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Parcellation of Cblns 1, 2, and 4 among different subpopulations of dorsal horn neurons in mouse spinal cord

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Abstract

The cerebellins (Cblns) are a family of secreted proteins that are widely expressed throughout the nervous system, but whose functions have been studied only in the cerebellum and striatum. Two members of the family, Cbln1 and Cbln2, bind to neurexins on presynaptic terminals and to GluR∂s postsynaptically, forming trans-synaptic triads that promote synapse formation. Cbln1 has a higher binding affinity for GluR∂s and exhibits greater synaptogenic activity than Cbln2. In contrast, Cbln4 does not form such triads and its function is unknown. The different properties of the three Cblns suggest that each plays a distinct role in synapse formation.

To begin to elucidate Cbln function in other neuronal systems, we used *in situ* hybridization to examine Cbln expression in the mouse spinal cord. We find that neurons expressing Cblns 1, 2, and 4 tend to occupy different laminar positions within the dorsal spinal cord, and that Cbln expression is limited almost exclusively to excitatory neurons. Combined *in situ* hybridization and immunofluorescent staining shows that Cblns 1, 2, and 4 are expressed by largely distinct neuronal subpopulations, defined in part by sensory input, although there is some overlap and some individual neurons co-express two Cblns.

Our results suggest that differences in connectivity between subpopulations of dorsal spinal cord neurons may be influenced by which Cbln each subpopulation contains. Competitive interactions between axon terminals may determine the number of synapses each forms in any given region, and thereby contribute to the development of precise patterns of connectivity in the dorsal gray matter.

Keywords

synapse formation; synaptic connections; neural circuitry

INTRODUCTION

The cerebellin, Cbln1, serves as a ‘synaptogenic’ protein that binds pre- and post-synaptically to stimulate synapse formation in a bidirectional manner. The four members of the Cbln family are secreted glycoproteins that are characterized by a conserved C-terminal globular C1q domain that mediates the formation of trimers (Urade et al., 1991; Pang et al., 2000) and an N-terminal cysteine motif by which the trimers assemble into hexamers (Bao et al., 2005). Cbln1, the first family member to be identified, is highly enriched in the

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Conflicts of Interest
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cerebellum, this providing the original basis for the name (Slemmon et al., 1984; Urade et al., 1991). Cbln1 is normally secreted from granule cells in the cerebellum and released from their parallel fiber terminals onto the dendritic spines of Purkinje cells. When Cbln1 is absent, the vast majority of dendritic spines (~80%) lack presynaptic contacts (Hirai et al., 2005). The synapses that do form are abnormal in structure, with the postsynaptic density extending beyond the apposed active zone, and in function, producing smaller than normal excitatory postsynaptic currents and lacking long-term depression.

The cellular events and molecular interactions underlying Cbln1's synaptogenic effects have recently been elucidated (Matsuda et al. 2010; Uemura et al., 2010; Lee et al., 2012). Cbln1 hexamers bind to the ‘orphan glutamate receptor’ GluR∂2 on Purkinje cells, promoting their clustering and the subsequent differentiation of the postsynaptic specialization. Cbln1 hexamers also bind to several isoforms of neurexin, which are expressed on the membrane of the presynaptic terminal, thereby stimulating the accumulation of synaptic vesicles. The neurexin-Cbln1-GluR∂2 triad thus bridges the synapse and promotes the differentiation of both the presynaptic terminal and the postsynaptic membrane. Cbln1 also binds to GluR∂1 and promotes synapse formation in tissue culture assays when GluR∂1, rather than GluR∂2, is present (Uemura and Mishina, 2008; Kuroyanagi et al., 2009; Ryu et al., 2012; Yasumura et al., 2012).

Much less is known about the function of the other Cblns. Cbln3 is restricted to the cerebellum and the dorsal cochlear nucleus (Miura et al., 2006) and is not secreted in the absence of Cbln1 (Bao et al., 2006). In contrast, Cbln2 and Cbln4 are widely expressed throughout the nervous system (Miura et al., 2006; Reiner et al., 2011) and can be secreted as homohexamers (Pang et al., 2000; Bao et al., 2005; 2006; Iijima et al., 2007). Cbln2 stimulates presynaptic differentiation in tissue culture, binds to GluR∂1, GluR∂2, and neurexin (Joo et al., 2011; Matsuda and Yuzaki 2011; Rong et al., 2012; Yasumura et al., 2012), and mimics the effects of Cbln1 in some, but not all, culture assays and parts of the brain (Rong et al., 2012). The function of Cbln4 remains obscure, as it has demonstrated little ability to promote presynaptic differentiation in tissue culture and shows weak, if any, binding to GluR∂1, GluR∂2, and neurexin, instead binding to the netrin-1 receptor, deleted in colorectal cancer (DCC) (Joo et al., 2011; Matsuda and Yuzaki 2011; Lee et al., 2012; Wei et al., 2012).

Our interest in Cblns started with a screen designed to reveal genes differentially expressed by specific subpopulations of developing chick DRG neurons, through which we identified the chicken homolog of Cbln2 (Yang et al., 2010). We subsequently found that Cbln2 is expressed by mechanoreceptive and proprioceptive DRG neurons, but not by nociceptive sensory neurons. We also observed that neurons in regions of the dorsal spinal cord where mechanoreceptive and proprioceptive afferents terminate express higher levels of Cbln2 than do those in regions receiving input from nociceptive afferents. This intriguing spatial pattern of Cbln2 expression suggested that Cbln2 may play a role in the establishment of sensory afferent connections in the dorsal horn. Studies examining Cbln function would be more readily undertaken in mice, however, because suitable mutants are available (Hirai et al., 2005; Rong et al., 2012; Wei et al., 2012) and knowledge of the morphological, electrophysiological, molecular properties of dorsal horn neurons and circuitry is greater.

The mammalian spinal cord has classically been divided into distinct regions based on cytoarchitectonic criteria that were first used by Rexed in the 1950's. It has since been realized that the dorsal part of the spinal cord is spatially organized according to the types of somatosensory input different regions receive. The most superficial layers of the dorsal horn, laminae I and II, receive input primarily from nociceptive, thermoceptive, and pruriceptive afferents. Laminae III and IV receive input primarily from mechanoreceptors,

while laminae V and VI receive input from proprioceptive afferents (Brown 1981). In addition, Aβ and Aδ sensory fibers terminate upon a population of 'wide-dynamic range' neurons in lamina V that respond to noxious, nonnoxious and visceral inputs (Basbaum et al., 2009). The recent use of genetic approaches is providing needed information about the organization of the dorsal horn (Braz and Basbaum, 2009; Li et al., 2011), but much remains to be learned, particularly with respect to identifying specific populations of dorsal horn neurons and delineating their patterns of synaptic connections.

Given the interestingly restricted distribution of Cbln2+ neurons in chick dorsal horn, we decided to extend our expression studies to all members of the Cbln family and to further define additional characteristics of Cbln+ neurons in the mouse. Here we show that Cblns 1, 2, and 4 are each expressed in largely separate populations of dorsal horn neurons and discuss how Cbln expression may contribute to the establishment of synaptic connectivity.

METHODS

Animals and tissue preparation

C57BL/6 mice were used for these studies. All procedures involving animals adhered to NIH guidelines and were approved by the Animal Care and Use Committee at the University of Tennessee Health Science Center. The day that newborn pups were observed in the morning was defined as P1. Mice younger than 7 days old were anesthetized with hypothermia, decapitated, and partially dissected to expose the spinal cord, which was then immersion fixed in 4% paraformaldehyde in 0.1M DEPC-treated phosphate buffer (PB; pH = 7.2–7.4) for 2 hr at 4°C. Older mice were anesthetized with intraperitoneal injection of avertin (0.2 ml per gram of body weight) and perfused transcardially with heparinized saline, followed by 15 ml of 4% paraformaldehyde in 0.1M DEPC-treated PB. The mice were then dissected to expose the spinal cord, and post-fixed for 2-4 hr at 4°C. The lumbar spinal cord was subsequently cryostat-sectioned transversely at 30 μm. The sections were mounted directly, in serial order, onto Superfrost/Plus slides (Fisher, Fair Lawn, NJ), and stored at −80°C until used.

