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Purkinje Cell Axon Collaterals Terminate on Cat-301+ Neurons in Macaca Monkey Cerebellum

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Abstract

The monoclonal antibody Cat-301 identifies perineuronal nets around specific neuronal types, including those in the cerebellum. This report finds in adult Macaca monkey that Basket cells in the deep molecular layer; granule cell layer (GCL) interneurons including Lugaro cells; large neurons in the foliar white matter (WM); and deep cerebellar nuclei (DCN) neurons contain subsets of Cat-301+ cells. Most Cat-301+ GCL interneurons are glycine+ and all are densely innervated by a meshwork of calbindin+/GAD+ Purkinje cell collaterals and their synapses. DCN and WM Cat-301+ neurons also receive a similar but less dense innervation. Due to the heavy labeling of adjacent Purkinje cell dendrites, the innervation of Cat-301+ Basket cells was less certain. These findings suggest that several complex feedback circuits from Purkinje cell to cerebellar interneurons exist in primate cerebellum whose function needs to be investigated.

Cat-301 labeling begins postnatally in WM and DCN, but remains sparse until at least 3 months of age. Because the appearance of perineuronal nets is associated with maturation of synaptic circuits, this suggests that the Purkinje cell feedback circuits develop for some time after birth.

Keywords

perineuronal net; chondroitin sulfate proteoglycan; Cat-301; cerebellum; glycine; primate

Perineuronal nets (PNN) are a specialized form of extracellular matrix that enwrap the cell soma and proximal processes of particular adult central nervous system neurons (Adams et al., 2001; Rhodes and Fawcett, 2004; Miyata et al., 2005; Carulli et al., 2007). PNNs are expressed after birth during critical periods of development for neuronal wiring (Kalb and Hockfield, 1990a,b; Pizzorusso et al., 2002; Yin et al., 2006). Probable functions include neuro-protection, organization of cortical compartments, stabilization of synaptic contacts and inhibition of axonal sprouting (Bruckner et al., 1993; Matthew et al., 2002; Carulli et al., 2006; Margolis and Margolis, 1997; Pizzorusso et al., 2002; Horn et al., 2003; Rhodes and Fawcett, 2004;; Yin et al., 2006). Conversely, the break down of PNNs has been shown in studies throughout the brain to be a necessary step to promote central and peripheral nervous system repair (Berardi

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et al., 2004; Kim et al, 2006; Barritt et al, 2006; Huang et al, 2006; Massey et al., 2006; Pizzorusso et al., 2006). Experimentally PNNs can be degraded with chondroitinase ABC, whose substrate is chondroitin sulfate proteoglycan (CSPG), which are comprised of a core protein and chondroitin-sulfate glycosaminoglycan chains (Lander et al., 1997; Celio et al 1998, for review).

Neurons expressing PNNs in the cerebellum have been described extensively in mouse and rat using antibodies, lectins, RNA probes and knockouts (Watanabe et al., 1994; Bruckner et al., 2000; Popp et al., 2003; Corvetto ad Rossi, 2005; Carulli et al., 2006; Carulli et al., 2007). PNNs are expressed postnatally on Golgi cells in the GCL and on excitatory deep cerebellar nuclei neurons (Carulli et al., 2006; Carulli et al., 2007). Double label experiments showed that Purkinje cells (PC) contact both these neurons and that the break down of PNNs induces 'vigorous outgrowth' of Purkinje collaterals (Corvetto and Rossi, 2005). A separate study in adult cat cerebellar cortex found that Lugaro cells, another class of interneurons in the granule cell layer (GCL), also express PNNs (Sahin and Hockfield, 1990).

