

# Phenotype of V2-derived interneurons and their relationship to the axon guidance molecule EphA4 in the developing mouse spinal cord

Line Lundfald,<sup>1</sup> C. Ernesto Restrepo,<sup>1</sup> Simon J. B. Butt,<sup>1,†</sup> Chian-Yu Peng,<sup>2</sup> Steven Droho,<sup>2</sup> Toshiaki Endo,<sup>1</sup> Hanns Ulrich Zeilhofer,<sup>3</sup> Kamal Sharma,<sup>2</sup> and Ole Kiehn<sup>1</sup>

<sup>1</sup>Mammalian Locomotor Laboratory, Department of Neuroscience, Karolinska Institutet, Retzius väg 8, 171 77 Stockholm, Sweden

<sup>2</sup>Department of Neurobiology, University of Chicago, Chicago, USA, and

<sup>3</sup>Institute of Pharmacology and Toxicology, University of Zurich, Switzerland

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## Abstract

The ventral spinal cord consists of interneuron groups arising from distinct, genetically defined, progenitor domains along the dorsoventral axis. Many of these interneuron groups settle in the ventral spinal cord which, in mammals, contains the central pattern generator for locomotion. In order to better understand the locomotor networks, we have used different transgenic mice for anatomical characterization of one of these interneuron groups, called V2 interneurons. Neurons in this group are either V2a interneurons marked by the postmitotic expression of the transcription factor Chx10, or V2b interneurons which express the transcription factors Gata2 and Gata3. We found that all V2a and most V2b interneurons were ipsilaterally projecting in embryos as well as in newborns. V2a interneurons were for the most part glutamatergic while V2b interneurons were mainly GABAergic or glycinergic. Furthermore, we demonstrated that a large proportion of V2 interneurons expressed the axon guidance molecule EphA4, a molecule previously shown to be important for correct organization of locomotor networks. We also showed that V2 interneurons and motor neurons alone did not account for all EphA4-expressing neurons in the spinal cord. Together, these findings enable a better interpretation of neural networks underlying locomotion, and open up the search for as yet unknown components of the mammalian central pattern generator.

## Introduction

The vertebrate spinal cord contains neuronal networks that can generate locomotion in the absence of sensory inputs. These networks, called central pattern generators (CPGs), are well characterized in lower vertebrates such as lamprey and tadpoles. Recent experiments have started to identify putative CPG interneurons in the mammalian spinal cord. Thus, the commissural intersegmental coordinating system is now well described (Bannatyne *et al.*, 2003; Butt & Kiehn, 2003; Jankowska *et al.*, 2003, 2005; Matsuyama & Jankowska, 2004) and a number of putative local, ipsilaterally projecting, excitatory CPG interneurons have also been identified (Shefchyk *et al.*, 1990; Angel *et al.*, 2005; Butt *et al.*, 2005; Hinckley *et al.*, 2005; Wilson *et al.*, 2005).

Previous electrophysiological experiments have demonstrated that the interneuron components of the CPG controlling hindlimb locomotion in rodents are localized in the ventral part of the lower thoracic and lumbar spinal cord (Kudo & Yamada, 1987; Bracci *et al.*, 1996; Kjaerulff & Kiehn, 1996; Cowley & Schmidt, 1997; Kremer & Lev-Tov, 1997; Bonnot *et al.*, 2002). Use of molecular biological techniques have identified classes of interneurons that settle in the

same region of the ventral spinal cord (Jessell, 2000; Lee & Pfaff, 2001; Goulding & Pfaff, 2005). These interneurons are derived from four progenitor domains (called p0–p3) in the ventricular zone, which develop into the postmitotic V0–V3 interneuron populations that are further defined by the expression of specific transcription factors. The V2 interneurons are derived from a common progenitor population expressing Lhx3 (Peng *et al.*, 2007), and are subdivided into two classes called V2a interneurons, which express Chx10, and V2b interneurons, which express Gata2 and Gata3 (Ericson *et al.*, 1997; Zhou *et al.*, 2000; Karunaratne *et al.*, 2002; Smith *et al.*, 2002).

In recent years, great effort has been put into relating these genetically defined groups of ventral interneurons to physiological locomotor-related populations, as expression of specific molecular markers provides a powerful tool for the identification and manipulation of discrete interneuron populations (Lanuza *et al.*, 2004; Gosgnach *et al.*, 2006). As a part of this characterization, the projection patterns and transmitter phenotypes have been identified for many of the ventral interneuron groups in embryonic mice. Recently it has been shown that the V0s are mixed  $\gamma$ -aminobutyric acid (GABA)- and glutamatergic commissural interneurons (Lanuza *et al.*, 2004), the V1s are ipsilaterally projecting interneurons that signal via GABA and glycine (Wenner *et al.*, 2000; Higashijima *et al.*, 2004; Li *et al.*, 2004; Sapir *et al.*, 2004; Gosgnach *et al.*, 2006) and the V3 interneurons are mixed ipsilateral and commissural excitatory interneurons (Geiman *et al.*, 2006). However, the exact projection pattern and

Correspondence: Professor Ole Kiehn, as above.

E-mail: O.Kiehn@ki.se

<sup>†</sup>Present address: Division of Neuroscience and Mental Health, Imperial College London, UK.

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neurotransmitter phenotype has not yet been described for the V2 interneurons in mammals. The present and accompanying paper (Al-Mosawie *et al.*, 2007) provide such a description covering ages from early embryonic life to adulthood.

We have previously described that mice with a knockout of the axon guidance molecule EphA4 have an abnormal hopping phenotype and that there is an increased crossover in the midline of axons from normally EphA4-expressing spinal interneurons (Kullander *et al.*, 2003; Butt *et al.*, 2005). This led us to propose the hypothesis that a group of excitatory, normally ipsilaterally projecting, CPG neurons have aberrant crossing in the *epha4*-null mice. In this paper we investigate the projection pattern and transmitter phenotype of the V2 population as well as the overlap of EphA4 expression with any of the V2 interneuron markers. Some of these data were presented earlier in abstract form (Lundfald *et al.*, 2006).

## Materials and methods

### Animals

Experiments were performed in wild-type C57BL/6 mice (Scanbur BK, Sollentuna, Sweden or Taconic, USA) or mice that incorporated a gene trap wherein *lacZ* is expressed under the control of the *epha4* promoter (*epha4<sup>lacZ</sup>*; Leighton *et al.*, 2001), kindly provided by Dr Marc Tessier-Lavigne, Genentech. For transmitter phenotyping, additional mice were a glycine transporter 2 (GlyT2)-enhanced green fluorescent protein (EGFP) mouse (Zeilhofer *et al.*, 2005) and a glutamic acid decarboxylase 67 (GAD67)-GFP mouse (kindly provided by Dr Yuchio Yanagawa, Gunma University Graduate School of Medicine; Tamamaki *et al.*, 2003). For Lhx3 cell tracing studies, additional mouse strains were Lhx3-cre mice (Sharma *et al.*, 1998) and mice with a ubiquitous yellow fluorescent protein (YFP) background (Rosa26 YFP/YFP; The Jackson Laboratory). For Hb9 interneuron study, we used transgenic mice expressing GFP under the control of the Hb9 promoter (Wilson *et al.*, 2005; kindly provided by Dr T. Jessell). Mice were bred for use in experiments and all animal experiment procedures were conducted in accordance with the European Communities Council Directive (86/609/EEC) and the National Institute of Health guidelines, and were approved by the local Animal Care and Use Committee.

### Dissections

Neonatal [postnatal day (P)0–3] or embryonic day (E)12.5–18.5 mice were used for all experiments. The embryonic stage was designated E0.5 on the morning of plug confirmation. Pregnant mice were anaesthetized deeply with isofluran (Abott Svandina, Solna, Sweden) before performing the caesarian section. The mother was killed with an overdose of isofluran as soon as all embryos were removed. Both embryos and neonatal mice were anaesthetized with isofluran, decapitated and eviscerated before the spinal cord was dissected out in ice-cold oxygenated (5% CO<sub>2</sub> in O<sub>2</sub>) 10% calcium Ringer's solution composed of (in mM): NaCl, 111.14; KCl, 3.09; NaHCO<sub>3</sub>, 25; KH<sub>2</sub>PO<sub>4</sub>, 1.1; MgSO<sub>4</sub>, 3.73; CaCl<sub>2</sub>, 0.25; and glucose, 11.1.

### Tracing studies in newborns

Fluorescein (FITC) dextran (MW 3000), Alexa Fluor 488 dextran (MW 10 000), Texas Red dextran (MW 3000) or Neurobiotin dextran (MW 3000; all from Molecular Probes) was applied to a fresh cut

along the ventral or dorsal midline or ~100 µm lateral to the midline (parasagittal application) to either the entire lumbar segment or to the L2 segment as previously described (Glover *et al.*, 1986; Stokke *et al.*, 2002; Nissen *et al.*, 2005). Alternatively, tracer was applied to a fresh cut in the ventrolateral funiculus. After application of tracer, the preparations were incubated for >4 h in the dark at room temperature in oxygenated (5% CO<sub>2</sub> in O<sub>2</sub>) 100% calcium Ringer's solution composed of (in mM): NaCl, 111.14; KCl, 3.09; NaHCO<sub>3</sub>, 25; KH<sub>2</sub>PO<sub>4</sub>, 1.1; MgSO<sub>4</sub>, 1.26; CaCl<sub>2</sub>, 2.52; and glucose, 11.1.

Spinal cords were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h and cryoprotected in 20–30% sucrose overnight prior to final embedding in Tissue-Tek (Sakura Finetek, Zoeterwoude, the Netherlands) and storage at –80 °C.

