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Localization of Cerebellin-2 in Late Embryonic Chicken Brain: Implications for a Role in Synapse Formation and for Brain Evolution

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Abstract

Cerebellin-1 (Cbln1), the most studied member of the cerebellin family of secreted proteins, is necessary for the formation and maintenance of parallel fiber–Purkinje cell synapses. However, the roles of the other Cblns have received little attention. We previously identified the chicken homolog of Cbln2 and examined its expression in dorsal root ganglia and spinal cord (Yang et al. [2010] *J Comp Neurol* 518:2818–2840). Interestingly, Cbln2 is expressed by mechanoreceptive and pro-prioceptive neurons and in regions of the spinal cord where those afferents terminate, as well as by preganglionic sympathetic neurons and their sympathetic ganglia targets. These findings suggest that Cbln2 may demonstrate a tendency to be expressed by synaptically connected neuronal populations. To further assess this possibility, we examined Cbln2 expression in chick brain. We indeed found that Cbln2 is frequently expressed by synaptically connected neurons, although there are exceptions, and we discuss the implications of these findings for Cbln2 function. Cbln2 expression tends to be more common in primary sensory neurons and in second-order sensory regions than it is in motor areas of the brain. Moreover, we found that the level of Cbln2 expression for many regions of the chicken brain is very similar to that of the mammalian homologs, consistent with the view that the expression patterns of molecules playing fundamental roles in processes such as neuronal communication are evolutionarily conserved. There are, however, large differences in the pattern of Cbln2 expression in avian as compared to mammalian telencephalon and in other regions that show the most divergence between the two lineages.

INDEXING TERMS

neural circuitry; neuronal projections; synaptic connections

The four cerebellins (Cblns) are secreted glycoproteins 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 that mediates the assembly of the trimers into hexamers (Bao et al., 2005; Iijima et al., 2007). Cblns 1, 2, and 4 can be secreted either as homo-hexamers or as hetero-hexamers, whereas Cbln3 can only be secreted when it is coexpressed with Cbln1 (Pang et al., 2000; Bao et al., 2005, 2006; Iijima et al., 2007). The name “cerebellin” comes from Cbln1, the first family member identified (Slemmon et al., 1984; Urade et al., 1991), which is highly enriched in the cerebellum.

Cbln1 is, to the best of our knowledge, the only family member whose function has been studied. Cbln1 is synthesized by granule cells and released from their axons (the parallel fibers, PF) onto the dendrites of Purkinje cells, starting at about the time synapses are first established (Pang et al., 2000; Hirai et al., 2005). In the absence of Cbln1, PF-Purkinje cell synapses exhibit a number of severe deficits. Specifically, most dendritic spines (~80%) appear “naked,” lacking presynaptic contacts with PFs. For the remaining 20% of spines, the length of the postsynaptic density frequently exceeds that of the active zone. PF stimulation produces smaller than normal excitatory postsynaptic currents and the synapses do not show long-term depression. The behavioral consequences of these defects are dramatic. Cbln1-null mice are severely ataxic, walk with an irregular gait, and do not maintain their balance on rotarod. These deficits can be rescued by exogenous Cbln1, either by inducing its expression in Purkinje cells through genetic manipulations (Wei et al., 2009) or by injection of recombinant Cbln1 (Ito-Ishida et al., 2008). It has been known for some time that mice lacking GluRδ2 exhibit the same deficits as Cbln1-null mice (Kashiwabuchi et al., 1995; Kurihara et al., 1997; Hirai et al., 2005). Indeed, Cbln1 hexamers have recently been shown to bind to GluRδ2 on the postsynaptic surface of Purkinje cells (Ito-Ishida et al., 2008; Miura et al., 2009; Matsuda et al., 2009, 2010; Uemura et al., 2010). Cbln1 hexamers also bind to β-neurexins (Uemura et al., 2010). Thus, Cbln1 forms a bridge between β-neurexin presynaptically and GluRδ2 postsynaptically, and it is this triad that is essential for the formation of parallel fiber-Purkinje cell synapses.

Cbln3 appears to be expressed only in mammals, with its absence in fish, frogs, and birds suggesting it is probably the family member to have evolved the most recently (Yang et al., 2010). Interestingly, Cbln3 expression is limited to only two areas in the mouse brain (the cerebellum and the dorsal cochlear nucleus) and starts at postnatal day (P)10, much later than any of the three other family members. In the cerebellum, Cbln3 is synthesized by granule cells (Pang et al., 2000; Bao et al., 2006) and released in heteromeric complexes with Cbln1 onto Purkinje cells (Miura et al., 2009). In the absence of Cbln1, Cbln3 gene expression appears normal but the Cbln3 protein is retained internally and most of it is ultimately degraded (Bao et al., 2006; Iijima et al., 2007; Wei et al., 2007). In contrast, in the absence of Cbln3, while Cbln1 mRNA levels are normal, Cbln1 protein levels are higher than in wildtype, due at least in part to changes in its degradation (Wei et al., 2007). However, Cbln3-null mice are phenotypically normal and their PF-Purkinje cell synapses do not exhibit any obvious deficits (Bao et al., 2006). It is thus believed that Cbln3 affects Cbln1's synthesis and/or degradation, but otherwise Cbln3 function remains obscure.

In contrast, Cbln2 and Cbln4, like Cbln1, are expressed widely throughout the brain and can first be detected early in development, at embryonic day (E)10 in the mouse (Pang et al., 2000; Miura et al., 2006). Given the high degree of sequence homology of Cbln2 (91%, based on the sequences for mice) and Cbln4 (85%) with Cbln1, they might be expected to play similar roles in synapse formation. However, to the best of our knowledge, their functions have not been studied.

We recently identified the chicken homolog of Cbln2 and found that it is expressed by specific subsets of spinal cord and dorsal root ganglia (DRG) neurons starting early in development, long before synapses are made, and expression is maintained as the animal matures (Yang et al., 2010). For example, Cbln2 is expressed by TrkB+ (mechanoreceptive) and TrkC+ (proprioceptive) DRG neurons and by neurons located in regions of the spinal cord where TrkB+ and TrkC+ afferents terminate. In contrast, Cbln2 is not expressed by any TrkA+ sensory neurons and by only a small proportion of the neurons in lamina I, which receives input nearly exclusively from TrkA+ afferents. Cbln2 is also expressed by preganglionic sympathetic neurons and their targets in the sympathetic chain ganglia. Thus, Cbln2 shows a tendency to be expressed by synaptically connected neuronal populations.

To further assess if expression by synaptically connected neuronal populations is a distinguishing characteristic of Cbln2, we decided to examine its expression in the brain, where we could evaluate a large number of discrete neuronal populations. The results presented here provide many more examples of Cbln2 expression in groups of synaptically connected neurons (and very few exceptions). The implications of this finding for Cbln2 function are discussed and compared to that for Cbln1. Finally, although not the original intent of this study, we found that the level of Cbln2 expression for many regions of the chicken brain is very similar to that of its mammalian homolog, consistent with the view that the expression patterns of molecules playing fundamental roles in processes such as neuronal communication tend to be evolutionarily conserved. However, for the pallium the patterns of Cbln2 expression are considerably different. We find this somewhat surprising since, although pallium has diverged the most between the two lineages, many genes that are enriched in mammalian pallium, such as Pax6, Emx2, Tbr1, and Lmo3, are similarly enriched in avian pallium (Fernandez et al., 1998; Puelles et al., 2000; Abellán et al., 2009).

MATERIALS AND METHODS

Tissue preparation, in situ hybridization, and immunohistochemical staining

For these studies we decided to use chicken embryos at Stage 40–41 (Hamburger and Hamilton, 1951), when their brains are mature in terms of organization but synapse formation is still ongoing. Our reasoning for using late embryos rather than hatched chicks was based on Cbln2 expression in the mouse brain, where the pattern of expression appears mature early in the postnatal period, but overall expression levels gradually decrease thereafter without any changes in overall distribution per se (Miura et al., 2006). Since chicks at 1–2 days after hatching are probably equivalent to weanling $\approx$ P21 mice in terms of behavior and, consequently, brain maturation, St. 40 chick embryos are roughly equivalent to P10–14 mice. The data presented here are based on brain sections from six St. 40–41 embryos.

Tissue preparation and processing was as previously described (Yang et al., 2010). White Leghorn chick embryos were removed from the egg at $\approx$ 14 days of incubation, decapitated, and staged. All procedures involving animals adhere to National Institutes of Health (NIH) guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Tennessee Health Science Center. Most of the skull was removed before immersion-fixing the head in 4% paraformaldehyde, 10% sucrose in 0.1 M phosphate buffer (PB; pH = 7.2–7.4) for $\approx$ 6 hours. The remaining skull casing was completely removed and coronal sections through the brain were cut at 30 μ m with a cryostat. To examine Cbln2 expression in the retina, we separately sectioned the eyes from two of the embryos. To evaluate Cbln2 expression in cranial ganglia, we used one additional embryo at St. 38, when the skull is softer, allowing us to section through the brain and the underlying skull, with the cranial ganglia in situ, still attached to the brain. The sections were mounted directly, in serial order, onto Superfrost/Plus slides (Fisher, Pittsburgh, PA), and stored at -80°C until use.

In situ hybridization was performed using an 1800 nt-long digoxigenin-labeled Cbln2 probe and labeling was visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The Cbln2 probe (directed to nucleotides 386–2218; GenBank accession number GU189513) includes 1,300 nt from the 3'UTR, which is unique to chicken Cbln2, and 500 nt from the coding region, which shares 78% and 71% identity with that of chicken Cbln1 and Cbln4, respectively. The stringent hybridization conditions we used, calculated to distinguish sequences of 80% homology, should preclude detection of Cbln1 and/or Cbln4. Further, a different Cbln2 probe, corresponding to nt 1453–2640 and thus not including any part of the coding region, yielded a similar labeling pattern, whereas the labeling pattern was

distinctly different using either a 674 nt-long chicken Cbln1 probe (directed to nucleotides 712–1385; GenBank accession number NM_001233212), or a 977 nt-long chicken Cbln4 probe (directed to nucleotides 724–1700; GenBank accession number NM_001079487). The Cbln1 and Cbln4 probes targeted the 3'UTR of their respective genes, which are each unique in that using the Basic Local Alignment Search Tool (BLAST) program to search the NCBI chicken nucleotide collection (nr/nt) database failed to detect any homologous sequences >100 nt long. There is no chicken Cbln3 homolog. Thus, the pattern of expression we report here should accurately represent that of Cbln2.

To assess neuronal densities in different brain regions, some sections from an additional St. 40 embryo were stained with an antibody that recognizes NeuN (see below and Table 1) and visualized with a DAB reaction product, as described in Yamamoto and Reiner (2005).

For some of the eye sections, in situ hybridization was followed by processing for immunofluorescence for islet-1 or TuJ1 (see below and Table 1). For these, the Cbln2 riboprobe was tagged with dinitrophenol-11-UTP (DNP; Perkin Elmer, Norwalk, CT) and visualized with rabbit anti-DNP (Invitrogen, La Jolla, CA; Cat. No. A-6430), followed by an anti-rabbit secondary antibody conjugated to Alexa 488 (Molecular Probes, Eugene, OR). The slides were subsequently incubated in primary antibody and then in secondary antibody conjugated to Alexa 594 or Alexa 647. Slides were coverslipped in Fluoromount-G (Southern Biotech, Birmingham, AL).