Probes

Antisense riboprobes were generated using standard procedures and transcribed using T7 or SP6 RNA polymerase with an NTP mixture containing either digoxigenin-11-UTP (DIG; Roche Applied Science, Indianapolis, IN) or dinitrophenol-11-UTP (DNP; Perkin-Elmer, Oakbrook, IL). For Cblns 1-4, the RNA probes were directed to all or part of the 3'UTR of the respective genes, specifically Cbln1 to nucleotides 932–2275 (GenBank accession number NM_019626) by using TGACTGGCACGTATCGGGAATG as the forward primer and CGGTACAATCAAAACTTCTGGACG as the reverse primer; Cbln2 to nucleotides 694–2083 (GenBank accession number NM_172633) by using TAGACTCAGAGCCACCAGGATGA as the forward primer and TGGGATTAGTGACGTTTCCTTGGC as the reverse primer; Cbln3 to nucleotides 661–1560 (GenBank accession number NM_019820) by using TGAGGACCCAAGCCTTTAAACGGA as the forward primer and TACAGGGAAATTAGCTGGGAGACAG as the reverse primer; and Cbln4 to nucleotides 1763–2575 (GenBank accession number NM_175631) by using TAGAACCCGACTTCTCCGTGATG as the forward primer and GCGATAGAGTATTCGATTTCCCACC as the reverse primer. The GAD1 probe was directed to nucleotides 1001–2028 (GenBank accession number NM_008077), which codes for the C-terminal half of the GAD1 protein, by using GTACAGCATCATGGCTGCTCGTTAC as the forward primer, and CTGTAAAGAGGGATTACAGATCCTG as the reverse primer. The TACR1 probe was

directed to nucleotides 1901–2900 (GenBank accession number NM_009313) by using GGTGATTATGAGGGGCTGGAAATGA as the forward primer, and ACCAGGAAACACAGACACTCAAAGA as the reverse primer. This probe corresponds to 236-nt coding for the C-terminal region of the TACR1 protein and 764nt of the 3'-UTR. The TrkB probe was directed to nucleotides 577-1330 (GenBank accession number NM_001025074) by using TTATGCCTGCTGGTCTTGGGCTTCT as the forward primer, and CTGCCACACAAGAGATTTGCTTTCC as the reverse primer. This probe corresponds to 754-nt coding for part of the extracellular domain of TrkB. This probe recognizes both the long and short isoforms. It does not have significant homology with mouse TrkA or TrkC. The TrkC probe was directed to nucleotides 155–975 (GenBank accession number NM_008746) by using GCAAATTGTGTCTGCAGCAAGACTG as the forward primer, and ACACGTGGAGGGTAGTAGACAGTGA as the reverse primer. This probe corresponds to 821-nt coding for part of the extracellular domain of TrkC. This probe recognizes both the long and short isoforms. It does not have significant homology with mouse TrkA or TrkB.

The overall pattern of labeling in the mouse brain for each of the Cbln riboprobes is similar to that shown in Miura et al. (2006) and in the Allen Brain Atlas. The labeling pattern in mouse spinal cord using the GAD1 riboprobe is similar to that shown by immunostaining for GABA and in a reporter GAD67 mouse (Dougherty et al., 2009; Restrepo et al., 2009). The TACR1 riboprobe reveals large neurons in lamina I, III-VI, similar to what is found with immunolabeling (Todd et al., 1998). The TrkB, and TrkC riboprobes label large and larger DRG neurons, respectively, as is found with immunolabeling (Bourane et al., 2009). In all cases, we observed no labeling when sense probes were used.

In situ hybridization and immunohistochemistry

The *in situ* hybridization protocol was modified slightly from the protocols described in Yang et al. (2010) and Reiner et al. (2011). Briefly, slides were postfixed in 4% paraformaldehyde, treated with proteinase K, and acetylated. Hybridization buffer was: 50% formamide, 1X standard saline citrate (SSC; pH 7.0), 1X Denhardt's solution, 1 mM EDTA, 10% dextran sulfate, 100 μg/ml salmon sperm DNA, 50 μg/ml yeast RNA, 50 μg/ml yeast tRNA, 1% blocking reagent (Roche Applied Science), and 0.1% sodium dodecyl sulfate (SDS). After hybridization overnight at 65°C, slides were washed with 50% formamide/2X SSC once, 0.5X SSC twice, and then treated with 500μg/ml RNaseA in TBS for 30 min at 37°C. Sections were blocked with 0.2% casein in TBS and then incubated overnight with anti-digoxigenin-AP conjugate Fab (Roche Applied Sciences, #11093274910) at 4°C and subsequently reacted in nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Fluorescent detection used digoxigenin (DIG)-and/or dinitrophenyl (DNP)-labeled riboprobes and tyramide signal amplification. Prior to hybridization, endogenous peroxidase activity was inactivated by treating the slides with 1% H_2O_2 in PB for ½ hr. Slides were incubated in sheep anti-DIG-POD-Fab (Roche Applied Science, #1333089) or rabbit anti-DNP (Invitrogen, #A-6430), followed by goat anti-rabbit HRP (Sigma, #A6154) and subsequently reacted with FITC-tyramide or Cy3-tyramide (PerkinElmer, #NEL753001KT). For double fluorescent *in situ* hybridization, any peroxidase activity remaining after the first reaction was inactivated by incubating the slides in 2% H_2O_2 in PB for 1 hr. To verify that this produced complete inactivation, some slides were not incubated with the second HRP-conjugated antibody but still underwent the second tyramide reaction. We typically used DNP-labeled riboprobes for single fluorescence detection and for the more difficult to detect riboprobe of any given pair for double fluorescence detection, since they generally showed greater sensitivity with these tyramide procedures than did the DIG-labeled riboprobes. However, for direct comparisons of Cbln1 and Cbln2 expression, we varied which tag was

used for each Cbln, or the sequence of detection, on different slides and still obtained similar results.

After the fluorescent *in situ* hybridization staining was complete, slides were processed for immunofluorescence. Sections were incubated in primary antibodies diluted in 3% bovine serum albumin (BSA) in 0.1 M PB overnight at 4°C, washed, and then incubated with appropriate species-specific secondary antibodies conjugated to Alexa 488, 594, or 647 (Invitrogen, Grand Island, NY) or to Cy3 (Jackson ImmunoResearch, West Grove, PA) for 1 hr at room temperature. Slides were washed and then coverslipped in Fluoromount-G (SouthernBiotech, Birmingham, AL).

Antibodies

The primary antibodies used are listed in Table 1, along with their respective immunogens, sources, and species in which they were raised. Immunofluorescence was carried out on slides processed for *in situ* hybridization to help in identifying the neurons expressing each Cbln. The HuC/D antibody was used to visualize the cytoarchitecture of the spinal cord and to reveal all neurons. Hu/C and Hu/D are RNA binding proteins that are expressed only in neurons, starting from the time they begin to differentiate (Wakamatsu and Weston, 1997). The overall pattern of labeling for HuC/D in the spinal cord is identical to that observed using another pan-neuronal antibody, NeuN, but the HuC/D antibody yields more intense cytoplasmic labeling and thus allowed better visualization of individual neurons.

The N52 neurofilament 200 antibody was used to evaluate Cbln expression in the DRGs. This antibody detects both phosphorylated and non-phosphorylated heavy neurofilaments and is widely used to label large DRG neurons (e.g. Fukuoka et al., 2008; Braz and Basbaum, 2009). It recognizes a single band at 200kDa in Western blots using lysates of rat spinal cord or DRGs.

The anti-Lmx1b antibody is directed against the full-length mouse protein. It specifically labels dI5 and dILB interneurons in the mouse spinal cord, and their progeny, as do other anti-Lmx1b antibodies (Muller et al., 2002; Cheng, et al., 2004).

The anti-Pax2 antibody is specific for the Pax2 protein and does not cross-react with other Pax family members (Dressler and Douglass, 1992). Immunoblotting, using mouse embryonic kidney lysates, reveals two bands: a major band at 46 kDa (Pax2b) and a minor band at 48 kDa (Pax2a), representing two isoforms of Pax2 from alternative splicing. This antibody also recognizes tumor cells in which Pax2 mRNA could be detected with *in situ* hybridization (Dressler and Douglass, 1992). This anti-Pax2 antibody yields the same pattern of expression shown with *in situ* hybridization (Cheng, et al., 2004).

The calbindinD28K (henceforth, referred to simply as calbindin) and calretinin antibodies were used to identify specific populations of dorsal horn interneurons. They each showed the same distribution pattern in the dorsal horn as has been reported previously using other antibodies to these two proteins (e.g. Ren and Ruda, 1994; Huang et al., 2005; Braz and Basbaum, 2009). In addition, double-labeling mouse spinal cord sections with the rabbit and mouse calbindin antibodies marked all of the same neurons, whereas we did not detect any coexpression when the sections were double-labeled with the mouse anti-calretinin antibody and the rabbit anti-calbindin antibody. Thus, despite the 58% homology between calbindin and calretinin, the three antibodies are each specific for the intended protein.

The PKCγ antibody labels axons in the main dorsal component of the mouse corticospinal tract and a population of lamina II neurons, as is found using other PKCγ antibodies (Braz and Basbaum, 2009; Seal et al., 2009). The antiserum stains two closely spaced bands at

about 80kDa in Western blots prepared from rat cerebellum and neocortex (Cardell et al., 1998) and does not detect PKCα, PKCβI or PKCβII when they are separately expressed in transfected cells. Staining in the corticospinal tract at caudal levels of the spinal cord disappears following lesioning of the dorsal funiculus or lateral hemisection in rat (Nielson et al., 2011).

Image capture and analysis

Brightfield images were obtained using a ScanScope digital slide scanner (Aperio Technologies; Vista, CA). Images of fluorescently labeled tissue sections were captured with a Zeiss 710 confocal laser-scanning microscope. Images were subsequently processed and analyzed using Adobe Photoshop. To help determine laminar location, we compared staining of our tissue for the pan-neuronal marker HuCD with images of Nissl stained sections, and the accompanying outlines, available at the Allen Brain Atlas website (Fig. 1D). In some cases, we used staining for PKCγ to identify the border between lamina II and lamina III.

To count labeled neurons, we created an overlying layer of the image in Adobe Photoshop, in which we marked neurons labeled with a particular fluorophore, using one color channel to visualize staining for the riboprobe and another channel to visualize the HuC/D labeling of all neurons (e. g. Fig. 2L) in enlarged images viewed on the computer monitor. Given that tyramide signal amplification produces a speckly reaction product, the HuC/D staining helped indicate when the tyramide labeling was associated with specific neuronal labeling. This was particularly useful for detecting lightly labeled neurons, such as those expressing Cbln4. Cbln4 labeling was typically less intense than that of Cbln1 and Cbln2 due, at least in part, to the shorter length of the Cbln4 riboprobe (813nt vs 1.3-1.4kb). Visualizing Cbln4+ neurons thus required a longer reaction time for an adequate signal to develop, with an accompanying increase in background staining. To assess the co-occurrence of two riboprobes, we marked the neurons labeled for each riboprobe independently, in two separate layers, and later reexamined the overlain images to ascertain that each pair of overlying marks indeed represented the same cell. To assess Cbln co-expression with calretinin, calbindin, or PKCγ, Cbln+ neurons were first identified and marked, as just described, and then examined for protein expression. For all counts of double-labeled neurons, a minimum of six sections was analyzed. The brightness and contrast of the images shown in the figures have been modified for increased clarity of presentation.