### Tracing studies in embryos

Embryos were dissected in 10% calcium Ringer's solution as described above except that a dorsal laminectomy was performed to expose the spinal cord. Embryos (E13.5 and E18.5) were then transferred to a dish with recirculating oxygenated Ringer's solution. A transverse cut was made at T11 on one side of the spinal cord and 2 µL of saturated Fluorescein dextran (MW 3000, Molecular Probes) was injected into the cut. Embryos were incubated at room temperature for 10 h, fixed for 2 h in 4% paraformaldehyde, washed with PBS, cryoprotected with 30% sucrose and embedded in OCT compound. Alternate frozen sections (14 µm) were collected on separate slides. One set was stained with guinea pig anti-Chx10 (1 : 20 000) and the other with guinea pig anti-Gata2/3 (1 : 6–8000) as described below. The signal was visualized with Cy3-conjugated secondary antibodies (Jackson Immuno Research). Double-labelled cells were counted in transverse sections.

### Lhx3 intracellular tracing studies

Lhx3 neurons were YFP-labelled by crossing Lhx3-cre mice (Sharma *et al.*, 1998) with mice with a homozygote ubiquitous YFP background (Rosa26 YFP/YFP). Dissection of P0–2 mice was performed as described above. The L1–L4 region was isolated and the pia carefully removed with fine scissors before immersing the spinal cord in warm agar (40 °C; see Takahashi, 1990). The agar was immediately cooled to 1 °C. A Leica VT1000 vibratome was used to cut slices (350 µm) from the L2 segment while immersed in ice-cold oxygenated (100% O<sub>2</sub>) HEPES-buffered Ringer's solution adjusted to pH 7.4 with 5 M NaOH. Slices were transferred to an incubation chamber and allowed to recover in 100% calcium Ringer for ~1 h at 30 °C. YFP-positive cells were identified using a combined fluorescence and differential interference contrast slice set-up, and cells were filled in whole-cell patch-clamp configuration with K-gluconate-based intracellular solution (in mM: K-gluconate, 138; HEPES, 10; ATP-Mg, 5; GTP-Li, 0.3; with CaCl<sub>2</sub>, 0.1 µM) containing 0.5% biocytin (Sigma). Post-tracing, slices were fixed with 4% paraformaldehyde in PBS, and cells filled with biocytin were visualized using the ABC method as described by Isa *et al.* (1998).

### Immunohistochemistry

Spinal cords were dissected out from neonatal or embryonic mice as described above before fixation in 4% paraformaldehyde in PBS for 1 h at 4 °C. Alternatively, the embryos were fixed whole in 4% paraformaldehyde in PBS for 2–3 h at 4 °C before the lumbar region was dissected out. Tissue was cryoprotected in 20–30% sucrose in

PBS overnight (4 °C) followed by embedding in Tissue-Tek as described above.

Serial 12- $\mu$ m cryosections were cut and were mounted separately on at least eight different SuperFrost Plus slides, such that each slide of nine sections represented a cross-section of the L2 neonatal spinal cord or a cross-section of the embryonic upper lumbar spinal cord.

Immunohistochemistry was performed according to one of two protocols: In Protocol A, tissue was blocked for 30 min with 1% heat-inactivated fetal calf serum (HI-FCS) in PBS and 0.1% Triton X-100 at room temperature. Primary antibody (see below) incubation was performed overnight at 4 °C in PBS with 1% HI-FCS. Species-specific secondary antibody incubation was performed at room temperature for 1 h in PBS with 1% HI-FCS. In Protocol B, primary antibody (see below) incubation was carried out overnight at 4 °C in 'incubation buffer' consisting of PBS with 0.1% Triton X-100, 0.02% Tween-20, 1% heat-inactivated normal donkey serum and 1% heat-inactivated normal goat serum. Species-specific secondary antibody incubation was performed at room temperature for 1–2 h in incubation buffer.

### Primary and secondary antibodies

The following primary antibodies were used: rabbit anti-Chx10, 1 : 1000, and guinea pig anti-Islet1 (Isl1), 1 : 1000 (both kindly provided by Dr Johan Ericson); mouse anti-Lhx3, 1 : 50 (Developmental Studies Hybridoma Bank); rabbit anti- $\beta$ -galactosidase ( $\beta$ -gal), 1 : 1000 (Cappel); mouse anti- $\beta$ -gal, 1 : 2000 (Promega); rat anti- $\beta$ -gal, 1 : 1000 (kindly provided by Dr Martyn Goulding); rabbit anti-Dbx1, 1 : 20 000 (kindly provided by Dr Alexandra Pierani); guinea pig anti-Evx1, 1 : 8000 (kindly provided by Dr Thomas Jessell); guinea pig anti-Gata2/3, 1 : 6–8000; guinea pig anti-Chx10, 1 : 20000; and rabbit anti-Hb9, 1 : 16000 (see Peng *et al.*, 2007). Species-specific fluorophore-conjugated secondary antibodies were purchased from Jackson Immuno Research (FITC, CY2, CY3, CY5, CY5 streptavidin) and Molecular Probes (Alexa Fluor 488) and used as recommended by the manufacturers.

### Images and analysing

Images were obtained with a Zeiss LSM510 Confocal microscope and analysed in Adobe Photoshop 7.0 (Adobe). Photographic images of the lumbar spinal cord transverse sections were analysed in Adobe Photoshop version 7.0 to estimate the number of double-labelled neurons. To assist counting, each image was divided into a grid. Immunopositive cells were selected according to their intensity levels as determined by the magic wand selection tool in Adobe Photoshop. In slices incubated with the DNA labelling reagent TO-PRO-3 (Molecular Probes), all cells in the ventral half of the cord immunopositive for  $\beta$ -gal were counted. To count a cell as  $\beta$ -gal-positive and thereby EphA4-positive we required that several  $\beta$ -gal-positive spots should be found surrounding the nucleus. This perinuclear position is typical of gene-trapped  $\beta$ -gal as previously described (Skarnes *et al.*, 1995). Often this was clearly seen in both embryos and newborn as a ring of  $\beta$ -gal-positive spots surrounding the nucleus. Nuclei were identified by antibodies against different transcription factors and/or by TO-PRO-3, which was included in all preparations. With this relatively conservative method it is possible that the real number of EphA4-positive cells was underestimated. If this was the case the underestimation should have been equal for all categories of cells and not have affected the relative distribution. A minimum of three slices was counted per animal and a minimum of three animals from each age group per staining protocol was

used. Unless otherwise stated, *n* is the number of animals used. All statistical values are expressed as mean  $\pm$  SD unless otherwise stated.

### In situ hybridization

Digoxigenin *in situ* hybridization was performed essentially as previously described (Schaeren-Wiemers & Gerfin-Moser, 1993) on 12- or 20- $\mu$ m cryosections using digoxigenin-labelled probe antisense to vesicular glutamate transporter 2 (VGLUT2; 1–811 bp; courtesy of Dr M. Goulding) or full-length GlyT2 antisense probe (Liu *et al.*, 1993). Hybridization was carried out at 60 °C and digoxigenin probes were visualized by development with BCIP/NBT from 6 h to overnight. The sections were processed after *in situ* hybridization with guinea pig anti-Chx10 or -Gata2/3 antibodies followed by fluorophore-conjugated antiguinea pig secondary antibody (Chx10) or biotinylated antiguinea pig secondary antibody. The biotin signal was detected using ABC reagent (Vector Laboratories, Inc) for signal amplification followed by DAB (Sigma) as the peroxidase substrate. Pictures of sections with *in situ* hybridization and fluorescence immunohistochemistry (IHC) were captured using a Nikon micro-photo-fx in combination with a Zeiss AxioCam, after which IHC staining was confirmed by overlapping of the resultant images in Adobe Photoshop version 7.0. Pictures of sections with *in situ* hybridization and biotin IHC was captured using conventional light microscopy.

## Results

### Projection patterns of the V2 population

V2 interneurons in mice are born from E10 to E13.5 (Ericson *et al.*, 1997; Nardelli *et al.*, 1999; Zhou *et al.*, 2000; Smith *et al.*, 2002; Peng *et al.*, 2007) and it has previously been suggested that they project ipsilaterally in embryonic mice (Saueressig *et al.*, 1999, Lee & Pfaff, 2001). Recently interneurons expressing Alx, the zebrafish homologue of Chx10, were also shown to be ipsilaterally projecting and mainly descending in zebrafish embryonic spinal cord (Kimura *et al.*, 2006). As an initial step in our characterization of V2 interneurons, we therefore investigated their projection patterns in E13.5 lower thoracic and upper lumbar spinal cord of embryonic mice. This was done by retrograde labelling of V2 interneuron cell bodies with fluorescent dextran amines injected into one hemisegment in the lower thoracic spinal cord (Th11–13). Alternating transverse sections were then recovered both rostral and caudal to the site of injection and stained with antibodies against Chx10 (V2a interneurons) and Gata2/3 (V2b interneurons), respectively (Fig. 1A–D).

Although only a fraction of the V2a and V2b interneurons in E13.5 were retrogradely labelled by the injection, all back-labelled V2 interneurons were ipsilaterally projecting. No V2 interneurons were ever labelled contralateral to the injection site at this time point. The ipsilateral projections extended for up to two segments away from the site of injection both rostrally and caudally, indicating that at this early age V2 interneurons have ascending, descending or bifurcating axons (see Fig. 1E).

