Published in final edited form as:

J Comp Neurol. 2010 December 1; 518(23): 4675–4701. doi:10.1002/cne.22441.

Target Selection of Proprioceptive and Motor Axon Synapses on Neonatal V1-Derived Ia Inhibitory Interneurons and Renshaw Cells

Valerie C. Siembab¹, Courtney A. Smith¹, Laskaro Zagoraiou², Maria C. Berrocal¹, George Z. Mentis³, and Francisco J. Alvarez^{1,*}

¹Department of Neuroscience, Cell Biology and Physiology, Boonshoft School of Medicine, Wright State University, Dayton, Ohio 45435

²Department of Neuroscience, Kavli Institute for Brain Science, Howard Hughes Medical Institute, Columbia University, New York 10032

³Section of Developmental Neurobiology, National Institute of Neurological Disorders and Stroke, NIH, Bethesda, Maryland 20892

Abstract

The diversity of premotor interneurons in the mammalian spinal cord is generated from a few phylogenetically conserved embryonic classes of interneurons (V0, V1, V2, V3). Their mechanisms of diversification remain unresolved, although these are clearly important to understand motor circuit assembly in the spinal cord. Some Ia inhibitory interneurons (IaINs) and all Renshaw cells (RCs) derive from embryonic V1 interneurons; however, in adult they display distinct functional properties and synaptic inputs, for example proprioceptive inputs preferentially target IaINs, while motor axons target RCs. Previously, we found that both inputs converge on RCs in neonates, raising the possibility that proprioceptive (VGLUT1-positive) and motor axon synapses (VACHT-positive) initially target several different V1 interneurons populations and then become selected or deselected postnatally. Alternatively, specific inputs might precisely connect only with predefined groups of V1 interneurons. To test these hypotheses we analyzed synaptic development on V1-derived IaINs and compared them to RCs of the same age and spinal cord levels. V1-interneurons were labeled using genetically encoded lineage markers in mice. The results show that although neonatal V1-derived IaINs and RCs are competent to receive proprioceptive synapses, these synapses preferentially target the proximal somato-dendritic regions of IaINs and postnatally proliferate on IaINs, but not on RCs. In contrast, cholinergic synapses on RCs are specifically derived from motor axons, while on IaINs they originate from Pitx2 V0c interneurons. Thus, motor, proprioceptive, and even some interneuron inputs are biased toward specific subtypes of V1-interneurons. Postnatal strengthening of these inputs is later superimposed on this initial preferential targeting.

Keywords

spinal cord; VGLUT1; VACHT; development; calbindin; parvalbumin; Ia afferent; motoneuron

© 2010 Wiley-Liss, Inc.

*CORRESPONDENCE TO: Department of Neuroscience, Cell Biology and Physiology, Boonshoft School of Medicine, Wright State University, 3640 Colonel Glenn Highway, Dayton, OH 45435. francisco.alvarez@wright.edu .

Current address for G.Z. Mentis: Center for Motor Neuron Biology and Disease, Columbia University, New York, NY 10032.

Additional supporting information may be found in the online version of this article.

The development of spinal cord local interneuronal circuits is currently poorly understood. Recent work has defined a relatively small number of cardinal interneurons in embryo that generates the much larger diversity of adult interneurons (Goulding, 2009). In the ventral horn just four embryonic subgroups (V0, V1, V2, V3) generate most adult premotor interneurons by diversification mechanisms that are currently largely unknown. Each group displays common properties that generally include origins from one type of progenitor cell (p0, p1, p2, p3), the direction taken by the primary axon, their cell migration and final location in the ventral horn with relation to motor pools, and their neurotransmitter phenotype. For example, V1 interneurons have common origins in the p1 progenitor area and are characterized by expression of the engrailed-1 (En-1) transcription factor. They migrate ventrolaterally, positioning themselves adjacent to motor pools, extend ascending axons that target ipsilateral motoneurons, and have an inhibitory phenotype (Matise and Joyner, 1997; Ericsson et al., 1997; Saueressig et al., 1999; Sapir et al., 2004; Alvarez et al., 2005). These canonical embryonic interneurons and their primary characteristics are conserved phylogenetically, but interestingly, the number and types of neurons derived from each class increases in complexity in parallel with the evolution of locomotion from fish to terrestrial animals (reviewed in Grillner and Jessell, 2009). V1-interneurons in zebrafish and tadpoles constitute a rather homogenous population that gates sensory-motor responses and are more effectively recruited at higher locomotor speeds (Higashijima et al., 2004; Li et al., 2004). In mammals, however, they give rise to several different types of adult premotor inhibitory interneurons with different functions (Alvarez et al., 2005). Interestingly, genetic deletion of all V1-interneurons in neonatal mice slowed fictive locomotor rhythms evoked in the spinal cord in vitro (Gosgnach et al., 2006). These observations suggest the possibility that from a primitive inhibitory interneuronal system adapted to adjust swimming speed there evolved in mammals several premotor inhibitory circuits for limb control and terrestrial locomotion (reviewed in Goulding, 2009). The ontogenetic mechanisms that diversify V1 interneurons in the mammalian spinal cord are unknown.

In mammals, at least two well-known adult inhibitory interneurons, Renshaw cells (RCs) and the Ia inhibitory interneurons that mediate reciprocal inhibition (IaINs), derive from V1 interneurons (Alvarez et al., 2005), and yet each develops a distinct synaptic connectivity that serves different functions (summarized in Fig. 1). Adult RCs mediate recurrent inhibition of homonymous and synergistic motoneurons and receive strong excitatory drive from recurrent collaterals of motor axons (Eccles et al., 1954; reviewed in Alvarez and Fyffe 2007). In contrast, IaINs mediate reciprocal inhibition between antagonistic motor pools and receive strong excitation from Ia afferents (Eccles et al., 1956; Jankowska and Roberts, 1972; Jankowska and Lindstrom, 1972; Rastad et al., 1990). The different sources of excitatory drive places each interneuron in a different functional position within the motor network and also serves as a method of identification. However, we recently reported that RCs also received functional Ia inputs in the neonate which appear to be reduced in density during late postnatal development (Mentis et al., 2006). In contrast, Ia afferent synapses strengthen on motoneurons postnatally in part by the addition of new synaptic boutons through mechanisms that are distinct from those controlling synaptic targeting (Shneider et al., 2009; reviewed in Mentis et al., 2010). At present there is no information on developing IaINs or the structure of their synaptic input in neonates or adult. Nonetheless, recurrent and reciprocal inhibition of motoneurons have both been described in neonates in mice (Sapir et al., 2004; Wang et al., 2008) and humans (McDonough et al., 2001), although they display many immature characteristics. Particularly, reciprocal inhibition is relatively weak, it is not detectable in all pairs of antagonistic motor pools, and shows initially a large extent of facilitation instead of inhibition (McDonough et al., 2001; Wang et al., 2008). Maturation of these interneuronal circuits seems to coincide with the transition to weight-bearing locomotion and adequate postural control in both species, but little effort has been devoted to analyze the development of interneuron properties during this period. A fundamental

question that remains to be resolved is the process that generates diverse connectivity on each type of interneuron.

These considerations raise the question of whether all embryonic V1 interneurons have common competencies to receive convergent inputs from motor and proprioceptive axons and whether these are later selected or deselected on different subgroups of V1-derived interneurons by differential strengthening or weakening during the maturation of locomotor activity. Alternatively, V1-derived IaINs and RCs might display different capabilities for receiving synapses from proprioceptive and motor axons and follow different developmental synaptogenetic programs from the start. At present, the degree of synaptic and functional differentiation between RCs and IaINs in the neonate is unknown. Therefore, here we specifically asked whether IaINs receive convergent inputs from motor and proprioceptive axons similar to RCs or if alternatively IaINs already show significant differences in their selection of particular inputs in neonates and the process of postnatal maturation of these inputs in each interneuron type.

To this purpose we characterized several transgenic mouse models that allowed us to follow the lineage of V1-derived neurons from neonate to adult and label their cell somata and dendritic arbors. We also confirmed during postnatal development the histological criteria used for identification of V1 derived RCs and IaINs and their primary afferents and motor axon inputs in more mature animals (Alvarez et al., ²⁰⁰⁴,2005; Mentis et al., 2006; summarized in Fig. 1). The results suggest that each interneuron forms discrete pools of V1-derived interneurons already clearly distinct from each other in the neonate. Moreover, each input, motor axon or proprioceptive, respectively, exhibits preferential selectivity for neonatal RCs or IaINs. Additionally, each input undergoes considerable postnatal maturation before reaching the adult synaptic organization characteristic of each cell.

These results have been presented in abstract form (Siembab et al., 2008).

MATERIALS AND METHODS

All animal procedures were performed according to National Institutes of Health (NIH) guidelines and were reviewed by the local Laboratory Animal Use Committees at Wright State University and NIH.

Animals

Three animal models were used to label, using Cre-lox recombination, the lineage of V1-derived interneurons (V1-INs) in the postnatal spinal cord. All animals were obtained by crossing *En1*^{Cre/+} heterozygotes (Sapir et al., 2004) with one of three reporter mice. *Thy1-loxP-STOP-loxP-YFP* (*Thy1-YFP*) mice (Buffelli et al., 2003) contain several copies of the reporter transgene in the genome and once crossed with *En1*^{Cre/+} results in strong yellow fluorescent protein (YFP) expression in V1-INs (*En1-Cre/Thy1-YFP* mice). Most of the study was carried out using these mice. In addition, *En1*^{Cre/+} heterozygotes were also crossed with *R26-loxP-STOP-loxP-LacZ* (*R26-lacZ*; Soriano, 1999) or *Tau-loxP-STOP-loxP-mEGFP-IRES-INLacZ* (*Tau-lacZ*; Hippenmeyer et al., 2005) reporter mice. In this last mouse line, the reporter cassette was knocked-in by targeted recombination in the *tau* gene and is passed to the progeny always in heterozygosis. Losing one copy of *tau* does not seem to affect development (Hippenmeyer et al., 2005). *En1-Cre/R26-LacZ* mice have been previously used and explained in full detail (Sapir et al., 2004; Alvarez et al., 2005). *En1-Cre/Tau-loxP-STOP-loxP-mEGFP-IRES-INLacZ* express, upon Cre recombination, a bicistronic transcript encoding a modified β -galactosidase (β -gal) with a nuclear localization signal (nls) and a modified membrane-bound enhanced green fluorescent protein (mEGFP) designed to best depict neurite morphology and surfaces (DePaola et al., 2003). In these

animals expression of the mEGFP reporter is relatively low and can only be visualized with antibody amplification. We mostly used β -gal expression to identify V1-INs in these animals (shortened to *En1-Cre/Tau-lacZ*). Pups with the desired genotypes were identified using standard polymerase chain reaction (PCR) analysis of tail clips with *Cre*, *lacZ*, and *GFP* primers.

Finally, we analyzed the possibility of interneuronal cholinergic inputs in V1-derived RCs and IaINs using spinal cords supplied by Dr. Tom Jessell (Columbia University) from double mutants expressing *pitx2::cre* and *En1::cre* crossed to the *Tau-loxP-STOP-loxP-mEGFP-IRES-INLacZ* reporter line described above. The *pitx2::cre* line has been thoroughly described (Liu et al., 2003; Zagoraoui et al., 2009).

The specificity of reporter expression in *En1^{Cre/+}* was previously assessed using several R26 reporter lines (Sapir et al., 2004). The *En1-cre* line was generated such that it will faithfully recapitulate endogenous expression patterns. The *cre* expression cassette was knocked-in the first coding exon of the *En1* gene downstream of the transcription start site such that the ATG of *En1* was replaced with the *Cre* ATG. The neocassette used for clonal selection was removed and therefore the only extra sequences that remain aside from *Cre* and poly A are a single FRT (≈ 37 – 40 bp) downstream of the polyA. This strategy has been quite successful in replicating endogenous expression patterns in a number of transgenic lines. We did not find the extra FRT sequences to interfere with endogenous regulation of *En1* and *Cre* expression. Thus, there was a perfect match between *En1* protein expression and reporter expression at embryonic day (E)11.5 when using two different R26 reporter lines. Unfortunately, similar experiments cannot be performed in the *Thy1* and *Tau* reporter lines because upregulation of reporter expression driven from these promoters occurs later in development than the downregulation of *En1* protein expression from V1 spinal cord interneurons. In particular, upregulation of YFP expression in the *Thy1* line occurs very late during embryogenesis and is not homogeneously upregulated in all V1 populations at the same time (for example, RCs are one of the last populations to express YFP in embryos; data not shown). To establish the adequacy of these lines to label postnatal V1-derived neurons we first compared the numbers and localization of neurons in the spinal cord that express the reporter proteins and compared them to an R26 line.

Tissue preparation

Mice of different postnatal ages (P0/1, P5, P10, P15, P20, and adult, i.e., P40 to 2 months) were deeply anesthetized with Nembutal or Euthasol (>50 mg/kg, intraperitoneally [i.p.]) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). After perfusion, the spinal cords were dissected and placed in fixative for 2–4 hours or overnight and then cryoprotected in 0.1 M PB (pH 7.4) with 15% or 30% sucrose and 0.01% sodium azide until the tissue was processed. All analyses were performed in lumbar segments 4 and 5 (L4–L5) unless otherwise noted. Most late postnatal (P10 to P20) and adult spinal cords were sectioned using a freezing sliding microtome (50- μ m thick) and processed free-floating. Due to the rather small size and fragile nature of neonatal mouse spinal cords, transverse sections of P1 and P5 spinal cords were obtained using a cryostat (20- μ m thick) and processed on slides.

Antibody and immunostaining characterization

The list of antibodies and some of their characteristics appears in Table 1. Antibodies against the nonmammalian reporter proteins green fluorescent proteins (GFP) and β -gal do not result in any immunostaining in spinal cord sections from animals that do not express these proteins. GFP antibodies extensively crossreact with YFP. In most of our tissues YFP

is the target protein recognized by GFP antibodies; therefore, we refer to this immunoreactivity as YFP throughout the text.

Marker antibodies

Vesicular glutamate transporter 1 (VGLUT1) antibody: The antibody used is a polyclonal antibody raised in guinea pig against the recombinant C-terminus region (104 aa) of the rat (see Table 1). This antibody labels a single band of appropriate molecular weight (≈ 62 kDa) in Western blots of synaptic vesicle fractions of rat brains (Synaptic Systems, Goettingen, Germany). In our spinal cord sections the laminar distribution of immunolabeled puncta (high densities in LIII–LV, LVII, and LIX; weaker densities in LI and LII and LVIII) is identical to that previously published by us (Alvarez et al., 2004; Mentis et al., 2006) and other authors using this or similar antibodies raised in different species (Todd et al., 2003; Oliveira et al., 2003).