RESULTS

Pattern of Cbln expression in the spinal cord

To characterize Cbln expression in the spinal cord, we started by carrying out *in situ* hybridization for individual DIG-labeled probes, visualizing each probe with a NBT reaction product. We used sections from P10-P17 mice because the spatial pattern of Cbln expression changes little, if at all, postnatally and yet, Cbln mRNA levels are higher than they are in adult animals (Miura et al., 2006). Moreover, the organization of the dorsal horn is mature by this time (Woodbury et al., 2000; Cavanaugh et al., 2011). As shown in Fig. 1A-C, neurons expressing Cbln1 and those expressing Cbln2 are abundant in the dorsal horn, while Cbln4+ neurons are less numerous. Cbln3+ neurons are lacking, as they are from most of the brain, except for the cerebellum and dorsal cochlear nucleus (Miura et al., 2006). Some, albeit fewer, neurons expressing Cbln1, Cbln2, or Cbln4 are also present in the ventral half of the spinal cord, but will not be discussed here.

The three classes of Cbln+ neurons present in the spinal cord primarily occupy different dorsal-ventral positions within the dorsal horn, although with some spatial overlap. Most

Cbln1+ neurons are located in the inner part of lamina II and the dorsal part of lamina III, whereas most Cbln2+ neurons are located in the ventral part of lamina III and in lamina IV, and most Cbln4+ neurons are located in laminae V and VI.

In addition to the overall dorsal-ventral stacking arrangement of Cbln1+, Cbln2+, and Cbln4+ neurons in the dorsal horn, all three Cblns are expressed in lamina I. The Cbln2+ neurons are intensely stained and seem to be the most numerous of the three in this most superficial lamina, whereas the Cbln4+ neurons are only lightly labeled and are the least numerous.

Coexpression of Cblns in the dorsal horn

The results described above suggested that each Cbln is largely restricted to a discrete set of dorsal horn neurons. To examine this directly, we carried out double fluorescent *in situ* hybridization, using DIG to tag one riboprobe and DNP to tag the other, followed by immunofluorescent detection of the riboprobes using tyramide signal amplification. The sections were also immune stained for the pan-neuronal marker, HuC/D, to help visualize individual neurons and the overall organization of the spinal cord.

Fig. 1E-G and Table 2 show that Cbln1 and Cbln2 are predominantly expressed by separate populations of dorsal horn neurons. Neurons double-labeled for Cbln1 and Cbln2 constitute 18.5% of all Cbln1+ neurons and 24.0% of the somewhat less numerous Cbln2+ neurons. Similarly, only 5.2% of Cbln1+ neurons and 24.3% of Cbln2+ neurons in the dorsal horn also label for Cbln4. Assuming that very few neurons express all three Cblns (see the following paragraph), the majority (76.3%) of Cbln1+ neurons would then express only Cbln1 and more than half (51.7%) of all Cbln2+ neurons would express only Cbln2. In contrast, given that 12.6% of Cbln4+ neurons also express Cbln1 and 44.4% also express Cbln2, it is likely that fewer than half (43.0%) of all Cbln4+ neurons express only Cbln4.

Most Cbln1+/Cbln2+ neurons are located in laminae II and III, whereas most Cbln1+/Cbln4+ neurons are situated in laminae IV and V and so these two types of double-labeled neurons are likely to represent two separate neuronal populations. Although Cbln1+/Cbln4+ neurons and Cbln2+/Cbln4+ neurons show some spatial overlap in laminae IV-VI, Cbln2+/Cbln4+ neurons typically lie deeper and are larger. Thus, these two types of double-labeled neurons are also likely to represent two separate populations of neurons. Together the differences in neuronal sizes and spatial distribution suggest that few (<5%), if any, Cbln+ neurons express all three Cblns, although without visualizing all three Cblns in individual sections, we cannot be certain of the precise percentage possessing all three. Interestingly, despite the expression of all three Cblns in lamina I, it contains exceedingly few double-labeled neurons. In contrast, double-labeled neurons comprise a more significant proportion of the total population in laminae IV and V.

Preferential expression of Cblns in excitatory dorsal horn neurons

We previously reported that Cbln2 is preferentially expressed by excitatory neurons in the dorsal horn of embryonic chicks (Yang et al., 2010). To determine if this is true for Cbln2+ neurons in the mouse dorsal horn, and for Cbln1+ and Cbln4+ neurons as well, we used two approaches. For the first approach, we took advantage of findings that Lmx1b and Pax2, homeodomain proteins that are expressed by different populations of progenitor cells in the developing spinal cord, also distinguish between glutamatergic excitatory neurons and GABAergic inhibitory neurons, respectively (Cheng et al., 2004). Accordingly, we combined *in situ* hybridization for Cbln1 or Cbln2 with immunofluorescent staining for Lmx1b and/or Pax2. We used P1-P6 mice for this analysis, because Lmx1b and Pax2 expression is downregulated postnatally (Rebelo et al., 2010), yet the pattern of Cbln1 and

Cbln2 expression in the dorsal horn is the same as at later times. The analysis was restricted to the dorsal one-third of the spinal cord, since nearly all Lmx1b+ and most Pax2+ neurons are situated in laminae I-III. (Excitatory neurons located more ventrally originate from different populations of progenitors expressing other transcription factors.) As shown in Fig. 2A-H, both Cbln1+ and Cbln2+ neurons are predominantly Lmx1b+. Cell counts (Table 3) showed that nearly all (89.8%) Cbln1+ neurons express Lmx1b, whereas exceedingly few (2.3%) express Pax2. Similarly, the vast majority (79.8%) of Cbln2+ neurons express Lmx1b and very few (7.5%) express Pax2. The dorsal horn is comprised of about 60% excitatory neurons and 40% inhibitory neurons (based on Lmx1b/Pax2 labeling; Cheng et al., 2004). The predominance of Cblns in excitatory neurons far exceeds those proportions.

Nearly all the remaining 7.9% of Cbln1+ neurons and 12.7% of Cbln2+ neurons (i.e. those not possessing either Lmx1b or Pax2) are situated in the ventral part of the analyzed region, corresponding to lamina IV, where Lmx1b+ and Pax2+ neurons are less abundant. Their excitatory vs inhibitory nature thus cannot be ascertained from this analysis. To overcome this limitation, we used a second approach, based on GAD1 expression. Inhibitory neurons in the spinal cord use either GABA or glycine for neurotransmission, but many dorsal horn neurons that use glycine also use GABA (nearly all in lamina I-III and about half in lamina IV-VI; Todd and Sullivan, 1990; Polgar et al., 2003). GABA is synthesized by either GAD67 (which is encoded by the GAD1 gene) or GAD65 (which is encoded by the GAD2 gene). GAD1 predominates in the dorsal horn (Feldblum et al., 1995; Restrepo et al., 2009) and is expressed at high levels in early postnatal mice (Dougherty et al., 2009). Accordingly, for the second approach, we carried out double fluorescent *in situ* hybridization for GAD1 and each Cbln, using mice at P10-12 before GAD1 levels are developmentally downregulated. As shown in Fig. 2I-L and Table 3, only ~2% of Cbln1+ and Cbln2+ neurons and <10% of Cbln4+ neurons in the dorsal half of the spinal cord express GAD1. Thus, consistent with the results described above for Cblns with Lmx1b and Pax2, each of the Cblns is expressed predominantly by excitatory neurons in the dorsal horn and only very rarely by inhibitory neurons.

Expression of Cblns in specific subpopulations of excitatory dorsal horn neurons

The dorsal horn contains diverse types of neurons that vary in their somatodendritic morphology, electrophysiological properties, connectivity, and neurochemistry. Several neuropeptides, calcium-binding proteins, and enzymes are restricted in their distribution within the dorsal horn and thus serve to distinguish different neuronal subpopulations (Todd, 2010). Some of these molecules are expressed only in inhibitory neurons (e.g. neuropeptide Y and parvalbumin), while others are expressed only in excitatory neurons. To further characterize Cbln-expressing dorsal horn neurons, we next combined fluorescent *in situ* hybridization with immunofluorescent labeling, using markers that define specific subpopulations of excitatory dorsal horn neurons. The three markers we used, calbindin, calretinin, and PKCγ, are especially abundant in lamina II, which is comprised of three sublaminae receiving different patterns of somatosensory input.

As shown in Fig. 3 and Table 4, 57.7% of Cbln1+ neurons express calbindin, nearly 20% express PKCγ, and less than 1% express calretinin. In accord with the lesser abundance of Cbln2+ neurons in lamina II, only a small percentage of Cbln2+ neurons express these markers (~5% are calbindin+, ~5% PKCγ+, and ~1% calretinin+). In contrast, Cbln4+ neurons in lamina II represent a completely separate subpopulation of interneurons, with nearly all, corresponding to about 12% of the total number of Cbln4+ neurons in the dorsal spinal cord, expressing calretinin. We did not combine *in situ* hybridization for Cbln4 with immunofluorescent detection of calbindin and PKCγ since Cbln4+ neurons are rarely found in lamina IIi, where those neurons predominate. Thus, each Cbln has a distinctive profile in

terms of its expression in calbindin+, calretinin+ and PKCγ+ neurons. Moreover, the vast majority of calbindin+ and PKCγ+ neurons in the dorsal horn express a Cbln, most commonly Cbln1 and to a lesser extent Cbln2, whereas while some calretinin+ neurons in the dorsal horn express Cbln4, most calretinin+ neurons completely lack all Cblns.

