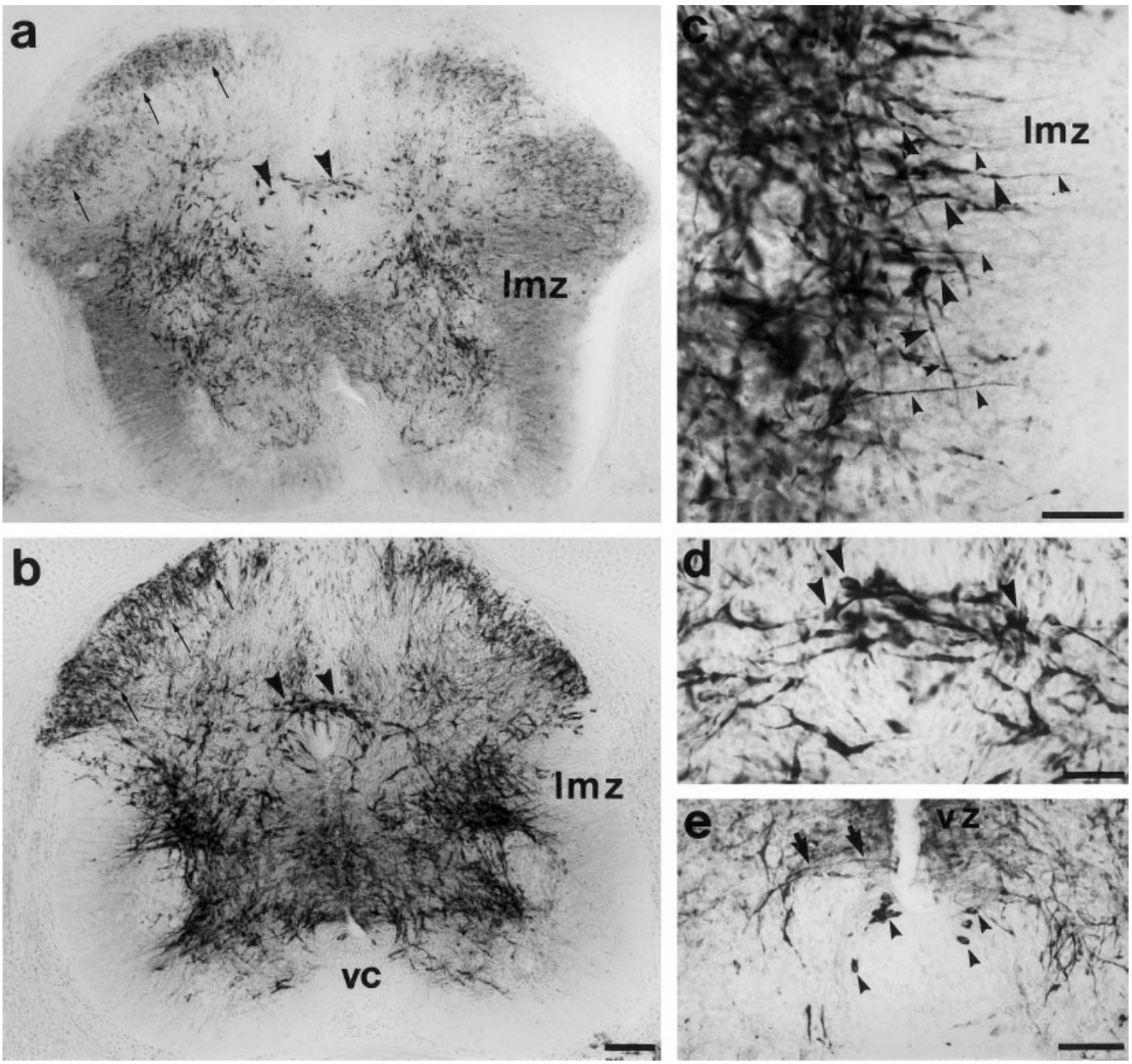

Fig. 7. Reelin expression in E16 rat slices grown for 1 day in vitro. Slices were grown in media without (a) or with (b–e) the addition of brefeldin A. All images are oriented with dorsal toward the top. **a:** A section of a slice shows Reelin localization in a pattern similar to that of E16 spinal cord. Numerous Reelin-positive cells are present in the intermediate gray, with fewer detected in the ventral and dorsal regions. Large Reelin-immunoreactive cells (arrowheads) occupy the dorsal commissure. Diffuse Reelin product was present in the superficial dorsal horn (arrows), the lateral marginal zone (lmz), and the central part of the spinal cord. **b:** A slice adjacent to that in a was exposed to brefeldin A. Note the lack of diffuse Reelin product in the lmz. Reelin-positive cell bodies and their processes in the superficial dorsal horn (arrows) and intermediate gray are intensely stained. Many Reelin-positive cells (arrowheads) are present in the dorsal commissure compared with very little immunoreactivity in the ventral commissure (vc). **c:** In a brefeldin-treated culture, Reelin-positive cell bodies in the intermediate gray matter project immunoreactive processes (small arrowheads) out into the lmz. Some of these Reelin-labeled processes display bulbous swellings (large arrowheads) and resemble axons. **d:** Enlargement of the dorsal commissure from a brefeldin-treated culture illustrates the large Reelin cells (arrowheads) and their processes. **e:** After brefeldin treatment, the floor plate and ventral commissure remain immunonegative, whereas a few Reelin-positive somata (arrowheads) appear to have exited the adjacent ventricular zone (vz). One labeled cell has a process (arrows) that is still attached to the lining of the ventricle. Scale bar = 100 μm in b (applies to a,b); 40 μm in c–e.

After brefeldin treatment, Reelin-labeled cells were distinct in the intermediate and ventral horns, in the dorsal commissure, and in a laminated pattern in the dorsal horn (Fig. 7b–d). Large Reelin-positive cell bodies were apparent along the border of lamina I in counterstained immunocytochemical sections (Fig. 5e,f). However, most Reelin-

positive cells in laminae I–II were small to medium in size. In addition, Reelin-positive cells were detected in the deep dorsal horn (Fig. 5e,f) and in the dorsal commissure (Fig. 7d). The diffuse Reelin product overlying the cells in the superficial dorsal horn (Fig. 7a) was replaced in brefeldin-treated slices with intensely immunoreactive somata and their processes (Fig. 7b).