The axonal projections of spinal V2a (Chx10) cells stayed ipsilateral at late embryonic time points and postnatally. This was seen in mice from E18.5 (*n* = 2; data not shown) and at P0 (Fig. 1F–G) where retrograde labelling was performed by applying dye to the ventral hemicord (*n* = 2; see Stokke *et al.*, 2002; Nissen *et al.*, 2005). In all of these cases the V2a population had exclusive ipsilateral projections. Finally, when parasagittal labelling was performed in

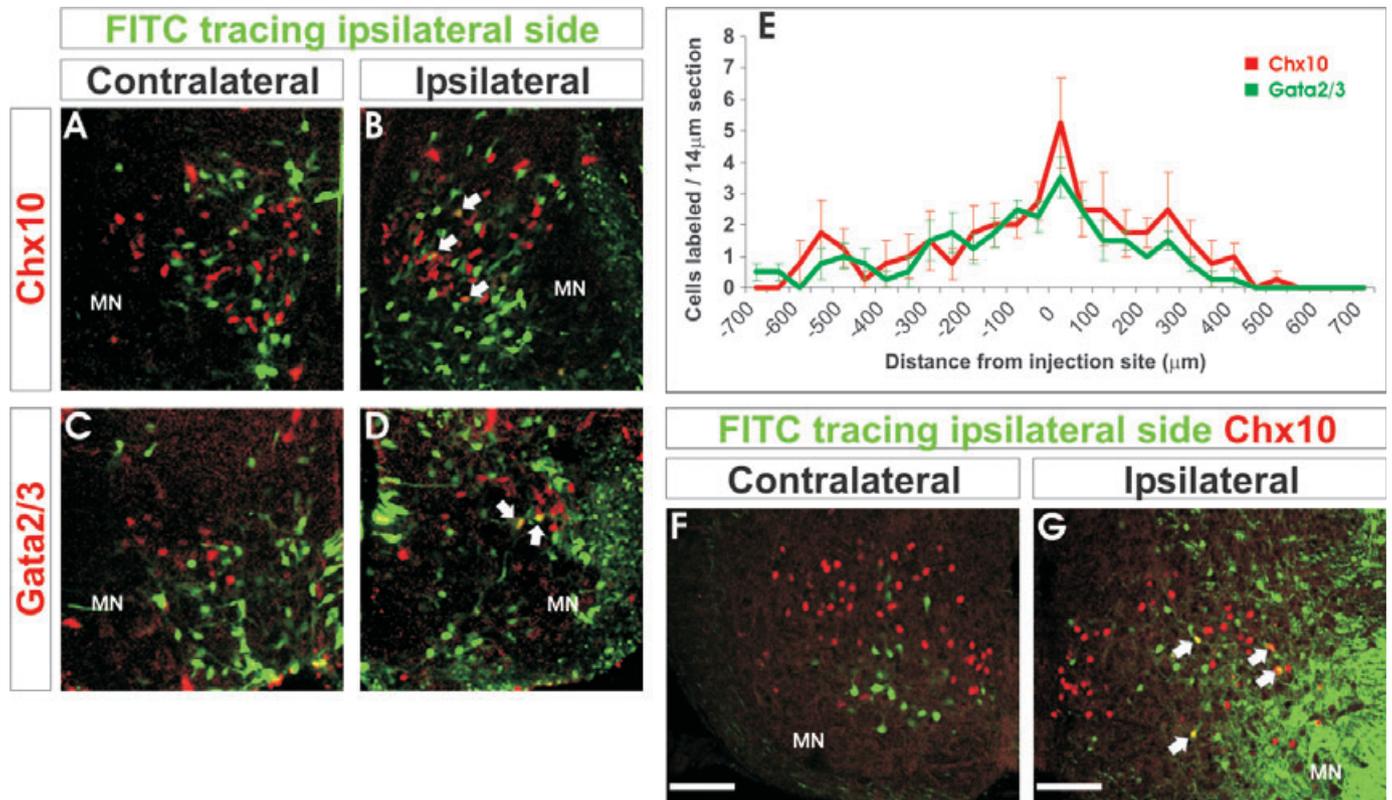


FIG. 1. Axonal projection of V2 cells. (A–D) Fluorescein dextran labeling (FITC tracing) on the ipsilateral side of E13.5 spinal cords fixed, cryosectioned and immunostained for (A and B) Chx10 and (C and D) Gata2/3. Neither Chx10 nor Gata2/3 are seen to colocalize with FITC on the contralateral side of the injection (A and C). Arrows in B and D point to ipsilaterally located cells double-labelled for FITC and Chx10 or Gata2/3, respectively. (E) Quantification of projection length on the ipsilateral side for Chx10 (red) and Gata2/3 (green) cells in E13.5 spinal cord. At this age V2 interneurons project up to two segments away from the injection site in both rostral (positive values) and caudal (negative values) directions. (F and G) FITC tracing of the ipsilateral side of P0 spinal cords followed by immunohistochemistry for Chx10 revealed no double-labelled cells on the contralateral side (F) while arrows in G point to double-labelled cells on the ipsilateral side. Scale bars, 100  $\mu$ m.

newborn mice where Lhx3-expressing interneurons, which include both V2a and V2b interneurons (Ericson *et al.*, 1997; Peng *et al.*, 2007), were marked with YFP there was no retrograde labelling (blue cells) of V2a (red cells or double-labelled yellow cells) on the side contralateral to the application (Fig. 2A;  $n = 2$ ). Note that Lhx3-cre only recombine to 40–60%, so some Chx10 cells are YFP-negative.

The developmental analysis of the V2a interneurons did show a clear change in projection pattern. At E18.5 (data not shown) and P0–1 (Fig. 1F–G) most V2a cells seem to project caudally. In two E18.5 animals, 13 out of 139 Chx10 cells located between 100 and 700  $\mu$ m rostral to the injection site could be retrogradely labelled, while caudal to the injection site only one out of 156 counted Chx10 cells was labelled.

The V2b (Gata2/3) neurons also appeared for the main part to project ipsilaterally at early postnatal ages. Thus, parasagittal labelling experiments in Lhx3-YFP mice showed that no Lhx3-YFP-positive and Chx10-negative cells were retrogradely labelled from the contralateral side (Fig. 2A;  $n = 99$  cells in two animals). However, detailed inspection of the Lhx3-YFP mice did reveal some YFP-positive (but Chx10-negative cells) adjacent to the midline, both above and below the central canal (Fig. 2B and C), that had processes crossing the midline (< 7% of the total population of YFP-positive and Chx10-negative cells). Although not confirmed immunologically, these processes looked like dendrites, akin to the midline crossing dendrites from medially located motor neurons (Fig. 2D). This midline crossing appeared to be specific to the more medially located Lhx3

cells. Thus, when a random population of YFP-labelled Lhx3-expressing interneurons in lamina VII were labelled intracellularly with biocytin ( $n = 10$ ) in acute slices from P0–2 mice, we found that none of the stained cells had processes which crossed the midline. In contrast, axonal processes were clearly seen to project toward the ipsilateral motor neuron region and into the ventromedial and/or ventrolateral funiculus (Fig. 3).

In summary, we found V2a interneurons in the lower thoracic and lumbar spinal cord of both embryonic and newborn mice project ipsilaterally. In addition, V2a interneurons exhibited a developmental shift in projection pattern over time, from projecting both rostrally and caudally at early embryonic times (E13.5) to projecting almost exclusively in a caudal direction later in development. Furthermore, while V2b interneurons were exclusively ipsilateral at E13.5, some Lhx3-YFP-positive, Chx10-negative and therefore possible spinal V2b interneurons postnatally extended processes across the midline.

#### Neurotransmitter phenotype of the V2 population

The next step was to investigate the neurotransmitter phenotype of V2a and V2b cells. As with the axonal projection experiments, we concentrated on the lower thoracic and lumbar region of the spinal cord and in order to enable us to relate the transmitter phenotype to physiological studies of locomotor network, we used newborn mice,

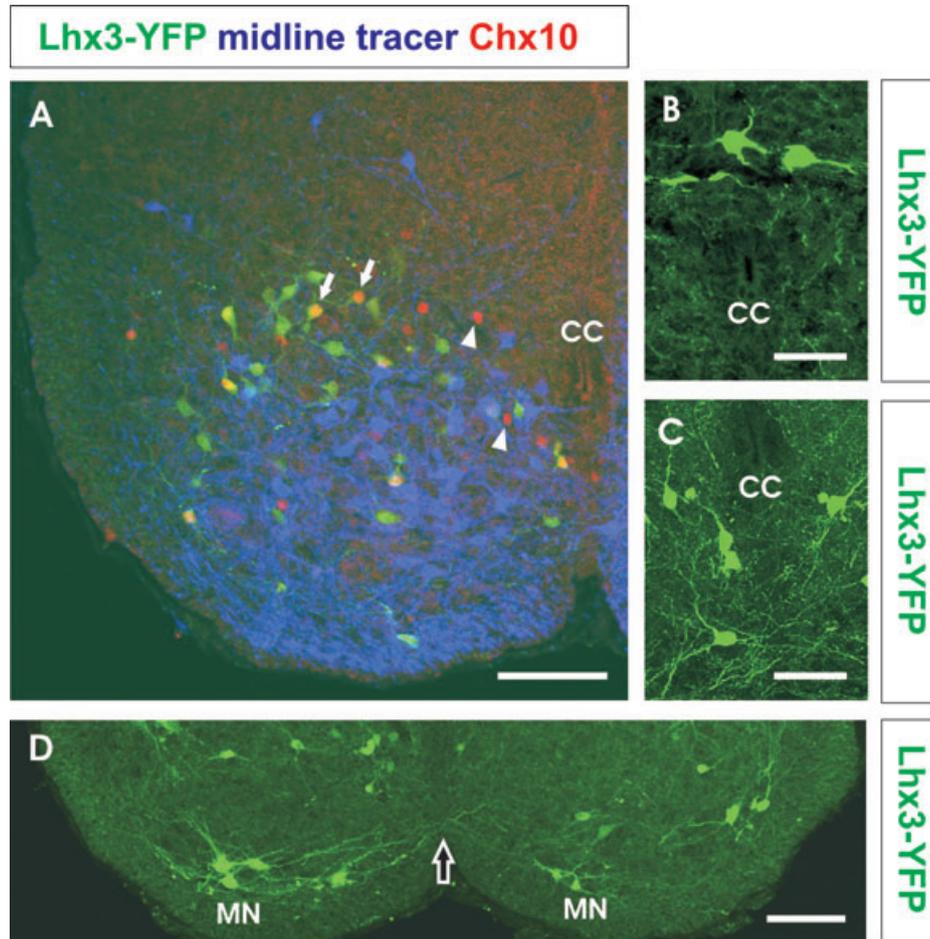


FIG. 2. Projection of Lhx3 neurons. (A) Section showing the ventral L2 spinal cord contralateral to parasagittal labelling with neurobiotin dextran (blue) in a Lhx3-cre-Rosa26-YFP mouse. Retrogradely labelled cells are blue, YFP-labelled cells green and Chx10-positive cells red (examples indicated by arrow heads). Arrows point to double-labelled YFP-Chx10 cells. Note that not all Chx10-positive cells are YFP-positive as Lhx3-cre only recombines 40–60% (Sharma *et al.*, 1998). (B and C) Lhx3-YFP-positive cells close to the midline with processes crossing the midline. (D) Large Lhx3-YFP-positive presumptive medial motor neurons with dendrites crossing the midline (arrow). CC, central canal; MN, motor neurons. Scale bars, 100  $\mu$ m (A and D), 50  $\mu$ m (B and C).

as spinal cord from mice at this stage generates locomotor rhythm *in vitro* (see Introduction for references).