Expression of Cblns in putative projection neurons

The analysis described above accounts for the majority of Cbln1+ neurons in the dorsal horn, but only ~10% of Cbln2+ and ~10% of Cbln4+ neurons. Most Cbln2+ and Cbln4+ neurons are situated in laminae III-VI, for which few neurochemically distinct subpopulations of neurons have been identified (Braz and Basbaum, 2009). Neurons in these laminae are instead typically distinguished by whether their projections are confined to the spinal cord or extend to the brain (projection neurons), with the projection neurons typically having larger cell bodies. Many Cbln2+ neurons in laminae III-VI are particularly large (see, for example, Fig. 1E, G) and are thus likely to be projection neurons.

Several types of projection neurons are present in the dorsal horn. Neurons forming the dorsal spinocerebellar tract are situated in Clarke's column at lower thoracic and upper lumbar levels and in the medial part of laminae IV-VI at mid-lumbar levels (e.g. Shrestha et al., 2012). Large neurons situated in these regions are often Cbln2+ (Figs. 1G, 3B). A second type of projection neuron extends to the caudal ventrolateral medulla, the lateral parabrachial area, and several thalamic nuclei (Todd 2010), and can be identified by the expression of the receptor for substance P, TACR1. *In situ* hybridization for TACR1 revealed labeled neurons in lamina I and laminae III-VI, as has been previously reported for rats (Littlewood et al., 1995; Todd et al., 1998; Fig. 3F, G). The double fluorescent *in situ* hybridization showed that 20.2% of Cbln2+ neurons in the dorsal horn also express TACR1, as do 23.3% of Cbln4+ neurons, but only 5.2% of Cbln1+ neurons (Table 5). The expression of TACR1 in Cbln-expressing neurons is consistent with our findings described above that nearly all Cbln-expressing neurons lack GAD1 and with reports by others that projection neurons are themselves excitatory (Littlewood et al., 1995). Our TACR1/Cbln double labeling studies further indicate that most TACR1+ neurons also express a Cbln; 10.5% also express Cbln1, 43% also express Cbln2, and 18.8% also express Cbln4. Depending on how many of these neurons also express a second Cbln, as few as 43% (if all do) or as many as 73% (if none do) of all TACR1+ neurons would also be Cbln+.

Expression of Cblns in lamina I

Although the neurons that express Cbln1, Cbln2, and Cbln4 largely occupy different positions within the dorsal spinal cord, they are intermixed in lamina I. Figs. 1, 2J, and 3B show that Cbln2+ neurons are more intensely stained and more numerous than other Cbln+ neurons in lamina I, whereas Cbln4+ neurons are stained the lightest and are the least numerous. Our counts show that about 10% of Cbln2 + neurons and ~6% of Cbln1+ neurons are situated in lamina I. Further, a higher number of neurons in lamina I express Cbln2 than express Cbln1, despite there being ~30% more Cbln1+ neurons than Cbln2+ neurons in the dorsal horn overall. Moreover, although Cbln1+, Cbln2+, and Cbln4+ neurons are all present in lamina I, the incidence of neurons expressing more than one Cbln is extremely low. We detected, at most, one neuron double-labeled for Cbln2 and Cbln4 in a given section (mean = 0.4 neurons/section), and no coexpression of Cbln1 and Cbln4. Thus, discrete populations of lamina I neurons express each of the three Cblns.

Lamina I contains both interneurons and projection neurons, many of the latter expressing high levels of TACR1 (Al Ghamdi et al., 2009; Todd, 2010). About 17% of all Cbln1+ lamina I neurons, ~34.4% of all Cbln2+ lamina I neurons, and ~10.3% of Cbln4+ lamina I neurons were found to express TACR1 (Fig. 3F, G). About 38.9% of the TACR1+ neurons

in lamina I are Cbln2+, while ~14.5% are Cbln1+ and another 13.9% are Cbln4+. Given that extremely few lamina I neurons express more than one Cbln, this indicates that the majority (~67.3%) of TACR1+ projection neurons in lamina I are likely to be Cbln-positive.

Expression of Cblns in DRGs

We also examined Cbln expression in the DRGs, given that they provide a large input to the dorsal horn. We found Cbln1 expression in a small subset of small DRG neurons. In contrast, Cbln2 is expressed at high levels in large DRG neurons. We then combined fluorescent *in situ* hybridization for each Cbln with detection of various subpopulations of DRG neurons. Based on their small size, Cbln1+ neurons could correspond to a subset of peptidergic nociceptors, nonpeptidergic nociceptors, pruriceptors, or C-fiber mechanoreceptors (Liu and Ma, 2011). However, because of the low level of Cbln1 expression in the DRGs, we were unable to obtain consistent fluorescent labeling and therefore unable to make this determination. To ascertain if the large Cbln2+ neurons correspond to mechanoreceptors or proprioceptors, we carried out double fluorescent *in situ* hybridization for Cbln2 and either TrkB or TrkC, and combined this with immunofluorescent detection of NF200, a marker of large neurons having heavily myelinated axons. We found that nearly all Cbln2+ DRG neurons are NF200+, with the vast majority (89%) expressing TrkB (Fig. 3I) and very few (1.9%) expressing TrkC. Thus, Cbln2+ DRG neurons correspond to mechanoreceptors, whereas proprioceptors are Cbln2-negative, in contrast to the situation in chick where both mechanoreceptors and proprioceptors express Cbln2 (Yang et al., 2009). Further, while the majority of TrkB+ DRG neurons in mouse express Cbln2, a sizable proportion (32%) do not. Three subtypes of TrkB + mechanoreceptors differing in their peripheral endings, physiological properties and expression of ret, CMaf, and MafA have recently been identified (Bourane et al., 2009; Reed-Geaghan and Maricich, 2011). Determining which of these subtypes of mechanoreceptors express(es) Cbln2 would require additional multiple-labeling studies.

DISCUSSION

The results reported here show that three of the four members of the Cbln family are expressed in the mouse dorsal horn, and by largely different subpopulations of neurons. We had previously found that in chick spinal cord Cbln2+ neurons are most abundant in regions that receive TrkB+ (i. e. mechanoreceptive) and TrkC+ (i. e. proprioceptive) input (Yang et al., 2010). The pattern of Cbln2 expression in the mouse spinal cord is generally similar to that in chick, despite considerable differences in the cytoarchitecture of the dorsal horn in chick as compared to other higher vertebrates. This, together with our previous observations that the level of Cbln2 expression in many regions of the chicken brain is very similar to that in the mammalian homologs (Reiner et al., 2011), supports the overall view that the expression patterns of molecules that play fundamental roles in processes such as the formation of specific synaptic connections are evolutionarily conserved.

Our previous work in chick had also shown that the majority of Cbln2+ neurons in the dorsal horn are excitatory. The same is true for Cbln1+, Cbln2+, and Cbln4+ neurons in the mouse, but to an even greater extent, with only ~2% of Cbln1+, ~2% of Cbln2+, and ~9% of Cbln4+ neurons being inhibitory. The nearly complete restriction of Cblns to excitatory neurons we find in the dorsal spinal cord does not, however, apply to Cbln expression in the brain. For example, Cbln1 is expressed by GABAergic neurons in the internal pallidal segment in the mouse (Miura et al., 2006; Wei et al., 2009) and Cbln2 by GABAergic neurons in parts of the amygdala, in chick (Reiner et al., 2011). Moreover, Cblns 1 and 2 stimulate the formation of inhibitory synapses in cortical neuron cultures, suggesting that their actions are not limited to excitatory synapses (Joo et al, 2011; Matsuda and Yuzaki

2011; Yasamura et al., 2012). Whether Cblns 1 and 2 induce excitatory or inhibitory synapses must instead depend on the type of presynaptic neuron and the particular neurexin isoforms that neuron expresses.

Cbln 1, 2, and 4 expression in different subpopulations of dorsal horn neurons

Neurons expressing Cblns 1, 2, and 4 tend to occupy different dorsal-ventral positions within the dorsal horn. The majority of Cbln1+ neurons are situated in lamina IIi and the dorsal part of lamina III. In contrast, most Cbln2+ neurons are located more ventrally, in the ventral part of lamina III and in lamina IV, and most Cbln4+ neurons are located even more ventrally, in laminae V and VI. Interestingly, this dorsal-ventral stacking of the three classes of Cbln+ neurons parallels the way afferent input corresponding to different sensory modalities is organized in the dorsal horn. Several sources of afferent input have additionally been shown to form synapses on specific subpopulations of neurochemically defined dorsal horn neurons (Braz and Basbaum, 2009). We have characterized the neurons expressing each Cbln with respect to these neurochemical features and also, as to whether they correspond to interneurons or projection neurons. Our analyses show that, even when there is some spatial overlap between neurons expressing the three different Cblns, they largely represent different neuronal populations. However, some dorsal horn neurons express more than one Cbln with a distinct subpopulation of Cbln1+/Cbln2+ neurons that is situated primarily near the border between lamina II and lamina III and another subpopulation of Cbln2+/Cbln4+ neurons that is situated in laminae IV and V. The location and identities of the various subpopulations of Cbln-expressing neurons are shown by the schematic drawings in Fig. 4 and discussed further below.