Vesicular acetylcholine transporter (VACHT) antibodies: We used two polyclonal antibodies raised in guinea pig and goat, respectively, each against a similar, but not identical, sequence in the C-terminus (see Table 1). By Western blot the guinea pig antibody reacted with a single band in rat brain homogenates that corresponded to the size predicted for VACHT (Chemicon, Temecula, CA). Both antibodies produced the same well-described populations of immunoreactive puncta in the spinal cord that can be differentiated according to their size: large boutons around motoneuron cell bodies (i.e., C-terminals), medium-size boutons at the exit of motor axons in ventral rootlets (synapses from motor axon recurrent collaterals), and small boutons throughout the spinal cord with slightly higher density in the dorsal horn (cholinergic boutons from local interneurons). This pattern is similar to that previously reported by us (Alvarez et al., 1999) and many others (see, for example: Arvidsson et al., 1997; Hellstrom et al., 1999). In dual immunofluorescence preparations immunoreactivity for these antibodies perfectly colocalized with each other (Valerie Siembab, unpubl. obs.). Moreover, dual immunofluorescence between guinea pig anti-VACHT and a choline acetyl-transferase antibody raised in goat (ChAT, dilution 1:1,000, Millipore, Bedford, MA; Cat. no. 144p; lot LV1450147) showed that all VACHT-immunoreactive (IR) neurons in the spinal cord were also positive to ChAT, as expected given the coregulation of both mRNA transcripts from a single locus (Erickson et al., 1996). Also as expected from the localization of VACHT to synaptic vesicles, VACHT immunolabeling of cell bodies was weaker than ChAT, but stronger in the cholinergic terminals (V. Siembab, unpubl. obs.). We therefore conclude that both VACHT immunolabelings are specific to cholinergic synapses in our spinal cord sections.

Calbindin antibodies: A mouse monoclonal antibody and a polyclonal antibody raised in rabbit were used. Both antibodies detect a single 28-kDa band in Western blots of brain homogenates of tissue originating from several species including rat, rabbit, guinea pig, mouse, chicken, and zebrafish and do not stain any structures in the brains of calbindin-D28k knockout mice (Swant, Bellinzona, Switzerland; Rabbit Calbindin (CB38), Mouse Calbindin (C300)). Staining of spinal cord sections with these antibodies immunostained the same cells (i.e., high density of cell bodies in upper lamina II–III and in the RCs ventrally) that are well described by us and many others (reviewed in Ren and Ruda, 1994; Alvarez and Fyffe, 2007; Watson et al., 2009).

Parvalbumin antibody: We used a mouse monoclonal antibody (see Table 1) that recognizes a 12-kDa protein in Western blots of mouse brain lysates (Chemicon). The staining pattern in this study was similar to the patterns of parvalbumin expression reported by us (Alvarez et al., 2004, 2005; Mentis et al., 2006) and by many others (review by Ren and Ruda, 1994) using a variety of antibodies in both neonates and adult. Parvalbumin

immunoreactivity in the adult is mainly localized in interneurons distributed at low density throughout the ventral horn and concentrated at higher densities in medial LV and superficial laminae (see Ren and Ruda, 1994). In the neonate no spinal cord cells are immunostained, but only the axons and terminals arborizations of proprioceptors travelling in the dorsal roots and dorsal columns and ending mostly in LV, LVII, and LIX.

NeuN antibody: We used a mouse monoclonal antibody raised against neuronal nuclei. Only one monoclonal antibody exists (A60, Mullen et al., 1992) and the antibody product we used was obtained through Chemicon (see Table 1). This monoclonal antibody recognizes a double band of 46–58 kDa in Western blots of mouse brain nuclear extracts (Mullen et al., 1992), which recently were shown to correspond to the splicing factor Fox-3 (Kim et al., 2009). In whole brain Western blots it additionally recognizes a band of 70 kDa, identified as synapsin I, but this crossreactivity disappears with paraformaldehyde fixation (Kim et al., 2009) and is not apparent in immunostained sections. Our immunostaining of neuronal nuclei and cell bodies in the spinal cord is identical to that reported previously by us (Alvarez et al., 2004, 2005) and that reported in spinal cord atlases (Watson et al., 2009).

Comparison of V1 lineage labeling in all three mice lines

One *En1-Cre/R26-LacZ*, one *En1-Cre/Tau-mEGFP-nlsLacZ*, and two *En1-Cre/Thy1-YFP* P40 animals were used to compare V1 genetic labeling in the different lines. The spinal cord sections were blocked with 10% normal donkey serum diluted in 0.01 M PBS with 0.3% Triton X-100 (PBS-T 0.3%; pH 7.4) and then immunolabeled for neuronal nuclear protein (NeuN; mouse monoclonal antibody, diluted 1:1,000, Chemicon) and one or the other reporter. YFP-IR was enhanced using a polyclonal sheep antibody against GFP that crossreacts with YFP (diluted 1:1,000 Biogenesis, Brentwood, NH) and lacZ expression revealed with chicken polyclonal antibodies against β -gal (1:1,000, AbCam, Cambridge, MA). The sections were incubated overnight at 4°C in primary antibodies mixtures and the next day the immunoreactive sites revealed using fluorochrome-conjugated secondary antibodies (FITC for YFP- or β -gal-IR; Cy3 for NeuN-IR) raised in donkey (1:50 dilution in PBS-T; Jackson Laboratories, West Grove, PA). The sections were coverslipped in Vectashield (Vector Labs, Burlingame, CA) and analyzed with a BX50 Olympus microscope with a motorized stage (Ludl Electronics, Harborne, NY) and coupled to a Neurolucida system (Micro-BrightField, Colchester, VT). Cell counts and plots were done on “live” fluorescence images visualized with a digital color camera (Microfire CCD, Optronics, Goleta, CA) and using the meander scan option in Neurolucida to plot at high magnification all NeuN immunofluorescent cells within a predefined area of interest (the ventral horn) and analyze their YFP or β -gal content. To define the ventral horn boundaries the central canal was first outlined and a line perpendicular to the dorsoventral axis drawn from the dorsal tip of the central canal straight across toward the white matter. This line, although arbitrarily chosen, allowed us to consistently delineate a border between dorsal and ventral horns in all spinal cord sections independent of age or labeling. The ventral horn was then outlined along the border between the white matter and gray matter and the cells with the appropriate labels plotted within the enclosed area. We analyzed 5–13 ventral horn slices per animal in the lumbar 4 and 5 segments and estimated the number of NeuN-IR cells with or without YFP or β -gal and their locations. Only neuronal profiles with a complete nucleus were included in the counts. No differences were noted between the data from the two *En1-Cre/Thy1-YFP* animals and therefore they were pooled together in the final analyses. The average percentages obtained in each of the three reporter animals was compared using one-way analysis of variance (ANOVA) (SigmaStat v. 2.0, Jandel, Sausalito, CA). Significance in this and all following analyses was set at $P < 0.05$. Images for presentation (Fig. 2) were obtained in an Olympus FV1000 confocal microscope (Olympus Optical, Tokyo, Japan).

Developmental expression of calbindin and parvalbumin in V1-derived interneurons

The expression of calbindin and parvalbumin at different developmental ages was studied in *En1-Cre/R26-LacZ* mice. *En1^{cre/+};R26-LacZ* and *En1^{+/+};R26-LacZ* mice were compared to examine any possible effects of *engrailed-1* (*En1*) heterozygosis. *En1-Cre* animals contain a neomycin-insertion cassette carrying *cre* recombinase in exon 1 of the *En1* gene rendering the *cre*-carrying allele null. Spinal cords of P1, P5, P10, P15, P20, and adult (1 year old) were cryostat sectioned (20 μ m) and processed for immunohistochemistry on slides. Spinal cords from two to three animals were studied for each age and genotype. The slides were blocked with normal donkey serum and processed for dual immunofluorescence using rabbit polyclonal antibodies against β -gal (1:10,000, Cappel, Malvern, PA) combined with mouse monoclonal antibodies against either calbindin (calbindin D-28K 1:500–1,000, Swant), parvalbumin (1:500, Swant) or NeuN (1:500 or 1,000, Chemicon). *En1^{+/+};R26-LacZ* mice were dual immunolabeled with the same mouse monoclonal parvalbumin antibody as before, combined with a rabbit polyclonal antibody against calbindin (1:1,000, Swant). Immunoreactivities were revealed using secondary antibodies coupled to Cy3 or FITC (1:50; Jackson Laboratories). The numbers of neuronal profiles with NeuN-IR, calbindin-IR, parvalbumin-IR, β -gal-IR, or colocalizing two of these markers were counted on digital images obtained with an Olympus BX60 epifluorescence microscope coupled to a Spot2 digital camera (Diagnostic Instruments, Sterling Heights, MI). The average number of cells per ventral horn expressing one or both markers was calculated from 10 ventral horn slices in each animal. Data from all the animals of the same age and genotype were averaged ($n = 20$ –30 ventral horns per data point). From these data we calculated the average proportions and numbers of β -gal-IR neurons at different postnatal ages and the expression of calbindin or parvalbumin-IR in V1-derived (β -gal-IR) and non-V1-derived INs (β -gal-negative). To analyze the dorsoventral location of immunoreactive cells the sections were reanalyzed in 100 μ m distance bins, being the first bin positioned at the ventralmost border between gray and white matter (see Alvarez et al., 2005). The numbers and percentages of single- or dual-labeled profiles across ages were compared using one-way ANOVAs (SigmaStat, v. 2.0). Fluorescence images shown in Fig. 3 were obtained with an Olympus FX confocal microscope.

Confocal analysis of VGLUT1 and VAcHT contact densities on YFP-IR Ia inhibitory interneurons and calbindin-IR Renshaw cells

Immunolabeling for VGLUT1 and VAcHT were respectively used to identify synapses from primary sensory afferents and putative cholinergic motor axons. The developmental maturation of VGLUT1 and VAcHT contacts on V1-derived Ia inhibitory interneurons (V1-IaINs) and RCs was studied using *En1-Cre/Thy1-YFP* mice. Spinal cords were analyzed at P0/1, P5, P10, P15, P20, and P40 ages. Sections were immunolabeled for YFP, calbindin, and either VGLUT1 or VAcHT. Tissue sections were blocked as before and incubated in the following primary antibodies: a polyclonal sheep antibody against GFP (1:1,000 Biogenesis) combined with polyclonal rabbit antibodies against calbindin (1:2,000, Swant) and guinea pig polyclonal antibodies against either VAcHT (1:1,000, Chemicon) or VGLUT1 (1:1,000, Synaptic Systems). Immunoreactive sites were revealed with fluorochrome-conjugated (FITC, Cy3 or Cy5) donkey species-specific secondary antibodies (1:50; Jackson Laboratories) and coverslipped as above.

For each of the two analyses (VGLUT1 or VAcHT contact densities) we included ≈ 10 RCs and 10 V1-IaINs per animal in spinal cord sections from lumbar 4 and 5 segments ($n = 2$ animals at each age except for P5 and P0/1, in which only one animal was analyzed). Imaging was done using an Olympus FV1000 confocal microscope (excitation: argon laser, 488 nm, krypton, 568 nm, and HeNe 635 nm). Triple-color immunofluorescent preparations were first imaged at low magnification (20 $\times$). From these low-magnification images a

sample of cells was selected being careful to include cells in which significant portions of the dendritic arbor was contained within the section. These cells were then reimaged using a 60× oil objective (N.A. 1.35) magnified 1.5× and a series of confocal optical sections (z-step 0.5 μm) obtained throughout their cell bodies and dendrites. The stacks of images were then loaded in Neurolucida and analyzed using the “neuron tracing” module. Dendritic arbors were reconstructed in 3D and VGLUT1 or VACHT synaptic contacts plotted on the reconstructed dendrites. From these reconstructions we estimated dendritic arbor morphology, synaptic densities and the distribution of synaptic contacts. Sholl analysis was performed in 50-μm increments from the cell soma in all cells (RCs and V1-IaINs) for both VGLUT1 and VACHT contacts and at all ages. Densities of contacts were calculated as the number of contacts per 10 μm of linear dendrite and compared across cell type at each age using one-way ANOVAs (Sigma Stat v. 2.0).

Estimates of VGLUT1 and VACHT synaptic densities on the cells somata were obtained by counting all contacts found on the cell bodies and estimating surface areas from ellipsoids with diameters equal to the maximum and minimum diameters calculated in the 2D projection of the whole cell body. Densities and statistical comparisons were obtained as above.

In some sections from P10 tissue we combined GFP, calbindin, and VGLUT1 antibodies with a mouse monoclonal antibody against parvalbumin (1:1,000, Chemicon) to identify proprioceptive sensory afferent axons. In these sections, calbindin-immunoreactive sites were revealed using a donkey biotinylated antirabbit IgG secondary antibody (diluted 1:100, Jackson Laboratories) followed by Alexa-405-conjugated streptavidin (Molecular Probes, Eugene, OR). These sections were used to confirm that VGLUT1 synapses arise from parvalbumin expressing proprioceptive primary afferents. High magnification (60×2) confocal image stacks of VGLUT1 contacts on five RCs (total 177 contacts) and five IaINs (total 215 contacts) were analyzed for parvalbumin content. Each VGLUT1-IR contact was outlined using Olympus Fluoview software to measure its average parvalbumin-immunofluorescence intensity. The traced outlines were kept in place while the image was moved up or down 3 μm in the z-axis to estimate background fluorescence from the adjacent neuropil. VGLUT1-IR contacts were considered positive when parvalbumin fluorescence was 2 standard deviations (SDs) above average neuropil background fluorescence. The average percentage of VGLUT1 contacts containing parvalbumin and their average parvalbumin/VGLUT1 fluorescence intensity was compared using t-tests between contacts on RCs and V1-IaINs.

Ventral root tracings in the neonatal spinal cord

To confirm the motor axon origins of VACHT-IR terminals, motor axon recurrent collaterals were retrogradely labeled from the fifth lumbar ventral root using Cascade Blue coupled dextrans (CB-Dxt; 10,000 MW; Invitrogen, Carlsbad, CA). One P7 *En1-Cre/Thy1-YFP* mouse was deeply anesthetized with Nembutal (50 mg/kg, i.p.) and after decapitation the spinal cord was quickly removed, pinned down to a sylgard base, and superfused with cold oxygenated ACSF (~18°C). The ventral root was placed in a suction electrode and backfilled with (Cascade Blue) CB-Dxt. Labeling was allowed to proceed overnight, after which the spinal cord was placed in 4% paraformaldehyde in 0.01 M PB for 4 hours and then placed in cryoprotectant. Fifty-micron-thick transverse frozen sections were obtained and immunolabeled with polyclonal rabbit antibodies against calbindin (1:2,000, Swant) and polyclonal goat antibodies against VACHT (1:500, Chemicon). Immunoreactive sites were revealed with Cy5- or Cy3-conjugated secondary antibodies as before. YFP was observed “naked” (i.e., without antibody enhancement) in these preparations. Immunofluorescent preparations were confocal imaged in the Olympus FV1000. Naked YFP was excited with the 515 nm argon line. Because the design of optical filter paths in the instrument, Cy3

fluorescence (excited with 568 nm krypton line) was imaged independently and then merged. All VACHT-IR contacts on 25 YFP expressing RCs (total 190 contacts) and 27 V1-IaINs (total 162 contacts) located in lumbar segments 4 and 5 were imaged at 60× to confirm CB-Dxt fluorescence. The percentage of retrogradely labeled VACHT-IR contacts on each cell type were compared using *t*-tests.