In transverse sections of L2 lumbar spinal cord taken from P0 mice, V2a (Chx10-positive) as well as V2b (Gata2/3-positive) cells are scattered in a broad band in lamina VII extending from the central canal to the lateral edges of the grey matter (see Fig. 4A–D). While the V2a cells were distributed in a continuum in this band, the V2b cells were separated into distinct locations with one population around the central canal in lamina X and another population spanning lamina VII. Some of the V2b cells in lamina X were located in the ependymal lining of the central canal.

To investigate the transmitter phenotype of Chx10-positive cells, we performed *in situ* hybridization for VGLUT2, which is the main vesicular glutamatergic transporter in neurons located in the ventral spinal cord (Kullander *et al.*, 2003). *In situ* hybridization was followed by IHC for Chx10 on transverse spinal cord slices from four P0 wild-type mice. Of the total number of Chx10-positive cells,  $80 \pm 14.5\%$  (mean  $\pm$  SD) were also VGLUT2-positive (Fig. 5A–C and I). Conversely, *in situ* hybridization for the neuronal GlyT2 (Zafra *et al.*, 1997; Betz *et al.*, 2006) revealed only  $5 \pm 1.1\%$  of the Chx10-positive cells to be glycinergic (data not shown). We also used two mouse lines where EGFP was expressed in glycinergic and GABAergic neurons:

the first was a transgenic line with EGFP under the control of the promoter for *GlyT2* (Zeilhofer *et al.*, 2005), while the second mouse line had EGFP knocked-in in the loci for GAD67, one of two enzymes responsible for GABA synthesis (Tamamaki *et al.*, 2003). IHC for both Chx10 and GFP revealed that at P0  $1 \pm 0.3\%$  were glycinergic (Fig. 5D and I, ‘GlyT2-GFP’), while 0% (not a single cell) were GABAergic (Fig. 5E and I, ‘GAD67-GFP’). We thus concluded that Chx10 cells were, for the most part, glutamatergic.

For V2b interneurons we first determined the percentage of Gata2/3-positive cells that colabelled with the inhibitory transmitters in (E)GFP mice. Performing IHC for both GFP and Gata2/3, we found that  $83 \pm 8.5\%$  of Gata2/3-positive cells in lamina VII were glycinergic (Fig. 5F and J, grey bars; ‘GlyT2-GFP’), while only  $23 \pm 5.1\%$  were GABAergic (Fig. 5G and J, grey bars; ‘GAD67-GFP’). Conversely, in lamina X around the central canal, the predominant transmitter in the Gata2/3 population was GABA as seen from the number of GAD67-positive cells ( $64 \pm 4.3\%$ ; Fig. 5G and J, white bars; ‘GAD67-GFP’). Only a minor fraction of the Gata2/3 population in lamina X was glycinergic as seen from the number of GlyT2-positive cells ( $4 \pm 7.2\%$ ; Fig. 5F and J, white bars; ‘GlyT2-GFP’). The Gata2/3 cells that were lining the central canal were either GAD67-positive or GAD67- and GlyT2-negative. To

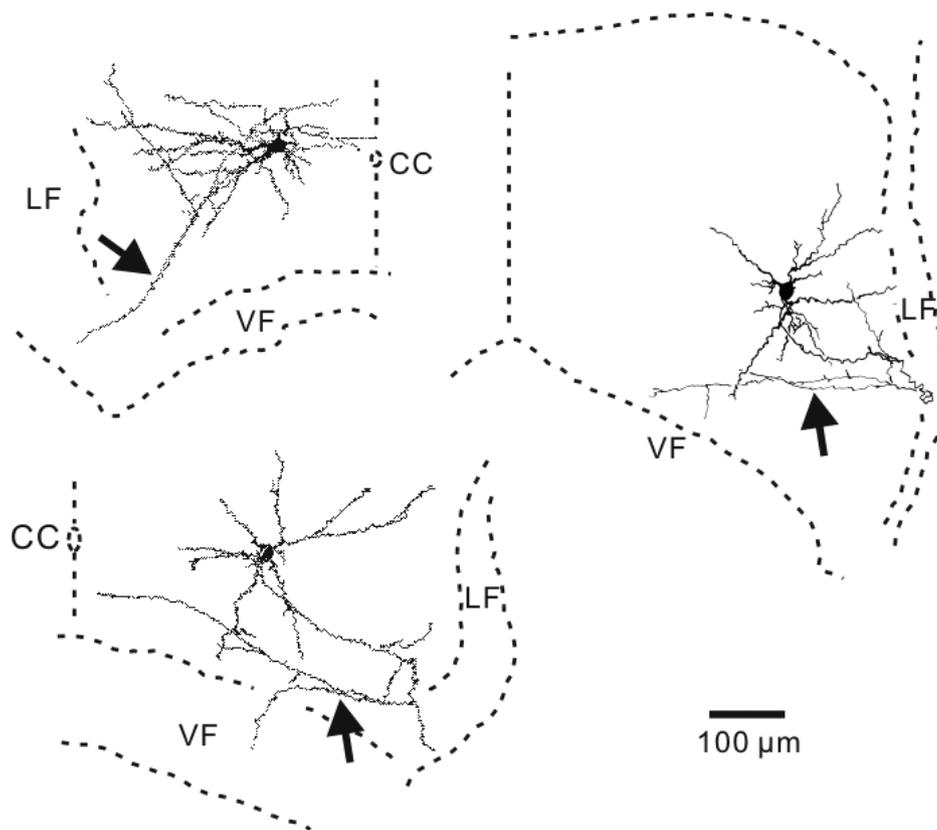


FIG. 3. Camera lucida drawings of Lhx3 neurons stained with biocytin. Putative axons are indicated by arrows. Dashed lines indicate midline and cutting edge of the slices. CC, central canal; LF, lateral funiculus; VF, ventral funiculus.

complete the picture of the transmitter content we performed *in situ* hybridization experiments for VGLUT2 combined with IHC for GATA2/3. These experiments showed that virtually none of the Gata2/3-expressing neurons expressed VGLUT2 at P0 ( $3.1 \pm 3.2\%$  in the intermediate area (Fig. 5H and J, grey bar; 'VGLUT2 *in situ*') and  $0.1 \pm 0.7\%$  in lamina X around the central canal (Fig. 5H and J, white bar; 'VGLUT2 *in situ*';  $n = 3$  animals, 12 sections per animal). Based on these data, we concluded that V2b cells were mainly inhibitory.

#### *EphA4* was expressed in many V2 interneurons

We have previously identified a group of ipsilaterally projecting EphA4-expressing interneurons that have glutamatergic inputs onto motor neurons (MNs; Butt *et al.*, 2005). As the V2 population contains ipsilaterally projecting excitatory cells, we sought to determine whether there was an overlap between EphA4 expression and the expression of V2 transcription factors. It has previously been shown that MNs are EphA4-positive during development (Eberhart *et al.*, 2000; Kania & Jessell, 2003). We therefore decided to investigate the expression pattern of EphA4 in the EphA4<sup>Lac/+</sup> mice to look for groups other than MNs expressing EphA4. We used the embryonic mouse time window of E12.5–15.5 because that is when the transcription factors defining the different ventral, postmitotic interneuron pools are readily identified (Jessell, 2000; Lee & Pfaff, 2001; Goulding & Pfaff, 2005) and when the interneuron classes are generated and start to migrate and send out projections (Nornes & Carry, 1978; Ericson *et al.*, 1997; Nissen *et al.*, 2005).

To mark MNs, we used antibodies against Isl1, which labels MNs in the lateral part of the lateral motor column (ILMC) as well as motor neurons in the medial motor column (MMC), and against Lhx3, which is a marker of MNs in the MMC and early V2 interneurons before they have differentiated into V2a and V2b populations (Tsuchida *et al.*, 1994; Pfaff *et al.*, 1996; Ericson *et al.*, 1997; Sharma *et al.*, 1998; Karunaratne *et al.*, 2002; Smith *et al.*, 2002; Peng *et al.*, 2007). As seen from Fig. 6, at E12.5 ~20% of the motor neurons in ILMC (MNs only expressing Isl1) are positive for EphA4 while as many as ~80% of the MNs in the MMC (Isl1-positive and Lhx3-positive) are EphA4-positive (Fig. 6A–C). Furthermore, in the group of interneurons expressing Lhx3 (Isl1-negative and Lhx3-positive neurons) ~40% also express EphA4 (arrows in Fig. 6B). These initial findings suggested that at least a proportion of the V2 interneurons expressed EphA4.

In order to further estimate the degree of EphA4 expression in the V2 population we went on to determine the coexpression of EphA4 (IHC for  $\beta$ -gal) and Chx10 (V2a) or Gata2/3 (V2b). This was done from E13.5 up until P0 in order to be able to extrapolate the data from embryos up until the age at which the locomotor experiments were performed in EphA4-mutated animals (Kullander *et al.*, 2003; Butt *et al.*, 2005). These data are presented in Fig. 7, which shows that the percentage of Chx10 cells coexpressing EphA4 was fairly constant at high levels between 67 and 87% throughout development until birth. For V2b, we only determined the percentage of Gata2/3 cells coexpressing EphA4 from E13.5 to E16.5. The percentage of coexpressing cells remained high (between 49 and 68%) throughout that developmental period. Thus, we concluded that a significant proportion of the V2 population do express EphA4.