Antibodies

The NeuN antibody stains all neuronal nuclei with the exception of mitral cells in the olfactory bulb and Purkinje cells in the cerebellum (Mullen et al., 1992) and is frequently used as a pan-neuronal marker. The antibody recognizes two bands at 46 and 48 kDa in immunoblots of nuclear extracts from adult mouse brain (Lind et al., 2005), which recently have been demonstrated to correspond to the protein Fox3 (Kim et al., 2009).

The anti-Islet1 antibody recognizes both Islet1 and Is-let2 (Tsuchida et al., 1994). The sequence of the immunogen (see Table 1) is 100% identical to that of chick Islet1, and 80% identical to the corresponding regions of rat and chick Islet2. The antibody labels motoneurons, sympathetic preganglionic neurons, and DRG neurons during early development, all of which express Islet1, as shown with in situ hybridization (Tsuchida et al., 1994). This anti-Islet1 antibody has also been used previously to identify retinal ganglion cells (Pimentel et al., 2000).

The TuJ1 antibody recognizes neuronal class III β -tubulin, but not the β -tubulin found in glial cells (Lee et al., 1990). It reveals a 50-kDa band in immunoblots prepared by using lysates from an immortalized sensory neuroblast cell line derived from mouse otocysts (Lawoko-Kerali et al., 2004). TuJ1 has been used previously as a marker of retinal ganglion cells (Pimentel et al., 2000).

Image capture and analysis

Brightfield images used for publication were obtained using a ScanScope digital slide scanner (Aperio Technologies, Vista, CA). Images of fluorescently labeled tissue sections were captured with a BioRad (Hercules, CA) MRC 1024 confocal laser-scanning microscope. Images were adjusted for brightness and contrast and small histological imperfections were removed using Adobe Photoshop (San Jose, CA).

Specific brain regions and nuclei were identified by reference to avian brain atlases (Karten and Hodos, 1967; Kuenzel and Masson, 1988). We used two criteria to characterize Cbln2 expression. First, the intensity of labeling was qualitatively assigned to one of three

categories: high, moderate, and low, with low corresponding to labeling that was unambiguously above background levels. Second, the approximate percentage of Cbln2+ neurons relative to the total number of neurons in a given region was classified as “most” (or “many”) for more than 65% (although this percentage was frequently much higher), “some” for 30–65%, and “few” for under 30%. Judgments about the number of neurons in a particular region were based on staining with the NeuN antibody (see above), unpublished micrographs from the Reiner laboratory, relevant figures from some of the cited references, and images of chicken brain sections from Harvey Karten available at www.brainmaps.org. The two senior authors separately assessed and then agreed upon the labeling descriptions for each region that are provided in Table 2. In the text, a high level of labeling is sometimes described as “rich”/“dark”/“intense.” A few labeled neurons are sometimes described as being “scattered.” A region described as “richly” or “intensely” labeled means that most of its neurons express high levels of Cbln2. Note that the overall appearance of regions described as Cbln2-rich can vary considerably, however, depending on whether the constituent neurons are densely or loosely packed. A region described as “poorly” labeled means that some of its neurons express low Cbln2 levels or a few of its neurons express moderate or low Cbln2 levels. Images providing examples of the varying levels of Cbln2 expression, taken at high magnification so that individual neurons can be visualized, are shown in Figures 1 and 2. Lower-magnification views illustrating the distribution of Cbln2+ neurons in the brain are shown in Figures 3–6.

RESULTS

Telencephalon

Pallium—Cbln2+ neurons were found in several parts of the pallium (Fig. 3). Most notably, all, or nearly all, mitral cells in the olfactory bulb (OB) expressed high levels of Cbln2, as did a few cells, presumably tufted cells, scattered nearby (Rugarli et al., 1993). A band of Cbln2-rich neurons (MDd) was present along the upper edge of the dorsal mesopallium (M) in its rostromedial aspect (Fig. 3B,C). At more caudal levels (Fig. 3D,E), this band of labeled neurons extended more laterally, appeared more dispersed, and was continuous with moderate to rich Cbln2+ neurons at the dorsolateral edge of the mesopallium in the caudal part of a region recently termed the dorsolateral (DL) pallium (Abellán and Medina, 2009). In turn, the band of Cbln2+ dorsolateral pallial cells was continuous with intensely labeled Cbln2+ neurons in the dorsolateral corticoid area (CDL; Fig. 3D,E). The Cbln2+ neurons in the CDL tended to be clustered, especially at very caudal telencephalic levels where the CDL receives olfactory bulb input (Reiner and Karten, 1985). A thin band containing some moderately labeled Cbln2+ neurons extended from DL pallium into the temporo-parieto-occipital area (TPO) lateral to the caudal nidopallium (N; Fig. 3E; also see Fig. 1A). Scattered Cbln2+ neurons were also observed in the hippocampal complex (Fig. 3D–I), especially in the hippocampus proper (Hp) and along its superficial surface dorsomedial to the parahippocampal area (APH). Finally, many moderately labeled Cbln2+ neurons were present in the dorsal arcopallium (AD) and the caudal part of the medial arcopallium (AM), the latter forming a crescent-shaped territory dorsal to the posterior amygdala (PoA; Figs. 3G–I, 4A). In contrast, the hyperpallium (Wulst; W) and the entopallium (E) contained only few faint Cbln2+ neurons (Fig. 3A–E). The nidopallium (N) proper, particularly the caudolateral nidopallium (NCL), was mostly devoid of labeled neurons, except for very rostromedial nidopallium, and along the lateral edge of the nidopallium, in the region of the piriform cortex (CPI; Fig. 3D,E,G; also see Fig. 1B), which contained some moderately to lightly labeled Cbln2+ neurons.

Subpallium—Cbln2+ neurons were found throughout much of the subpallium (Figs. 3C–I, 4A). Numerous Cbln2-rich neurons were found scattered in the lateral forebrain bundle

(LFB), whereas in the intrapeduncular nucleus (INP), Cbln2-rich neurons constituted a small proportion of the total cell population. For both LFB and INP, the Cbln2+ neurons were similar in size and frequency to the cholinergic neurons found in those regions (Fig. 1D). Numerous Cbln2-rich neurons were observed in the olfactory tubercle (TuO), the taenia of the amygdala (TnA), and the subpallial amygdala (SpA). A few moderately labeled Cbln2+ neurons were present in the lateral bed nucleus of the stria terminalis (BSTL), the medial and lateral striatum (MSt and LSt), globus pallidus (GP; Fig. 1C), and ventral pallidum (VP), whereas the posterior amygdala (PoA) and the septum contained only a few lightly labeled Cbln2+ neurons.

Diencephalon

Epithalamus—Many cell groups of the diencephalon showed intense neuronal labeling for Cbln2. In the epithalamus, the neurons of the medial habenula (HbM), the lateral habenula (HbL), the superficial parvocellular nucleus (SPC), and in and around stria medullaris (SMe) were intensely labeled for Cbln2 (Fig. 4A–E).

Thalamus—In the upper part of the thalamus, most neurons of the anterior part of dorsolateral anterior pars medialis nucleus (DLM) were intensely labeled for Cbln2, whereas most neurons of posterior DLM and the dorsolateral anterior pars lateralis nucleus (DLL), the avian homolog of mammalian dorsal lateral geniculate nucleus (GLd) (Karten et al., 1973; Güntürkün and Karten, 1991), were moderately labeled for Cbln2 (Figs. 3I, 4D). The dorsomedial anterior (DMA) and dorsomedial posterior (DMP) nuclei contained some moderately labeled Cbln2+ neurons, whereas the neurons of the dorsointermediate posterior (DIP) and dorsolateral posterior (DLP) nuclei were intensely labeled (Fig. 4B–G). Neurons in nucleus semilunaris parovoidalis (SPo), which lies ventral to ovoidalis (Ov), and neurons forming the shell of ovoidalis, specifically those in the anterior, posterior, medial, and lateral shell nuclei of Durand et al. (1992), were extremely rich in Cbln2 (Fig. 4D). In contrast, ovoidalis itself (which receives midbrain auditory input) was more moderately stained, resulting in a core and shell pattern of labeling to ovoidalis and its surround (Fig. 4D–G). Similarly, the tectorecipient visual nucleus rotundus (Rt) appeared more lightly stained than did the surrounding region. The neurons in rotundus proper, which are widely spaced, were richly labeled in its dorsal and medial sectors and more moderately labeled ventrally (Fig. 1F). Rotundus was surrounded by a thin Cbln2-rich band laterally, and intensely labeled Cbln2+ neurons in the dorsointermediate ventral anterior area (DIVA) and the ventrointermediate area (VIA) medially (Fig. 4B–E). Nucleus subrotundus (SRt) was also rich in Cbln2+ neurons, as was nucleus posteroventralis (PV) posterior and lateral to subrotundus (Fig. 2E–G; note that the medial edge of PV in panel F may be a posterior continuation of SRt).

Ventral thalamus—The majority of neurons in the inner layer of the ventral lateral geniculate nucleus (GLv), the lateral anterior nucleus (LA), the stratum cellulare internum (SCI; not shown), and the ventrolateral thalamus (VLT) were well labeled for Cbln2 (Fig. 1E). In contrast, the stratum cellulare externum (SCE) contained some moderately labeled, and the subthalamic nucleus (STN) and thalamic reticular nucleus (TRN) only a few moderately labeled Cbln2+ neurons (Figs. 4, 5A).

Hypothalamus—A very prominent pair of vertically oriented bands of Cbln2+ neurons was present at the medial edge of the caudal septum at and just rostral to the anterior commissure (AC). This nucleus appears to be the median preoptic nucleus (MePO). More ventrally, many neurons of the preoptic area (POA) were very intensely labeled for Cbln2. Caudal to the anterior commissure, most neurons in the medial suprachiasmatic nucleus (medSCN) and in the supramammillary region (SpMam) were rich in Cbln2. In contrast,

neurons in the lateral (visual) supra-chiasmatic nucleus (latSCN), lateral and medial hypothalamic areas (LHy and MHy), the ventromedial hypo-thalamic region (VMH), the tuberal hypothalamus (TuHy), and the paraventricular hypothalamus (PVN; not shown) tended to be weakly labeled for Cbln2 (Figs. 3F–I, 4, 5A–B).

Pretectum—The pretectal nuclei varied considerably in their levels of Cbln2 expression (Figs. 4D–G, 5A–B). Most neurons of the tectal gray (GT) were intensely Cbln2+, whereas most neurons in the nucleus precommissuralis principalis (PPC) were moderately Cbln2+. Some neurons in area pretectalis (AP) and in the nucleus of the basal optic root (nBOR) were moderately Cbln2+. The other major pretectal nuclei contained a few moderately Cbln2+ neurons (spiriformis medialis: SpM), a few weakly Cbln2+ neurons (spiriformis lateralis, SpL; subpretectalis, SP) or no Cbln2+ neurons (nucleus pretectalis, PT).