Analysis of V1-derived IaINs in upper and lower lumbar segments

In this work we defined IaINs as neurons receiving dense projections from calbindin-IR V1 axons and therefore most likely originated in RCs. In previous work we noted that these cells are parvalbumin-positive in the mature spinal cord (Alvarez et al., 2005). However, not all parvalbumin-IR interneurons with dense input from calbindin-IR RC axons were labeled by the V1 genetic markers. To study the proportion of IaINs (defined by their RC inputs) that express V1 lineage markers we used one *En1-Cre/Thy1-YFP* and three *En1-Cre/Tau-nls lacZ* P15 animals. The sections were triple immunolabeled for calbindin (rabbit polyclonal, Swant), parvalbumin (mouse monoclonal, Swant), and YFP (sheep polyclonal, 1:1,000, Biogenesis) or β -gal (chicken polyclonal, 1,1000, AbCam) as described above. We analyzed with confocal microscopy (Olympus FV1000) at medium magnification (20×) sections from lower (L4 and L5) and upper (L1 and L2) lumbar segments. Five to 14 ventral horns were analyzed per animal for a total of 187 putative parvalbumin-IR IaINs analyzed in *En1-Cre/Thy1-YFP* and 1,338 in *En1-Cre/Tau-nls lacZ* animals, 667 in lower lumbar, and 671 in upper lumbar. The percentages of parvalbumin-IR IaINs expressing lineage markers was calculated in each animal and their average ($n = 3$ animals) compared between upper and lower lumbar segments using *t*-tests.

Figure composition

Figures were composed using CorelDraw (v. 12.0). Graphs were composed in SigmaPlot (v. 9.0, Jandel). Microscope image modifications for presentation such as adjusting contrast and brightness were carried out in Image Pro Plus (v. 5.0 Media Cybernetics, Bethesda, MD) and always preserved all the information content of the images. Some images were sharpened using a “high gauss” filter. Quantification was always carried out in original unprocessed images. Images containing only green and red are also supplied as supplemental figures using magenta-green colors for better visualization by readers with color-impaired vision. Triple and quadruple immunolabeled images could not be transferred into this palette without loss of image clarity.

RESULTS

Animal models for lineage labeling V1-interneurons in the postnatal spinal cord

The *En1* transcription factor defines V1-interneurons but is expressed only in a restricted temporal window during early embryogenesis (Saueressig et al., 1999). To identify V1-derived interneurons (V1-INs) in postnatal animals we therefore used the Cre-lox conditional system to obtain permanent expression of reporter labels (lacZ, YFP) in the V1 cell lineage. For this purpose we established three mouse lines derived from the crossing of *En1^{Cre/+}* mice (*En1-Cre*; Sapir et al., 2004) with different conditional reporter mice. Originally we used *R26-lacZ* reporter mice (Soriano et al., 1999) to label the cellular lineage of V1 interneurons (Sapir et al., 2004; Alvarez et al., 2005). There was a very good correlation between the expression of the endogenous *En1* protein and reporter labeling at E11.5 (Sapir et al., 2004). However, labeling of dendrites, axons, and cellular surfaces is limited in this animal, hampering detailed study of synaptic connections. Two additional reporter mice were therefore used. The *Tau-mEGFP-nls lacZ* reporter line (Hippenmeyer et al., 2005) expresses from the *Tau* locus and upon Cre-recombination β -gal with a nuclear localization signal (nls) and aEGFP variant, mEGFP, designed to best depict cellular

surfaces (DePaola et al., 2003). The second line, *Thy1-YFP* (Buffeli et al., 2003), carries several copies of a transgene that expresses YFP upon Cre recombination. The characteristics of the *Thy1* promoter resulted in mosaicism of reporter expression in other neuronal populations (Feng et al., 2000). As a first step we compared V1-IN labeling in these three animal models. Unfortunately, reporter gene expression in these two new lines is upregulated after the period of maximal expression of the *En1* protein in embryo (data not shown); therefore, we first compared the numbers and localization of labeled cells in these new lines with the previously characterized *R26-lacZ* line.

Dual immunolabeling with the neuronal marker NeuN was used for laminar identification and to estimate the proportion of ventral horn interneurons labeled in each animal (Fig. 2A–C). In *En1-Cre/R26-lacZ* and *En1-Cre/Tau-mEGFP-nls lacZ* mice β -gal-IR was mainly restricted to the nucleus with additional labeling of small intracytoplasmic inclusions in *En1-Cre/R26-lacZ* mice (insets in Fig. 2A1,B1). Nuclear β -gal-IR was more intense in *En1-Cre/Tau-mEGFP-nls lacZ* animals compared to *En1-Cre/R26-lacZ*. Additionally, in *En1-Cre/Tau-mEGFP-nls lacZ* mice mEGFP was preferentially targeted to axons (not shown) and could not be used to depict dendritic arbors. The distribution of V1 mEGFP-IR axons was similar to that previously described using a *En1-Cre/R26-GAP43-EGFP* reporter mouse (Alvarez et al., 2005) and to axonal labeling in *En1-Cre/Thy1-YFP* mice. YFP (visualized “naked” or amplified using an anti-GFP antibody, see Materials and Methods) filled the cell bodies, dendrites, and axons of V1-derived interneurons in *En1-Cre/Thy1-YFP* mice (Fig. 2C1–2,E). In all three animal models, labeled V1-INs in lumbar 4 and 5 spinal cord segments were mostly located laterally in lamina VII (LVII) forming a dorsoventral arc surrounding lamina IX (LIX) motoneurons. In *En1-Cre/Thy1-YFP* mice V1 axons are present in the lateral and ventral funiculi and project throughout the ventral horn with lower density in lamina VIII and highest in LIX (Fig. 2C1). In the *En1-Cre/Tau-mEGFP-nls lacZ* and *En1-Cre/Thy1-YFP* mice we observed a few labeled interneurons in the dorsal horn with some variability between animals, segmental levels, and sections. Their significance is presently unknown.

To compare the efficiency of V1-IN labeling in each mouse line we used Neurolucida plots to determine the percent and locations of labeled and unlabeled ventral horn NeuN-IR interneurons with and without V1-lineage markers (Fig. 2A3,B3,C3). The boundary between ventral and dorsal horns was arbitrarily set from the dorsal tip of the central canal. The percentage of NeuN-IR neurons with V1-lineage markers was not significantly different between *En1-Cre/R26-lacZ* ($24.5 \pm 1.49\%$) ($\pm$ SEM) and *En1-Cre/Tau-mEGFP-nls lacZ* ($23.3 \pm 1.45\%$; $P = 0.203$, t -test), but in both these animal genotypes the percentage was larger than in *En1-Cre/Thy1-YFP* mice ($18 \pm 1.23\%$; $P < 0.001$, one-way ANOVA, $P < 0.05$, post-hoc Dunn’s tests). Therefore, $\approx 25\%$ fewer V1-INs are labeled in *En1-Cre/Thy1-YFP* mice. Optimal visualization of somatic and dendritic surfaces was however, obtained in *En1-Cre/Thy1-YFP* mice (Fig. 2E); thus, we used this animal model to analyze postnatal development of synaptic connectivity.

Developmental regulation of calbindin and parvalbumin expression in V1-derived interneurons

V1-derived RCs and IaINs were previously identified in mature spinal cords using criteria based on their differential expression of the calcium buffering proteins calbindin (CB) and parvalbumin (PV) (Alvarez et al., 2005). Mature RCs display strong CB-IR and weak PV-IR, while some V1-derived IaINs express PV only. However, CB and PV expression in spinal cord are both developmentally regulated (Zhang et al., 1990), raising questions about the adequacy of these markers in neonatal spinal cords. To confirm the presence of these markers in the neurons and postnatal period of interest we analyzed in *En1-Cre/R26-lacZ* mice the development of CB and PV expression in V1-INs (Fig. 3). First, we confirmed that

there are no changes in the number and localization of V1-INs during postnatal development. Since cellular density decreases with development as neuropil (the area occupied by dendrites, axons, synapses, and glia) increases in size, we normalized at different ages the percentages of V1-IN profiles to the total number of NeuN-IR ventral interneurons detected per section. Overall, V1-INs represent 20–30% of all NeuN-IR ventral interneurons from P1 to adult with no significant differences among different ages. Their distribution was analyzed by comparing cell numbers in 100- μ m thick dorsoventral bins (see Alvarez et al., 2005). The percent of V1-INs to all NeuN-IR cells remained relatively constant in all bins ($P = 0.13$, one-way ANOVA) except the ventral-most 100 μ m ($P < 0.01$, one-way ANOVA) that showed progressive enrichment in V1-INs from P1 to P10. This region is occupied by motor axons exiting the spinal cord and their postnatal growth expands the neuropil and, hence, results in a larger than normal developmental reduction in neuronal density in this region. V1-derived RCs are selectively retained in this area while non-RCs become progressively excluded, explaining the enrichment in V1s during postnatal growth.

Calbindin and parvalbumin expression were differentially regulated during development. In general, CB is downregulated from ventral interneurons while PV becomes upregulated (Fig. 3A–D). *En1^{Cre/+}* heterozygosity did not affect the developmental regulation of calcium-buffering protein expression in ventral horn neurons (Fig. 3B1,D1). Interestingly calbindin-IR was specifically retained in RCs during postnatal development while its expression decreased in other V1-INs, similar to the overall pattern in the ventral horn (Fig. 3B2). Thus, CB-IR was downregulated after P5 in V1-INs located in all dorsoventral bins except for the first 100 μ m containing RCs (Fig. 3B3). PV-IR INs were not present at P1 and were rare at P5. At these ages PV-IR in the spinal cord is restricted to proprioceptive sensory afferent axons (Fig. 3C1). PV expression in ventral horn INs and V1-INs is simultaneously upregulated after P5 (Fig. 3C2–3,3D2). In general, PV upregulation appears slightly faster in V1-INs located in the first 100 μ m bin corresponding to RCs (Fig. 3D3), although PV-IR in RCs is comparatively less intense than in other PV-IR INs.

In conclusion, calbindin-IR is an adequate marker for RCs throughout postnatal development; however, parvalbumin-IR is not expressed in putative IaINs until after the first postnatal week.

Identification of RCs and V1-IaINs in *En1-Cre/Thy1-YFP* mice; not all Ia inhibitory interneurons are V1-derived

Renshaw cells were identified in *En1-Cre/Thy1-YFP* mice by their ventral location in the “Renshaw cell area,” their relatively intense CB-IR throughout development and V1-derivation (Fig. 4A1; see Alvarez and Fyffe, 2007, for a critical review of these criteria).

Criteria for IaIN identification in histological sections was designed to parallel electrophysiological criteria (Alvarez et al., 1997,2005). Ia inhibitory interneurons are the only known ventral interneurons strongly modulated by RC inputs (Hultborn et al., 1971; Wang et al., 2008) and therefore convergence of RC and Ia sensory inputs reliably defines IaINs involved in reciprocal inhibition, at least in lumbar segments (Jankowska, 1992). Therefore, V1-derived IaINs (V1-IaINs) were recognized in this study as YFP V1-INs receiving a dense pericellular basket innervation from RC axons. RC axons were identified by their CB-IR and V1-origins (YFP-IR) (Fig. 4). Accurate quantification of these pericellular baskets was unreliable because their density and structure is highly variable from cell to cell, depending on their location with respect to regions containing different densities of RC axons; however, as shown in the figures they are relatively easy to identify over many neurons. We included in our sample only cells displaying very clear pericellular baskets. All neurons surrounded by these RC axons also received convergent inputs from

primary afferents labeled by VGLUT1 and/or parvalbumin immunoreactivity. YFP-labeled V1-INs with these characteristics can be recognized from P0 and 25% of them express strong PV-IR by P15.

We also found that not all PV-IR IaINs identified at P15 by these criteria were V1-derived. In P15 *En1-Cre/Thy1-YFP* lower lumbar segments (L4–L5) only 59% of 187 putative PV-IR IaINs expressed YFP. As mentioned earlier, we expected labeling in 75% of V1-INs in *En1-Cre/Thy1-YFP* mice. Therefore, we repeated the analysis in *En1-Cre/Tau-mEGFP-nlslacZ* mice (Fig. 5). We also compared upper (L2–L3) and lower lumbar levels (L4–L5) to test possible differences at different rostrocaudal lumbar segments. In L4–L5, 68% of PV-IR cells receiving RC inputs ($n = 667$) were V1-derived (β -gal-IR). Significantly fewer (49%, $n = 671$; $P < 0.01$, t -test) were V1-derived in L2–L3 segments. These results suggest that not all neurons identified as putative IaINs according to their RC input are V1-derived. This conclusion agrees with a recent report suggesting that Ia reciprocal inhibition between quadriceps and posterior biceps motoneurons is functional in E18 and P0 *Pax6^{-/-}* mice (Wang et al., 2008) which lack V1-interneurons (Burrill et al., 1997). Taken together, the results suggest that, in contrast to lumbar RCs, lumbar IaINs are a heterogeneous population and originate from more than one type of embryonic precursor.

In summary, IaINs appear to be not uniform regarding their embryonic origins. The group of IaINs we focus on this study is the lower lumbar V1-IaINs that can be recognized by pericellular baskets of V1 CB-IR axons. Other populations of IaINs, either non-V1-derived or V1-derived but with a lesser RC input are therefore not included in the study.