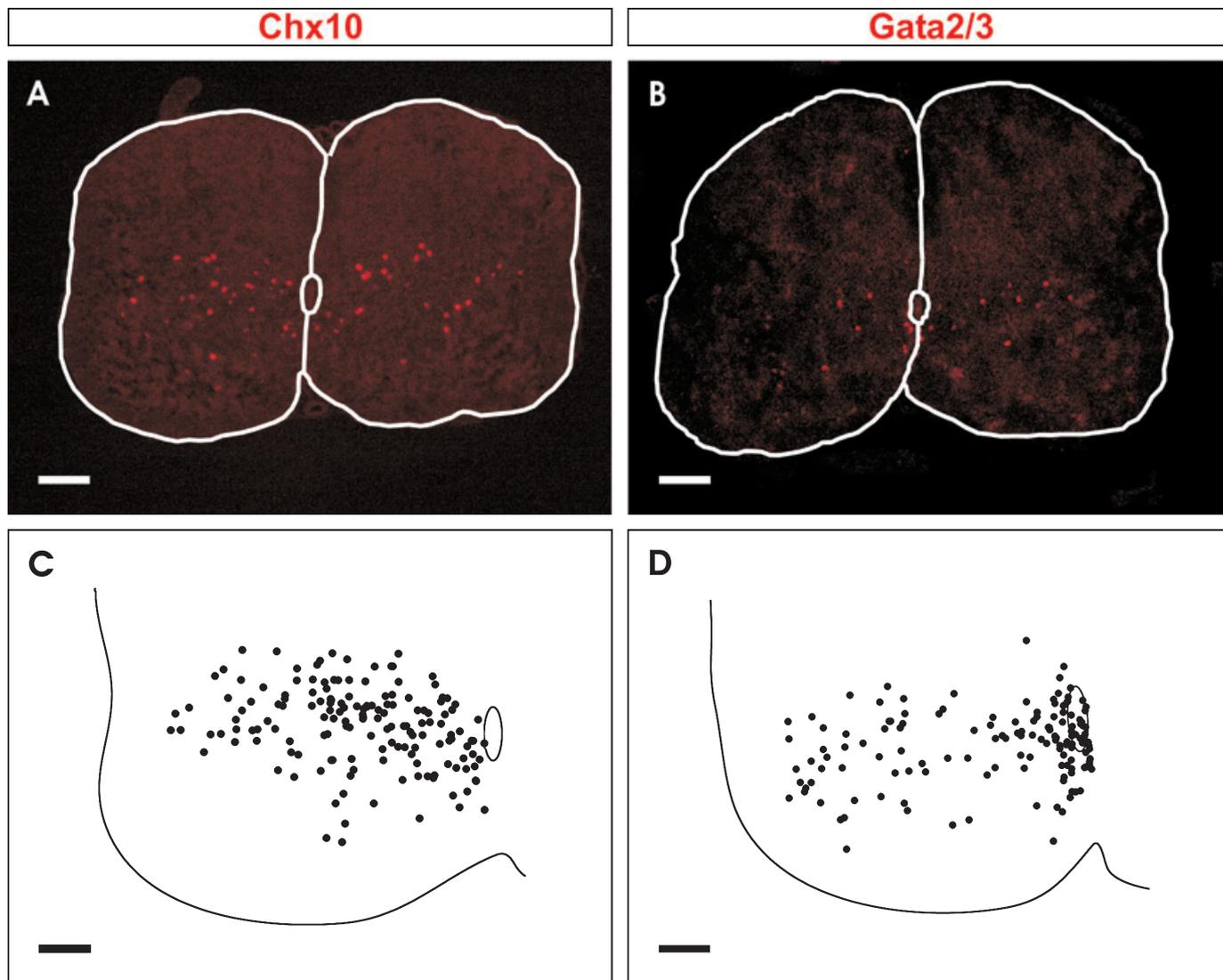


FIG. 4. Location of V2 cells at P0. Overview picture of a P0 spinal cord from a wild-type mouse showing immunostaining for (A) Chx10 and (B) Gata2/3 cells in the lumbar segment 2 (L2). Summary diagrams of location of (C) Chx10-positive and (D) Gata2/3-positive cells in L2 in a P0 wild-type mouse. Diagrams are composites of five 12- $\mu$ m sections from (C) two or (D) three animals. Scale bars, 100  $\mu$ m (A and B), 200  $\mu$ m (C and D).

As a small cluster of medially located interneurons situated in L1–2 of the lumbar mouse spinal cord are glutamatergic and express the motor neuron marker Hb9 (Hinckley *et al.*, 2005; Wilson *et al.*, 2005; Hinckley & Ziskind-Conhaim, 2006) we also looked for coexpression of Hb9 and EphA4 ( $\beta$ -gal) in these interneurons. Because the Hb9-GFP mouse also expresses GFP in non-Hb9 cells (Hinckley *et al.*, 2005; Wilson *et al.*, 2005), we combined the use of the Hb9-GFP mouse with antibody staining with a rabbit anti-Hb9 antibody (see Materials and methods) in order to positively identify Hb9 interneurons. However, none of the Hb9 interneurons expressed EphA4 in P0 animals ( $n = 41$  cells in six animals; see Fig. 8).

*The large majority of EphA4-expressing cells were motor neurons or belonged to the V2 population*

In order to determine the extent to which the EphA4 cell population could be accounted for by MNs and V2 interneurons, we counted

all the EphA4-expressing cells in the ventral part of the spinal cord at E13.5 and determined the fraction of cells identified as MNs (by the expression of the motor neuron marker Hb9; Arber *et al.*, 1999; Thaler *et al.*, 1999), V2a interneurons (Chx10 expression) and V2b interneurons (Gata2/3 expression). From these counts, we found that MNs comprised 36.0% of all EphA4-expressing cells at E13.5 while the V2 interneurons comprised 34.8% of the EphA4 cells, divided into V2a (22.1%) and V2b (12.7%;  $n = 3$ –5 slices from at least three animals; data not shown). Thus, at this age,  $\sim 30\%$  of the EphA4 cells could not be accounted for as V2 interneurons or MNs.

To further determine the other classes of E13.5 ventral interneurons that express EphA4 we used IHC or *in situ* hybridization to visualize V0 (Dbx1 or Evx1 commissural excitatory and inhibitory interneurons), V1 (En1, ipsilateral inhibitory interneurons) and V3 (Sim1, excitatory ipsilateral and commissural interneurons) populations. However, Sim1 is down-regulated early and we were not able to see any expression after E11.5. For En1 we were also unable to obtain