In E16 embryos, the floor plate and the ventral commissure of slice cultures contained little to no Reelin, in contrast to the adjacent ventral ventricular zone (Fig. 6e,f). In brefeldin-treated slices, distinct Reelin-positive cells were detected in the ventral ventricular zone, and occasionally Reelin-labeled processes were still attached to the ventricular lining (Fig. 7e, arrowheads and arrows). These findings suggest that at least some of the Reelin product associated with the ventral ventricular zone was contained within postmitotic cells in the process of migrating out of the ventricular zone.

DISCUSSION

Previously Reelin was reported to surround the borders of the two populations of preganglionic neurons as well as the Islet-positive motoneurons (Carroll et al., 2001; Phelps et al., 2002; Yip et al., 2000). In the present study, we identified a similar pattern of Reelin-labeled cells within the superficial dorsal horn, the dorsal commissure, and the intermediate and ventral spinal cord in both mice and rats, demonstrating the conservation of Reelin across species. In addition, we determined that Reelin expression is detected throughout all segmental levels of the spinal cord. Furthermore, findings from in vitro perturbation experiments suggested that the diffuse Reelin product present in the lateral marginal zone was most likely secreted by varicose Reelin-containing axons emanating from more medially located immunoreactive cells. It is likely that the diffuse Reelin product concentrated in the lateral marginal zone functions to maintain most of the wild-type preganglionic neurons in the intermediate horn. Finally, the continued presence of Reelin in postnatal spinal cord implies that Reelin may function in other roles in addition to that of regulating cell migration.

Conservation of Reelin expression in rat and mouse spinal cord

In addition to the strong conservation of the *reeler* gene between species (Bar et al., 2000; Bermier et al., 2000; Perez-Garcia et al., 2000), the cell types that contain Reelin are known to share many similarities. For example, the marginal zone of the developing cerebral cortex and the molecular layer of the adult laminated cortex of mammals and reptiles both contain Reelin-expressing cells (Bar et al., 2000; Perez-Garcia et al., 2000). In addition, the spontaneous rat *Creeping* mutation was mapped to the *reeler* homolog and displayed a phenotype similar to that of the *reeler* mouse (Yokoi et al., 2000). Together these findings prompted us to compare the pattern of Reelin-secreting cells in the mouse spinal cord with those of the rat. Populations of novel Reelin-positive cells in the dorsal horn and dorsal commissure were virtually identical between these two rodents. Similarly, the pattern of diffuse Reelin expression detected in the ventricular and marginal zones was found in both species. Thus, the conservation of the overall pattern of Reelin-containing cells in the spinal cord, coupled with the experimental advantages of working with rat tissue, supports the rat as a good model for further studies of Reelin function.