VGLUT1-IR synaptic contacts label proprioceptive sensory inputs on V1-IaINs and Renshaw cells

VGLUT1-IR was used as a marker of sensory afferent synapses (Todd et al., 2003; Oliveira et al., 2003; Alvarez et al., 2004; Mentis et al., 2006) on developing V1-IaINs and RCs in *En1-Cre/Thy1-YFP* mice. However, some ventral horn VGLUT1-IR synapses might originate from the corticospinal tract (Alvarez et al., 2004; Persson et al., 2006) or perhaps other yet unknown descending projections. To confirm that VGLUT1-IR contacts on V1-IaINs and RCs originate from sensory afferents we analyzed their colocalization with parvalbumin, which labels proprioceptive sensory afferents in neonatal spinal cords (Arber et al., 2000). To diminish confounding effects from interneuron parvalbumin axons we performed the analysis at P10. Colocalization of PV inside VGLUT1-IR clusters was determined as PV-immunofluorescence intensity 2 SDs above background. At P10, 82% and 85% of VGLUT1-IR contacts, sampled respectively on five RCs ($n = 177$ This contacts) and five V1-IaINs ($n = 215$), contained PV. small difference was not significant ($P = 0.587$, t -test). Similarly, no differences were detected on average PV immunofluorescence intensity inside P10 VGLUT1-IR contacts on each cell type (1701.69 ± 591.61 on V1-IaINs and 1600.73 ± 707.27 on RCs; average $\pm$ SD, arbitrary units from 24 bit images). The remaining 15–18% of PV-negative boutons could originate from other sources, but other explanations are also possible. They might result in part because of PV underestimation with our stringent criteria (for example, 92% of boutons in both cells were labeled at 1 SD above background) or might represent primary afferent boutons that started to downregulate their PV-content (note that primary afferent synaptic boutons in the ventral horn downregulate PV with development, see Fig. 3C). These alternative possibilities were not pursued further in our study. We can conclude, however, that the large majority of VGLUT1-IR synaptic inputs on V1-IaINs and RCs originate from proprioceptive sensory afferents. This conclusion is also supported by recent results reported by Betley et al. (2009) in which using genetic labeling they found that less than 2% of VGLUT1 synapses in the ventral horn originated from the corticospinal tract.

VGLUT1-IR synaptic numbers are larger on V1-IaINs than on RCs throughout postnatal development

Because most VGLUT1-IR contacts target dendrites, densities of VGLUT1-IR synapses on YFP-labeled IaINs and RCs (Fig. 6) were analyzed using NeuroLucida-based reconstructions of the YFP-labeled dendritic trees. Within the 50- μ m-thick sections we were able to analyze transversely oriented dendrites up to 150–200 μ m distance from the cell body in most neurons. No significant differences were found between cells sampled from different animals and therefore all cells of the same type from two different animals in each age group were pooled together. Approximately 20 V1-IaINs and 20 V1-RCs were analyzed at each age from P5 to adulthood (P10, P15, P20, and adult (P40)); at P5 only 10 cells of each type were analyzed and only in one animal). Synaptic densities were calculated as VGLUT1-IR contacts per 10 μ m of linear dendrite (Fig. 7A,B). At P5 V1-IaINs already displayed a larger number and density of VGLUT1-IR contacts on their dendrites compared to RCs. With postnatal development VGLUT1-IR contact density on V1-IaINs increased further ($P < 0.001$, one-way ANOVA; Fig. 7C). Post-hoc tests indicated that the most significant increase occurred from P10 to P15 ($P < 0.05$, post-hoc Dunn's test) and no differences in density were detected between P15, P20, and adult ($P = 0.488$, one-way ANOVA). Estimation of VGLUT1-IR contact density on developing YFP-labeled RCs sampled in the same sections as V1-IaINs replicated our previous findings on CB-IR RCs (Mentis et al., 2006). VGLUT1-IR contact density significantly increased in these cells from P5 to P15 and then significantly decreased in P20 and adult ($P < 0.001$, one-way ANOVA; VGLUT1-IR density at P15 was significantly higher than in all other ages $P < 0.05$, post-hoc Dunn's test). When compared to V1-IaINs, VGLUT1-IR density was significantly lower in RCs at all ages (P5 $P = 0.005$; P10 $P < 0.001$; P15 $P = 0.024$; P20 $P < 0.001$; P40 $P < 0.001$, *t*-tests) (Fig. 7C). Interestingly, a significant number of VGLUT1-IR contacts target the somata of V1-IaINs but not of RCs. Approximately 20% of all VGLUT1-IR boutons sampled on V1-IaINs from P15 to adult contact the cell body, while this percentage is lower for RCs (<5%). Differences in contact numbers and density between V1-IaINs and RCs were therefore large when comparing the cell bodies of adult (P40) cells (Fig. 7D).

We attempted to study V1-derived IaINs at P0/P1 but consistent VGLUT1-IR at this age was only obtained with an alternative antibody raised in rabbit (see Mentis et al., 2006). The most likely explanation is sensitivity differences between both antibodies being VGLUT1 expressed at low levels in the spinal cord of newborns and dramatically upregulated after birth (Mentis et al., 2006). Unfortunately, rabbit VGLUT1 antibodies could not be combined with the calbindin rabbit antibodies used in this study for IaIN identification (mouse monoclonal antibodies against calbindin labeled RC axons weakly). Nonetheless, in a previous study we found no significant difference in VGLUT1 contacts on RCs between P1 and P5 and, similarly, few differences were observed in VACHT contacts on RCs or IaINs between these two ages in this study. It appears that the peak in postnatal synaptogenesis occurs after P5.

The possibility that differences exist in the proximodistal distributions of VGLUT1-IR boutons on dendrites was investigated using Sholl analysis. The dendritic arbor was divided in three proximodistal compartments that were well visualized with YFP labeling in the majority of cells (0–50 μ m, 50–100 μ m, and 100–150 μ m) (Fig. 7E). A higher percentage of VGLUT1-IR boutons were located in the first 50 μ m of dendrite and cell body on V1-IaINs (70%) than on RCs (52%) (Fig. 7F). Despite this predominant proximal location no significant differences in density were detected ($P > 0.05$, one-way ANOVAs; Fig. 7E) because the larger number of dendritic segments and branches found proximally in both cell types. At each distance-bin VGLUT1-IR contact density on V1-IaINs was always significantly higher compared to RCs ($P < 0.001$, *t*-tests), although the differences became smaller with increasing distance from the cell body.

In addition to differences in density and proximodistal location we also found differences in the development of VGLUT1-IR cluster sizes (Table 2). VGLUT1-IR clusters represent the labeling of presynaptic vesicles and therefore differences in their apparent size likely reflect differences in presynaptic vesicle pools. VGLUT1-IR clusters increase similarly in size on both cell types from P5 to P10, but they are significantly larger on V1-IaIN dendrites and somata by P15 (IaINs: 4.5 ± 1.7 and RCs: $4.0 \pm 1.3 \mu\text{m}^2$, average $\pm$ SD; $P = 0.003$, t -test). After P15 VGLUT1-IR cluster size continues to increase on V1-IaINs, while on RCs they become slightly smaller (Table 2).

In summary, V1-IaINs receive a higher density of VGLUT1-IR contacts than RCs at any postnatal age. In addition, after P15 the contact density is maintained on developing V1-IaINs while it decreases on RCs. The larger size, higher density, and more proximal locations of VGLUT1-IR contacts on V1-IaINs suggest a higher input strength on these cells compared to RCs.

VACHT-IR bouton density on V1-IaINs is lower than on RCs of all ages and preferentially target the cell soma and initial segment of primary dendrites

VACHT, which packages acetylcholine into synaptic vesicles for exocytosis, is an effective synaptic marker of motor axon synapses on RCs but there are additional sources of VACHT-IR synapses in the ventral horn (Alvarez et al., 1999; Mentis et al., 2005; Miles et al., 2007; Zagoraiou et al., 2009). Some VACHT-IR contacts were observed on V1-IaIN dendrites at P20 (Alvarez et al., 2005); therefore, we investigated their origins and the possibility that this input, similar to the VGLUT1 input on RCs, was initially stronger in neonatal V1-IaINs and then deselected (Fig. 8A–C). We analyzed the distribution and density of VACHT-IR contacts on 20 V1-IaINs and 20 RCs, pooled from two animals at each age from P10 to P40 and 10 cells from a single animal at P0/1 and P5. Neurolucida analysis of the traced neurons (Fig. 9A,B) indicated that VACHT-IR contacts were sparse on the dendrites of V1-IaINs of any age and their density was always much lower than on RCs ($P < 0.001$, t -tests paired comparisons at each age) (Fig. 9C). In addition, VACHT-IR contact density on V1-IaIN dendrites significantly decreased from P0/1 to P15 ($P < 0.05$, one-way ANOVA). VACHT-IR contact density on RC dendrites was always much larger, increased significantly from P0/1 to P20 ($P < 0.05$, one-way ANOVA), and was maintained in the adult (P40).

Surprisingly, the somata of V1-IaINs received a 3-fold larger number of VACHT-IR contacts compared to RCs ($P < 0.001$, t -test). Indeed, 44% of all VACHT-IR contacts sampled on V1-IaINs target the cell body; in contrast, RC somata received less than 5% of the total VACHT-IR input. V1-IaIN somata are larger than RC somata; nevertheless, the larger number of boutons represents a significant higher VACHT-IR contact density on V1-IaINs compared to RCs ($P < 0.001$, t -test) (Fig. 9D). Most VACHT-IR contacts on RCs ($\approx 89\%$) were located in the first 100 μm of dendrite (Fig. 9F), while VACHT-IR contacts on V1-IaINs were located on the soma and together with the first dendritic compartment (first 50 μm) they account for 82% of all VACHT-IR inputs on V1-IaINs. Other than in one V1-IaIN with one VACHT-IR contact, no contacts were found at distances on the dendrite beyond 100 μm from the cell body. In conclusion, V1-IaINs display a proximal cholinergic input that preferentially targets the cell soma while cholinergic synapses on RCs target dendritic locations.

Further differences were found on the size of VACHT-IR clusters on each cell type. The average size of VACHT-IR clusters contacting mature P20 V1-IaINs was one-third the size of those contacting P20 RCs (1.3 ± 0.4 and $3.7 \pm 1.3 \mu\text{m}^2$, respectively; $P < 0.001$, t -test). During postnatal development VACHT-IR clusters on RCs are initially larger than those on IaINs and then progressively increase in size during postnatal maturation, while those on IaINs decrease in size (Table 3). VACHT-IR synapses of different sizes have been previously proposed to originate from different sources (Alvarez et al., 1999). To investigate

if VACHT-IR terminals on V1-IaINs were of motor axon origin, Cascade Blue fluorescent dextran was applied to the L5 ventral root of a P7 *En1-Cre/Thyl-YFP* mouse and the percentages of retrogradely labeled VACHT-IR boutons on V1-IaINs and CB-IR RCs estimated (Fig. 10A–D). The large majority of VACHT-IR boutons on CB-IR RCs (80.2%, $n = 190$ boutons) contained retrograde labeling. This is an impressively high percentage, considering that some motor axon inputs on RCs might enter through adjacent unlabeled roots and that the efficiency of retrograde labeling of motoneuron somata is at best 80–85% using ventral root fills (GZ Mentis, unpubl. obs.). In contrast, only 6.9% of VACHT-IR boutons ($n = 162$ boutons) on V1-IaINs were labeled. These analyses were not repeated at more mature ages because the efficiency of retrograde labeling dramatically decreases after P10 (discussed in Mentis et al., 2006). To investigate whether cholinergic terminals on IaINs arise from cholinergic Pitx2 (V0c) interneurons, which also give rise to the C-terminals associated with motoneurons (Zagoraïou et al., 2009), we analyzed sections from P15 dual *pitx2::cre* and *En1::cre* animals crossed to the Tau-lacZ-mEGFP reporter line. In these sections the cell nuclei and axons of both Pitx2 and En1 (V1) cells are labeled by lacZ and mEGFP (Fig. 10E1). Since we did not detect any cholinergic synapses originating from V1 axons in *En1::cre* animals we assumed that EGFP-labeled VACHT-IR boutons in double *pitx2::cre* and *En1::cre* animals must come from the Pitx2 (V0c) cells. In lumbar 5 we analyzed 167 VACHT-IR contacts on eight V1-RCs and only seven (4.2%) contained EGFP. In contrast, 89.1% of 46 VACHT-IR contacts sampled on 10 PV-IR V1-IaINs were EGFP-positive (see examples in Fig. 10E–G).

In summary, V1-IaINs do not receive a significant VACHT-IR input on their distal dendrites. However, their somata and most proximal dendrites are targeted by small VACHT-IR boutons that originate from Pitx2 (V0c) neurons. In conclusion, cholinergic synapses on V1-IaINs differ from those on RCs in their origins and organization. These differences are already established in neonates and maintained into adulthood.

DISCUSSION

In this study we validated several mouse models to trace the lineage of V1 interneurons into their adult derivatives and confirmed histochemical criteria to identify developing RCs, IaINs, and synapses from proprioceptive and motor axons. Using these tools we describe the development of these inputs on V1-IaINs and compared them to RCs. Comparisons were made within the same sections that were triple or quadruple immunolabeled for simultaneous identification of both types of interneurons. In doing so, we provide the first analysis of the organization of proprioceptive synapses on IaINs and describe a novel cholinergic input on this cell type.

The results also allow us to reject the hypothesis that neonatal IaINs receive convergent inputs from motor axons and sensory afferents. Their synaptic organization and development is therefore distinct from that of neonatal RCs, which initially receive convergent inputs from both sources (Mentis et al., 2006). Interestingly, motor axons exhibited a great degree of specificity in their targeting, suggesting fundamental differences with proprioceptive axons in recognizing different classes of developing V1 interneurons. The data also show that although proprioceptive inputs initially target both interneurons, there is a clear bias toward V1-IaINs revealed by differences in the number, density, and location of VGLUT1 contacts. Then during postnatal maturation proprioceptive synapses proliferate more numerous on V1-IaINs. After P15 proprioceptive synaptic densities are maintained on V1-IaINs but not on RCs. Moreover, VGLUT1-IR presynaptic vesicle clusters on V1-IaINs grow significantly larger than those on RCs, in agreement with previous electron microscopy observations indicating that the synaptic apparatus of VGLUT1-IR boutons partially regresses on RC dendrites from P15 to adult (Mentis et al.,

2006). The compound effects of larger presynaptic vesicle clusters, higher density, and more proximal locations of proprioceptive synapses on IaINs, all suggest the maturation of Ia inputs of higher strength on IaINs compared to RCs. Indeed, Ia afferents elicit relatively large monosynaptic EPSPs on adult cat spinal IaINs and evoke firing in these neurons more consistently and effectively than on other interneurons, at both low and high stimulation frequencies (Hultborn et al., 1971, 1976; Jankowska, 1992). In contrast, similar studies in the cat concluded that adult RCs lack monosynaptic activation by Ia or any other dorsal root afferent (Renshaw, 1946; Curtis and Ryall, 1966; Ryall et al., 1972; but see also Frank and Fuortes, 1956). Thus, monosynaptic activation of RCs by sensory afferents appears to be restricted to neonates (Mentis et al., 2006) and at this stage this input already shows quantitative differences in bouton density compared to IaINs.