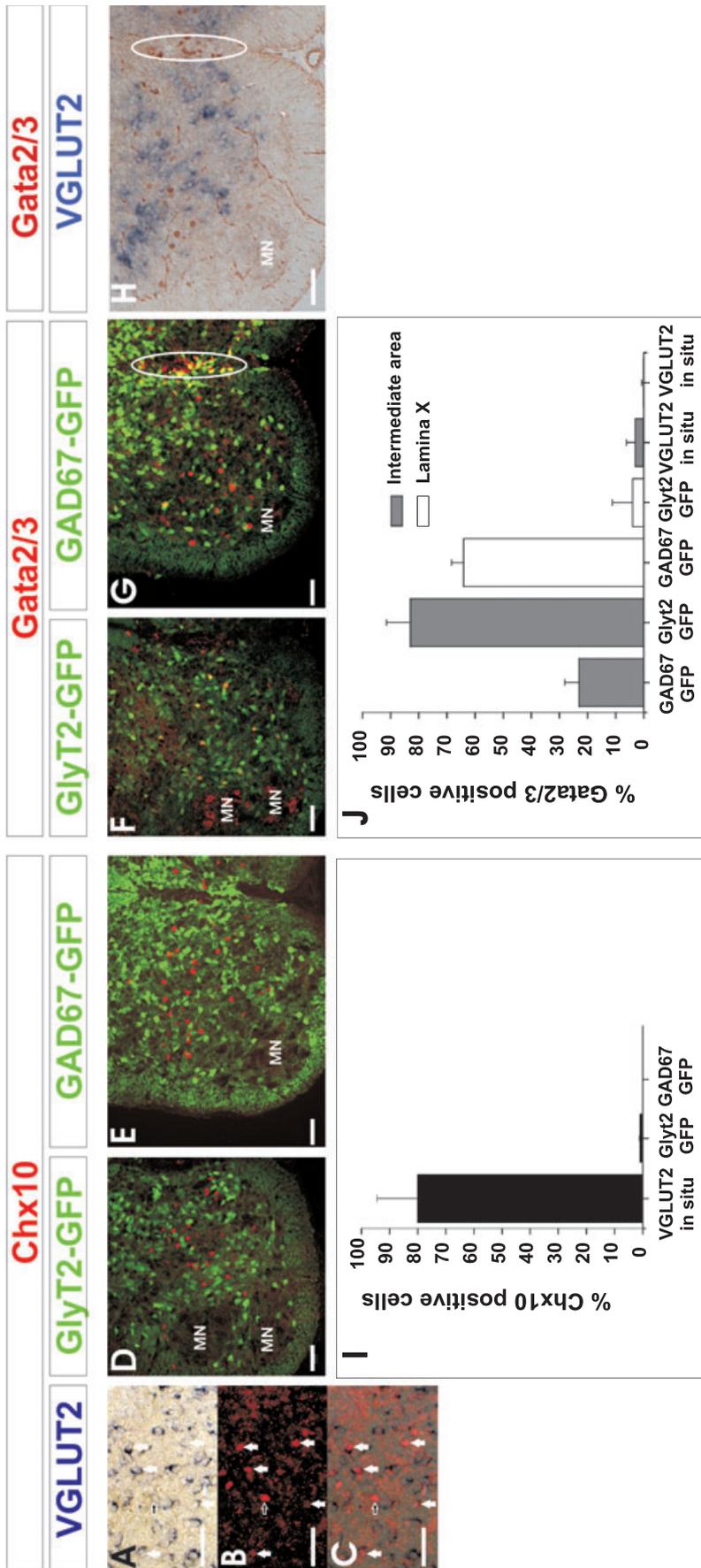


FIG. 5. Transmitter content of Chx10- and Gata2/3-positive cells in P0 wild-type mouse spinal cord segment L2. (A) *In situ* hybridization for VGLUT2 revealed glutamatergic cells in the ventral horn. (B) Immunostaining for Chx10 after *in situ* hybridization showed Chx10-positive neurons in the same area. (C) Overlaying A and B reveals VGLUT2-positive Chx10 cells. Solid white arrows point to VGLUT2-positive Chx10 cells while open (black) arrow points to a Chx10 cell not positive for VGLUT2. (A–C and I) 80 ± 14.5% (mean ± SD) of Chx10 cells were glutamatergic as determined by *in situ* hybridization against VGLUT2 (*n* = 4) while (D and I) 1 ± 0.3% of Chx10 cells were glycinergic as determined by Chx10 immunostaining in GlyT2-GFP mice (*n* = 3). (E and I) No Chx10 cells were GABAergic (*n* = 4) as determined by Chx10 immunostaining in GAD67-GFP P0 mice. (F, G and J) In the intermediate area of the spinal cord, the majority of Gata2/3 cells were glycinergic (83 ± 8.3%; *J*; *n* = 3) as determined by Gata2/3 immunostaining in GlyT2-GFP mice while 23 ± 5.1% of the Gata2/3-positive cells are GABAergic (immunostaining on GAD67-GFP mice; G and J; *n* = 5). This is in contrast to the Gata2/3 population in lamina X around the central canal (white circle) where GABA dominated with 64 ± 4.3% of all Gata2/3 cells being GAD67-positive while only 4 ± 7.2% of the Gata2/3 population positive (white bars in J). (H and J) Few Gata2/3 cells expressed VGLUT2 both in the intermediate area (3.1 ± 3.2%; H and grey bar in J) and around the central canal (1 ± 0.7%; H and white bar in J) as determined by Gata2/3 immunostaining and VGLUT2 *in situ* hybridization. All scale bars, 50 µm.

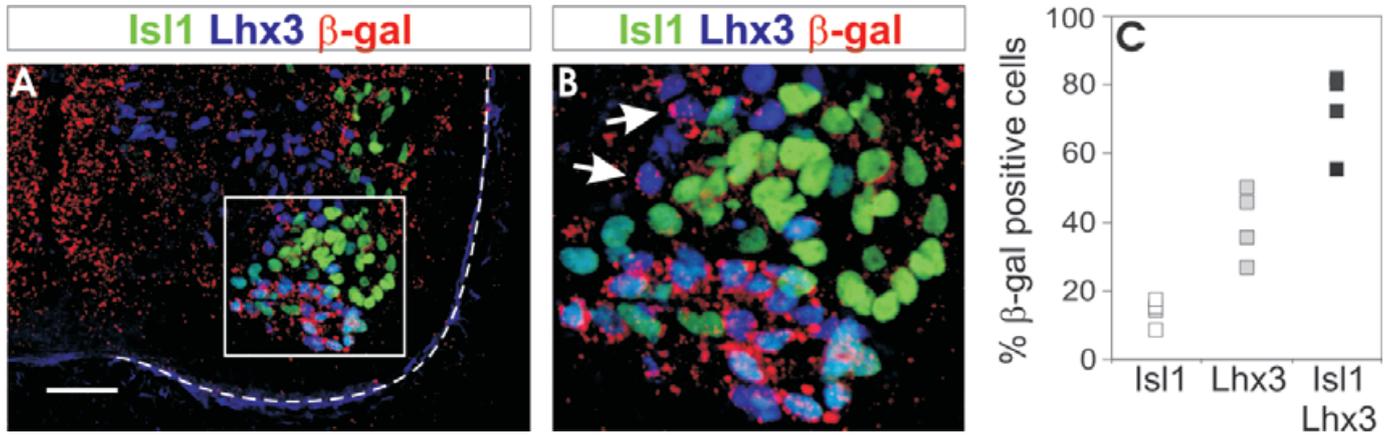


FIG. 6. EphA4-expressing cells included both motor neurons and V2 interneurons at E12.5. (A) Overview of the ventral quadrant of lumbar *ephA4<sup>LacZ/+</sup>* mouse spinal cord at E12.5 stained for Isl1 (green), Lhx3 (blue) and β-gal (red). (B) Closeup of insert in A. Arrows point to putative V2 interneurons located next to the motor neuron pools. (C) In the lumbar ventral horn at E12.5 the majority ( $72 \pm 12.2\%$ ) of medial motor column neurons labelled by Isl1 and Lhx3 were EphA4-positive as determined by staining for β-gal ( $n = 4$ ; datapoints shown for each animal). Isl1-positive profiles of the lateral motor column were largely negative whereas a proportion of the Lhx3-only cells that include laterally migrated V2 interneurons were also positive for EphA4 ( $39 \pm 10.6\%$  of the Lhx3 cells). Scale bar, 50 μm.

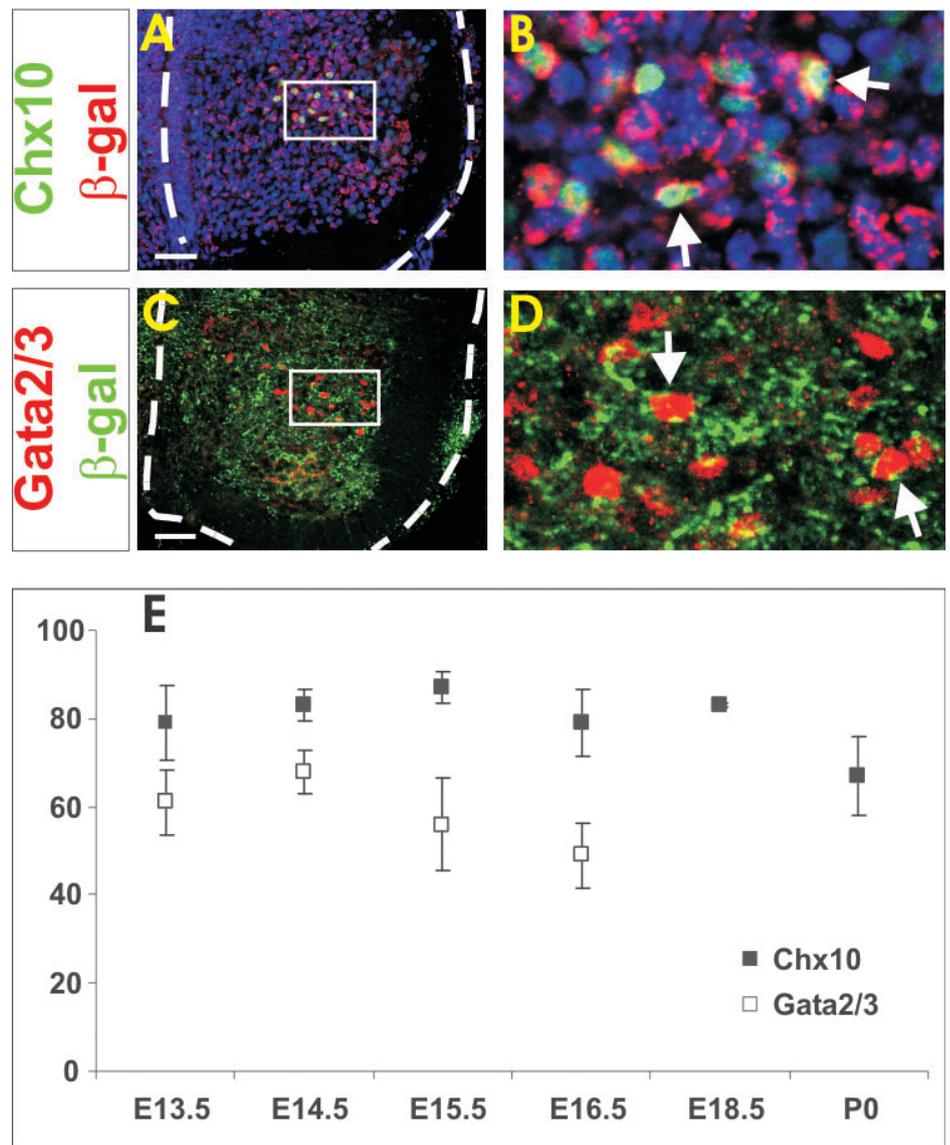


FIG. 7. V2 cells expressed EphA4. (A–D) Ventral quadrant of an E14.5 *ephA4<sup>LacZ/+</sup>* mouse lumbar spinal cord immunostained for β-galactosidase (β-gal) and Chx10 (A and B) or Gata2/3 (C and D). Cell nuclei were labelled with TO-PRO-3 (blue) to assist in cell analysis. (B) Close-up of the insert in A. Arrows point to cells clearly labelled for both β-gal and Chx10. (D) Close-up of the insert in C. Arrows point to cells clearly labelled for both β-gal and Gata2/3. (E) The high coexpression levels of Chx10 (black squares) or Gata2/3 (white squares) with EphA4 is illustrated when plotting the age against the percentage of Chx10 or Gata2/3 cells that express EphA4. At any given age from E13.5 to P0, 67–87% of Chx10 cells expressed EphA4 while the corresponding number for Gata2/3 and EphA4 coexpression was 49–68%. For each age and immunostaining investigated, at least three sections from 2–5 animals were used. Scale bars, 50 μm.