This study reports for the first time that several types of neurons in adult Macaca monkey cerebellum have PNNs containing CAT-301. These are identified using a monoclonal antibody (mab) Cat-301 that recognizes aggrecan, a member of the CSPG family (Lander et al., 1998; Yin et al., 2006). In the current study, double label experiments illustrate that many glycine+ (Gly) interneurons in the GCL are Cat-301+ (Crook et al., 2006). Moreover, these have an unusual relationship with PC axons and their collaterals that labeled with an antibody to the calcium-binding protein calbindin-D28k (CalB) a well-known label for PC in many species (Sahin and Hockfield, 1990; Fortin et al., 1998; Laine and Axelrad, 2002; Corvetto ad Rossi, 2005). In our earlier paper (Crook et al., 2006) it was noted that a subset of Gly+ GCL interneurons were coated with a dense layer of large glutamic acid decarboxylase (GAD)+ puncta. This study finds that the Gly+/GAD+ interneurons are those which are Cat301+ and densely innervated by GAD+/CalB+ PC collaterals. This relationship shows an underappreciated role for local feedback of PC activity in the cerebellar cortex.

MATERIALS AND METHODS

Tissue preparation

Seven Macaca monkey brains aged fetal day (Fd) 55, Fd70, Fd80, Fd90, Fd145 (birth Fd170), postnatal (P) 5 days and P 3 months were compared to immunolabeling in three adult Macaca monkeys between 7.6 to 13 years of age. All procedures were approved by the University of Washington Animal Care Committee. Fetal monkeys were delivered by caesarian section under surgical anesthesia. All monkeys were deeply anesthetized with Nembutal (50 mg/kg; i.p.) and perfused through the ascending aorta via the left ventricle. All fetuses and one adult were perfused with 2% paraformaldehyde, one adult with 4% paraformaldehyde and one with 4% paraformaldehyde containing 0.2% glutaraldehyde. All fixatives were prepared in 0.1M phosphate buffer pH7.4 (PB). Brains were postfixed for 1–4 hours and then cryoprotected in 10%, 20% and finally 30% sucrose in PB. Selected cerebellum pieces at known orientation were frozen in OCT (Tissue-Tek, Sakura Finetek, Torrence, CA), sections cut at 12–25 μ m in either the parasagittal or coronal plane and mounted on glass slides or held in 30% sucrose.

Immunohistochemistry

Sections were blocked for 1 hour in 10% Chemiblocker (Chemicon, Temecula, CA) diluted in standard medium (0.01M phosphate-buffered saline (PBS) containing 0.05% sodium azide and 0.5% Triton) and then incubated overnight in two primary antisera from different species diluted in standard medium containing 5% Chemiblocker. Primary antisera were: mouse mabs to Cat-301 (1/10–1/40; gift of R. Mathews, U. Syracuse, NY), GAD65 (1:500; Developmental

Hybridoma Bank mab6) and CalB (1/5000; SWant, Bellazona Switzerland); rabbit polyclonal antibodies to GAD67 (1:500; gift of A. Tobin, UCLA, Los Angeles CA), CalB (1/5000; SWant, Bellazona Switzerland); and CalR (1/2000; SWant, Bellazona Switzerland); and rat polyclonal antibody to Gly (1:2,000; gift of D.Pow, U.Newcastle, Australia). Sections were then washed in PBS, followed by a 1 hour incubation with species-specific IgG conjugated to either Alexafluor 594 (mouse or rat), or Alexafluor 488 (mouse or rabbit; Molecular Probes, Eugene OR) diluted 1/500 in the standard medium. Sections were then washed in PBS and cover slipped with Aqua Polymount (Polysciences Inc, Warrington, PA).

Free floating 25µm sections were incubated in tissue culture wells using the same protocol as above but for 72hrs in the primary antisera, 36hrs in wash, and 2hrs in Alexafluors. For ABC Vectastain labeling, sections were incubated free floating in a single primary antiserum for 72hrs, washed overnight, and then incubated sequentially in species-specific biotinylated IgG (1/200) for 2hrs, washed for 2hrs and then incubated in ABC reagent (VECTAstain Elite kit) for 1hr. After a 1hr wash they were preincubated in 2% diaminobenzidine (DAB) in 0.1M phosphate buffer pH7.4 for 10 mins followed by a 5–15min incubation in 0.05% DAB containing 0.003% hydrogen peroxide. Sections were washed in distilled water, mounted on slides and coverslipped with Aqua Polymount.