Mesencephalon and isthmic derivatives

Neurons in several regions within the chick midbrain expressed Cbln2 (Figs. 5, 6A–C). Moderate to rich Cbln2+ neurons were present in several of the deeper layers of the optic tectum (Fig. 5E). Most of the neurons in tectal layers 10 and 14 were Cbln2+. Roughly half the neurons in layer 13 were Cbln2+, and roughly a third in layer 8. A few scattered Cbln2+ neurons were found in layers 11 and 15, and layer 9 contained lightly labeled cells. Within the subtectal optic lobe, the nucleus isthmi pars parvocellularis (Ipc; Fig. 5F–I; also see Fig. 2A) and the rostral part of the nucleus semilunaris (SLu; Fig. 5F,G) were the most intensely labeled, with all or nearly all neurons expressing high levels of Cbln2. Most neurons of the lateral and medial reticular formation (FRL and FRM) expressed Cbln2 at high to moderate levels, whereas neurons in the caudal part of SLu (Figs. 5H,I; 6A,B; also see Fig. 2B) were moderate in Cbln2, a few neurons in the isthmo-optic nucleus (ION) were lightly labeled, and those in nucleus isthmi pars magnocellularis (Imc; Fig. 5F–H; also see Fig. 2A) were devoid of Cbln2. The core of nucleus mesencephali lateralis pars dorsalis (MLd) was poor in Cbln2+ neurons and the MLd periphery contained scattered Cbln2+ neurons (Fig. 5A–D). In contrast, the shell surrounding MLd, which is formed by the nucleus externus (Next) laterally, the nucleus superficialis (Nsuf) along the tectal ventricle, and the nucleus intercollicularis (ICo) medially, was rich in Cbln2+ neurons. Medial to ICo, the central periaqueductal gray (Gct) was moderate in Cbln2 labeling. Within the tegmentum, most neurons of the ventral tegmental area (VTA), the medial part of the dorsal raphe (RaD), and nucleus ruber (Ru) were rich in Cbln2, as were some of the neurons of the linear part of the mesencephalic raphe (RaL). Some neurons of the A8 region, substantia nigra pars compacta (SNc), and reticu-lata (SNr) were labeled for Cbln2, at levels ranging from high to moderate. The medial part of the interpeduncular nucleus (IP) was moderate in Cbln2, but the lateral part of IP, the oculomotor nucleus (M3), the trochlear motor nucleus (M4), and all but perhaps the lateralmost part of the nucleus of Edinger–Westphal (EW) contained only a few lightly labeled Cbln2+ neurons. More caudally, most neurons in a region we identify as the ventral nucleus of the lateral lemniscus (LLv) (based on Arends and Zeigler, 1986, fig. 1D,E; see also Wild, 1987, fig. 1F and Wild, 1995, fig. 6B), and in a region we identify as the caudal part of the intermediate nucleus of the lateral lemniscus (LLi) (based on Arends and Zeigler, 1986, fig. 1B–D) were rich in Cbln2 (Figs. 5H,I, 6A–C). In contrast, neurons in rostral LLi and the dorsal nucleus of the lateral lemniscus (LLd) were poor in Cbln2 (Figs. 5H,I, 6A,B). (LLd is situated just dorsal to LLi in Figs. 5H,I, 6A,B, but because they abut and both were unlabeled, we could not clearly distinguish between them. See Arends and Zeigler (1986) and Krützfeldt et al. (2010b) for a discussion about the terminology used for the lateral lemniscal nuclei.)

Hindbrain

Metencephalon—The vast majority of neurons in the parabrachial region (Pb), the principal nucleus of the trigeminus (Pr5; Fig. 2C), the nucleus of the descending tract of the trigeminus (TTd), the linear part of the pontine raphe (RaL), the medial and lateral pontine nuclei (PM and PL), the superior vestibular nucleus (VeS), the lateral vestibular nucleus (VeL), and the lateral cerebellar nucleus (CbL) were rich in Cbln2 (Fig. 6A–F). In comparison, a smaller proportion of neurons in the tegmenti ventralis (TV) and dorsal and ventral subcoeruleus (SCd and SCv) expressed moderate (SCd, SCv) to high levels of Cbln2 (TV). Many lightly Cbln2+ neurons were found in the trigeminal motor nucleus (M5) and the facial motor nucleus (M7), whereas the medial cerebellar nucleus (CbM), the locus coeruleus (LoC), and the constituent regions of the pontine reticular formation (i.e., reticularis pontis oralis RPo, reticularis pontis gigantocellularis RPg, reticularis pontis RP; Figs. 5G–I, 6A–F) contained few moderately Cbln2+ neurons. The cerebellum proper was generally poor in Cbln2, except for scattered cells in the granule cell layer expressing moderate levels of Cbln2 (Fig. 6G–J).

Myelencephalon—The vast majority of neurons in numerous parts of the myelencephalon expressed high levels of Cbln2 (Fig. 6I–N; also see Fig. 2D–I). These included the nucleus of the descending tract of the trigeminus (TTd), the inferior olive (OI), the nucleus of the solitary tract (NTS), the motor nucleus of the vagus (M9/10), the gracile and cuneate nuclei (GC), the external cuneate nucleus (ECu), the lateral reticular nucleus (RL), and the hindbrain auditory nuclei angularis (An) and laminaris (La). Some neurons in the medial vestibular (VeM) nucleus and in the linear part of the medullary raphe (RaL) were rich in Cbln2. Most neurons in the dorsal part of the central medullary nucleus (Cnd) were moderately labeled, as were some neurons in the descending vestibular nucleus (VeD), nucleus reticularis parvocellularis (Rpc), and nucleus reticularis gigantocellularis (Rgc). In contrast, the ventral part of the central medullary nucleus (Cnv) contained few light to moderate Cbln2+ neurons, while the superior olive (SO), the hypoglossal motor nucleus (M12), and nucleus mag-nocellularis (Mc) were nearly, or completely, devoid of Cbln2.

Sensory input to the brain

The results described above show that Cbln2 is expressed in many sensory areas of the brain. We therefore wondered if Cbln2 is also expressed in primary sensory neurons.

Retina—Within the retina, Cbln2+ neurons were found in the inner nuclear layer (INL) and in the ganglion cell layer (GCL; Fig. 7A,B). The Cbln2+ cells in the INL are likely to include both amacrine cells and bipolar cells, but not displaced retinal ganglion cells, based on their location and size. In the GCL, Cbln2+ cells tended to be large, suggesting that they are retinal ganglion cells (RGCs), rather than displaced amacrine cells. Moreover, in double-labeled material, many Cbln2+ neurons in the GCL also expressed islet-1 or TuJ1 (data not shown), which label RGCs (Pimentel et al., 2000). Overall, ~5% of RGCs appeared to be Cbln2+, although they were not uniformly distributed throughout the retina, being more common peripherally than centrally.

Cranial ganglia—As shown in Figure 7C–H, the majority of neurons in the trigeminal (V), geniculate (facial, VII), and jugular (vagal, X) ganglia expressed very high levels of Cbln2. Approximately half of the neurons in the petrosal (glosso-pharyngeal, IX) ganglion, were intensely labeled for Cbln2, and those were clustered together in small groups, especially around the periphery of the ganglion. In contrast, Cbln2 expression was low in the vestibular and cochlear ganglia, with only a few neurons expressing low to moderate levels of Cbln2.

DISCUSSION

The results described above show that Cbln2⁺ neurons are widely distributed throughout the chicken brain, as they are in mice (Miura et al., 2006). The pattern of Cbln2 expression is generally similar for chick and mouse, except for pallial parts of the telencephalon where Cbln2 is expressed at lower levels and is less widespread in chick than in mice. Cbln2 expression is especially high in a few brain regions in the chick, for example, the lateral habenula, nucleus isthmi pars parvocellularis (Ipc), and the shell of ovoidalis, where seemingly every neuron was intensely labeled for Cbln2. For other regions, the level of Cbln2 expression is lower due to a smaller proportion of neurons expressing Cbln2 and/or to individual neurons staining less intensely.

Based on the recent demonstration that Cbln1 binds to GluR82, it has been suggested that other members of the Cbln family also bind to GluR82, and to GluR81 as well. Since the GluR8s are orphan glutamate receptors, one might then expect Cbln expression to be limited to glutamatergic neurons, presynaptic to the postsynaptic GluR8s. However, we find that Cbln2 is not restricted to glutamatergic neurons; rather, it is expressed by neurons in the amygdala, including TnA and SpA, which are GABAergic (Sun et al., 2005); by neurons in the basal forebrain bundle and Ipc, which are cholinergic (Medina and Reiner, 1994); and by neurons in the raphe nuclei, which are serotonergic (Yamada et al., 1984). Similarly, Cbln1 is expressed by GABAergic neurons in the internal pallidal segment (GPI) in the mouse (Miura et al., 2006; Wei et al., 2009) and in cholinergic Ipc neurons and serotonergic raphe neurons in the chick (unpubl. obs.).

Cbln2 expression by synaptically connected neuronal populations

We initiated these studies to determine whether Cbln2 is expressed by synaptically connected neuronal populations in the brain, as we had found for mechanoreceptive and proprioceptive DRG neurons and their target inter-neurons in the dorsal horn (Yang et al., 2010). Indeed, we did find numerous neuronal populations for which this is true. For example, the Cbln2-rich lateral habenula projects to the Cbln2-rich ventral tegmental area (VTA) and pontine linear raphe (Kitt and Brauth, 1986; Marcinkiewicz et al., 1989; Montagnese et al., 2003), and receives input from the Cbln2-rich supramammillary nucleus of the hypothalamus (Berk and Hawkin, 1985); the Cbln2-rich medial habenula projects to the Cbln2-rich supramammillary hypothalamus and the Cbln2-rich medial interpeduncular nucleus (Montagnese et al., 2003) and receives input from the Cbln2-rich olfactory tubercle (TuO; Székely et al., 1994); and the Cbln2-rich supramammillary hypothalamus projects to the Cbln2-rich dorsolateral corticoid area (CDL; Berk and Hawkin, 1985). On the other hand, while the Cbln2-rich ovoidalis shell receives input from the Cbln2-rich MLd shell and projects to the Cbln2-rich CDL and to a Cbln2-rich band in the dorsal mesopallium, it also projects to the Cbln2-moderate outer temporo-parieto-occipital area, medial arcopallium, and DL pallium, and to the Cbln2-poor field L and dorsal nidopallium (Karten, 1968; Durand et al., 1992). Similarly, the substantia nigra pars compacta, in which about half of the neurons are Cbln2⁺, shares reciprocal connections with the Cbln2-poor medial striatum (Brauth et al., 1978; Kitt and Brauth, 1981, 1986a), but also receives some input from the Cbln2-rich lateral habenular nucleus (Herkenham and Nauta, 1979; Montagnese et al., 2003). Trying to interpret the pattern of Cbln2 expression thus becomes complicated by the prevalence of convergent and divergent inputs and outputs for most brain regions. Moreover, individual neurons located within any given brain region may vary in the presynaptic inputs they receive and the postsynaptic targets they innervate. Determining if intermixed Cbln2⁺ and Cbln2[−] neurons actually differ in their connectivity would therefore be interesting and might provide insight into how neurons establish cell-type-specific identities and synaptic connections. Nonetheless, all or nearly all neurons in many of the connected cell groups express Cbln2, indicating that, in these cases at least, the connected