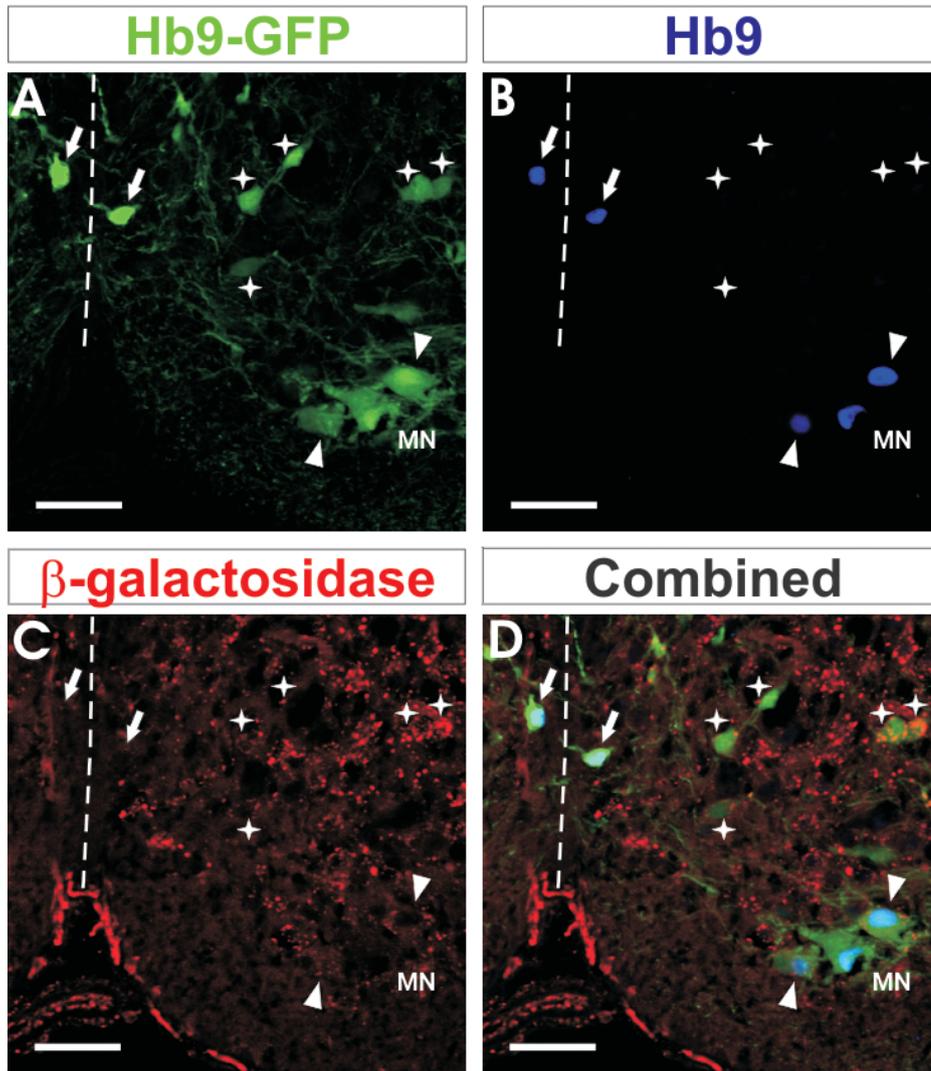


FIG. 8. Hb9 interneurons were not EphA4-positive. (A) Cells labelled by GFP in the upper lumbar segment of a P0 Hb9-GFP  $\times$  *ephA4<sup>LacZ/+</sup>* mouse. (B) Immunostaining for Hb9 protein. (C) Immunostaining for  $\beta$ -gal. (D) Overlay of A–C. Arrows point to Hb9 interneurons while arrowheads indicate motoneurons (MN) of the medial motor column. Stars indicates cells that are labelled by the Hb9-GFP protein but are not MNs or Hb9 interneurons. Dashed line indicates the midline. Scale bars, 50  $\mu$ m.

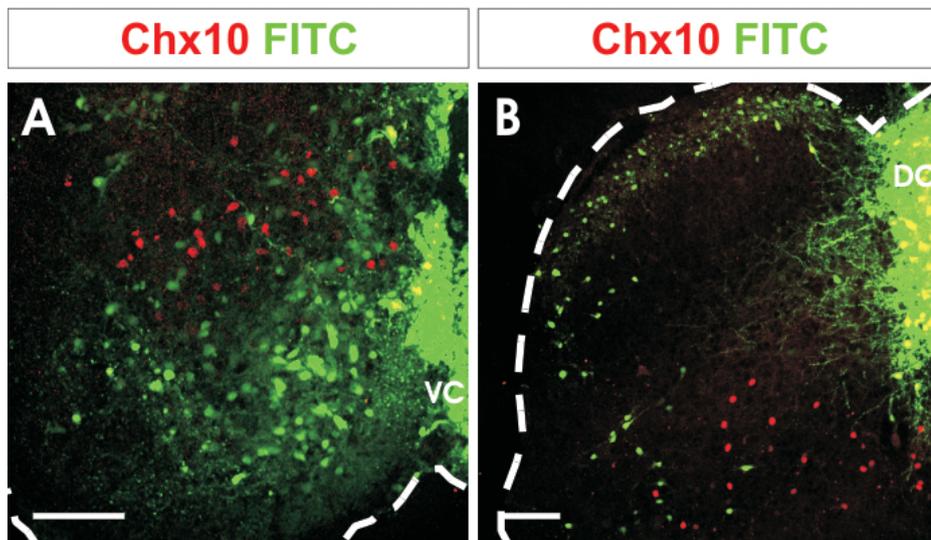


FIG. 9. Chx10-expressing cells did not project contralaterally in *ephA4<sup>LacZ/-</sup>* mice. (A) P0 *ephA4<sup>LacZ/-</sup>* mice ( $n = 4$ ) stained with Fluorescein (FITC) dextran along the entire lumbar segment (L1–6) in the ventral midline and immunostained for Chx10. (B) P5 *ephA4<sup>LacZ/-</sup>* mice ( $n = 2$ ) stained with Fluorescein (FITC) dextran along the entire lumbar segment (L1–6) in the dorsal midline and immunostained for Chx10. Chx10 staining was not seen to overlap with Fluorescein (FITC) dextran-traced cells. VC, ventral commissure; DC, dorsal commissure. Scale bars, 100  $\mu$ m.

reliable strong labelling at E13.5; similar to what others have reported (Dr M. Goulding, personal communication). In the V0 population marked with either Dbx1 or Evx1 antibodies, 25.4% ( $n = 6$ ; Dbx1) or

24.2% ( $n = 7$ ; Evx1) of the cells were also EphA4-positive; this makes up  $<10\%$  of the total population of EphA4 cells in the ventral cord.

### *Chx10-expressing V2 interneurons did not project over the midline in EphA4<sup>lacZ/-</sup> mice*

Given that the V2 population represents a large proportion of the EphA4-positive cells at embryonic and newborn ages, and that MN axons never cross the midline, it is probable that this class of interneuron contributes to the observed aberrant excitatory crossover and hopping phenotype in *ephA4*-null mice (Kullander *et al.*, 2003). If this is the case, the excitatory V2a interneurons should have aberrant axon projections in *ephA4*-null mice. To test this hypothesis, we performed midline staining with fluorescein dextran-amine (Stokke *et al.*, 2002; Kullander *et al.*, 2003) in *ephA4<sup>LacZ/+</sup>* (control) and *ephA4<sup>LacZ/-</sup>* (hopping-gait) mice aged E13.5, E15.5 and newborn, in whom the hopping phenotype is fully developed (Kullander *et al.*, 2003; Butt *et al.*, 2005). In embryonic heterozygotes ( $n = 1$  for E13.5 and  $n = 3$  for E15.5) a population of retrogradely labelled commissural interneurons was found in the ventromedial area at all ages (Nissen *et al.*, 2005). Very few of these cells were EphA4-positive ( $\beta$ -gal-positive; data not shown), as also previously reported from newborn animals (Kullander *et al.*, 2003). In order to identify whether V2a EphA4-expressing cells form aberrant connections in *ephA4*-null mice, we tested dextran amine-labelled sections with the Chx10 antibody. As expected, very few of the cells labelled from the midline (1–4 cells in the lumbar cord of each animal) at any age tested were Chx10-positive in heterozygote mice, consistent with V2a interneurons being ipsilateral interneurons (data not shown). Somewhat surprising, however, a similar picture was also found in homozygote animals tested both in embryos ( $n = 1$  for E13.5) and postnatally ( $n = 4$  for P0 and  $n = 2$  for P5). Very few, if any, interneurons colabelled for dextran amine and Chx10 antibody. This was evident when labelling both from the ventral (Fig. 9A) and from the dorsal midline (Fig. 9B). This suggests that the aberrant crossing previously observed in newborns (Kullander *et al.*, 2003) was not a result of misguided axons across the midline originating from Chx10-expressing V2a interneurons. Therefore, it would appear that neither motor neurons (see Kullander *et al.*, 2003) nor V2a interneurons, two major classes of ventral neurons that express EphA4 at a crucial time point during network development, extend aberrant axons across the midline in the *ephA4* mutant.

## Discussion

In this paper, we have characterized the murine V2 population anatomically, in terms of location, in the region of the spinal cord that is involved in controlling hindlimb locomotion, as well as their transmitter phenotype and projection patterns. Such an anatomical characterization is of paramount importance for a further understanding of their possible functional role in the mammalian CPG when using specific deletions of spinal cord interneurons (Lanuza *et al.*, 2004; Gosgnach *et al.*, 2006; see also Kiehn, 2006). Furthermore, we have shown that, although EphA4 is expressed in the V2a ipsilateral population, these cells do not cross the midline in *ephA4*-null mice, suggesting that nonoverlapping groups of excitatory EphA4 neurons participate in ipsilateral CPG activity.

### *Distribution and projection pattern of the V2 population*

In newborn animals, we found the distribution of the V2 cells in the lower thoracic and upper lumbar spinal cord to be located in a broad band extending from the central canal to the lateral edge of the grey

matter (Laminae X and VII). This distribution is in line with previously reported locations of embryonic postmitotic V2a (Ericson *et al.*, 1997; Peng *et al.*, 2007) and V2b interneurons (Zhou *et al.*, 2000; Karunaratne *et al.*, 2002; Smith *et al.*, 2002; Peng *et al.*, 2007) in chick and mouse embryos. The distribution is also similar to the distribution of the Alx population in zebrafish embryos (Kimura *et al.*, 2006) although, due to the difference in size and shape of the spinal cord in these two species, a direct comparison is more difficult. A Gata3 population has also been described in the ventral half of zebrafish embryos (Neave *et al.*, 1995) but has not been characterized in detail.