In addition, we found that cholinergic synapses on IaINs and RCs have different origins. Those on IaINs are mostly ($\approx 90\%$) originated from Pitx2 (V0c) cholinergic interneurons (Zagoraïou et al., 2009). Very few might have a motor axon origin. There are no known long descending cholinergic projections in the mouse spinal cord (VanderHorst and Ulfhake, 2006). A common source of cholinergic inputs in the somata of motoneurons and IaINs is consistent with the general rule of common inputs on IaINs and motoneurons sharing Ia afferent inputs from the same muscle (Jankowska, 1992). However, cholinergic synapses on IaINs likely display a different structure than on motoneurons since the cholinergic contacts observed here were much smaller than C-terminals and synaptic inputs with morphological characteristics of C-terminals have not been found on spinal interneurons (Johnson and Sears, 1988). Cholinergic inputs on IaINs most likely operate through muscarinic mechanisms since we found little evidence of postsynaptic nicotinic currents in ventral interneurons other than RCs (Gonzalez-Forero and Alvarez, unpubl. obs.; reviewed in Alvarez and Fyffe, 2007). Activation of muscarinic receptors on the somatodendritic surface of spinal motoneurons and dorsal horn inhibitory interneurons increase their excitability (Baba et al., 1998; Miles et al., 2007), but potential muscarinic effects have not yet been tested, to our knowledge, on IaINs.

The results therefore argue against the existence of “generic” embryonic V1 cells competent to receive similar complements of synaptic inputs, which are thereafter selected or deselected on individual interneuron subclasses. On the contrary, the results favor a model of significant input selectivity and organization before birth on different interneurons or even neuronal compartments (somata, proximal, or distal dendrites). Although in this model input selection is necessarily predetermined in embryo, nonetheless, significant strengthening or weakening of these synaptic inputs occurs postnatally (as indicated by changes in numbers, density, and sizes). It appears that mechanisms that differentially favor motor or proprioceptive synaptogenesis on different interneurons can be dissociated temporally from factors that developmentally modulate changes in input strengths. Both, however, are necessary for organizing effective inputs on interneurons. Avoidance of synapse formation or postnatal weakening both result in lack of functional inputs, as exemplified, respectively, by lack of monosynaptic excitation of IaINs by motor axons or RCs by proprioceptors. Postnatal modulation of synaptic strengths can result in significant circuit alterations. Disproportionate postnatal strengthening of Ia afferent central synapses, because of excessive supply of peripheral neurotrophin 3 (NT3), can overwhelm initial connectivity patterns and result in loss of specificity in the connections with motoneurons (Wang et al., 2007) or abnormal maintenance of high densities of VGLUT1 synapses on RCs (Mentis et al., 2010).

In summary, development of specific inputs on IaINs and RCs likely result from an interplay of postnatal maturation mechanisms that adjust proprioceptive and motor axon synaptic

densities on IaINs and RCs superimposed on (and likely influenced by) initial patterns of preferential synaptogenesis on each cell type.

Two phases of postnatal synaptic maturation and the ontogeny of motor development

Our results suggest two phases in the postnatal maturation of proprioceptive and motor axon inputs on IaINs and RCs. The first 2 postnatal weeks are characterized by synaptogenesis, with proprioceptive synapses proliferating on both IaINs and RCs and motor axons specifically on RCs. This period is followed at the end of the second week and into the third postnatal week by stabilization or weakening of specific inputs. The initial phase coincides with a period of continuous increase in motoneuron activity and the parallel maturation of the stretch reflex and increases in Ia afferent activity (Vejsada et al., 1985; Westerga and Gramsbergen, 1993, 1994; Brocard et al., 1999). This suggests a correlation between overall motor activity and synaptogenesis in the ventral horn. During the second postnatal week rodents gradually attain optimal postural control through extensor facilitation of all three hindlimb joints (hip, knee, ankle) allowing transition from crawling to weight-bearing locomotion (Altman and Sudarshan, 1975; Westerga and Gramsbergen, 1990; Brocard et al., 1999). This process starts around P8–P10 but locomotion remains rather immature and ataxic, displaying wide stance, un-coordination and tremor until P13–P14. By P15 locomotion becomes adult-like (Gramsbergen, 1998). Therefore, stabilization of synaptic densities from Ia afferents and motor axons, respectively on IaINs and RCs, and deselection of proprioceptive synapses on RCs coincides with the phase of adult-like locomotion. It is presently difficult to establish causal relationships between increased synaptic organization in premotor interneurons and the maturation of motor behavior because too little is known on motor network function at these stages of development. However, our data clearly demonstrate a temporal correlation between presynaptic input development on key premotor inhibitory interneurons and postnatal development of locomotion. In humans similar postural adjustments and maturation of walking occur during a longer period (first 18 months) and have been suggested to depend on strengthening of descending influences that coincide with and perhaps triggers the maturation of many segmental reflexes (reviewed in Altman and Bayer, 2001) including strengthening of recurrent and reciprocal inhibition of motoneurons (McDonough et al., 2001).

Neonatal V1-derived Ia inhibitory interneurons and the ontogeny of reciprocal inhibition

In mature spinal cords Ia inhibitory interneurons (IaINs) were previously identified histologically by PV expression and convergent RC and proprioceptive inputs, labeled respectively by CB and VGLUT1 (Alvarez et al., 2005). We confirmed here that CB and VGLUT1 axons converging on YFP-labeled neonatal V1-INs originate from V1-derived RCs and proprioceptive afferents. Convergent RC and Ia afferent inputs are hallmark criteria for identification of interneurons mediating reciprocal inhibition in lumbar spinal cord (Hultborn et al., 1971; Jankowska and Lindstrom, 1972; Rastad et al., 1990; Alvarez et al., 1997). In our study V1-IaINs defined anatomically by these convergent inputs can be recognized in neonates at P1 and a proportion upregulate PV postnatally with a time course similar to other spinal cord interneurons. At present, no independent markers for IaINs that are stably expressed throughout development are known. Therefore, it is difficult to confirm whether the criteria we used allow recognition of all or subpopulations of IaINs. Nevertheless, these criteria do permit positive identification of some neonatal IaINs and demonstrate that key synaptic connections of the reciprocal inhibitory circuitry are present at birth. This conclusion agrees with a recent report demonstrating reciprocal inhibition between quadriceps (Q) afferents and posterior biceps-semitendinosus motoneurons (PBSt) in neonatal mice and their modulation by RC-mediated recurrent inhibition (Wang et al., 2008). Similarly, recurrent inhibitory synaptic potentials can be elicited in embryonic and neonatal mouse motoneurons (Sapir et al., 2004). The present anatomical study provides

direct evidence for the presence in the newborn of the basic synaptic connectivity between RCs, IaINs, and motoneurons and suggests that recurrent and reciprocal inhibition of motoneurons is mediated by similar interneurons and circuits in newborn and adult. A similar conclusion was reached previously in human infants using more indirect tests (McDonough et al., 2001); this, study however, also highlights considerable postnatal maturation in the strength of these connections and this is also in good agreement with the anatomical results presented in our study.

Interestingly, deletion of the transcription factor Pax6, which is required for V1-IN development (Burrill et al., 1997), abolished recurrent inhibition (Sapir et al., 2004) but did not disturb the reciprocal inhibition observed in neonates between Q afferents and PBSt motoneurons (Wang et al., 2008), suggesting sparing of at least some IaIN populations in animals lacking V1-INs. Here we generated anatomical evidence supporting the conclusion that lumbar IaINs are a heterogeneous population originated from V1 and non-V1 neuronal groups. This contrasts with RCs, which appear to be homogeneously derived from V1 interneurons (Sapir et al., 2004). Alternative ventral interneurons with ipsilateral inhibitory axons are derived from the V2b group (Al-Mosawie et al., 2007; Lundfald et al., 2007). The possible derivation of some populations of IaINs from the V2b group is currently under investigation. The significance of various populations of lumbar interneurons involved in reciprocal inhibition is presently unknown. This diversity could result, perhaps, in variable developmental schedules of maturation of reciprocal inhibition when mediated by different interneurons. For example, reciprocal inhibition between Q afferents and PBSt motoneurons can be detected in neonates but not in the opposite direction, i.e., from PBSt afferents on Q motoneurons (Wang et al., 2008).

Diversification of V1 interneuronal populations

Our data strongly suggests that the basic RC and IaIN alternate phenotypes and their different synaptogenic competence are acquired within V1 populations before the birth of the animal. Diversification of other ventral horn interneuronal populations has also been noted during embryonic development. V0 interneurons are divided in embryo into dorsal and ventral groups characterized by differential expression of the transcription factor *Evx1* and neurotransmitter content (V0d are inhibitory while V0v are glutamatergic; Lanuza et al., 2004; Goulding, 2009). More recently, two more interneuronal groups were found to originate from the p0 domain, one cholinergic (V0c) and one glutamatergic (V0g), both characterized by the expression of the transcription factor *Pitx2*. *Pitx2* neurons are also already recognizable in early embryos (E12) within the V0 population (Zagoraoui et al., 2009). V2 interneurons diversify into excitatory Chx10-positive V2a and inhibitory GATA3-positive V2b interneurons (Lundfald et al., 2007). Finally, V3 interneurons also diversify in embryo into two groups with distinct migration patterns becoming located in two different spinal cord regions (Zhang et al., 2008). The mechanisms of differentiation are mostly unknown except for the case of the split between V2a and V2b interneurons. In this case p2 progenitors were shown to alternatively generate V2a or V2b interneurons based on Notch-delta signaling within the progenitor zone (Peng et al., 2007). Activation of notch resulted in diversification of transcription factor expression within mitotic p2 progenitors resulting in two different populations generating either V2a or V2b interneurons without temporal differences in their embryonic production. The diversification of RC and IaINs from the V1 group is under active investigation, but recent data indicate that a different mechanism is at play. Bromodeoxyuridine birthdating clearly show distinct generation times for RCs and IaINs from p1 progenitors in the early embryo (Benito-Gonzalez et al., 2008).

In conclusion, there is significant refinement and strengthening/weakening of connections in local interneuron circuits during postnatal maturation of locomotion. However, the fundamental interneuronal diversity and connectivity adapted to the different forms of

locomotion in each species might originate from genetic/molecular mechanisms that occur in early embryo and before actual locomotion starts.

Overall, the present study indicates that 1) input specificity on interneuronal subpopulations results from the combined action of initial preferential synaptogenesis with particular interneuron types followed by postnatal strengthening or weakening of specific inputs; 2) discrete populations of V1 interneurons, distinct from RCs, can be defined by their inputs as IaINs at birth; 3) development of reciprocal inhibition involves several different groups of ventral interneurons; and 4) there is considerable maturation of the density and size of proprioceptive synapses on IaINs from P1 to P15, suggesting significant changes in synaptic strength in the reciprocal inhibitory circuit during this period.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Martyn Goulding (Salk Institute) who provided the *En1-cre* animals and was a continuous source of input during the development of this study. We also thank Dr. Silvia Arber (Biozentrum, University of Basel, Switzerland), Dr. Joshua Sanes (Harvard University, Boston, MA), and Dr. Robert Brownstone (Dalhousie University, Halifax, Nova Scotia) for sharing different strains of reporter mice from their colonies. We thank, Dr. Tom Jessell, and Ms. Staceyann Doober (Columbia University, New York, NY) for help with the double *pitx2::cre* and *en1::cre* lines. The *Pitx2::cre* line was originally established by Dr. James F. Martin (Texas A-M, Health Science Center, Houston, TX).

Grant sponsor: National Institutes of Health (NIH); Grant number: NS047357 (to F.J.A.); Grant sponsors: Biomedical Sciences PhD program at Wright State University (to V.C.S.); Intramural Funds of NINDS (to G.Z.M.); Grant from project ALS (to L.Z.); Grant sponsor: NINDS; Grant number: NS33245 (to L.Z.).

LITERATURE CITED

- Al-Mosawie A, Wilson JM, Brownstone RM. Heterogeneity of V2-derived interneurons in the adult mouse spinal cord. *Eur J Neurosci.* 2007; 26:3003–3015. [PubMed: 18028108]
- Altman J, Sudarshan K. Postnatal development of locomotion in the laboratory rat. *Anim Behav.* 1975; 23:896–920. [PubMed: 1200422]
- Altman, J.; Bayer, SA. Development of the human spinal cord: an interpretation based on experimental studies in animals. Oxford University Press; New York: 2001.
- Alvarez FJ, Fyffe RE. The continuing case for the Renshaw cell. *J Physiol.* 2007; 584(Pt 1):31–45. [PubMed: 17640932]
- Alvarez FJ, Dewey DE, Harrington DA, Fyffe RE. Cell-type specific organization of glycine receptor clusters in the mammalian spinal cord. *J Comp Neurol.* 1997; 379:150–170. [PubMed: 9057118]
- Alvarez FJ, Dewey DE, McMillin P, Fyffe RE. Distribution of cholinergic contacts on Renshaw cells in the rat spinal cord: a light microscopic study. *J Physiol.* 1999; 515(Pt 3):787–797. [PubMed: 10066905]
- Alvarez FJ, Villalba RM, Zerda R, Schneider SP. Vesicular glutamate transporters in the spinal cord, with special reference to sensory primary afferent synapses. *J Comp Neurol.* 2004; 472:257–280. [PubMed: 15065123]
- Alvarez FJ, Jonas PC, Sapir T, Hartley R, Berrocal MC, Geiman EJ, Todd AJ, Goulding M. Postnatal phenotype and localization of spinal cord V1 derived interneurons. *J Comp Neurol.* 2005; 493:177–192. [PubMed: 16255029]
- Arber S, Ladle DR, Lin JH, Frank E, Jessell TM. ETS gene *Er81* controls the formation of functional connections between group Ia sensory afferents and motor neurons. *Cell.* 2000; 101:485–498. [PubMed: 10850491]