neurons themselves must express Cbln2. The results presented here show that Cbln2 is especially prevalent in many kinds of sensory neurons and in areas of the brain that receive sensory information. For example, Cbln2+ trigeminal and jugular ganglia neurons (which are primarily somatosensory) project to the Cbln2+ principal sensory nucleus of the trigeminus (PrV) and the nucleus of the descending tract of the trigeminal nerve (TTd) (Dubbeldam and Karten, 1978; Dubbeldam, 1984; Wild and Zeigler, 1996), the latter of which in turn projects to the Cbln2+ parabrachial region (PB) (Arends et al., 1984). Cbln2+ geniculate ganglia neurons (most of which innervate taste buds) project to the Cbln2+ NTS, which shares reciprocal connections with the Cbln2+ PB. The petrosal ganglion (which carries primarily visceral sensory information) contains a mixture of Cbln2+ and Cbln2– neurons and, like the geniculate ganglion, projects to the nucleus of the solitary tract (NTS). Notably, many brain regions that receive direct input from NTS and/or PB, including the dorsal and linear raphe, POA, SpA, TnA, and TuO, and in turn some of their targets, such as intercollicularis, the supramammillary region, and VTA (Berk and Hawkin, 1985; Arends et al., 1988; Wild et al., 1990), are rich in Cbln2. This highly interconnected network of Cbln2-rich brain structures is schematized in Figure 8. An interesting aside is that the expression of Cbln2 by nearly all neurons in the trigeminal, facial, and jugular ganglia indicates that Cbln2+ neurons subserve a variety of sensory modalities (Fontaine-Perus et al., 1985), and come from different embryological origins (the jugular from the neural crest, the geniculate from placodes, and the trigeminal from both the neural crest and placodes; Fontaine-Perus et al., 1985).

Cbln2 is also highly expressed by neurons in the DRGs and in spinal cord laminae III–IV (Yang et al., 2010) that project to the gracile, cuneate, and external cuneate nuclei (Giesler et al., 1984; Wild, 1985; Funke and Necker, 1986), which in turn project to the Cbln2-rich DIVA and DLP in the thalamus (Wild, 1989). However, DIVA and DLP then project to the Cbln2-poor Wulst (Wild, 1987b) and the Cbln2-poor dorsal ventricular ridge (Gamlin and Cohen, 1986), respectively. Thus, higher-order sensory areas of the pallium in chick are not characterized by high Cbln2 expression, as will be discussed again below.

In contrast to the above-mentioned Cbln2-rich somato-sensory and gustatory neurons, only ≈5% of ganglion cells in the retina express Cbln2. RGCs vary in their physiological properties and general morphology, and >20 types of RGCs are distinguishable by the combinatorial expression of molecules such as adhesive proteins, calcium-binding proteins, neuropeptides/neurotransmitters, and their receptors (Yamagata et al., 2006). Different types of RGCs also have different projections within the brain (Yamagata et al., 2006), but these have not been fully characterized. Accordingly, we did not try to determine which kind(s) of RGCs express Cbln2, and so we next consider retinal projections as a whole.

RGCs project to several brain regions, which vary in their expression of Cbln2. These regions include the Cbln2-rich ventral lateral geniculate (GLv) and tectal gray (GT), the Cbln2-moderate dorsal lateral geniculate (DLL), nBOR, and area pretectalis (AP), and the Cbln2-poor lateral (visual) SCN (Repérant, 1973; Güntürkün and Karten, 1991; Shimizu et al., 1994). RGCs also innervate the tectum, where their axons arborize in layers 2–7, forming synapses on the dendrites of neurons with cell bodies situated in layers at least as deep as layer 13 (Repérant, 1973; Hayes and Webster, 1985). Cbln2 is expressed by most neurons in tectal layers 10 and 14, by some neurons in tectal layers 8 and 13, by a few neurons in layer 15, and is largely absent from layers 2–7. Without knowing which type(s) of RGCs express Cbln2 and which tectal neurons those RGCs innervate, it is not possible to determine if Cbln2+ RGCs project to Cbln2+ tectal neurons.

The layers of the tectum that contain Cbln2+ neurons also vary in the other inputs they receive and in their outputs. Tectal layer 10 neurons share reciprocal connections with the

Cbln2-rich nucleus isthmi pars parvocellularis (Ipc) and GLv (Hunt and Künzle, 1976b; Hellmann et al., 2004). Tectal layer 10 may also project to nucleus semilunaris (SLu), which is Cbln2-rich anteriorly and Cbln2-moderate posteriorly, although the laminar source of the tectum's projection to SLu has not been precisely determined (Hellmann et al., 2001). Layer 8–13 neurons receive input from the Cbln2-moderate SNr and the Cbln2-poor spiriformis lateralis (Reiner et al., 1982a,b, 1998). Layer 13 projects to the Cbln2-rich DLP, the Cbln2-moderate rotundus core, and the Cbln2-poor sub-pretectalis and nucleus pretectalis (Hunt and Künzle, 1976a; Benowitz and Karten, 1976; Gamlin and Cohen, 1986). Neurons in layers 14 and 15 receive input from neurons in upper tectal layers (Stone and Freeman, 1971; Leresche et al., 1986), although precisely which neurons provide this input is not known, and together with neurons in layers 8–13, project to the Cbln2-rich pontine nuclei (Reiner and Karten 1982; Hellmann et al., 2004). Interestingly, neurons along the border between tectal layers 9 and 10, which are Cbln2-poor, project to the isthmo-optic nucleus (Woodson et al., 1991), which is also poor in Cbln2. Thus, Cbln2 levels for the central inputs and projections of neurons in several different tectal layers frequently correspond. If a similar correspondence also pertains to retinal input to specific tectal neurons cannot be determined at the present time.

In the cochlear ganglia, only a small proportion of neurons express Cbln2, and only at low to moderate levels. However, the related auditory areas of the brain show intriguing patterns of Cbln2 expression as illustrated by the schematic in Figure 9. Nucleus magnocellularis, one of the main targets of the cochlear ganglion (Boord and Rasmussen, 1963), expresses little Cbln2, although it in turn projects to the Cbln2-rich nucleus laminaris (Boord, 1968; Young and Rubel, 1983; Takahashi and Konishi, 1988a), whereas the other major primary auditory area, nucleus angularis (Boord and Rasmussen, 1963), itself expresses high levels of Cbln2. The Cbln2-rich nucleus laminaris projects to the ventral nucleus of the lateral lemniscus (LLv), which we have identified as Cbln2-rich, and to the Cbln2-poor superior olive and nucleus mesencephali lateralis pars dorsalis (MLd; Boord, 1968; Conlee and Parks, 1986; Takahashi and Konishi, 1988a,b; Wild, 1995; Krütfeldt et al., 2010a,b). The Cbln2-rich nucleus angularis projects to the apparently Cbln2-rich LLv, to LLi (which is Cbln2-rich at caudal levels, but Cbln2-poor rostrally), to both the Cbln2-rich MLd shell (comprised of Next, Nsupf, and ICo) and the Cbln2-poor MLd, and to the Cbln2-poor superior olive (Boord, 1968; Conlee and Parks, 1986; Takahashi and Konishi, 1988a,b; Wild, 1995; Krütfeldt et al., 2010a,b). In addition, the Cbln2-rich gracile/cuneate nuclei project to the Cbln2-rich LLv, the Cbln2-rich MLd shell, and the Cbln2-poor MLd (Wild, 1995), and the Cbln2-rich LLv in turn projects to the Cbln2-rich nucleus semilunaris paravoidalis (SPo; Wild, 1987a) and to the Cbln2-poor MLd (Wild et al., 2010). This system thus provides several examples of synaptically connected neurons expressing different levels of Cbln2. Nonetheless, each Cbln2-rich cell group projects to at least one other Cbln2-rich cell group, and Cbln2 levels are often similar in synaptically connected neuronal populations, for example, the Cbln2-rich LLv and its inputs from the Cbln2-rich laminaris, angularis, and gracile/cuneate nuclei. Further, and most strikingly, the Cbln2-rich MLd shell in the midbrain projects to the Cbln2-rich ovoidalis shell in the thalamus, whereas the Cbln2-poor MLd projects to the more moderately Cbln2+ ovoidalis core (Karten, 1968; Durand et al., 1992). Neurons in ovoidalis and its shell then innervate several parts of the pallium, some of which are Cbln2-rich, while others are Cbln2-poor.

The vestibular ganglia, like the cochlear ganglia, are Cbln2-poor, and project to vestibular nuclei that are Cbln2-rich, namely, VeS and VeL, and to a lesser extent, VeM and VeD (Correia et al., 1983; Dickman and Fang, 1996). VeS, VeL, and VeM in turn project to the Cbln2-rich thalamic dorsolateral posterior nucleus (Wild, 1988). VeL also projects to the Cbln2-rich nucleus ruber, while VeD projects primarily to ventral regions of the spinal cord

(Correia et al., 1983) where motoneurons, some of which are Cbln2-rich (Yang et al., 2010), are situated.

Cbln2 is heavily expressed by mitral and tufted cells in the chick olfactory bulb. Mitral and tufted cells receive direct input from olfactory receptor neurons situated in the olfactory epithelium, which we did not examine, and project to several brain regions. Those regions include the Cbln2-rich olfactory tubercle (TuO), the taenia of the amygdala (TnA), the subpallial amygdala (SpA), HbL, and CDL (Reiner and Karten, 1985). Mitral/tufted cells also project to the piriform cortex (Reiner and Karten, 1985), which contains some neurons expressing low to moderate levels of Cbln2, and which in turn projects to the Cbln2-rich TnA, SpA, TuO, and CDL, and to the Cbln2-moderate dorsal arcopallium (Bingman et al., 1994). Interestingly, the SpA, TnA, and TuO also receive input from the Cbln2-rich PB and NTS (Arends et al., 1988; Wild et al., 1990). Finally, mitral/tufted cells also project to the ventrolateral edge of the lateral striatum (Reiner and Karten, 1985), which appears to represent a part of the olfactory tubercle (Abellan and Medina, 2009) and is rich in Cbln2+ neurons.

Thus, taken together, it appears that numerous sensory areas are characterized by high levels of Cbln2 expression. For some sensory systems (e.g., trigeminal, facial, jugular), Cbln2 is expressed in the primary sensory neurons and also in the related secondary and tertiary sensory regions in the brain. For other systems (e.g., visual, auditory, and vestibular), intense Cbln2 expression is limited to some of the higher-order sensory regions of the brain, but those regions that are Cbln2-rich are commonly interconnected. In comparison, most motor areas tend to be relatively Cbln2-poor (e.g., the striatum, globus pallidus, the trigeminal and facial motor nuclei, the medial cerebellar nucleus, and the cerebellum per se), with only a few motor areas (e.g., DIP in the thalamus, nucleus ruber, and the lateral cerebellar nucleus) being Cbln2-rich. Interestingly, DIP and nucleus ruber are both innervated by the Cbln2-rich lateral cerebellar nucleus (Arends and Zeigler, 1991; Wild, 1992). Thus, Cbln2-rich motor areas receive input from other Cbln2-rich motor areas, although DIP also receives input from the Cbln2-moderate globus pallidus (Medina and Reiner, 1997), and projects to the Cbln2-poor striatum (Veenman et al., 1997). Finally, as described above, it is our impression that, more often than not, Cbln2-rich neuronal subpopulations are synaptically interconnected. In the section below, we assess whether this tendency also applies to the other Cblns and then discuss possible functional implications.