Our studies show that lumbar V2a interneurons are exclusively ipsilaterally projecting while some lumbar Lhx3-positive, Chx10-negative and therefore possible V2b interneurons have contralateral processes that are likely to be dendrites. These processes were mostly confined to cells close to the midline. In no cases did we find V2a cells that had contralateral projections, even though commissural interneurons were readily labelled in the same labelling protocol (see also Stokke *et al.*, 2002; Nissen *et al.*, 2005). The ipsilateral projection has previously been suggested on the basis of experiments in embryos (Saueressig *et al.*, 1999; Lee & Pfaff, 2001). Here, we have performed extensive tracing studies using both retrograde and intracellular labelling of V2 interneurons at different developmental ages. We have shown that there is a developmental shift in the ipsilateral projection of V2a interneurons, from being both rostrally and caudally projecting in E13.5 mice to becoming mainly caudally projecting later in development. Meanwhile, V2b interneurons may shift from being ipsilateral at E13.5 to having at least some processes extending over the midline at the time of birth. However, while these midline-crossing processes are found in the V2b population they do not seem to be abundant (see also the accompanying paper Al-Mosawie *et al.*, 2007).

In all ipsilateral retrograde tracing experiments only a small proportion (10–20%) of the V2 population was retrogradely labelled, even close to the site of dye application and even when the entire hemicord was filled with dye. This has been a concern to us as it indicated that we, in spite of our efforts, were not able to trace more than a fraction of the total number of ipsilaterally projecting V2 cells and therefore were unable to accurately determine their projection patterns. We do not have a clear explanation for the relatively low percentage of retrogradely labelled ipsilaterally projecting V2 cells. One possibility is that V2 interneurons have very small axons that do not easily take up the dextrans. We observed similar difficulties earlier in an attempt to retrograde label partition cells (Sherriff & Henderson, 1994) with dextrans injected into the motor nucleus. These cells have relatively thin axons and, despite the fact that the partition cells were not retrogradely labelled with dextran amines, they were readily labelled when using transsynaptic labelling (C. E. Restrepo and O. Kiehn, unpublished observations).

### *Distinct transmitter phenotypes for V2a and V2b cells*

While the V2a and V2b populations have overlapping distribution patterns in the cord, the two populations have largely different transmitter phenotypes. On average 80% of the V2as were glutamatergic while only a minority of cells were identified as glycinergic (5% at the RNA level and only 1% at the protein level) and none were GABA-ergic. As *in situ* hybridization experiments always give a conservative estimate of the actual numbers of positive cells, our data suggest that the vast majority of V2a interneurons are indeed excitatory. This could also include those cells that expressed GlyT2

as it has been shown in retinal ganglion cells, which also express Chx10 during development (Edqvist *et al.*, 2006), that glycine and glutamate are colocalized in ~10% of the cells (Sun & Crossland, 2000). We did not test for this possibility directly in the present study but preliminary studies using mouse line that express Cre under the VGLUT2 promoter (L. Borgius, unpublished observations) show that there is no overlap between VGLUT2 and GlyT2 in P0 spinal cord cells ( $n = 2$ ), suggesting that such colocalization is not present in the spinal cord.

In contrast to the V2a population, the V2b population appeared to mainly express neuronal GABA or glycine, based on their expression of GFP under the control of the *gad67* or *glyt2* genes (Tamamaki *et al.*, 2003; Zeilhofer *et al.*, 2005) and not VGLUT2. We did not systematically test for the expression of GAD65, so we cannot exclude the possibility that some Gata2/3 cells will express GAD65 at perinatal and early postnatal ages or later in adults, as suggested in the companion paper (Al-Mosawie *et al.*, 2007). We noted that the number of cells expressing GlyT2-GFP or GAD67-GFP add up to 106%, indicating that there may be a population of V2b cells that express both GABA and glycine. Coexpression of GABA and glycine has previously been described in the rat dorsal horn (Polgar *et al.*, 2003) and may indicate that either a change of transmitter phenotype is taking place or, alternatively, the V2b cells may use both transmitters, as has previously been reported for spinal interneurons (Jonas *et al.*, 1998).

We also observed that the cluster of V2b cells located around the central canal in lamina X contained a higher percentage of GABAergic cells than those located more laterally in lamina VII, indicating that these differentially located V2b cells delineate two different functional and/or developmental classes of cells. Moreover, some of the Gata2/3-positive cells in lamina X were lining the central canal and some of them also expressed GAD67. It is possible that these later Gata2/3-positive GABAergic cells in the ependyma are indeed neurons while non-GABAergic ependymal Gata2/3-positive cells are likely to be non-neuronal (Russo *et al.*, 2004).

In summary, there is a clear distinction between the V2a and V2b cells in terms of neurotransmitter content and, thereby, possibly also function (see below). In the zebrafish embryo, Alx interneurons are also glutamatergic and a large number of Alx cells have been shown to have excitatory monosynaptic connections onto MNs (Kimura *et al.*, 2006). Whether Chx10 cells also have monosynaptic connections to motor neurons in mammals remains to be demonstrated directly (see the companion paper Al-Mosawie *et al.*, 2007). It is therefore not impossible that the group of mammalian V2a cells is a remnant of the original design from fish. The V2b interneurons exist in fish but have not been characterized extensively (Peng *et al.*, 2007). Although GABA and glycine can act as excitatory or depolarizing transmitters in embryonic life, their main role during network activity in postnatal mammals is to act as inhibitory transmitters during network activity (see Kiehn, 2006 for references). We have therefore designated the V2b population an inhibitory interneuron population.

#### *The V2 population overlapped with the axon guidance molecule EphA4*

We have previously found that, in EphA4-knockout mice, a group of normally EphA4-expressing neurons aberrantly project over the midline and we have shown that many of the EphA4-expressing cells are glutamatergic (Kullander *et al.*, 2003; Butt *et al.*, 2005). Furthermore, we were able to attribute the hopping phenotype to an increased aberrant commissural excitatory drive, as increasing the normal bilateral inhibition (Kullander *et al.*, 2003) or decreasing the

excitatory drive (S. J. B. Butt and O. Kiehn, unpublished observations) would revert the hopping gait to alternating walking. This is in agreement with observations by Akay *et al.* (2006) reporting that adult *ephA4*-null mice will walk normally for a few minutes after waking up from anaesthetics, which are known to increase the inhibitory drive. In this context EphA4-expressing glutamatergic V2a interneurons seemed a good candidate for being the neurons that aberrantly project over the midline in *ephA4*-null mice. One aim of the present study was therefore to provide evidence for or against this hypothesis. What we found was that the V2a cells do not cross the midline aberrantly in *ephA4*-null mice. Despite intensive efforts to label V2a cells from the midline in *ephA4*-null mice, we never saw crossing axons from V2a cells. Furthermore, as the aberrantly overcrossing axons observed in *ephA4<sup>LacZ</sup>/-* mice were labelled by the same type of dextrans (Kullander *et al.*, 2003) as used in this study, we feel confident that V2a interneurons are still confined to the ipsilateral side and do not cross aberrantly in *ephA4<sup>LacZ</sup>/-* mice.

Two other interneuron groups, V2b interneurons and V0 interneurons (as determined by IHC for Dbx1 and Evx1), were also shown to express EphA4 at E13.5. The V2b interneurons seem from our study to be, if not exclusively then mainly, inhibitory and therefore lack the required neurotransmitter content to explain the increased aberrant commissural excitatory drive in the *ephA4*-null mice. Interneurons positive for both Dbx1/Evx1 have been shown to be involved in the normal alternation system (Lanuza *et al.*, 2004), which is overridden in the *ephA4*-null mice. These observations makes it less likely that the V2b and V0 cells are key components for the changed phenotype in the EphA4 mutant.

Taken together, our evidence therefore suggests that there is another ipsilateral excitatory EphA4 population which does not overlap with the V2a population that has aberrant commissural projections in the *ephA4*-null mice. Given that we were unable to determine the identity of ~20% of all the EphA4-positive cells in the ventral half of the spinal cord because of the time-dependent development of the transcription factor profile, this would appear plausible. It is likely that the EphA4-positive, excitatory and ipsilaterally projecting interneurons recorded from in Butt *et al.* (2005) belong to this unknown group. At the moment the only other population of excitatory ipsilateral interneurons that we have excluded is the Hb9-positive interneurons located close to the midline in the upper lumbar spinal cord (Hinckley *et al.*, 2005; Wilson *et al.*, 2005), as none of the Hb9 neurons express EphA4.

#### *Concluding remarks*

In our present work we have described the anatomical characteristics of V2 cells located in the lower thoracic and upper lumbar spinal cord, although V2 cells are distributed along the entire spinal cord as well as in supraspinal structures (Liu *et al.*, 1994; Nardelli *et al.*, 1999; Peng *et al.*, 2007). The reason for concentrating on this area is that it contains the CPG networks that generate hindlimb locomotion. The location of the V2 population in the ventral spinal cord, where the CPG networks are concentrated, suggests that V2 interneurons may be members of the CPG networks, a situation similar to that described for V2a-like Alx neurons in zebrafish (Kimura *et al.*, 2006). The characterization of the V2 interneurons opens up the possibility of defining the role of these cells in the CPG network by loss- or gain-of-function experiments. Our initial experiments on the Chx10 cells indeed suggest that these cells serve a specific role in coordinating CPG activity (Droho *et al.*, 2006). The data also suggest that there are further Chx10-negative, EphA4-positive excitatory interneuron populations important for normal CPG activity that are as yet uncharacterized.

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## Abbreviations

$\beta$ -gal,  $\beta$ -galactosidase; CPG, central pattern generator; E, embryonic day; EGFP, enhanced GFP; FITC, fluorescein; GABA,  $\gamma$ -aminobutyric acid; GAD, glutamic acid decarboxylase; GlyT2, glycine transporter 2; GFP, green fluorescent protein; IHC, immunohistochemistry; Isl1, Isl1; lLMC, lateral part of the lateral motor column; MMC, medial motor column; MN, motor neuron; P, postnatal day; PBS, phosphate-buffered saline; VGLUT2, vesicular glutamate transporter 2; YFP, yellow fluorescent protein.

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