- Arvidsson U, Riedl M, Elde R, Meister B. Vesicular acetylcholine transporter (VACHT) protein: a novel and unique marker for cholinergic neurons in the central and peripheral nervous system. *J Comp Neurol.* 1997; 378:454–467. [PubMed: 9034903]
- Baba H, Kohno T, Okamoto M, Goldstein PA, Shimoji K, Yoshimura M. Muscarinic facilitation of GABA release in substantia gelatinosa of the rat spinal dorsal horn. *J Physiol.* 1998; 508(Pt 1):83–93. [PubMed: 9490821]
- Benito-Gonzalez, A.; Berrocal, MC.; Goulding, M.; Alvarez, FJ. 2008 Neuroscience Meeting Planner. Society for Neuroscience; Washington, DC: 2008. Renshaw cells are V1-derived neurons with a very early birth date. Program No. 374.8. 2008. Online
- Betley JN, Wright CVE, Kawaguchi Y, Erdélyi F, Szabó G, Jessell TM, Katschmidt JA. Stringent specificity in the construction of a GABAergic presynaptic inhibitory circuit. *Cell.* 2009; 139:161–174. [PubMed: 19804761]
- Brocard F, Vinay L, Clarac F. Development of hindlimb postural control during the first postnatal week in the rat. *Brain Res Dev Brain Res.* 1999; 117:81–89.
- Buffelli M, Burgess RW, Feng G, Lobe CG, Lichtman JW, Sanes JR. Genetic evidence that relative synaptic efficacy biases the outcome of synaptic competition. *Nature.* 2003; 424:430–434. [PubMed: 12879071]
- Burrill JD, Moran L, Goulding MD, Saueressig H. PAX2 is expressed in multiple spinal cord interneurons, including a population of EN1+ interneurons that require PAX6 for their development. *Development.* 1997; 124:4493–4503. [PubMed: 9409667]
- Curtis DR, Ryall RW. The synaptic excitation of Renshaw cells. *Exp Brain Res.* 1966; 2:81–96. [PubMed: 5921135]
- De Paola V, Arber S, Caroni P. AMPA receptors regulate dynamic equilibrium of presynaptic terminals in mature hippocampal networks. *Nat Neurosci.* 2003; 6:491–500. [PubMed: 12692557]
- Eccles JC, Fatt P, Koketsu K. Cholinergic and inhibitory synapses in a pathway from motor-axon collaterals to motoneurons. *J Physiol.* 1954; 126:524–562. [PubMed: 13222354]
- Eccles JC, Fatt P, Landgren S. Central pathway for direct inhibitory action of impulses in largest afferent nerve fibres to muscle. *J Neurophysiol.* 1956; 19:75–98. [PubMed: 13286723]
- Erickson JD, Weihe E, Schafer MK-M, Neale E, Williamson L, Bonner TI, Tao-Cheng J-H, Eiden LE. The VACHT/ChAT “cholinergic gene locus”: new aspects of genetic and vesicular regulation of cholinergic function. *Prog Brain Res.* 1996; 109:69–82. [PubMed: 9009694]
- Erickson J, Rashbass P, Schedl A, Brenner-Morton S, Kawakami A, van Heyningen V, Jessell TM, Briscoe J. Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell.* 1997; 90:169–180. [PubMed: 9230312]
- Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JM, Lichtman JW, Sanes JR. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron.* 2000; 28:41–51. [PubMed: 11086982]
- Frank K, Fuortes MG. Unitary activity of spinal interneurons of cats. *J Physiol.* 1956; 131:424–435. [PubMed: 13320344]
- Gosgnach S, Lanuza GM, Butt SJ, Saueressig H, Zhang Y, Velasquez T, Riethmacher D, Callaway EM, Kiehn O, Goulding M. V1 spinal neurons regulate the speed of vertebrate locomotor outputs. *Nature.* 2006; 440:215–219. [PubMed: 16525473]
- Goulding M. Circuits controlling vertebrate locomotion: moving in a new direction. *Nat Rev Neurosci.* 2009; 10:507–518. [PubMed: 19543221]
- Gramsbergen A. Posture and locomotion in the rat: independent or interdependent development? *Neurosci Biobehav Rev.* 1998; 22:547–553. [PubMed: 9595568]
- Grillner S, Jessell TM. Measure motion: looking for simplicity in spinal locomotor networks. *Curr Opin Neurobiol.* 2009; 19:572–586. [PubMed: 19896834]
- Hellstrom J, Arvidsson U, Elde R, Cullheim S, Meister B. Differential expression of nerve terminal protein isoforms in VACHT-containing varicosities of the spinal cord ventral horn. *J Comp Neurol.* 1999; 411:578–590. [PubMed: 10421869]
- Higashijima S, Masino MA, Mandel G, Fetcho JR. Engrailed-1 expression marks a primitive class of inhibitory spinal interneuron. *J Neurosci.* 2004; 24:5827–5839. [PubMed: 15215305]

- Hippenmeyer S, Vrieseling E, Sigrist M, Portmann T, Laengle C, Ladle DR, Arber S. A developmental switch in the response of DRG neurons to ETS transcription factor signaling. *PLoS Biol.* 2005; 3:e159. [PubMed: 15836427]
- Hultborn H, Jankowska E, Lindstrom S. Recurrent inhibition from motor axon collaterals of transmission in the Ia inhibitory pathway to motoneurons. *J Physiol.* 1971; 215:591–612. [PubMed: 4326306]
- Hultborn H, Illert M, Santini M. Convergence on interneurons mediating the reciprocal Ia inhibition of motoneurons. I. Disynaptic Ia inhibition of Ia inhibitory interneurons. *Acta Physiol Scand.* 1976; 96:193–201. [PubMed: 1258669]
- Jankowska E. Interneuronal relay in spinal pathways from proprioceptors. *Prog Neurobiol.* 1992; 38:335–378. [PubMed: 1315446]
- Jankowska E, Lindstrom S. Morphology of interneurons mediating Ia reciprocal inhibition of motoneurons in the spinal cord of the cat. *J Physiol.* 1972; 226:805–823. [PubMed: 4118049]
- Jankowska E, Roberts WJ. Synaptic actions of single interneurons mediating reciprocal Ia inhibition of motoneurons. *J Physiol.* 1972; 222:623–642. [PubMed: 5033026]
- Johnson IP, Sears TA. Ultrastructure of interneurons within motor nuclei of the thoracic spinal cord of the adult cat. *J Anat.* 1988; 161:171–185. [PubMed: 3254890]
- Kim KK, Adelstein RS, Kawamaoto S. Identification of neuronal nuclei (NeuN) as Fox-3, a new member of the Fox1 gene family of splicing factors. *J Biol Chem.* 2009; 284:31052–31061. [PubMed: 19713214]
- Lanuza GM, Gosgnach S, Pierani A, Jessell TM, Goulding M. Genetic identification of spinal interneurons that coordinate left-right locomotor activity necessary for walking movements. *Neuron.* 2004; 42:375–386. [PubMed: 15134635]
- Li WC, Higashijima S, Parry DM, Roberts A, Soffe SR. Primitive roles for inhibitory interneurons in developing frog spinal cord. *J Neurosci.* 2004; 24:5840–5848. [PubMed: 15215306]
- Liu W, Selever J, Lu MF, Martin JF. Genetic dissection of Pitx2 in craniofacial development uncovers new functions in branchial arch morphogenesis, late aspects of tooth morphogenesis and cell migration. *Development.* 2003; 130:6375–6385. [PubMed: 14623826]
- Lundfald L, Restrepo CE, Butt SJ, Peng CY, Droho S, Endo T, Zeilhofer HU, Sharma K, Kiehn O. Phenotype of V2-derived interneurons and their relationship to the axon guidance molecule EphA4 in the developing mouse spinal cord. *Eur J Neurosci.* 2007; 26:2989–3002. [PubMed: 18028107]
- Matisse MP, Joyner AL. Expression patterns of developmental control genes in normal and Engrailed-1 mutant mouse spinal cord reveal early diversity in developing interneurons. *J Neurosci.* 1997; 17:7805–7816. [PubMed: 9315901]
- McDonough SM, Clowry GJ, Miller S, Eyre JA. Reciprocal and Renshaw (recurrent) inhibition are functional in man at birth. *Brain Res.* 2001; 899:66–81. [PubMed: 11311867]
- Mentis GZ, Alvarez FJ, Bonnot A, Richards DS, Gonzalez-Forero D, Zerda R, O'Donovan MJ. Noncholinergic excitatory actions of motoneurons in the neonatal mammalian spinal cord. *Proc Natl Acad Sci U S A.* 2005; 102:7344–7349. [PubMed: 15883359]
- Mentis GZ, Siembab VC, Zerda R, O'Donovan MJ, Alvarez FJ. Primary afferent synapses on developing and adult Renshaw cells. *J Neurosci.* 2006; 26:13297–13310. [PubMed: 17182780]
- Mentis GZ, Alvarez FJ, Shneider NA, Siembab VC, O'Donovan MJ. Mechanisms regulating the specificity and strength of muscle afferent inputs in the spinal cord. *Ann N Y Acad Sci.* 2010 (in press).
- Miles GB, Hartley R, Todd AJ, Brownstone RM. Spinal cholinergic interneurons regulate the excitability of motoneurons during locomotion. *Proc Natl Acad Sci U S A.* 2007; 104:2448–2453. [PubMed: 17287343]
- Mullen RJ, Buck CR, Smith AM. NeuN, a neuronal specific nuclear protein in vertebrates. *Development.* 1992; 116:201–211. [PubMed: 1483388]
- Oliveira AL, Hydling F, Olsson E, Shi T, Edwards RH, Fujiyama F, Kaneko T, Hokfelt T, Cullheim S, Meister B. Cellular localization of three vesicular glutamate transporter mRNAs and proteins in rat spinal cord and dorsal root ganglia. *Synapse.* 2003; 50:117–129. [PubMed: 12923814]

- Peng CY, Yajima H, Burns CE, Zon LI, Sisodia SS, Pfaff SL, Sharma K. Notch and MAML signaling drives Scl-dependent interneuron diversity in the spinal cord. *Neuron*. 2007; 53:813–827. [PubMed: 17359917]
- Persson S, Boulland JL, Aspling M, Larsson M, Fremereau RT Jr, Edwards RH, Storm-Mathisen J, Chaudhry FA, Broman J. Distribution of vesicular glutamate transporters 1 and 2 in the rat spinal cord, with a note on the spinocervical tract. *J Comp Neurol*. 2006; 497:683–701. [PubMed: 16786558]
- Rastad J, Gad P, Jankowska E, McCrea D, Westman J. Light microscopical study of dendrites and perikarya of interneurons mediating Ia reciprocal inhibition of cat lumbar alpha-motoneurons. *Anat Embryol (Berl)*. 1990; 181:381–388. [PubMed: 2346230]
- Ren K, Ruda MA. A comparative study of calcium-binding proteins calbindin-D28K, calretinin, calmodulin and parvalbumin in the rat spinal cord. *Brain Res Rev*. 1994; 19:163–179. [PubMed: 8061685]
- Renshaw B. Central effects of centripetal impulses in axons of spinal ventral roots. *J Neurophysiol*. 1946; 9:191–204. [PubMed: 21028162]
- Ryall RW. Excitatory convergence on Renshaw cells. *J Physiol*. 1972; 226:69P–70P.
- Sapir T, Geiman EJ, Wang Z, Velasquez T, Mitsui S, Yoshihara Y, Frank E, Alvarez FJ, Goulding M. Pax6 and engrailed 1 regulate two distinct aspects of rensaw cell development. *J Neurosci*. 2004; 24:1255–1264. [PubMed: 14762144]
- Saueressig H, Burrill J, Goulding M. Engrailed-1 and netrin-1 regulate axon pathfinding by association interneurons that project to motor neurons. *Development*. 1999; 126:4201–4212. [PubMed: 10477289]
- Shneider NA, Mentis GZ, Schustak J, O'Donovan MJ. Functionally reduced sensorimotor connections form with normal specificity despite abnormal muscle spindle development: the role of spindle-derived neurotrophin 3. *J Neurosci*. 2009; 29:4719–4735. [PubMed: 19369542]
- Siembab, VC.; Richards, D.; Berrocal, MC.; Goulding, M.; Alvarez, FJ. 2008 Neuroscience Meeting Planner. Society for Neuroscience; Washington, DC: 2008. Distinct synaptic development in Ia inhibitory interneurons compared to Renshaw cells. Program No. 374.7. 2008. Online
- Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet*. 1999; 21:70–71. [PubMed: 9916792]
- Todd AJ, Hughes DI, Polgar E, Nagy GG, Mackie M, Ottersen OP, Maxwell DJ. The expression of vesicular glutamate transporters VGLUT1 and VGLUT2 in neurochemically defined axonal populations in the rat spinal cord with emphasis on the dorsal horn. *Eur J Neurosci*. 2003; 17:13–27. [PubMed: 12534965]
- VanderHorst VG, Ulfhake B. The organization of the brainstem and spinal cord of the mouse: relationships between monoaminergic, cholinergic, and spinal projection systems. *J Chem Neuroanat*. 2006; 31:2–36. [PubMed: 16183250]
- Vejsada R, Hnik P, Payne R, Ujec E, Palecek J. The postnatal functional development of muscle stretch receptors in the rat. *Somatosens Res*. 1985; 2:205–222. [PubMed: 3159063]
- Wang Z, Li LY, Taylor MD, Wright DE, Frank E. Prenatal exposure to elevated NT3 disrupts synaptic selectivity in the spinal cord. *J Neurosci*. 2007; 27:3686–3694. [PubMed: 17409232]
- Wang Z, Li L, Goulding M, Frank E. Early postnatal development of reciprocal Ia inhibition in the murine spinal cord. *J Neurophysiol*. 2008; 100:185–196. [PubMed: 18463181]
- Watson, C.; Paxinos, G.; Hayalioglu, G.; Hesie, C. Atlas of the mouse spinal cord. In: Watson, C.; Paxinos, G.; Hayalioglu, G., editors. *The spinal cord*. A Christopher and Dana Reeve Foundation Text and Atlas. Elsevier; Oxford: 2009.
- Westerga J, Gramsbergen A. The development of locomotion in the rat. *Brain Res Dev Brain Res*. 1990; 57:163–174.
- Westerga J, Gramsbergen A. Development of locomotion in the rat: the significance of early movements. *Early Hum Dev*. 1993; 34:89–100. [PubMed: 8275886]
- Westerga J, Gramsbergen A. Development of the EMG of the soleus muscle in the rat. *Brain Res Dev Brain Res*. 1994; 80:233–243.

- Zagoraïou L, Akay T, Martin JF, Brownstone RM, Jessell TM, Miles GB. A cluster of cholinergic premotor interneurons modulates mouse locomotor activity. *Neuron*. 2009; 64:645–662. [PubMed: 20005822]
- Zhang JH, Morita Y, Hironaka T, Emson PC, Tohyama M. Ontological study of calbindin-D28k-like and parvalbumin-like immunoreactivities in rat spinal cord and dorsal root ganglia. *J Comp Neurol*. 1990; 302:715–728. [PubMed: 2081815]
- Zhang Y, Narayan S, Geiman E, Lanuza GM, Velasquez T, Shanks B, Akay T, Dyck J, Pearson K, Gosgnach S, Fan CM, Goulding M. V3 spinal neurons establish a robust and balanced locomotor rhythm during walking. *Neuron*. 2008; 60:84–96. [PubMed: 18940590]

Figure 1.