Comparison with other Cblns and functional implications

To compare the pattern of Cbln2 expression with that of other Cblns, we focused on Cbln1, which is widely distributed throughout the brain, in contrast to Cbln3 and Cbln4, which are limited to relatively few brain regions. Interestingly, Miura et al. (2006) noted that Cblns are expressed at high levels by some neuronal populations but tend to be absent from their postsynaptic targets. They specifically pointed out, for example, that Cbln1 is highly expressed by cerebellar granule cells but is absent from Purkinje cells, is highly expressed in the entorhinal cortex but is absent from the hippocampus, and is highly expressed in the intralaminar thalamic nuclei but is absent from the caudate-putamen. However, these particular examples ignore other contributors to the connectivity of the nuclei in question, such as Cbln1-rich mitral cells, which provide input to the entorhinal cortex (Insausti et al., 2002), and the Cbln1-rich GPi and deep cerebellar nuclei, which provide input to the intralaminar thalamic nuclei (Reiner et al., 1998). Further, Morgan and colleagues (Wei et al., 2007) maintained, in contrast to Miura et al. (2006), that synaptically connected neuronal populations frequently express similar levels of Cbln1, noting, for example, that the Cbln1-rich nucleus ruber receives input from several Cbln1-rich nuclei, including the posterior thalamic, deep cerebellar, parabrachial, and cuneate nuclei.

The incidence of Cbln1 expression in synaptically connected neuronal populations appears to be less than for Cbln2, however, perhaps simply because Cbln1 is expressed in fewer regions than is Cbln2 (Miura et al., 2006 for mouse; our unpubl. obs. for chick). In addition, Cbln1 tends to be less prevalent in primary sensory neurons and in second-order sensory regions in the brain than is Cbln2. For example, in mice, Cbln1 is expressed in the olfactory bulb, parabrachial region, gracile/cuneate nuclei, cochlear nuclei, and vestibular nuclei, but is absent from NTS, the ventral lateral geniculate nucleus, the medial geniculate nucleus, SCN, superior olive, and inferior colliculus (Miura et al., 2006; Wei et al., 2007). We obtained generally similar results for Cbln1 in chick and, in addition, found that retinal ganglion cells and neurons in the trigeminal and vestibular ganglia do not express Cbln1.

The functional importance of these expression patterns is uncertain. Function per se of course requires that mRNAs be translated into proteins. This has been shown for Cbln1 in mouse brain, based on the correspondence between the patterns of Cbln1 mRNA expression (Miura et al., 2006) and Cbln1 protein expression (Wei et al., 2007). However, this has not (and cannot, at least at present) be demonstrated for Cbln2, since, to the best of our knowledge, antibodies specific for Cbln2 (from either chicken or mouse) do not exist.

Assuming that Cbln2 mRNA is translated into protein, the tendency for Cbln1 and Cbln2 to be expressed in synaptically connected neuronal populations may be coincidental, and Cbln released from presynaptic terminals may simply act at successive synapses along a given neuronal pathway. Alternatively, Cbln expression in synaptically connected neuronal populations may be of functional significance if, perhaps, Cbln released presynaptically and Cbln released postsynaptically act at the same synapse. Cbln1 has recently been shown to bind β -neurexins on presynaptic terminals and GluR δ 2 on postsynaptic surfaces, thereby forming a bridge (Uemura et al., 2010). Cbln2 has been hypothesized to similarly bind β -neurexins and GluR δ s (Uemura et al., 2010). Moreover, when Cbln1 is expressed postsynaptically, i.e., by Purkinje cells in L7-Cbln1 transgenic mice which otherwise lack Cbln1, it promotes the formation of synapses made by granule cell parallel fibers (Wei et al., 2009). In this situation, Cbln1 is presumably released from Purkinje cell dendrites and participates in forming this “molecular” bridge at sites of synaptic contact. Accordingly, if endogenous Cblns are released from postsynaptic cell bodies and/or dendrites within the brain, the triad consisting of neurexin-Cbln-GluR δ would provide a mechanism by which Cbln originating postsynaptically could promote the formation of synapses neurons receive. Whether Cblns released presynaptically and Cblns released postsynaptically indeed act on the same synapses and whether they interact synergistically is presently unknown.

Finally, it is interesting to note that, although they have distinct patterns of expression (Miura et al., 2006), Cbln1 and Cbln2 are both highly expressed in several of the same areas of the brain. In a few such cases, for example, mitral cells, the Ipc in chick, and the parafascicular nucleus in mouse, all neurons appeared labeled for Cbln1 and for Cbln2, strongly suggesting that individual neurons coexpress both Cblns. For such neurons, whether Cbln1 and Cbln2 are actually secreted as heterohexamers (as happens for transfected heterologous cells and for Cbln1/Cbln3 in granule cells) and, if so, whether the heterohexamers differ from homohexamers in their binding properties and/or downstream signaling is not known.

Comparison of Cbln2 labeling between birds and mammals

Since the avian and mammalian lineages diverged 300 million years ago, several extensive changes have taken place that are reflected in the differences in the brains of extant birds and mammals, especially in the telencephalon. Hence, in the discussion below, in which we compare the expression of Cbln2 in chick and mouse brains, we start with the hindbrain and move rostrally, ending with the telencephalon. The information on Cbln2 expression in the

mouse brain comes primarily from images of in situ hybridization shown in the Allen Brain Atlas, as well as from Miura et al. (2006) and our own unpublished observations. Note also that we use mouse as a representative of mammals, and chicks as a representative of birds, but more detailed evolutionary considerations would require studying Cbln2 expression in additional mammalian and avian species, and in reptiles as well.

Hindbrain—Nearly all structures in the chick hindbrain have clear mammalian homologs. Cbln2 is highly expressed in both chick and mouse in many of these structures, including the gracile/cuneate nuclei, descending trigeminal nucleus, solitary tract nucleus, the vestibular nuclei (medial, superior, and lateral), ventral parts of the nuclei of the lateral lemniscus, and the linear raphe. In contrast, the superior olive in both chick and mouse lacks Cbln2.

For other parts of the hindbrain, Cbln2 expression differs between homologous structures in chick and mouse. Notably, the pontine nuclei and inferior olive in birds, but not in mice, are rich in Cbln2, perhaps reflecting the independent expansion of the cerebellum and its afferent systems from the much simpler cerebellum of the common ancestor stem amniotes (Larsell, 1967).

In addition, the primary auditory nucleus angularis (and the secondary auditory nucleus laminaris) in chick are Cbln2-rich, whereas the primary auditory dorsal and ventral cochlear nuclei in mice are both Cbln2-poor, as is the mammalian medial superior olive, which is similar to laminaris in function, but has a different evolutionary history (Grothe et al., 2005). The evolutionary relationship of avian and mammalian cochlear nuclei has been much debated and remains unresolved. Some investigators have suggested that these structures evolved independently in birds and mammals, based primarily on middle and inner ear differences and on differences in physiologically defined cell types in the cochlear nuclei themselves (see discussion in Grothe et al., 2005). However, there is also evidence supporting homologies between avian and mammalian cochlear nuclei. For example, nucleus angularis and the dorsal cochlear nucleus are similar in topography, rhombomeric origin, neuronal typology, connectivity, and role in auditory frequency detection (Boord, 1969; Cramer et al., 2000; Diaz et al., 2003; Maklad and Fritzsche, 2003; Grothe et al., 2005; Farago et al., 2006; Yang and Feng, 2007). Nucleus angularis and the dorsal cochlear nucleus are also similar in their projections to both the core and the shell of MLD and inferior colliculus, respectively (Boord, 1968; Willard and Martin, 1983; Conlee and Parks, 1986; Takahashi and Konishi, 1988a,b; Wild, 1995; Cant and Benson, 2003). Interestingly, while chick nucleus angularis and mouse dorsal cochlear nucleus differ in terms of Cbln2 expression, they both highly express two Cblns: nucleus angularis, Cbln1 and Cbln2; the dorsal cochlear nucleus, Cbln1 and Cbln3. The expression of a different second Cbln (Cbln2 in chick, Cbln3 in mouse), in addition to Cbln1, may reflect the absence of Cbln3 in chick, as well as the extensive evolutionary changes in the peripheral auditory apparatus and in the related central target areas that have occurred independently in birds and in mammals, from the simpler condition in stem amniotes, whose frequency range was more limited. On the other hand, neurons of the mammalian ventral cochlear nucleus appear to match neurons of the nucleus magnocellularis-laminaris complex in their physiology, typology, and projections (Willard and Martin, 1983; Conlee and Parks, 1986; Takahashi and Konishi, 1988a,b; Wild, 1995; Carr and Soares, 2002; Cant and Benson, 2003; Grothe et al., 2005; Yang and Feng, 2007). However, laminaris does not receive cochlear nerve input directly (Boord and Rasmussen, 1963; Boord, 1968), but rather receives auditory input indirectly, via the nucleus magnocellularis, which is Cbln2-poor, like the ventral cochlear nucleus. It has been suggested that nucleus laminaris and magnocellularis differentiated from a single nucleus devoted to auditory spatial localization in stem amniotes (Grothe et al., 2005). The observation that both the ventral cochlear nucleus and nucleus laminaris express Cbln4 (Allen Brain Atlas; our unpubl. obs.) is consistent with the possibility that laminaris and

magnocellularis together correspond to the mammalian ventral coch-lear nucleus, and suggest Cbln2 expression by laminaris may have emerged as laminaris differentiated into an individual nucleus in the sauropsid lineage.

Mesencephalon and isthmus—For structures in the midbrain and isthmus, there is a very high degree of correspondence between the levels of Cbln2 expression in chick and the homologous regions in mouse.

The midbrain roof, termed the superior colliculus in mammals and the optic tectum in birds, is a major visual area in the mesencephalon (Karten, 1969; Reiner and Karten, 1982). The tectum and the superior colliculus both receive direct retinal input, project to a thalamic relay nucleus, and are laminated. Tectal layer 13 in birds is comparable to the deepest sublayer of the superficial layer (stratum griseum superficiale, SGS3) and the upper stratum opticum of the superior colliculus in mammals in terms of connectivity, neurochemistry, and the distinctive bottlebrush dendritic morphology of their constituent neurons (Luksch et al., 1998; Major et al., 2000; Yamamoto and Reiner, 2005). Consistent with these similarities, tectal layer 13 and SGS3 both contain numerous Cbln2-rich neurons. Neurons in tectal layers 10 and in the second sublayer of SGS (SGS2) share similarities in their connectivity and neurochemistry (Yamamoto and Reiner, 2007). Cbln2+ neurons are present in both, although they are more abundant in chick tectal layer 10 than in mouse SGS2, possibly in keeping with the relative hypertrophy of two major targets of tectal layer 8–10 neurons, specifically the ventral lateral geniculate nucleus (GLv) and nucleus isthmi pars parvocellularis (Ipc), in chick. The level of Cbln2 expression is also very high in both the chick GLv and Ipc, and in their mammalian homologs, the GLv and the parabigeminal nucleus.