The pattern of Reelin is common throughout the spinal cord and is maintained after birth

The Reelin expression pattern was common to all segmental levels examined despite the fact that the only migrational errors identified to date have been restricted to thoracic and sacral segments. The intensity of Reelin immunoreactivity was highest during embryonic development and then fell dramatically by early postnatal ages. However, neurons within the superficial dorsal horn and the intermediate spinal cord continued to express Reelin long after migration had ceased, findings suggesting that Reelin may fulfill another role postnatally.

Reelin-positive cells are found in the dorsal but not the ventral commissure

Prominent Reelin-labeled cells were positioned in the dorsal commissure as early as E16 in rat sections and slice cultures. This is 1 day earlier than when the first dorsal commissural axons project into and through the dorsal commissure (Orlino et al., 2000; Smith, 1983; Snow et al., 1990). Inhibitory molecules, such as keratan sulfate, initially may form a barrier at the roof plate that blocks axonal invasion into the region until these molecules are lost (Snow et al., 1990). The Reelin-labeled cells may be less sensitive to inhibitory molecules of the roof plate, in that they are able to occupy the dorsal commissure 1 day before primary sensory afferents (Smith, 1983) or L1- and GAD65-positive axons (Orlino et al., 2000) are detected in the dorsal commissure. Currently, little is known about the Reelin-positive dorsal commissural cells other than that they are large and easy to detect during late embryonic development. The relationship between the E13–14 Reelin-positive cells next to the rat roof plate and the immunoreactive cells found in the E16 dorsal horn is under investigation.

Except for a few immunoreactive axons in the early ventral commissure, the floor plate and ventral commissure generally were devoid of Reelin-labeled cells or processes. The absence of Reelin in the embryonic floor plate argues that Reelin does not function as a chemotrophic molecule during early spinal cord development, an interpretation consistent with the findings of Jossin and Goffinet (2001) showing that Reelin does not directly influence cortical axonal outgrowth.

Reelin-secreting cells of the superficial dorsal horn

Reelin expression has been detected within laminated areas of the CNS, including the cerebral cortex, olfactory bulb, cerebellum, and retina (for reviews see Caviness and Rakic, 1978; Lambert de Rouvrout and Goffinet, 1998; Rice and Curran, 2001). One of the best studied abnormalities associated with the *reeler* mutation is the disrupted lamination pattern of the cerebral cortex when the Cajal-Retzius cells fail to secrete Reelin (Lambert de Rouvrout and Goffinet, 1998; Rice and Curran, 1999). The effect that Reelin has on the migration and patterning of neurons throughout laminated CNS, coupled with the presence of a distinct band of Reelin-secreting cells in laminae I and II of the dorsal horn, implies that Reelin may play a role in