Cbln1+ neurons—Cbln1 is expressed nearly exclusively by interneurons and very rarely (5.2%) by putative projection neurons. The majority of Cbln1+ neurons are located in lamina IIi, which is comprised solely of interneurons. Cbln1+ neurons situated in other laminae also appear to be interneurons in that they rarely express TACR1 and tend to be smaller than most of their neighboring neurons. Nearly 60% of Cbln1+ dorsal horn neurons belong to the calbindin+ cell population and ~20% to the population of PKCγ+ interneurons in lamina IIi. Likewise, the majority of calbindin+ and PKCγ+ interneurons in lamina IIi are Cbln1+.

Calbindin+ lamina IIi interneurons have been shown to receive input from unmyelinated, nonpeptidergic, IB4+ nociceptive afferents (Braz and Basbaum, 2009). By contrast, PKCγ+ lamina IIi interneurons (which are implicated in allodynia; Miraucourt et al., 2007) receive input from myelinated vGlut1+ mechanoreceptive afferents (Neumann et al., 2008), as would Cbln1+ neurons situated in the dorsal part of lamina III (Alvarez et al., 2004). In addition, both C-fiber vGlut3+ low-threshold mechanoreceptors (LTMRs), which are nonpeptidergic and IB4-negative, express tyrosine hydroxylase, and mediate intense mechanical pain and the mechanical hypersensitivity caused by inflammation and nerve injury (Seal et al., 2009; Li et al., 2011) and Aδ-LTMRs, also known as D-hair cells (Light and Perl, 1979) and recently identified by their expression of TrkB (Li et al., 2011), project to the region occupied by PKCγ+ interneurons, and may thus provide additional sources of somatosensory input to Cbln1+ neurons.

Cbln2+ neurons—The vast majority of Cbln2+ neurons are situated in the ventral part of lamina III, lamina IV, and the dorsal part of lamina V, which receive extensive input from myelinated vGlut1+ mechanoreceptive afferents. Cbln2+ neurons are also abundant in medial lamina V, where myelinated vGlut1+ proprioceptive afferents terminate on Ia interneurons (Alvarez et al., 2004). Laminae III-VI contain a mix of interneurons and projection neurons, the latter being larger in size, as are most of the Cbln2+ neurons. Some

of the large Cbln2+ neurons are situated far medially, near the dorsal funiculus at the juncture of laminae IV and V, and are thus likely to correspond to dorsal spinocerebellar tract neurons (Miesegaes et al., 2009; Hantman and Jessell, 2010). Numerous other large Cbln2+ neurons are situated in laminae III-V and express TACR1. Many TACR1+ neurons in laminae III-V possess dendrites that extend dorsally into lamina I, where they receive input from peptidergic afferents (Naim et al., 1997; Todd et al., 2002), and axons that project to the lateral parabrachial area, the thalamus and the periaqueductal gray (Basbaum et al., 2009; Todd 2010), and thereby transmit pain information to the brain.

Cbln4+ neurons—Cbln4+ neurons are primarily situated in laminae V-VI, and so are spatially segregated from Cbln1+ neurons in laminae II and III. Cbln4+ neurons and Cbln2+ neurons, however, exhibit considerable spatial overlap, and in fact ~40% of Cbln4+ neurons are also Cbln2+. Cbln2+/Cbln4+ neurons are predominantly located in laminae IV and V and probably correspond to the TACR1+ projection neurons discussed above. Most other Cbln4+ neurons are intermediate in size and lie even deeper, in the ventral part of lamina V and in lamina VI, largely separate from the Cbln2+ neurons. Another distinct population of Cbln4+ neurons is situated in lamina IIo, where Cbln1+ and Cbln2+ neurons are scarce. Lamina IIo receives input primarily from unmyelinated peptidergic afferents and thinly myelinated A∂ afferents (Braz and Basbaum, 2009; Todd, 2010). The Cbln4+ neurons in lamina IIo express calretinin (Ren and Ruda, 1994; Huang et al., 2005). Approximately 12% of Cbln4+ neurons in dorsal horn belong to this population of calretinin+ interneurons, although the vast majority of calretinin+ interneurons lack Cbln4.

Lamina I—In contrast to other layers of the dorsal horn, lamina I contains neurons that express each of the three Cblns. Cbln2+ neurons are the most abundant, with ~40% expressing high levels of TACR1 and thus probably corresponding to projection neurons. In contrast, the vast majority (>85%) of Cbln1+ neurons and Cbln4+ neurons in lamina I lack TACR1 and are likely to be interneurons. Although Cbln1+, Cbln2+, and Cbln4+ neurons are intermixed in lamina I, very few express more than one Cbln. Lamina I receives input from unmyelinated peptidergic, unmyelinated nonpeptidergic vGlut3+, and thinly myelinated A∂ sensory afferents (Braz and Basbaum, 2009; Seal et al., 2009; Li et al., 2011). One possibility is that the three Cbln-specific neuronal populations differ from one another with respect to the sensory input they receive. However, the input from peptidergic and A∂ afferents appears to converge on lamina I neurons (Braz and Basbaum, 2009; but note that roughly one-third of the A∂ afferents are peptidergic (McCarthy and Lawson, 1990). Lamina I contains neurons selective for specific sensory modalities (i.e. nociceptive, thermoreceptive, histamine-dependent and histamine-independent itch; Craig 2003; Sun et al., 2009; Liu and Ma, 2011), neurons responding to multiple modalities (i.e. polymodal nociceptive neurons, which detect heat, cold, and pinch) and neurons exhibiting four different types of responses to injection of depolarizing current (i.e. tonic, phasic, delayed onset, or single spikes; Prescott and DeKoninck, 2002). Finally, four distinct morphological classes of lamina I neurons have been identified based on the shape of their cell bodies and dendritic arbors (i.e. fusiform, multipolar, pyramidal, and flattened; Lima and Coimbra, 1986). Whether the individual Cbln-specific neuronal populations correspond to any of these functional or morphological classes of lamina I neurons remains uncertain.

The role of Cblns in promoting synapse formation

Very little is known about the mechanisms by which different functionally important subpopulations of dorsal horn neurons become integrated into specific neuronal circuits. Results showing that Cblns 1, 2, and 4 vary in their binding affinities for their receptors, and thereby in their ability to promote synapse formation, suggest the possibility that Cblns may contribute to the establishment of appropriate patterns of connectivity. Synapse formation is

a highly dynamic process, with continuous bidirectional signaling between axons and dendrites, starting from the time they initially contact one another and ending with the stabilization and maturation of some contacts and the loss of others. Competitive interactions among multiple presynaptic inputs for postsynaptic targets or among multiple postsynaptic targets for presynaptic input have been shown to determine the final outcome in some neuronal systems. For example, cortical neurons expressing higher concentrations of neuroligin receive more synaptic contacts than do their neighbors, suggesting that they have competed more successfully for limited amounts of neurexin, neuroligin's binding partner, on presynaptic terminals (Kwon et al., 2012). Given that connectivity patterns can be influenced by the relative abundances of pre- and post-synaptic adhesion proteins, it seems likely that the relative binding affinities of different Cblns for their receptors may do so as well. Below we describe how competitive interactions among neurons expressing different Cblns may affect the establishment of precise patterns of synaptic connectivity in the dorsal spinal cord.

Cbln1—Current understanding of Cbln function is largely based on studies of parallel fiber synapses onto Purkinje cells, and more recently, of cerebellar and heterologous cell culture systems. Cbln1 is secreted from granule cells in hexameric form (Bao et al, 2005). Two such Cbln1 hexamers bind to tetrameric GluR∂2 on the post-synaptic surface and to four monomeric neurexins on the presynaptic terminal membrane, forming a “synaptogenic triad” (Lee et al., 2012). Cbln1 binding to neurexins stimulates their clustering, the accumulation of synaptic vesicles and the ultimate formation of presynaptic boutons (Ito-Ishida et al., 2012). Cbln1 binding to GluR∂2 promotes its clustering, which in turn recruits the scaffolding proteins, homer and shank, and AMPA receptors to the postsynaptic surface (Matsuda et al., 2010). Cbln1 thereby stimulates the differentiation of both the presynaptic terminal and the postsynaptic density. Cbln1 can also bind to GluR∂1 (Wei et al., 2012; Yasamura et al., 2012) and such binding can stimulate presynaptic and postsynaptic differentiation in cultured heterologous, cortical or hippocampal cells (Uemura and Mishina, 2008; Kuroyanagi et al., 2009; Ryu et al., 2012; Yasumura et al., 2012).

Similar events may explain how Cbln1+ neurons form synapses in the dorsal horn. The majority of Cbln1+ dorsal horn neurons are excitatory lamina II interneurons that contain either calbindin or PKCγ and whose axons arborize in laminae I-III (Morris et al., 2004). Neurexins, GluR∂1 and GluR∂2 are all expressed in the spinal cord (Allen Brain Atlas). Cbln1 binds to both GluR∂s with higher affinity and exhibits greater synaptogenic activity than either Cbln2 or Cbln4. Cbln1+ interneurons would thus have a competitive advantage over neurons expressing the other (or no) Cblns in establishing synapses within the dense neuropil of lamina II.