The avian MLd is considered homologous to the core of the mammalian inferior colliculus based on their similar connectivity (receiving ascending auditory input from the cochlear nuclei via the lateral lemniscus and projecting to a thalamic auditory nucleus) and their location deep to the optic tectum/superior colliculus (Boord and Rasmussen, 1963; Karten, 1967, 1968, 1969; Boord, 1968). Chick MLd is Cbln2-poor, as is the central nucleus of the inferior colliculus in mouse, whereas both the shell surrounding MLd and the shell of inferior colliculus are Cbln2-rich.

Several additional midbrain and isthmial regions that are clearly homologous in birds and mammals are also similarly enriched in Cbln2+ neurons, including the para-brachial region, nucleus ruber, VTA, the linear raphe, and the medial part of the dorsal raphe.

Diencephalon—For most of the diencephalon, Cbln2 levels in specific chick nuclei are similar to what is found for the mammalian homologs. For example, in the epithalamus, Cbln2 is very highly expressed in the lateral habenular nuclei and at slightly lower levels in the medial habenular nuclei, as also observed in mammals. The habenular nuclei are evolutionarily ancient and seemingly conservative in their connectivity (Butler and Hodos, 2005). Similarly, several hypothalamic regions are Cbln2-rich in chick, including the preoptic area, the medial suprachiasmatic nucleus, and the supramammillary region, as also true in mouse. Hypothalamic organization in birds and mammals show many similarities (Kuenzel and Tienhoven, 1982; Butler and Hodos, 2005), and conservation of Cbln2 in homologous neuronal populations appears to be a reflection of this similarity. In contrast, many of the nuclei in the pre-tectum express little, if any, Cbln2 in both chick and mouse. An exception to this appears to be area pretectalis, which like its mammalian homolog the olivary pretectal nucleus, contains numerous Cbln2+ neurons.

For the thalamus, Cbln2 levels are similar for most of the specific chick nuclei that have clear mammalian homologs. For example, VIA, like its mammalian homolog, the motor thalamus (the ventromedial nucleus) is rich in Cbln2, as are avian DIP and its mammalian homolog, the parafascicular nucleus (Veenman et al., 1997). Further, DMA and DMP, which have been suggested to be homologous to the Cbln2-poor mammalian midline thalamic and mediodorsal nuclei, respectively, based on connectivity and neurochemistry (Veenman et al., 1997) and on ErbB4 expression (Bruce et al., 2002), contain some moderately labeled Cbln2+ neurons. In contrast, however, the chicken homolog (DLL) of the mammalian dorsal lateral geniculate nucleus (GLd) contains many moderately labeled Cbln2+ neurons, whereas mammalian GLd (Takatsuji and Tohyama, 1989; Güntürkün and Karten, 1991) is extremely poor in Cbln2.

The core of the avian nucleus ovoidalis (Ov) is generally considered homologous to the ventral part of the mammalian medial geniculate nucleus (Cajal, 1911; Morest, 1965; Karten, 1967; Karten, 1968), based on their heavy input from the core of the auditory midbrain (Karten, 1967, 1969), similar neurochemistry (Yamamoto and Reiner, 2007), and low expression of ErbB4 (Bruce et al., 2002). The core of chick ovoidalis and the ventral part of mouse medial geniculate are both poorer in Cbln2 than are their surrounding shells.

The mammalian homolog of the avian nucleus rotundus is currently unresolved. Some researchers consider avian nucleus rotundus to be homologous to the mammalian caudal pulvinar nucleus (Hall and Ebner, 1970; Karten and Shimizu, 1989; Major et al., 2000). These researchers note that the input that rotundus receives from tectal layer 13 (Karten and Revzin, 1966; Karten and Hodos, 1970) is similar to the input that the mammalian caudal pulvinar nucleus receives from lower stratum griseum superficiale (SGS3) and upper stratum opticum of mammalian superior colliculus (Albano et al., 1979; Graham and Casagrande, 1980; Abramson and Chalupa, 1988). Related to this comparison is the evidence, described earlier in the Discussion, that tectal layer 13 neurons and SGS3/stratum opticum neurons are homologous. Other researchers maintain that avian nucleus rotundus is homologous to the mammalian posterior thalamic nucleus because they consider tectal layer 13 to be homologous to the stratum griseum intermedium (SGI) of mammalian superior colliculus (Dávila et al., 2000). We find that tectal layer 13 is rich in Cbln2 (as are SGS3 and stratum opticum, whereas SGI is moderate) and that rotundus is moderate to rich in Cbln2 (as is caudal pulvinar, whereas the posterior thalamic nucleus is Cbln2-poor). Thus, the pattern of Cbln2 expression is more consistent with the hypothesis that rotundus is homologous to the caudal pulvinar nucleus than it is to the alternative, that rotundus is homologous to the posterior thalamic nucleus.

Based on connectivity and neurochemistry, Veenman et al. (1997) suggested that DLM and DLP in birds are homologous to the lateral intralaminar nuclei in mammals. ErbB4 mRNA expression patterns support this interpretation (Bruce et al., 2002). Others have suggested that avian DLP (in part or in its entirety) is homologous to the mammalian posterior thalamic nucleus (Gamlin and Cohen, 1986; Korzeniewska and Güntürkün, 1990; Kröner and Güntürkün, 1999). Chick DLM and DLP are rich in Cbln2, whereas mouse lateral intralaminar and posterior nuclei are both Cbln2-poor. Thus, for these structures, Cbln2 expression differs between chick and mouse, and does not support either of the proposed homologies or help suggest alternative possibilities.

Telencephalon—Most regions of the subpallium are Cbln2-poor in both chick and mouse, including the BSTL, chick dorsal striatum (Mst and LSt), and its mammalian homolog, the caudate-putamen. Chick globus pallidus contains a few moderately Cbln2+ neurons, whereas mouse globus pallidus internus (GPi) is Cbln2-rich and globus pallidus externus (GPe) is Cbln2-poor. One possibility is that the Cbln2+ neurons in chick globus pallidus

correspond to GPi neurons. However, GPe-like and GPi-like neurons are intermingled in chick, and so testing this idea would require combining in situ hybridization with immunostaining to determine if the Cbln2+ neurons in globus pallidus receive substance P+ GPi-type input or enkephalin+ GPe-type input (Reiner et al., 1998). In contrast, in the lateral forebrain bundle of both chick and mouse, Cbln2+ neurons are fairly numerous and are likely to correspond to the cholinergic neurons.

Two subpallial amygdaloid nuclei, TnA and SpA, have been identified in birds, and are widely accepted as being homologous to the mammalian medial amygdaloid nucleus and a sublenticular part of the extended amygdala, respectively (Kitt and Brauth, 1981; Wild et al., 1990; Thompson et al., 1998; Roberts et al., 2002; Absil et al., 2002a; Reiner et al., 2004; Sun et al., 2005). However, TnA and SpA contain more intensely labeled Cbln2+ neurons than do their mammalian homologs, which contain only scattered moderately labeled Cbln2+ cells.

For pallial parts of the telencephalon, chick and mouse differ considerably in their expression of Cbln2. Cbln2 is expressed by neurons in the glomerular, mitral, and granule cell layers in mouse olfactory bulb. In contrast, Cbln2+ neurons are largely confined to the mitral cell layer in chick, whose olfactory bulb is small and poorly developed in comparison to that of rodents. Mitral cells project to the piriform cortex, which for both birds and rodents receives dorsomedial thalamic and ventral tegmental area inputs, in addition to olfactory bulb input, and projects to the hippocampus (Bingman et al., 1994). Mouse piriform cortex, which is three-layered (Price, 1973; Haberly and Price, 1978; Haberly and Bower, 1984; Ojima et al., 1984), contains many Cbln2-rich neurons in the olfactory-recipient layer 2. In contrast, in chick piriform cortex, which consists of a single olfactory-recipient cell plate (Reiner and Karten, 1985; Bingman et al., 1994), Cbln2 is moderately expressed by a smaller proportion of neurons. The lower number of Cbln2+ neurons in chick than in mouse piriform cortex may be a reflection of the smaller size of the avian olfactory circuit in general.

The avian hippocampal complex is considered homologous to the hippocampal complex in mammals, based on similarities in its dorsomedial pallial location (Ariëns-Kappers et al., 1936), connections with hypothalamus and septum (Krayniak and Siegel, 1978a; Casini et al., 1986), and role in spatial memory (Olton and Papas, 1979; Bingman et al., 1984; Sahgal, 1984; Bingman et al., 1985; Morris et al., 1989). Although the hippocampal complexes in chick and mouse are very different in their cytoarchitecture, they are similar in their low expression of Cbln2, with scattered Cbln2+ neurons being present in the parahippocampal area and the hippocampus proper in chick and in mouse. The correspondences of specific hippocampal subdivisions between birds and mammals are unresolved (Benowitz and Karten, 1976b; Witter, 1989; Krebs et al., 1991; Erichsen et al., 1991; Amaral, 1993; Székely and Krebs, 1996; Atoji et al., 2002; Kahn et al., 2003; Atoji and Wild, 2004) and the pattern of Cbln2 expression does not provide any additional clarification.

The Wulst is considered to be homologous to mammalian cerebral cortex medial to the temporal sulcus (Medina and Reiner, 2000), and both are thought to have originated from a sector of dorsal pallium in stem amniotes. Further, subregions of the Wulst are homologous to mammalian primary somatosensory cortex (Wild, 1987b; Funke, 1989) and primary visual cortex (Karten et al., 1973; Reiner and Karten, 1983) in terms of their connectivity. However, only a small proportion of neurons in the Wulst express any Cbln2, and these only at low levels, while layer 2/3 and 5/6 neurons of the dorsomedial cerebral cortex are Cbln2-rich. This difference is likely to reflect the extensive changes between the Wulst and dorsomedial cerebral cortex in their development and adult morphology that occurred as they evolved independently from a common ancestor.

In contrast, Cbln2-expressing neurons are present in the CDL, medial arcopallium, and dorsal arcopallium of the chick brain, and so in this respect these regions are similar to mammalian cerebral cortex. However, except for a Cbln2+ band within the upper mesopallium, the avian mesopallium and nidopallium (together called the dorsal ventricular ridge, or DVR) are poor in Cbln2. There are three main hypotheses regarding DVR's mammalian homolog. One hypothesis is that DVR is homologous to the part of mammalian cerebral cortex lateral to the temporal sulcus (Karten, 1969, 1991; Karten and Shimizu, 1989; Reiner, 2000). However, neurons of temporal cortex layers 2/3 and 5/6 are rich in Cbln2. A second hypothesis is that DVR is homologous to the claustrum, endopiriform nucleus, and lateral amygdaloid nucleus (Striedter, 1997; Puelles et al., 1999, 2000). However, those areas express moderate levels of Cbln2. A final hypothesis is that the nidopallium is homologous to the mammalian lateral amygdaloid nucleus (Bruce and Neary, 1995). However, mammalian lateral and basolateral amygdaloid nuclei are both moderate in Cbln2. Thus, Cbln2 expression differs considerably between chick DVR and each of its suggested mammalian homologs.