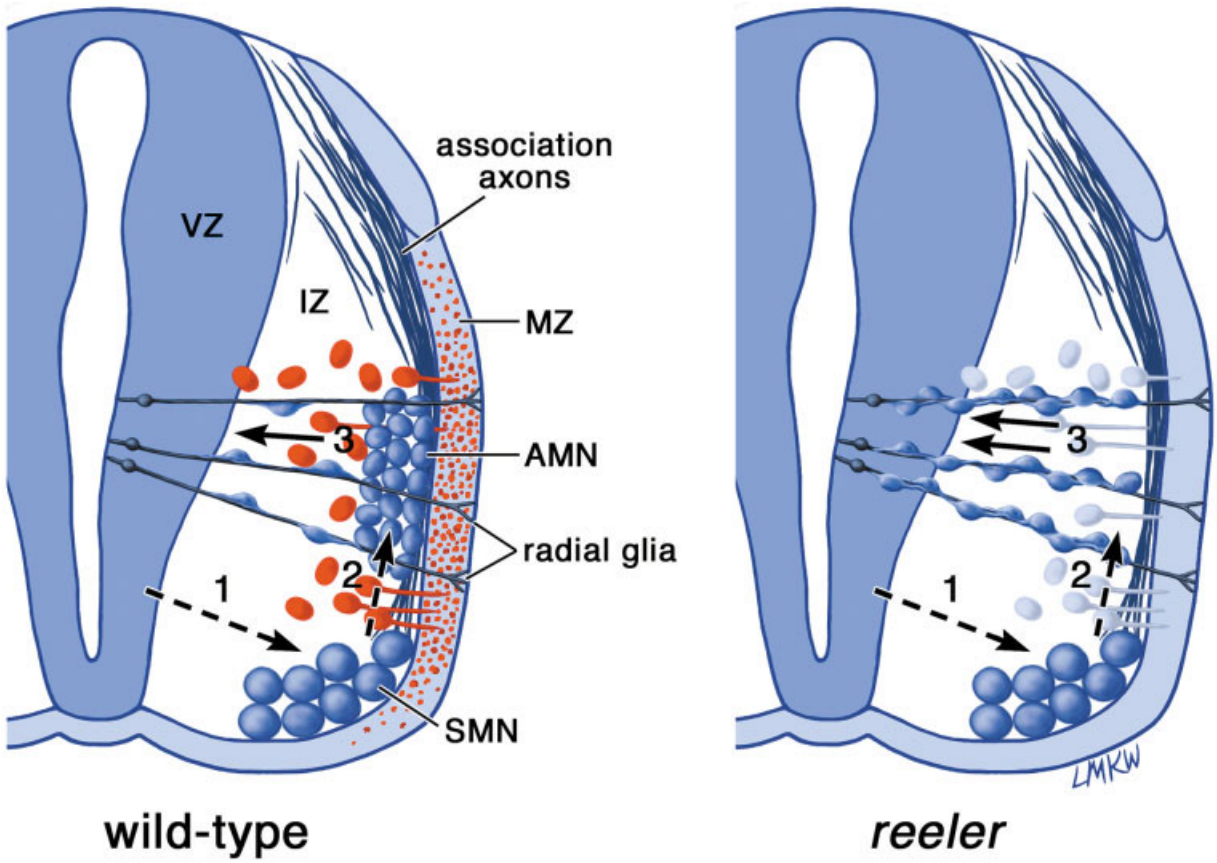

Fig. 8. Summary diagram comparing the migration of the autonomic motor neurons (AMN) in wild-type and *reeler* mice. During the first step (1), somatic and autonomic motor neurons migrate out to form the primary motor column. While the somatic motor neurons (SMN) remain ventrally, the AMNs migrate dorsally (2) near the association axons. Reelin acts on only the third (3) medial migration along radial glia in wild-type mice. Reelin-positive cells (red cells) project processes into the lateral marginal zone (mz), a major site of Reelin secretion (red dots). Reelin functions to maintain most of the AMNs in the intermediolateral horn. In *reeler* mice, the lack of Reelin protein allows most of the AMNs to migrate medially.

establishing the lamination pattern of the dorsal horn. Large Reelin-secreting cells were present in the outer edge of lamina I (present results), and they resembled the classical Waldeyer's cells in their size, location, and process orientation (Lima and Coimbra, 1983, 1986; Willis and Coggeshall, 1991). Altman and Bayer (1984) proposed that Waldeyer's cells may play a role in establishing the outer limits of the dorsal horn, and, if they do, one would predict that there may be defects in the *reeler* dorsal horn. To date, only the small groups of diaphorase-positive and cholinergic dorsal horn interneurons have been evaluated in *reeler* mutants and found to be relatively normal (Phelps et al., 2002). Reelin-secreting dorsal horn cells also are present at late postnatal ages and thus may be maintained for a function unrelated to neuronal migration.

Axonal secretion of Reelin in the presumptive lateral funiculus

A diffuse immunoreactive product was present within both mouse and rat lateral marginal zones, areas that contain primarily axons. Because Reelin is a secreted extracellular matrix protein (D'Arcangelo et al., 1995, 1997), localization of a diffuse product was anticipated, but we were surprised to find it in a presumptive white matter region at some distance from any immunoreactive somata. Furthermore, after blocking of secretion in vitro, distinct immunoreactive processes that morphologically resembled axons were detected in place of the diffuse product. Thus, when secretion was blocked, we were able to visualize cells and their processes filled with Reelin that were otherwise difficult to detect.