Diagram of the major cellular elements and neurochemical markers of the mature premotor circuits involving Ia inhibitory interneurons and Renshaw interneurons. A pair of antagonist flexor and extensor motoneurons are depicted. Motoneurons make synapses within the spinal cord with RCs that are distributed in pools preferentially coupled to extensor or flexor motor pools (only one cell shown here). These motor axon synapses express VAcHt-IR and can be retrogradely labeled from the ventral roots during early development. RCs themselves provide recurrent inhibition to the same motoneurons they receive input from and can be identified since they are ventral V1-interneurons expressing high levels of CB-IR in their cell body and axons. Ia inhibitory interneurons establish reciprocal inhibition (IaINs) and innervate motor pools projecting to muscles that are antagonists of the muscles of origin of their primary afferent (Ia) input. Ia afferent synapses are immunoreactive for VGLUT1 and PV in the neonate, but slowly reduce PV-IR during postnatal maturation. In the adult, Ia afferents contact motoneurons in lamina IX and IaINs in LVII. RCs also receive functional synapses in the neonate (Mentis et al., 2006). PV-IR increases during development within a population of V1 cells that correspond to IaINs (this study). IaINs can be recognized because they receive convergent synapses from VGLUT1 Ia afferent and V1 CB-IR axons from RCs. RCs inhibit IaINs and motoneurons that receive a common Ia afferent input.

Figure 2.

Distribution of V1-derived neurons in *En1-Cre/R26-lacZ*, *En1-Cre/Tau-lacZ* and *En1-Cre/Thy1-YFP* mice. **A1,B1,C1:** Low-magnification confocal images of lumbar spinal cord sections immunolabeled with NeuN (Cy3) and either β -gal (FITC, A1,B1) or YFP (FITC, C1) in *En1-Cre/R26-lacZ* (A1), *En1-Cre/Tau-lacZ* (B1), and *En1-Cre/Thy1-YFP* (C1) P40 mice. In all three animal models V1-derived interneurons (V1-INS) are concentrated in lamina VII (VII) surrounding lamina IX (IX) motor pools (large NeuN-IR cells). V1-IN axons are labeled in *En1-Cre/Thy1-YFP* animals (C1) and travel in the ventral and lateral funiculi (VF, LF). V1-IN axonal projections project to laminae VII and IX and are sparse in lamina VIII (VIII) and the dorsal horn. The dotted lines indicate laminae borders in the ventral horns (CC, central canal). **A2,B2,C2:** Distribution of V1-INS in the ventral horn at higher magnification. Insets show labeling patterns for each reporter (NeuN is shown in red, arrowheads in A2 indicate intracytoplasmic inclusions of β -gal immunoreactivity). **A3,B3,C3:** Neurolucida plots showing the distribution of V1-INS in the ventral horn of *En1-Cre/R26-lacZ* (A3), *En1-Cre/Tau-lacZ* (B3), and *En1-Cre/Thy1-YFP* (C3) mice (50- μ m thick sections). These plots were used to estimate the percentage of NeuN-IR interneurons derived from the V1 lineage. NeuN-IR cells were also plotted but not shown for simplicity. **D:** Percentages of ventral NeuN-IR interneurons expressing reporter labeling for the V1-lineage in the three mouse models. Error bars indicate SEMs. Significant differences were detected in the percentages of NeuN-IR cells expressing V1 lineage markers between *En1-Cre/R26-lacZ* or *En1-Cre/Tau-lacZ* mice when compared to *En1-Cre/Thy1-YFP* mice (asterisk, $P < 0.05$, one-way ANOVA). No differences were found between *En1-Cre/R26-lacZ* and *En1-Cre/Tau-lacZ* mice. **E:** High-magnification of a 2D projection of V1-INS shown in C2 inset and rotated 90°. YFP fills their somata, dendrites, and axons. Arrows indicate YFP-IR axons contacting a large LIX NeuN-IR cell (presumably a motoneuron). (A color version of this figure using the magenta-green palette is provided as Fig. S1 in the Supporting materials.) Scale bars = 100 μ min A–C; 20 μ min in sets in A2–C2,E.

Figure 3.

Developmental regulation of calbindin and parvalbumin expression in V1-derived interneurons. **A1–3:** Low-magnification confocal images of calbindin-IR (CB-IR; Cy3, red) and β -gal-IR (LacZ; FITC, green) neurons in the ventral horns of P5 (A1), P10 (A2), and P15 (A3) spinal cords from *En1-Cre/R26-lacZ* mice. Blue arrowheads indicate CB-IR cells that are not V1-derived (β -gal-negative; red only). White arrowheads indicate CB-IR V1-INs (appearing yellow due to double labeling between Cy3-calbindin-IR and FITC- β -gal-IR). Single arrowheads denote CB-IR V1-INs in the RC region; double arrowheads point dorsal V1-INs that preserved some weak CB-IR at P15. CB-IR V1-INs outside the RC region are likely non-RCs. During postnatal development, CB-IR in the ventral horn becomes largely restricted to V1-derived RCs (located in the ventralmost 100 μ m, see scale bar). **B1–3:** The number and percentages of V1-INs and ventral interneurons expressing CB decreases with development. B1: Comparison of the total number of CB-IR ventral interneurons in *En1* heterozygotes (*En1*^{Cre/+}) and wildtype littermates (*En1*^{+/+}) of different ages. In both groups of animals there was a similar downregulation in the total number of CB-IR cells with age. B2: The percentage of V1-INs that are CB-IR significantly decreased from P5 to P10 ($P < 0.05$, one-way ANOVA). B3: CB-IR downregulates in V1-INs at all dorsoventral locations except in the ventralmost 100 μ m, where CB-IR RCs are found. **C1–3:** Low-magnification images of parvalbumin-IR (PV-IR; Cy3, red) and β -gal-IR (FITC, green) in ventral horns at P5 (C1), P10 (C2), and P15 (C3). No PV-IR interneurons are present at P5, at this age PV-IR is restricted to the axons and terminals of primary afferents (white arrows in C1). In P10 (C2) and P15 (C3) images white arrowheads indicate colocalization of β -gal-IR and PV-IR. Blue arrowheads indicate PV-IR interneurons that are not V1-derived (appearing in red only). PV-IR in primary afferent axon appears downregulated at these ages compared to P5. **D1–3:** The number and percentages of V1 and non-V1 INs expressing PV increases with development. D1: Similar developmental upregulation of PV expression in *En1*^{Cre/+} heterozygote animals and *En1*^{+/+} wildtypes. D2: PV upregulation in V1 interneurons is first detected at P10 and thereafter their proportion significantly increases with age ($P < 0.05$, one-way ANOVA). D3: PV-IR upregulation in V1-INs is more rapid in cells located in the ventralmost 100 μ m corresponding with the RCs. (A color version of this figure using the magenta-green palette is provided as Fig. S2 in the Supporting materials.) Scale bars = 100 μ m.

Figure 4.

Histochemical identification of V1-derived Ia inhibitory interneurons in *En1-Cre/Thy1-YFP* mice. **A1:** Low-magnification confocal image of the ventral horn of an *En1-Cre/Thy1-YFP* P15 mouse immunostained for CB (Cy5, blue) and YFP (FITC, green). The dotted white line indicates the border between the ventral horn and white matter. A box outlines one YFP-IR neuron shown at higher magnification in A2 and A3. **A2,A3:** High-magnification superimposition of a series of confocal optical sections used to reconstruct this YFP-IR V1-IN (green in A2) and the dense innervation from V1-derived CB-IR axons (blue and green in A3). These cells were identified as V1-IaINs throughout the study. In A3 the intensity of green was lowered to improve visibility of blue axons and the inset shows a proportion of these contacts at higher magnification. **B1:** Low-magnification confocal image of an *En1-Cre/Thy1-YFP* P15 ventral horn immunolabeled for PV (Cy3, red) and CB (Cy5, blue). IaINs were previously identified as V1-derived PV-IR cells receiving dense proximal innervation from CB-IR RC axons (Alvarez et al., 2005), similar to the one outlined. **B2:** Medium magnification confocal image of the PV-IR (red) neuron outlined in B1 showing in the inset the boxed region at higher magnification. This cell received many CB-IR contacts (blue arrows) from V1-RC origin and also large PV-IR contacts (red arrows), most likely originated in proprioceptive axons. **B3:** Superimposed YFP-IR (FITC, green) on the same PV-IR cell demonstrating its V1 origin. Scale bars = 100 μ m in A1,B1; 20 μ m in A2,B2 (applies to A3,B3).

Figure 5.

Some PV-IR IaINs are V1-derived while others are non-V1 derived. **A1–3:** Low-magnification single plane confocal images of a ventral horn section from lumbar 5 segment of a P15 *En1-Cre/Thy1-YFP* mouse spinal cord immunolabeled for YFP (FITC, green) (A1), calbindin (Cy5, blue) (A1,A2), and parvalbumin (Cy3, red) (A2). Superimposition in A3. The dotted line indicates the border between the ventral horn and the white matter. Boxes surround the neurons shown at higher magnification in B,C. **B1–3:** High-magnification of the neuron indicated in the top box in A reconstructed through several optical planes. This cell is V1-derived (YFP-IR, green, B1) contains PV-IR (red, B2) and receives many CB-IR contacts (blue B1 and B2). Thus is classified as a V1-derived IaIN. Superimposition in B3. **C1–3:** High-magnification of the neuron indicated in the bottom box in A, also reconstructed from several optical planes. This cell is PV-IR (red, C2) but does not contain YFP (asterisk, C1), although it is surrounded by CB-IR contacts (blue, C1,C2). Superimposition in C3. This cell is classified as non-V1-derived IaIN. **D1–3:** Low-magnification single confocal plane images of a ventral horn section from the lumbar 4 segment of an *En1-Cre/Tau-lacZ* P15 mouse showing β -gal-IR (FITC, green) (D1), calbindin-IR (Cy5, blue) (D1,D2), and PV-IR (Cy3, red) (D2). Superimposition in D3. Boxes indicate the cells shown at higher magnification in E and F. **E1–3,F1–3:** High-magnification confocal images of β -gal-IR (green; E1,F1), CB-IR (blue; E1,E2,F1,F2), and PV-IR (red; E2,F2). Superimpositions in E3 and F3. The cells were reconstructed through superimposition of several confocal planes. The cell in E is PV-IR and β -gal-IR (thus V1-derived) receiving a dense innervation from CB-IR axons (thus classified as V1-IaIN). F shows a PV-IR neuron that also receives inputs from CB-IR axons, but it is not V1-derived (β -gal-negative, asterisk in F1). **G1–3:** Low-magnification single plane confocal images similar to D but from the L3 segment. Immunolabeling for β -gal (FITC, green) (G1), CB (Cy5, blue) (G1 and G2) and PV (Cy3,

red) (G2). Superimposition in G3. Boxed areas indicate the location of the cells shown in H,I. **H1–3,I1–3**: Superimposition of several high-magnification images through multiple confocal planes of PV-IR cells receiving a dense innervation from CB-IR axons. The two cells shown in H are V1-derived (β -gal-IR) while the cell in I is not (β -gal-negative, asterisk in I1). Scale bars = 100 μ m in A1,D1,G1 (applies to A,D,G); 20 μ m in B1,C1,E1,F1,H1,I1 (applies to B,C,E,F,H,I).

Figure 6.

Development of VGLUT1-IR contacts on V1-derived Ia inhibitory interneurons and RCs in *En1-Cre/Thy1-YFP* mice. **A1,B1,C1:** Low-magnification images of VGLUT1 (Cy3, red), YFP (FITC, green), and calbindin (CB-IR; Cy5, blue) immunoreactivities in the ventral horn of P5 (A1), P15 (B1), and P40 (C1) *En1-Cre/Thy1-YFP* mice. Yellow dotted line indicates the border between the ventral horn and the white matter. Boxes indicate cells shown at higher magnification in A2–A3, B2–B3, and C2–C3. **A2,B2,C2:** High-magnification images of P5 (A2), P15 (B2), and P40 (C2) YFP-IR V1-IaINs. All receive a dense innervation from V1-derived CB-IR RC axons (green-YFP-IR and blue-CB-IR) and all receive VGLUT1-IR contacts (red, indicated with white arrowheads). Some of the VGLUT1-IR contacts are shown at higher magnification in the insets below. The number of VGLUT1-IR contacts on V1-IaINs increases from P5 to P15. **A3,B3,C3:** VGLUT1-IR contacts on V1-derived RCs (CB-IR and YFP-IR) at P5 (A3), P15 (B3), and P40 (C3). Arrowheads indicate VGLUT1-IR contacts on RC dendrites. Insets below show VGLUT1-IR contacts at higher magnification. There are more contacts on RCs at P15 compared to either P5 or P40. Because the 3D complexity of the images there are a different numbers of confocal planes superimposed in each image. Confocal planes were added or deleted as needed at high magnification with respect to the low-magnification images to better isolate the structures highlighted and improve clarity. Scale bars = 100 μ m in A1,B1,C1; 20 μ m in A2,A3,B2,B3,C2,C3; 5 μ m in insets.

Figure 7.

Density and distribution of VGLUT1-IR contacts on developing V1-Ia inhibitory interneurons and RCs. **A,B:** 3D Neurolucida reconstructions of a V1-IaIN (A) and a V1-derived RC (B) from a P40 *En1-Cre/Thy1-YFP* indicating the position of VGLUT1-IR contacts on dendrites and somata (dots). **C:** Linear density of VGLUT1-IR contacts (number per 10 μm of dendrite) on V1-IaINs (black bars) and V1 CB-IR RCs (gray bars) of P5, P10, P15, P20, and adult (P40) ages. VGLUT1-IR contact density increased significantly in V1-IaINs from P5 to P15 (black asterisk, $P < 0.05$, one-way ANOVA, post-hoc Tukey tests). VGLUT1-IR contact density on V1-IaINs is maintained after P15 and no significant differences were detected between P15, P20, and adult cells ($P = 0.488$, one-way ANOVA). VGLUT1-IR input density on CB-IR RCs increases significantly from P5 to P15 and then decreases at P20 (gray asterisks; $P < 0.05$, post-hoc Tukey tests). No significant differences were seen between P20 and adult RCs. At all ages, VGLUT1-IR contact density on V1-IaINs was significantly higher when compared to RCs (open white asterisks, $P < 0.05$, t -tests). **D:** The number and density (contacts per 100 μm^2) of VGLUT1-IR contacts on the cell soma of P40 V1-IaINs was significantly greater than on RCs (asterisks; $P < 0.001$, t -test). **E:** VGLUT1-IR contacts, expressed as linear densities (per 10 μm of linear dendrite), in three dendritic compartments at Sholl distance bins of increasing 50 μm from the cell body center. Numbers in parentheses indicate the number of cells with dendritic data in each distance bin. There were no significant differences in the density of VGLUT1-IR contacts among the three dendritic compartments in V1-IaINs ($P = 0.318$, one-way ANOVA) or RCs ($P = 0.336$, one-way ANOVA). However, at all distance bins the density of VGLUT1-IR contacts on IaINs is always significantly higher when compared to RCs (asterisks, $P < 0.05$, one-way ANOVA). **F:** Percentage of VGLUT1-IR contacts (from the cell totals) found on the soma or each 50 μm Sholl bin in V1-IaINs and RCs. Most VGLUT1-IR contacts on V1-IaINs and RCs are located on proximal dendrites less than 100 μm distant from the soma. Significantly more VGLUT1-IR contacts were present in the first 50 μm Sholl dendrite bin compared to the soma and the most distal dendritic bin in both V1-IaINs and RCs (one asterisk; $P < 0.05$, one-way ANOVA). P -values below the graph indicate significant differences in the percentage of contacts between V1-IaINs and RCs. VGLUT1-IR contact densities tended to be significantly larger proximally on V1-IaINs and distally on RCs. Error bars in all graphs indicate SEMs.