In summary, Cbln2 expression is not conserved in regions of the avian brain that have undergone extensive changes during evolution as compared to mammals. In the hindbrain, regions with differing Cbln2 expression include the pontine, inferior olive, and primary auditory nuclei, which are rich in Cbln2 in birds, but Cbln2-poor in mice. For the telencephalon, the opposite is true, with the mouse cerebral cortex expressing much higher levels of Cbln2 than do the Wulst and DVR in chick. In contrast, Cbln2 expression is highly conserved between chick and mouse for brain regions that have diverged less during evolution, consistent with it playing a fundamental and thus a conserved role in synapse formation.

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Abbreviations

A	arcopallium
AC	anterior commissure
AD	dorsal arcopallium
AL	ansa lenticularis
AM	medial arcopallium
An	nucleus angularis
AP	area pretectalis
APH	parahippocampal area
BSTL	lateral bed nucleus of the stria terminalis
Cb	cerebellum
CbL	lateral cerebellar nucleus
CbM	medial cerebellar nucleus

CDL	dorsolateral corticoid area
Cnd	dorsal part of the central medullary nucleus
Cnv	ventral part of the central medullary nucleus
CPi	piriform cortex
CuE	external cuneate nucleus
DIP	dorsointermediate posterior nucleus
DIVA	dorsointermediate ventral anterior area
DL (pallium)	dorsolateral (pallium)
DLL	dorsolateral anterior, pars lateralis nucleus
DLM	dorsolateral anterior, pars medialis nucleus
DLP	dorsolateral posterior nucleus
DMA	dorsomedial anterior nucleus
DMP	dorsomedial posterior nucleus
DVR	dorsal ventricular ridge
E	entopallium
EW	nucleus of Edinger-Westphal
FLM	fasciculus longitudinalis medialis
FRL	lateral reticular formation
FRM	medial reticular formation
GC	gracile and cuneate nuclei
GCL	ganglion cell layer (retina)
GCt	central periaqueductal gray
GLd	dorsal lateral geniculate nucleus
GLv	ventral lateral geniculate nucleus
GP	globus pallidus
GPe	globus pallidus externus
GPi	globus pallidus internus
GT	tectal gray
HbL	lateral habenular nucleus
HbM	medial habenular nucleus
Hp	hippocampus proper
ICo	nucleus intercollicularis
Imc	nucleus isthmi, pars magnocellularis
INL	inner nuclear layer (retina)
INP	intrapeduncular nucleus
IO	inferior olive

ION	isthmo-optic nucleus
IP	interpeduncular nucleus
Ipc	nucleus isthmi, pars parvocellularis
LA	lateral anterior nucleus
La	nucleus laminaris
latSCN	lateral suprachiasmatic nucleus
LFB	lateral forebrain bundle
LHy	lateral hypothalamic area
LLd	dorsal nucleus of the lateral lemniscus
LLi	Intermediate nucleus of the lateral lemniscus
LLv	ventral nucleus of the lateral lemniscus
LoC	locus coeruleus
LSt	lateral striatum
M	mesopallium
M3	oculomotor nucleus
M4	trochlear motor nucleus
M5	trigeminal motor nucleus
M7	facial motor nucleus
M9/10	motor nucleus of the vagus
M12	hypoglossal motor nucleus
Mc	nucleus magnocellularis
MDd	dorsal mesopallium, dorsal band
medSCN	medial suprachiasmatic nucleus
MePO	median preoptic nucleus
MHy	medial hypothalamic area
MLd	nucleus mesencephalicus lateralis, pars dorsalis
MSt	medial striatum
N	nidopallium
nBOR	nucleus of the basal optic root
NCL	caudolateral nidopallium
Next	nucleus externus
Nsupf	nucleus superficialis
NTS	nucleus of the solitary tract
OB	olfactory bulb
OMT	occipitomesencephalic tract
Ov	nucleus ovoidalis

Pb	parabrachial region
PL	lateral pontine nucleus
PM	medial pontine nucleus
POA	preoptic area
PoA	posterior amygdaloid nucleus
PPC	nucleus precommisuralis principalis
Pr5	principal nucleus of the trigeminus
PT	nucleus pretectalis
PV	nucleus posteroventralis
PVN	paraventricular hypothalamus nucleus
RaD	dorsal raphe
RaL	linear part of the raphe
RGC	retinal ganglion cells
Rgc	nucleus reticularis gigantocellularis
RL	lateral reticular nucleus
RP	nucleus reticularis pontis
Rpc	nucleus reticularis parvocellularis
RPgc	nucleus reticularis pontis gigantocellularis
RPo	nucleus reticularis pontis oralis
Rt	nucleus rotundus
Ru	nucleus ruber
SCd	dorsal subcoeruleus
SCE	stratum cellulare externum
SCI	stratum cellulare internum
SCv	ventral subcoeruleus
SGI	stratum griseum intermedium
SGS	stratum griseum superficiale
SLu	nucleus semilunaris
SMe	stria medullaris
SNc	substantia nigra, pars compacta
SNr	substantia nigra, pars reticulata
SO	superior olive
SP	nucleus subpretectalis
SpA	subpallial amygdaloid nucleus
SPC	superficial parvocellular nucleus
SpL	nucleus spiriformis lateralis

SpM	nucleus spiriformis medialis
SpMam	supramammillary region
SPo	nucleus semilunaris paravoidalis
SRt	nucleus subrotundus
STN	subthalamic nucleus
TnA	taenia of the amygdala
TPO	temporo-parieto-occipital area
TRN	thalamic reticular nucleus
TTd	nucleus of the descending tract of the trigeminus
TuHy	tuberal hypothalamus
TuO	olfactory tubercle
TV	tegmenti ventralis
VeD	descending vestibular nucleus
VeL	lateral vestibular nucleus
VeM	medial vestibular nucleus
VeS	superior vestibular nucleus
VIA	ventrointermediate area
VLT	ventrolateral thalamus
VLv	ventrolateral nucleus of the lateral lemniscus
VMH	ventromedial hypothalamic nucleus
VP	ventral pallidum
VTa	ventral tegmental area
W	Wulst (hyperpallium)

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Figure 1.

Cbln2+ neurons in the forebrain. High-magnification views are shown to illustrate neuronal morphology, varying levels of Cbln2 expression, and varying proportions of Cbln2+ neurons in different brain regions. **A–D:** From the low-magnification image shown in Figure 3E. **A:** The DL pallium and TPO contain some moderate to rich Cbln2+ neurons. **B:** Some lightly to moderately labeled Cbln2+ neurons are situated in Cpi, which occupies the entire lateral edge of the part of the telencephalon shown in the image. **C:** GP and LSt contain a few moderately labeled Cbln2+ neurons. Arrows in A–C indicate scattered neurons in the nidopallium or entopallium expressing low to moderate levels of Cbln2. **D:** The rostral LFB contains numerous Cbln2-rich neurons, which by size, abundance, and location are likely to correspond to the cholinergic neurons of the LFB. **E:** The majority of neurons in GLv, LA, and VLT express high levels of Cbln2. This image is from the section shown in Figure 4A. **F:** Most neurons in Rt express moderate to high levels of Cbln2. This image is from the section shown in Figure 4C. The density of Cbln2+ neurons in Rt appears lower than in GLv, LA, VLT because overall neuronal packing density is less. Scale bar = 200 μ m.

Figure 2.

Cbln2⁺ neurons in the midbrain and hindbrain. High-magnification views showing that the vast majority of neurons in numerous brain nuclei express high levels of Cbln2, including Ipc (A), PrV (C), La and An (D), NTS (E), TTd (G), IO (H), M9/10 (I). In contrast, Imc (A) and Mc (D) do not contain any Cbln2⁺ neurons, and M12 (I) only a few lightly labeled neurons. Most neurons in caudal parts of Slu express moderate levels of Cbln2 (B), as do some neurons in Rgc (F), although a few large neurons in Rgc are Cbln2-rich. The image in (A) comes from Figure 5F, (B) from Figure 5H, (C) from Figure 6D, (D) from Figure 6I, (E,F) from the right side of Figure 6K, (G) from the right side of Figure 6L, (H) from the left side of Figure 6M, (I) from the right side of Figure 6N. Scale bar = 200 μ m, except (D), 250 μ m.

Figure 3.

Cbln2 expression in the telencephalon and rostral diencephalon. Cbln2 expression is generally low in the pallium, although it is heavily expressed by mitral cells in the olfactory bulb. Cbln2+ neurons are also present in a band (MDd) extending across much of the dorsal mesopallium that is continuous with the DL pallium, which is in turn continuous with the CDL. In the subpallium, Cbln2 expression is highest in the LFB, ThA, and SpA. Cbln2 expression is also high in the DLM of the thalamus and in MePo, POA and medSCN of the hypothalamus. For this as well as for Figures 4–6, brains were sectioned coronally, and the right side of brain is shown. Scale bar = 1 mm, except (A inset), 367 μ m.

Figure 4.

Cbln2 expression in caudal telencephalon and diencephalon. A crescent-shaped band of moderately labeled Cbln2+ neurons (AM band) is present in the caudal part of the telencephalon (A). Cbln2 is highly expressed by HbL, HbM, SMe, and SPC in the epithalamus. In the thalamus, Cbln2 expression is high in DLM, DIP, DLP, SPo, the shell of ovoidalis, and the neurons surrounding rotundus, including those in DIVA and VIA. GLv, LA, SCI, and VLT in the ventral thalamus are also rich in Cbln2, as are SpMam in the hypothalamus and GT in the pretectum. The section shown in (A) is from a different animal than the other panels; the small difference in sectioning planes is responsible for the apparent discontinuity in HbL and HbM between (A) and (D). Scale bar = 1 mm.

Figure 5.

Cbln2 expression in caudal diencephalon and midbrain. Cbln2 is highly expressed by SpMam in the caudal part of the dience-phalon and by GT in the pretectum. In the subtectal optic lobe, neurons forming the shell surrounding MLd are rich in Cbln2 as are all, or nearly all, neurons in Ipc. In the tectum, Cbln2 is heavily expressed by neurons in layers 10, 13, and 14. Ru, VTA, RaD, and upper part of RaL in the tegmentum are Cbln2-rich, as is LLv in the isthmus. Scale bar = 1 mm for A–C, F–I; 500 μ m for D; 167 μ m for E.

Figure 6.

Cbln2 expression in the isthmus and hindbrain. **A–C:** Cbln2 is heavily expressed by LLv and, at more caudal levels, by LLi. **C–F:** Numerous metencephalic structures are rich in Cbln2, including Pb, Pr5, RaL, PM, PL, CbL, VeL, VeS, and TTd, which extends into the myelencephalon. The ventral part of the brain is not included in (E), so that CbM and CbL can be shown, and to provide space for the high-magnification view of the cerebellum in H. **G,H:** Moderately labeled Cbln2+ neurons are found scattered in the granule cell layer of the cerebellum. **I–N:** In the myelencephalon, An, La, GC, CuE, IO, M9/10, NTS, RL, and TTd are rich in Cbln2. Scale bar = 1 mm for A–F, I–N; 500 μ m for G; 132 μ m for H.