Cbln2—Cbln2 stimulates presynaptic differentiation in a variety of neuronal culture systems (Joo et al., 2011; Matsuda and Yuzaki, 2011; Yasumura et al., 2012), although not as effectively as Cbln1. In accord with this, Cbln2 binds GluR∂1, GluR∂2, and neurexin, but with a lower affinity than Cbln1 for each binding partner (Joo et al., 2011; Matsuda and Yuzaki, 2011; Yasumura et al., 2012; Rong et al., 2012; Wei et al., 2012). Within the dorsal horn, the majority of Cbln2+ neurons are putatively projection neurons that lie in laminae III-V or, to a lesser extent, in lamina I. These neurons may not form synapses as their axons grow through the dense neuropil of the dorsal horn toward white matter tracts ascending to the brain, because, given Cbln2's lower synaptogenic activity, they may be outcompeted by the nearby axons of Cbln1-containing interneurons.

Another subpopulation of Cbln2+ neurons, situated primarily in laminae II and III, coexpresses Cbln1. Heterologous cells transfected with two Cblns secrete a combination of homohexamers comprised of each Cbln and heterohexamers comprised of both Cblns

(Iijima et al., 2007). Neurons coexpressing Cbln1 and Cbln2 should similarly release both kinds of homohexamers, as well as heterohexamers, and should thus have a synaptogenic potential midway between that of the Cbln1+ interneurons and the Cbln2+ projection neurons. Accordingly, Cbln1+/Cbln2+ interneurons may be more limited in their ability to form synapses in lamina II, by competition from the many Cbln1+ interneurons present, than they would be elsewhere, for example, in lamina III or IV.

TrkB+ DRG neurons, which probably correspond to mechanoreceptive afferents whose synapses are located primarily in lamina III (Li et al., 2011), also express Cbln2. These afferent fibers grow through the superficial dorsal horn (laminae I and II) without branching (Ozaki and Snider, 1997). That Cbln2+ DRG bypass numerous potential post-synaptic targets in lamina II may reflect the presence of many Cbln1+ interneurons, whose axons would possess a higher level of synaptogenic activity. Upon reaching lamina III, where few Cbln1+ interneurons are present, Cbln2+ central processes would face less competition for post-synaptic targets and would therefore be more likely to form synapses.

Cbln4—In contrast to Cbln1 and Cbln2, Cbln4 stimulates presynaptic differentiation very weakly, if at all, in the various culture systems in which it has been tested (Joo et al., 2011; Matsuda and Yuzaki, 2011; Yasumura et al., 2012). Cbln4 also does not bind to GluR∂1, GluR∂2, or neurexins (Joo et al., 2011; Matsuda and Yuzaki, 2011; Lee et al., 2012; Rong et al., 2012; Wei et al., 2012; Yasumura et al., 2012) and instead binds to DCC (Wei et al., 2012). DCC has recently been shown to contribute to synaptogenesis and synaptic plasticity; in its absence, hippocampal neurons have shorter dendritic spines and long-term potentiation is impaired (Horn et al., 2013). Cbln4-null mice show no obvious impairments (Wei et al., 2012), however, and thus, whether Cbln4 binding to DCC promotes synapse formation is currently uncertain.

In the dorsal horn, Cbln4 is expressed by a subset of calretinin+ neurons in lamina IIo. Interneurons in lamina II make synapses locally and so DCC, which is expressed by moderate levels by neurons in the dorsal horn (Allen Brain Atlas), may contribute to the formation of synapses made by those Cbln4+ neurons. Cbln4 is also expressed by intermediate-sized neurons in laminae V and VI, whose synaptic targets, to the best of our knowledge, have not been identified. Interestingly, the majority of Cbln4+ dorsal horn neurons express another Cbln. Cbln4 is most frequently coexpressed with Cbln2 in large projection neurons located in laminae IV and V. These projection neurons would then secrete three kinds of Cbln hexamers: Cbln4 homohexamers, which would bind to DCC; Cbln2-Cbln4 heterohexamers, which would exhibit moderate levels of binding to both neurexin-GluR∂ and to DCC (Wei et al., 2012); and Cbln2 homohexamers, which would bind to neurexin-GluR∂ and thereby promote synapse formation.

The results presented here show that several discrete subpopulations of dorsal horn neurons can be distinguished based on the individual Cblns or combination of Cblns they express. These neuronal subpopulations would then vary in their ability to form synapses, with the expression of neurexins, GluR∂1 and GluR∂2 on different pre- and post-synaptic neurons also influencing the number and the strength of the synapses that are made and maintained. Determining whether Cblns indeed contribute to the development of precise patterns of connectivity in the dorsal horn will require manipulating their expression.

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Role of Authors

MCC performed experiments and analyzed data. MGH designed the study, performed experiments, analyzed and interpreted the data, and wrote the article.

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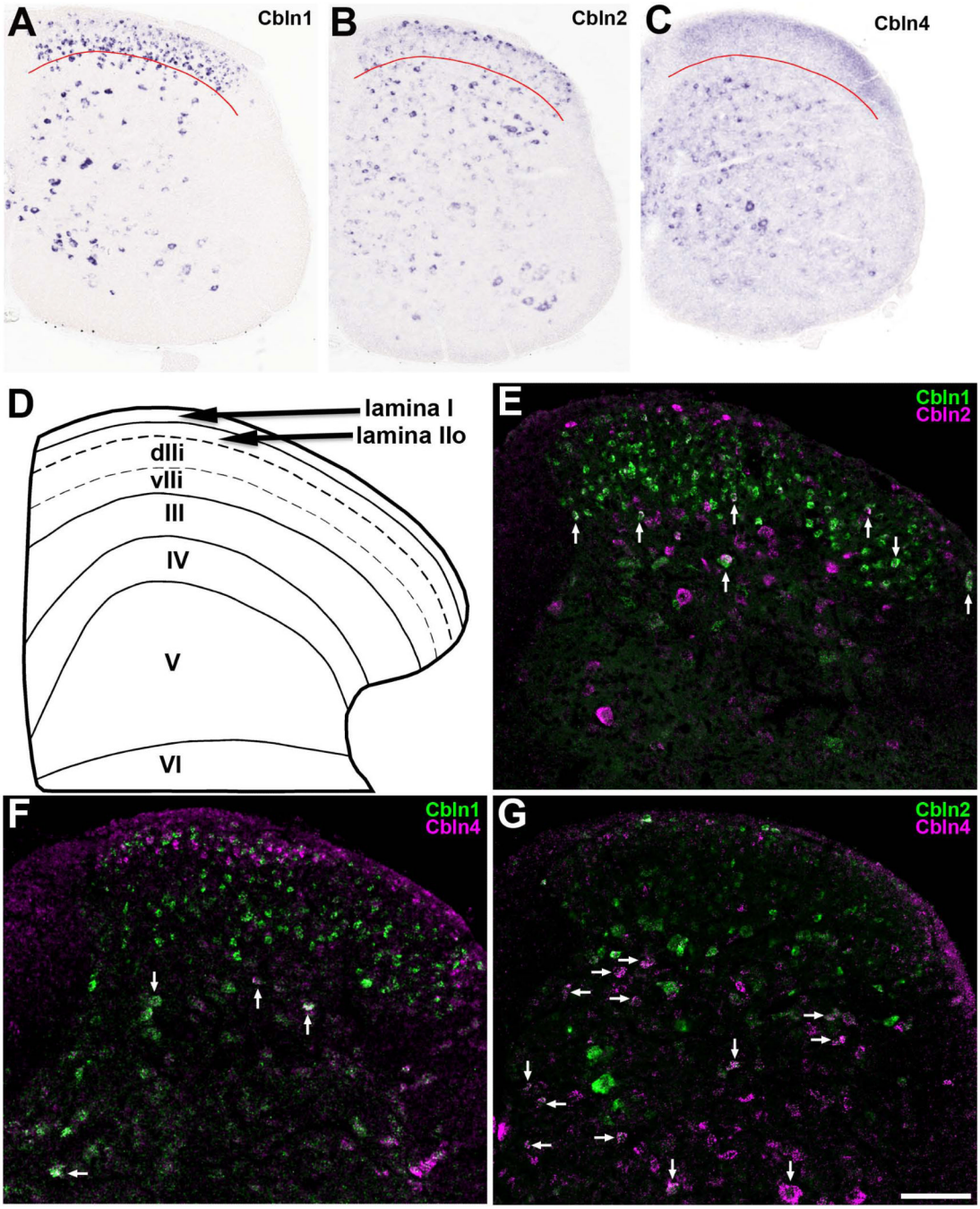

Figure 1. Expression of Cblns in mouse spinal cord

A-C) *In situ* hybridization for Cblns 1, 2, and 4 in mouse lumbar spinal cord. Only one side of the spinal cord is shown here and in all other figures. Medial is to the left, dorsal is toward the top. Location of the lamina II-III border is indicated by a red line. D) Schematic of mouse dorsal horn laminae based on drawings for juvenile mice from the Allen Brain Atlas and immunofluorescent staining of lumbar spinal cord sections. E-G) Coexpression of Cblns in the dorsal half of the spinal cord, corresponding to the area shown in D. The vast majority of dorsal horn neurons express only one Cbln. Examples of neurons expressing two Cblns are indicated with arrows. Double-labeled neurons typically appear white, except in

cells for which the intensity of one of the labels dominates the other. Scale bar = 210μm for A-C, 100μm for E-G.