The long axon-like processes in our brefeldin-treated slices actually closely resemble axons of Cajal-Retzius cells in the *Orleans reeler* mutants ($Reln^{Orl}$), animals that can make an almost full-length protein but are unable to secrete it (de Bergeyck et al., 1997; Takahara et al., 1996). Thus, in both $Reln^{Orl}$ mutants and brefeldin-treated slices, Reelin-positive neurons can make, but cannot secrete, Reelin. A recent study of the Cajal-Retzius cells in the $Reln^{Orl}$ mutant suggested that Reelin accumulates in large, smooth, spherical cisterns within their beaded axons (Derer et al., 2001). Derer et al. (2001) named these smooth cisterns "axonal Reelin reservoirs" and suggested that Cajal-Retzius cells secrete Reelin at these sites. Similar swellings were common along the axons within our

brefeldin-blocked slices. Therefore, Reelin may be secreted at these varicosities, along the proximal part of the axons in the spinal cord, as well as in the cerebral cortex. Together with the work of Derer et al. (2001), findings from our brefeldin experiments suggest that Reelin is likely to be secreted from varicose axons during early spinal cord development and that these axons are concentrated in the lateral marginal zone, at a distance from their Reelin-positive somata.

Role of Reelin in spinal cord development

Reelin is known to be an extracellular matrix protein, so the functional form of Reelin most likely is the diffuse product. By comparing cultures processed with and without brefeldin, we were able to localize the secreted product with both the G10 and the CR-50 antibodies. Both of these antibodies are known to bind to the N-terminus of Reelin at different epitopes in the unique region between the area of F-spondin homology and the first Reelin repeat (de Bergeyck et al., 1998). The CR-50 antibody has been reported to block Reelin activity in a number of in vivo and in vitro experiments (D'Arcangelo et al., 1999; Miyata et al., 1997; Nakajima et al., 1997; Ogawa et al., 1995; Yip et al., 2000) and to inhibit Reelin–Reelin interactions needed for proper assembly, dimerization, and signaling (Kubo et al., 2002; Utsunomiya-Tate et al., 2000). Although the mechanism by which CR-50 activity inhibits Reelin is still unclear, it is likely that the N-terminus of the molecule is closely associated with Reelin signaling.

After localization of the secreted form of Reelin just lateral to the preganglionic neurons, the final step in our previously proposed model of Reelin function in spinal cord requires revision (Phelps et al., 2002). Reelin does not affect the initial migration (Fig. 8, step 1) or the second dorsal migration (Fig. 8, step 2) of either SPN or PPN. However, it is likely that it is the secreted Reelin in and around the lateral marginal zone rather than the medial Reelin containing somata that interferes with the third or medial migration of the preganglionic neurons (Fig. 8, step 3). In that the last part of the SPN migration was proposed to occur along radial glia (Phelps et al., 1993), this laterally located Reelin may interfere with the ability of the preganglionic neurons to attach their leading processes along the radial glia. In this manner, Reelin may function to maintain neurons with somata and/or processes near the lateral marginal zone in the intermediolateral horn. This interpretation also is consistent with the proposal of Hack et al. (2002), who reported that Reelin is a detachment signal for the neurons that migrate out to the olfactory bulb. Other studies also have led to the proposal that Reelin may interfere with the ability of neurons to engage or disengage from radial glia, possibly by changing the adhesive properties between the migrating cells and their glial substrates (Dulabon et al., 2000; Lambert de Rouvrout and Goffinet, 1998; Ogawa et al., 1995; Rice and Curran, 1999). Such suggestions are consistent with our previous study that demonstrated defects in radially migrating preganglionic neurons but not in those groups that migrated tangentially (Phelps et al., 2002).

In addition to its role in embryonic migration, Reelin also may have a functional role during later periods of development. For example, previously we reported that the length of the somatic motor neuron dendrites in postnatal *reeler* mutants was stunted in thoracic, but not in sacral, spinal levels (Phelps et al., 2002). One possible explanation for this difference is that only somatic motor neurons with dendrites in the proximity of the diffuse Reelin in the lateral marginal zone might have their dendritic arborizations affected. In conclusion, the substantial levels of diffuse Reelin expressed during late embryonic development and throughout the first month of postnatal development may be associated with an additional role of the Reelin protein.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Drs. Thomas Curran and Susan Magdaleno of St. Judes Children's Hospital for their generous gift of Reelin antibodies, Dr. Ellen Carpenter for her guidance with genotyping, and Drs. Carpenter and Xian-Jie Yang for suggestions on the article.

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