Figure 7.

Cbln2 expression in the retina and cranial ganglia. **A:** Cbln2+ neurons are found in the inner nuclear layer (INL) and in the ganglion cell layer (GCL) in the retina. Two images of the retina are shown, to illustrate that the distribution of Cbln2+ RGCs is not uniform. The vast majority of neurons in the trigeminal (**B**), geniculate (**C**), and jugular (**F**) ganglia, and about half of the neurons in the petrosal ganglion (**G**) express high levels of Cbln2. Cbln2 expression is low in the vestibular (**D**) and cochlear (**E**) ganglia. All the ganglia shown are from the animal's right side. **D,E:** Thick arrows point to the ganglia, thin arrows to cranial nerve trunks. Scale bar = 100 μ m.

Figure 8.

Schematic overview of Cbln2 expression in the trigeminal, geniculate, jugular, and petrosal ganglia, the olfactory bulb, and in the major brain structures receiving the related sensory information. Structures are represented in roughly a caudal to rostral sequence from the top to the bottom. The intensity of the gray shading indicates the relative level of Cbln2 expression for a given structure. Solid gray indicates that many of the neurons in the structure are Cbln2+, while the thick gray stripes indicate that only some neurons are Cbln2+. Note that nearly all of the structures in this highly interconnected network are Cbln2-rich.

Figure 9.

Schematic overview of Cbln2 expression in the cochlear ganglion and in the major auditory regions of the brain. Structures are represented in roughly a caudal to rostral sequence, with the brainstem near the top and the telencephalon at the bottom. Soma-to-sensory input, relayed via the gracile and cuneate nuclei, to LLv and MLd is also shown. L1, L2, and L3 are contiguous parts of field L, which is the primary auditory cortical area in birds. For simplicity, LLi and LLd have been omitted and some connections (for example, of LLv to An, La, Mc, and SO) are not shown. The intensity of the gray shading for a given structure indicates its relative level of Cbln2 expression. Solid gray indicates that many of the neurons are Cbln2+, while the thin gray stripes indicate that Cbln2 is expressed by only a few neurons. The rectangle corresponding to TPO, AM, and DL pallium is shown in a medium shade of gray, rather than with stripes, since these three targets vary somewhat in their levels of Cbln2 expression levels and the relationship between the precise location of the Cbln2+ neurons relative to where Ov and Ov shell neurons actually terminate is not known. While the schematic shows some examples of synaptically connected neurons expressing different levels of Cbln2, it also provides some examples of synaptically connected neurons expressing similar levels of Cbln2. Most strikingly, the Cbln2-rich MLd shell projects to the Cbln2-rich ovoidalis shell while the Cbln2-poor MLd projects to the more moderately Cbln2+ ovoidalis core.

TABLE 1

Antibodies Used

Antigen	Immunogen	Source, catalog, or clone number	Host	Dilution
Islet1	C-terminal portion of rat Islet1, aa178–349	DSHB, contributed by T. Jessell, clone 39.4D5	mouse	1:100
TuJ1	neuronal class III beta-tubulin	Covance, Cat.#MMS-435P	mouse	1:1,000
NeuN	purified cell nuclei from mouse brain	Chemicon, Cat.#MAB377, clone #A60	mouse	1:500

TABLE 2

Cbln2 Expression

Brain region	Expression
Telencephalon	
Pallium	
olfactory bulb (OB): mitral cells	most+++
piriform cortex (CPi)	some +,++
dorsolateral corticoid area (CDL)	most+++
dorsolateral pallium (DL pallium)	some ++,+++
temporo-parieto-occipital area (TPO)	some++
caudolateral nidopallium (NCL)	few+
entopallium (E)	few+
nidopallium (N)	few+,++
hippocampus proper (Hp)	few+++
parahippocampal area (APH)	few++
dorsal arcopallium (AD)	most++
medial arcopallium (AM) crescent	most++
dorsal mesopallium, dorsal band (MDd)	most+++
hyperpallium (Wulst; W)	few+
Subpallium	
posterior amygdala (PoA)	few+
subpallial amygdala (SpA)	most+++
taenia of the amygdala (TnA)	most+++
lateral striatum (LSt)	few++
medial striatum (MSt)	few++
globus pallidus (GP)	few++
ventral pallidum (VP)	some++
intrapuduncular nucleus (INP)	few+++
lateral bed nucleus of the stria terminalis (BSTL)	few++
lateral forebrain bundle (LFB)	rostral most+++ / caudal –
olfactory tubercle (TuO)	most+++
septum	few+
Diencephalon	
Epithalamus	
lateral habenula (HbL)	most+++
medial habenula (HbM)	most+++
stria medullaris (SMe)	most+++
superficial parvocellular nucleus (SPC)	most+++
Thalamus	
dorsointermediate posterior (DIP)	most+++
dorsointermediate ventral anterior area (DIVA)	most+++
dorsolateral anterior, pars lateralis nucleus (DLL)	most++

Brain region	Expression
dorsolateral anterior, pars medialis nuclei (DLM)	rostral most+++ / caudal most++
dorsolateral posterior nuclei (DLP)	most+++
dorsomedial anterior (DMA)	some++
dorsomedial posterior nucleus (DMP)	some++
nucleus posteroventralis (PV)	most+++
nucleus rotundus (Rt)	most++,+++
nucleus subrotundus (SRt)	most+++
ovoidalis (Ov)	most++
ovoidalis shell (Ov shell)	most+++
nucleus semilunaris paravoidalis (SPo)	most+++
ventrointermediate area (VIA)	most+++
Ventral thalamus	
lateral anterior nucleus (LA)	most+++
stratum cellulare externum (SCE)	some++
stratum cellulare internum (SCI)	most+++
subthalamic nucleus (STN)	few++
thalamic reticular nucleus (TRN)	few++
ventral lateral geniculate nucleus (GLv)	most+++
ventrolateral thalamus (VLT)	most+++
Hypothalamus	
lateral hypothalamic area (LHy)	some+,++
lateral suprachiasmatic nucleus (latSCN)	none
medial hypothalamic area (MHy)	some+,++
median preoptic nucleus (MePO)	most+++
medial suprachiasmatic nucleus (medSCN)	most+++
paraventricular hypothalamus (PVN)	few+
preoptic area (POA)	most+++
supramammillary region (SpMam)	most+++
tuberal hypothalamus (TuHy)	few+
ventromedial hypothalamic region (VMH)	some+
Pretectum	
area pretectalis (AP)	some++
nucleus of the basal optic root (nBOR)	few++
nucleus precommissuralis principalis (PPC)	most++
nucleus pretectalis (PT)	none
spiriformis lateralis (SpL)	few+
spiriformis medialis (SpM)	few++
subpretectalis (SP)	few+
tectal gray (GT)	most+++
Mesencephalon	
Optic tectum	
layer 8	few+++

Brain region	Expression
layer 9	some+
layer 10	most+++
layer 11	few+++
layer 13	some++,+++
layer 14	most++,+++
layer 15	few+,++
Subtectal optic lobe	
lateral and medial reticular formation (FRL, FRM)	most++,+++
MLd periphery	few+++
nucleus externus (Next)	most+++
nucleus intercollicularis (ICo)	most+++
nucleus isthmi, pars magnocellularis (Imc)	none
nucleus isthmi, pars parvocellularis (Ipc)	all+++
nucleus mesencephali lateralis, pars dorsalis (MLd)	few+
nucleus semilunaris (SLu)	rostral most+++ / caudal most++
nucleus superficialis (Nsupf)	most+++
Tegmentum	
A8 region	some++,+++
central periaqueductal gray (GCT)	some++
dorsal raphe (RaD)	most+++
interpeduncular nucleus (IP)	some++
linear part of the mesencephalic raphe (RaL)	upper most+++ / lower some++
nucleus of Edinger-Westphal (EW)	none
nucleus ruber (Ru)	most+++
oculomotor nucleus (M3)	none
substantia nigra, pars com dorsal raphe (RaD) paxa and reticulata (SNc, SNr)	some++,+++
trochlear motor nucleus (M4)	few+
ventral tegmental area (VTA)	most+++
Isthmus	
dorsal nucleus of the lateral lemniscus (LLd)	none
intermediate nucleus of the lateral lemniscus (LLi)	rostral most+ / caudal most+++
isthmo-optic nucleus (ION)	few+
ventral nucleus of the lateral lemniscus (LLv)	most+++
Metencephalon	
cerebellum: granule cell layer	few++
dorsal and ventral subcoeruleus (SCd and SCv)	some++
facial motor nucleus (M7)	most+
lateral cerebellar nucleus (CbL)	most+++
lateral vestibular nucleus (VeL)	most+++
linear part of the pontine raphe (RaL)	most+++
locus coeruleus (LoC)	few++

Brain region	Expression
medial and lateral pontine nuclei (PM and PL)	most+++
medial cerebellar nucleus (CbM)	few++
nucleus of the descending tract of the trigeminus (TTd)	most+++
parabrachial region (Pb)	most+++
principal nucleus of the trigeminus (Pr5)	most+++
reticularis pontis (RP)	few++
reticularis pontis gigantocellularis (RPgc)	few++
reticularis pontis oralis (Rpo)	few++
superior vestibular nucleus (VeS)	most+++
tegmenti ventralis (TV)	some+++
trigeminal motor nucleus (M5)	most+
Myelencephalon	
angularis (An)	most+++
descending vestibular nucleus (VeD)	some++
dorsal part of the central medullary nucleus (Cnd)	most++
external cuneate nucleus (ECu)	most+++
gracile and cuneate nuclei (GC)	most+++
hypoglossal motor nucleus (M12)	few+
inferior olive (OI)	most+++
laminaris (La)	most+++
lateral reticular nucleus (RL)	most+++
linear part of the medullary raphe (RaL)	some+++
magnocellularis (Mc)	none
medial vestibular nucleus (VeM)	some+++
motor nucleus of the vagus (M9/10)	most+++
nucleus of the descending tract of the trigeminus (TTd)	most+++
nucleus of the solitary tract (NTS)	most+++
nucleus reticularis gigantocellularis (Rgc)	some++
nucleus reticularis parvocellularis (Rpc)	some++
superior olive (SO)	few++
ventral part of the central medullary nucleus (Cnv)	few+,++
Retina	
inner nuclear layer (INL)	most+++
ganglion cell layer: RGCs	few+++
Cranial ganglia	
trigeminal ganglion	most+++
geniculate ganglion	most+++
vestibular ganglion	few+,++
cochlear ganglion	few+,++
jugular ganglion	most+++
petrosal ganglion	some+++

Expression was qualitatively assessed. The approximate percentage of Cbln2+ neurons relative to the total number of neurons in a given region was classified as most for 365%, some for 30–65%, few for <30%, and none. The labeling intensity for the neurons in each region was identified as being high+++, moderate ++, or low+.