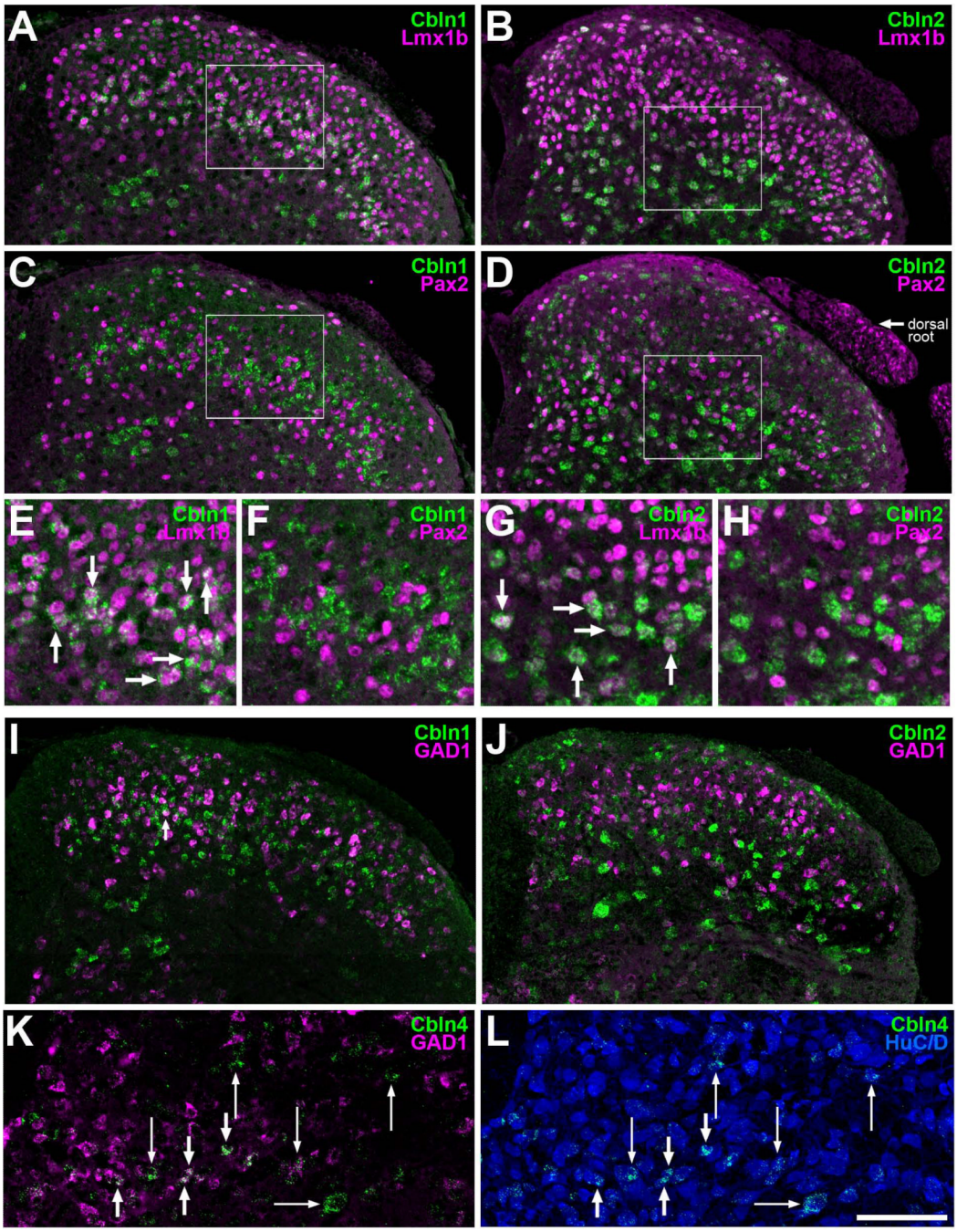

Figure 2. Cbln-expressing neurons are excitatory

A-H) Many Cbln1+ and Cbln2+ neurons express the excitatory marker, Lmx1b, whereas very few express the inhibitory marker, Pax2. Panels A, C, E, and F are views of the same section for which *in situ* hybridization for Cbln1 was combined with double immunofluorescent staining for Lmx1b and Pax2. Similarly Panels B, D, G, and H are all views of the same section stained for Cbln2, Lmx1b, and Pax2. For clarity, images show different combinations of labeling with two fluorophores rather than all three fluorophores, and Lmx1b and Pax2 are both shown as magenta. Sections are from P6 mice. Only the dorsal third of the spinal cord, extending into lamina IV is shown, since very few Lmx1b+ and Pax2+ neurons are situated more ventrally. Panels E-G are higher magnification views

of the areas enclosed by the boxes in panels A-D. Arrows in E and G point to examples of Cbln+/Lmx1b+ neurons. Cbln+/Pax2+ neurons are very rare; none are present in the fields of view shown in F and H. I-K) The vast majority of Cbln-expressing neurons do not express the inhibitory neuron marker, GAD1, as shown by double *in situ* hybridization for each Cbln and for GAD1. The one Cbln1+/GAD1+ neuron in panel I is marked with an arrow; no Cbln2+/GAD1+ neurons are present in panel J. Panels I-J show laminae I-V, where nearly all Cbln1+ and Cbln2+ neurons are located. Panels K-L, at a higher magnification than panels I-J, show laminae IV-VI, where most Cbln4+ neurons are located. GAD1+ neurons are not as intensely labeled in this region as they are in laminae II-III; the brightness of the red channel was increased accordingly in panel K to make them more readily visible. Panel L shows the same field of view as panel K, stained for the pan-neuronal marker, HuC/D, to show the perikaryal labeling for Cbln4. The four Cbln4+/GAD1+ neurons present are marked by short arrows; several Cbln4+neurons that are not GAD+ are marked by long, thin arrows. The HuC/D labeling helps distinguish specific Cbln4 labeling from background staining. Scale bar = 120μm for A-D, 60μm for E-H, 150μm for I-J, 100μm for K-L.

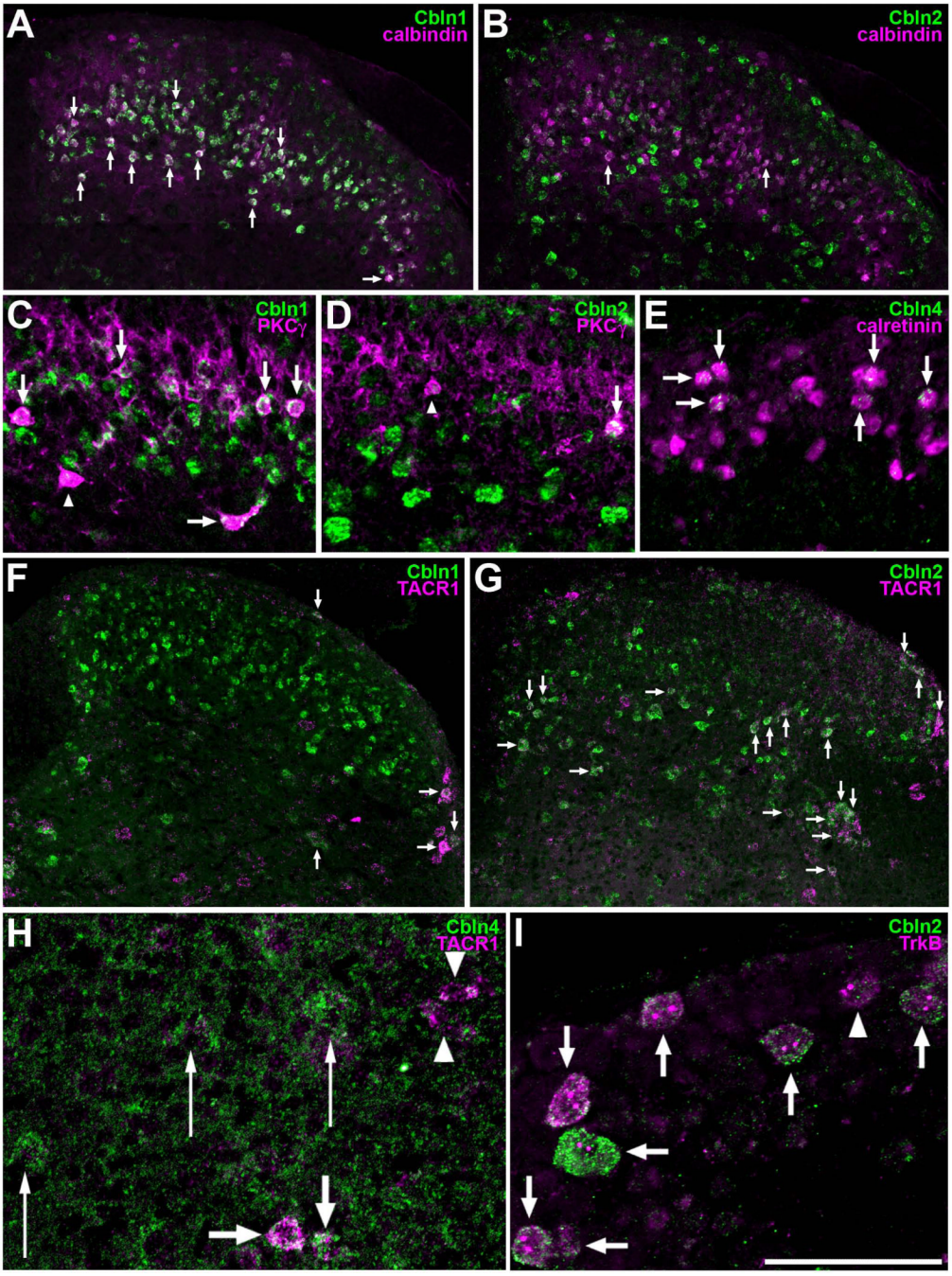

Figure 3. Cell-type classification of Cbln-expressing neurons

A-E) *In situ* hybridization for Cblns 1, 2, and 4 combined with immunofluorescent labeling for calbindin, PKCγ, and calretinin. A, B. Many Cbln1+ neurons, but relatively few Cbln2+ neurons, express calbindin. Calbindin+ neurons are abundant in the inner lamina II. C, D. In ventral lamina IIi and dorsal lamina III, where PKCγ+ neurons are located, a few Cbln1+ neurons and a single Cbln2+ neuron are double-labeled. Note that much of the PKCγ immunolabeling is found in neuronal processes rather than cell bodies, and that PKCγ+ neurons are much less abundant than are neurons expressing Cbln1, Cbln2, or calbindin. E. Several Cbln4+ neurons are labeled for calretinin in outer lamina II, where calretinin+ neurons are abundant. In A, arrows mark examples of the numerous Cbln1+/calbindin+