Figure 8.

Development of VACHT-IR contacts on V1-derived Ia inhibitory interneurons and RCs in *En1-Cre/Thy1-YFP* mice. **A1,B1,C1:** Low-magnification images of *En1-Cre/Thy1-YFP* mouse spinal cords at P5 (A1), P15 (B1), and P40 (C1) immunolabeled for calbindin (CB-IR; Cy5, blue), YFP (FITC, green) and VACHT (Cy3, red). Yellow dotted line indicates border between the ventral horn and the white matter. Boxes indicate cells shown at higher magnification in A2,A3,B2,B3,C2,C3. **A2,B2,C2:** High-magnification images showing VACHT-IR inputs (red) on P5 (A2), P15 (B2), and P40 (C2) YFP-IR V1-IaINs (green) densely innervated by V1-derived CB-IR RC axons (green and blue). Arrowheads indicate VACHT-IR contacts on V1-IaINs. VACHT-IR contacts on V1-IaINs occur predominantly on the cell soma, are frequently of very small size, and their number decreased from P5 to P15. Insets show VACHT-IR contacts on V1-IaIN somata and dendrites at higher magnification. **A3,B3,C3:** VACHT-IR contacts on CB-IR and YFP-IR RCs at P5 (A3), P15 (B3), and P40 (C3). Arrowheads indicate the position of the contacts. VACHT-IR contacts on RCs mostly target dendrites and are relatively large. The number of VACHT-IR contacts on RCs increase from P5 to P40. Insets show the contacts at higher magnification. Confocal planes were added or deleted as needed at high magnification with respect to the low-magnification images to better isolate the structures highlighted and improve clarity. Scale bars 100 = μm in A1,B1,C1; 20 μm in A2,A3,B2,B3,C2,C3; 5 μm in insets.

Figure 9.

Density and distribution of VACHT-IR contacts on V1-derived IaINs and RCs. **A,B:** Neurolucida 3D reconstruction of a V1-IaIN (A) and a RC (B) from a P40 *En1-Cre/Thy1-YFP* mouse with VACHT-IR contacts plotted on the reconstructed dendrites and cell bodies (dots). **C:** VACHT-IR contacts per 10 μm of linear dendrite on V1-IaINs and CB-IR RCs in *En1-Cre/Thy1-YFP* mice of P5, P10, P15, P20, and adult (P40) ages. VACHT-IR contact density significantly decreases on V1-IaINs from P10 to P15 (black asterisk, $P < 0.05$, one-way ANOVA, post-hoc Tukey tests), whereas no significant differences were detected between P5 and P10 or P15, P20 and adult. In contrast, VACHT-IR input density on CB-IR RCs increases significantly from P5 to P20 (gray asterisk, $P < 0.05$) and is maintained after P20 with no significant differences between P20 and adult. At all ages, VACHT-IR contact density on V1-IaINs is significantly lower compared to RCs (open white asterisks, P5-P40 $P < 0.001$, t -tests). **D:** VACHT-IR contacts on P40 V1-IaINs and RC cell bodies. V1-IaIN cell bodies are larger than RCs and they receive 3-fold more VACHT-IR contacts compared to RCs. This difference in the number of VACHT-IR contacts correlates with a 2-fold difference in their surface densities. Differences between both cell types in the number or surface density of somatic contacts were significant (asterisks; $P < 0.001$, t -test). **E:** Sholl analysis of VACHT-IR contacts on the dendrites of P40 V1-IaINs and CB-IR RCs. Sholl analysis is estimated in 50 μm bins of increasing distance from the center of the cell body. V1-IaINs displayed significant fewer VACHT-IR contacts in all distance bins compared to RCs (asterisks, $P < 0.05$, one-way ANOVA). Within a single cell type there were no significant differences in the density of VACHT-IR contacts in different dendritic compartments at P40. **F:** Percentage of VACHT-IR contacts (referred to the cell total) detected on the cell body and at each dendritic Sholl bin in P40 V1-IaINs and RCs. VACHT-IR contacts on V1-IaINs are more abundant on the cell body and more proximal dendrites (first 50 μm bin), then they are significantly reduced (asterisks, $P < 0.005$, One-way ANOVA) at dendritic distances beyond 50 μm . VACHT-IR contacts on RCs were found in the first 100 μm of dendrite and very few were on the cell body. VACHT-IR contact density was similar in the 0–50 and 50–100 μm bins and these were significantly denser compared to the cell soma and the distal dendritic bin (beyond 100 μm ; asterisks; $P < 0.05$, one-way ANOVA). P -values below graph indicate the significance of differences in the percentage of contacts between V1-IaINs and RCs in each cell compartment. V1-IaINs display larger densities of VACHT-IR contacts on their cell bodies and lower densities on dendrites. Error bars in all graphs indicate SEMs.

Figure 10.

VACHT-IR boutons on V1-derived Ia inhibitory interneurons and RCs have different origins. **A1–5:** P7 *En1-Cre/Thy1-YFP* spinal cord showing motoneurons and motor axons retrogradely labeled from the L5 ventral root (arrow in A1; Cascade blue dextran, blue, Cb-dxt) (A1–A3) superimposed with VACHT-IR (Cy3, red) (A2,A4), naked YFP (green) (A3–A4), and calbindin-IR (Alexa-405, white) (A3,A4). All four fluorochromes superimposed in A5. Box with one asterisk corresponds to the RC area shown in B,D. The area contained in the box with two asterisks is shown at higher magnification in C and represents the region enriched with V1-IaINs. Yellow dotted lines indicate the border between the ventral horn and white matter. **B1–5:** Higher magnifications of the RC region (top panel medium magnification, bottom panels high magnification). This area contains many motor axons (blue) converging into the ventral roots. Some collaterals of these axons leave varicosities in this region (arrows in bottom B1 panel corresponding to the area boxed in B1 upper panel) and contain VACHT-IR (B2, upper and bottom panels). These varicosities contact RCs (white, B3,B4,B5) and contain VACHT-IR (red, in B4, yellow arrows). All four fluorochromes are superimposed in B5 (upper and bottom panels). White axons indicate a few appositions from retrogradely labeled profiles lacking VACHT. **C1–3:** Region containing YFP V1-IaINs (green) surrounded by CB-IR axons (white). Blue cells are retrogradely labeled motoneurons. Lower panels show high magnifications of the areas boxed in the upper panels. Few retrogradely labeled motor axon collaterals (blue) or VACHT-IR boutons (red) are present in this region. VACHT-IR boutons (red) on V1-IaINs (green) (red in C2,C3) did not contain retrograde labeling (blue in C1 and C3, upper and lower panels). **D:** Non-RC YFP V1-INs (green) located in the RC area (just medial to the field shown in B panels) are contacted by CB-IR (white) and VACHT-IR boutons (red), but their VACHT-IR contacts are not retrogradely labeled from the ventral roots either (yellow arrows), with a few exceptions (white arrow indicates one VACHT-IR contact with some retrograde labeling). Thus, motor axons seem to avoid non-RCs within the V1 population, irrespective of location in the ventral horn. **E:** Low-magnification confocal images of a

ventral horn section of a P15 *Pitx2-Cre/En1-Cre/Tau-lacZ-mGFP* mouse spinal cord immunolabeled for GFP and β -gal (both in FITC, green) (E1,E3), parvalbumin (Cy5, blue) (E2,E3), calbindin (Alexa-405, white) (E2,E5) and VACHT (Cy3, red) (E4). Superimposition in E5. Box with one asterisk indicates RC shown at higher magnification in F. Box with two asterisks corresponds to the IaIN shown at higher magnification in G. **F1–F2:** High-magnification images of the cell indicated in the box with one asterisk in E. Bottom row panel show the dendritic boxed regions at the highest magnification. This cell is calbindin-IR (CB-IR, white, F1,F2) and receives VACHT-IR contacts (red, F1), which are not GFP-IR (green, F2). **G1–G3:** High-magnification images of a PV-IR (blue, G1–G3) receiving a dense innervation of CB-IR axons (white, G1) and VACHT-IR contacts (red, G2,G3) that contain GFP (green, G1,G3). Scale bars = 100 μ m in A1 (applies to all A); 20 μ m in B1 (applies to B–D,F,G); 10 μ m in B1 inset (applies to insets in B–D); 5 μ m in F1 inset (applies to insets in F,G).

Antibodies Used in the Study

TABLE 1

Antigen	Immunogen	Host / type	Manufacturer	Specificity	Dilution
β-gal	β-gal purified from <i>Escherichia coli</i>	Chicken polyclonal	AbCam Inc. (Cambridge, MA), cat. no. ab9361, lot no. 470013,	Did not immunostain spinal cord sections lacking β-gal expression	1:1,000
β-gal	β-gal purified from <i>Escherichia coli</i>	Rabbit polyclonal	Cappel (Aurora, IL), cat. no. ab6645, lot no. 03023,		1:10,000
Calbindin	Calbindin D28k purified from chicken gut	Mouse monoclonal	Swant (Bellinzona, Switzerland), cat. no. CB300, lot no. 18F,	Both antibodies detect a single 28kDa band in WB and produce no staining in calbindin ^{-/-} mice ²	1:500-1:1,000
Calbindin	Rat recombinant Calbindin D28k	Rabbit polyclonal	Swant (Bellinzona, Switzerland), cat. no. CB-38a, lot no. 9.03,		1:1,000-1:2,000
GFP	Native GFP from jellyfish	Sheep polyclonal	Biogenesis (Brentwood, NH), cat. no. 4745-1051, lot no. 4321MC1,	Did not immunostain spinal cord sections lacking GFP expression ¹	1:1,000
NeuN	Purified cell nuclei from mouse brain	Mouse monoclonal	Chemicon (Temecula, CA), cat. no. MAB377B, lot no. 25040759,	Labels specifically Fox-3 in tissue sections and a corresponding 46-58 kDa band in WB ³	1:500-1:1,000
Parvalbumin	Purified from frog muscle	Mouse monoclonal	Chemicon (Temecula, CA), cat. no. MAB1572, lot no. 070105877,	Detects single 12kDa band in WB ⁴	1:500-1:1,000
VACHT	Synthetic peptide from rat VACHT (11 aa: 519-530)	Guinea pig polyclonal	Chemicon (Temecula, CA), cat. no. AB1588, lot no. 0512017297, 1	Detects a single band of 55-60 kDa band in WB ⁵ Co-localizes with Chat in spinal cord sections ⁶	1:1,000
VACHT	Synthetic peptide from rat VACHT (20 aa: 511-530)	Goat polyclonal	Chemicon (Temecula, CA), cat. no. AB1578, lot no. 0602021588,	Perfect colocalization with VACHT (AB1588) immunoreactivity ⁷	1:500
VGLUT1	Purified recombinant rat VGLUT 1 (104 aa: 456-560)	Guinea pig polyclonal	Synaptic Systems (Goettingen, Germany), cat. no. 135 304, lot no. 135 304/3	Detects single band 62 kDa in WB ⁸	1:1,000

β-gal, β-galactosidase; GFP, Green Fluorescent Protein; NeuN, Neuronal Nuclear Protein; VACHT, Vesicular Acetylcholine Transporter; VGLUT1, Vesicular Glutamate Transporter 1; WB, Western Blot (species and preparations tested in the WBs are fully described in Materials and Methods).

¹ Valerie Siembab, observations not shown.

² Rabbit Calbindin (CB38): <http://www.swant.com/pdf/Rabbit%20anti%20Calbindin%20D-28k%20CB38.pdf>; Mouse Calbindin (CB300): <http://www.swant.com/pdf/Monoclonal%20anti-calbindind28k%20300.pdf>

³ Kim et al., 2009 J. Biol. Chem. 284:31052-31061.

⁴ Mouse Parvalbumin (MAB1572): <http://www.millipore.com/catalogue/item/mab1572>

⁵ Guinea pig VACHT (AB1588): <http://www.millipore.com/catalogue/item/ab1588>

⁶ Valerie Siembab, observations not shown.

⁷ Valerie Siembab, observations not shown.

⁸ Guinea pig VGLUT1 (135 304): <http://www.sysy.com/products/vglut1/135304-biot.html> For further information on all these antibodies (with the exception of GFP ab4745-1051 from Biogenesis) please consult the *Journal of Comparative Neurology* database at: http://wileyonlinelibrary.com/homepages/31248/ICN_Antibody_Database_V3.xls

TABLE 2

Developmental Increases in Presynaptic VGLUT1-IR Cluster Sizes

Age	Renshaw cell	n	Ia inhibitory interneuron	n
P5	2.00 ± 0.08	100	2.01 ± 0.08	100
P10	2.35 ± 0.09	100	2.35 ± 0.08	100
P15	4.03 ± 0.82	268	4.51 ± 0.12	214
P20	3.61 ± 0.14	100	4.73 ± 0.18	100
Adult	3.85 ± 0.20	100	4.84 ± 0.18	100

Average ± SEM.

n, number of boutons analyzed.

TABLE 3

Developmental Increases in Presynaptic VACHT-IR Cluster Sizes

Age	Renshaw cell	n	Ia inhibitory interneuron	n
P5	1.98 ± 0.06	100	1.52 ± 0.07	84
P10	2.51 ± 0.10	100	1.40 ± 0.09	34
P15	3.41 ± 0.07	340	1.45 ± 0.03	143
P20	3.51 ± 0.14	100	1.30 ± 0.06	41
Adult	3.66 ± 0.13	100	1.29 ± 0.05	77

Average ± SEM.

n, number of boutons analyzed.