neurons present. In B-E, all double-labeled neurons in the field of view are marked with arrows. In C and D, arrowheads mark PKCγ+ neurons that are Cbln-negative. F-H) Double *in situ* hybridization for each Cbln and for TACR1. Panels F and G show laminae I-VI. Arrows indicate double-labeled neurons. Panel H shows part of laminae IV-V, at a higher magnification than panels F and G. Short arrows mark Cbln4+/TACR1+ neurons; long, thin arrows mark neurons expressing only Cbln4; arrowheads mark neurons expressing only TACR1. I) Double *in situ* hybridization for Cbln2 and TrkB on sections of lumbar DRGs. Arrows mark Cbln2+/TrkB+ neurons; the arrowhead marks one neuron expressing TrkB but not Cbln2. About ten additional large neurons were present in this field of view, but were not labeled for either Cbln2 or TrkB, as were larger numbers of small DRG neurons. Scale bar = 267μm for A, B, F, G; 110μm for C and D; 80μm for E; 100μm for H and I.

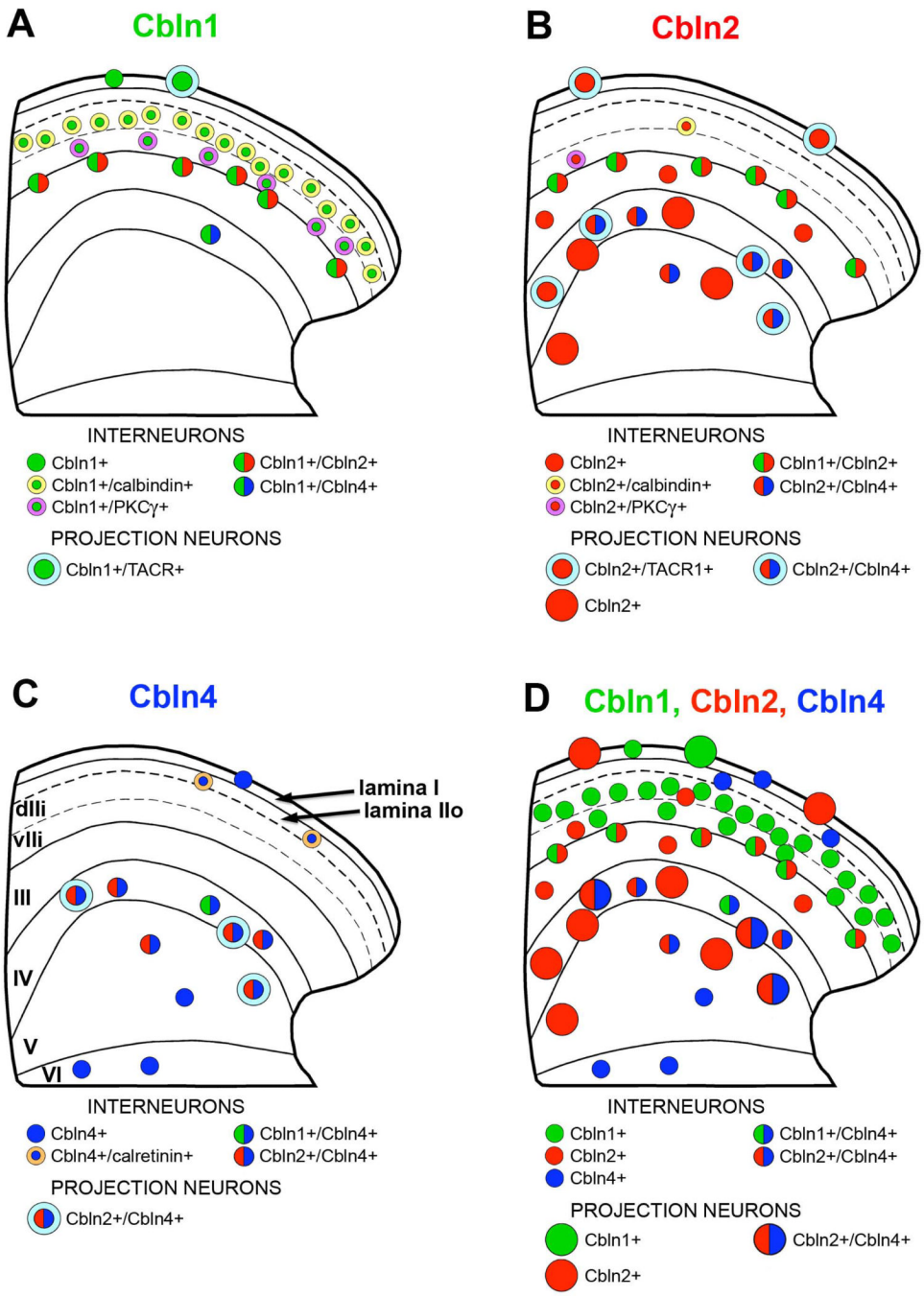

Figure 4. Schematic representation of the spatial distribution of various subsets of Cbln-expressing neurons in the dorsal spinal cord

Cblns 1, 2 and 4 are represented by green, red, and blue circles, respectively. The outer colored rings indicate co-expression of calbindin, calretinin, PKCγ, or TACR1, as shown by the code for each panel. Neurons expressing two Cblns are shown as half-circles filled with the appropriate colors. Interneurons are represented by small circles and putative projection neurons by larger circles. The number of circles denoting each type of neuron is roughly proportional to its actual frequency. The number of Cbln2+/Cbln4+/TACR1+ neurons is inferred from the sizes of the labeled neurons and the results from the three combinations of double labeling. D shows the spatial distribution of all Cbln+ neurons in the dorsal spinal

cord. It is a composite of A-C, and, for purposes of clarity, the various neurochemically-defined subsets of interneurons are not indicated in D. Neurons expressing Cblns 1, 2, and 4 largely occupy different parts of the dorsal spinal cord, except for lamina I, where neurons expressing each of the three Cblns are found, and lamina V, where there is some overlap between Cbln2+ and Cbln4+ neurons.

TABLE 1

Antibodies used in this Study

Antibody	Immunogen	Source, catalogue or clone number	Host
calretinin	recombinant rat calretinin	Millipore, MAB1568, clone 6B8.2	mouse monoclonal
calbindinD28k	purified bovine kidney calbindin	Sigma, C9848	rabbit polyclonal
calbindinD28k	recombinant mouse calbindin	Millipore, AB1778	mouse monoclonal
HuC/D	aa 240-251 of human HuD (QAQRFRLDNLLN)	Invitrogen, Cat.# A-21271, clone 16A11	mouse monoclonal
Lmxlb	full-length mouse Lmxlb	T. Müller and C. Birchmeier; Max-Delbrück-Centrum for Molecular Medicine. Berlin, Germany	guinea pig polyclonal
NF200	carboxyterminal tail segment of dephosphorylated pig neurofilament 200	Sigma, N 0142, clone N52	mouse monoclonal
Pax2	mouse Pax2 C-terminal domain, aa-188-385	Zymed, Cat.# 71-6000	rabbit polyclonal
PKCγ	synthetic peptide corresponding to residues 679-697 of mouse PKCγ (DFVHPDARSPTSPVPVPVM)	Santa Cruz, sc-211	rabbit polyclonal

TABLE 2

Co-expression of Cblns

Cbln	% Cbln1+ neurons co-expressing	% Cbln2+ neurons co-expressing	% Cbln4+ neurons co-expressing
Cblnl	-	24.0%	12.6%
Cbln2	18.5%	-	44.4%
Cbln4	5.2%	24.3%	-

TABLE 3

Expression of excitatory and inhibitory markers by Cbln+ neurons

Marker	% Cbln1+ neurons co-expressing	% Cbln2+ neurons co-expressing	% Cbln4+ neurons co-expressing
Lmx1b (excitatory)	89.8%	79.8%	ND
Pax2 (inhibitory)	2.3%	7.5%	ND
GAD1 (inhibitory)	2.2%	1.8%	9.3%

TABLE 4

Cbln+ neurons double-labeled for markers of subsets of excitatory neurons

Marker	% Cbln1+ neurons co-expressing	% Cbln2+ neurons co-expressing	% Cbln4+ neurons co-expressing
calbindin	57.7%	4.0%	ND
calretinin	0.7%	0.5%	12.3%
PKCγ	19.6%	4.3%	ND

TABLE 5

Cbln expression in TACR1+ projection neurons

	% Cbln1+ neurons co-expressing	% Cbln2+ neurons co-expressing	% Cbln4+ neurons co-expressing		% TACR1+ neurons co-expressing
TACR1	5.2%	20.2%	23.3%	Cbln1	10.5%
				Cbln2	43.0%
				Cbln4	18.8%