Controls and Data analysis

All antisera labeled a subset of neurons with minimal background. There was no specific soma labeling in the absence of primary antiserum, or when secondary IgG was absent. Labeling reported below was consistent across concentrations which were varied several fold except that there was an increase in nonspecific background with the highest concentrations. There was no major difference in labeling between the three monkey brains. Regardless of antiserum type or in its absence, a nonspecific immunofluorescence which resembled puncta was present between Purkinje cell bodies.

Labeled sections were studied and drawn using a camera lucida, and photographed in a Nikon Optiphot microscope using a mercury bulb source (Zeiss Pascal LSM 5) and narrow band filters (Omega Optical). Selected sections were imaged in a Zeiss Pascal confocal laser scanning microscope with sequential acquisition of two immunomarkers in separate color channels. Z-stack photos were used to trace the processes of some cells, and cell body sizes were determined using Pascal software. Images were processed for contrast using Adobe Photoshop.

Cell density was determined by counting Cat-301+ GCL interneurons from seven parasagittal sections from three adult monkeys. The GCL was divided into equal internal and external halves and the total number of cells in each subdivision was averaged for each section. Each Cat-301+ cell was characterized as to cell body, shape and number of processes.

RESULTS

Cat-301 Labeling in Adult *Macaca* Monkey Cerebellum

Cat-301 stained neuronal elements in all layers and all folia. There was no noticeable difference in cell number or distribution between the vermis or hemispheres, or between labeling patterns in parasagittal vs. coronal sections. There were considerable differences in the distribution and staining intensities of Cat-301 labeling similar to other reports (Bruckner et al., 2000).

Cat-301 cell body labeling was most pronounced in the GCL. Figure 1A shows a typical distribution in a parasagittal section through adult monkey cerebellum with each black dot representing a Cat-301+ GCL interneuron. The average linear density was 6 cells/centimeter. Even though they were very sparse, the cells occasionally appeared in small clusters. In the GCL Cat-301 labeled triangular (Fig. 1B,C), fusiform (Fig. 1E), and round/oval (Fig. 1F) somas

that ranged in size from 15–40 μm in their longest dimension. More than half of the Cat-301+ somas were fusiform in shape, with round/oval next in frequency and triangular the least frequent (Fig. 2A). The vast majority (93%) of the Cat-301+ somas were located in the upper half of the GCL with the remainder in the lower half.

Labeled somas and proximal dendrites in the GCL were outlined by Cat-301 labeling so that the primary dendritic tree's shape often could be determined. Cat-301+ cells have 2–6 visible dendrites (Fig. 1B–F;2B). Typically one or more dendrites exit from the opposite poles of a fusiform shaped soma, the corners of a triangular shaped soma, or from a number of points on a round or oval shaped soma. Dendrites begin to label with Cat-301 at their origin, but the length labeled varied from ending near the cell body to extending hundreds of microns (see Double Labeling of Cat-301 and Glycine below). The majority of Cat-301+ primary dendrites have few branches and course horizontally within the GCL (Fig. 1F), but some run vertically (Fig. 1C,E). Dendrites run long distances, often bifurcate, and occasionally make a 90 degree turn toward the lower GCL. Dendrites running vertically can cross the entire GCL and reach but do not run into the WM. Cat-301+ dendrites infrequently entered the Purkinje cell layer (PCL) where they ran just above the PC bodies. Occasionally a short segment of what was believed to be the axon of a Cat-301+ cell was identified based on its size and trajectory. These putative axons were at least half the width of a dendrite and either branched directly from the soma or from a primary dendrite (Fig. 3A,B). Of thirty-seven Cat-301+ presumed axons, 35% were directed toward the molecular layer (ML) and 65% toward the lower GCL.

In addition to the GCL labeling described above, three other populations of Cat-301+ neurons were observed. In the lower third of the ML, small circular cell bodies 10–16 μm in diameter and their initial dendrites were outlined (Fig. 1B,C, arrowheads). Cat-301 showed that the multiple primary dendrites either arose from opposite sides of a round or oval soma, or branched in all directions. Based on their location, soma shape and dendritic distribution, these are likely to be Basket cells. The second group consisted of a large number of somas in the deep cerebellar nuclei (DCN) which exhibited a prominent Cat-301+ surface labeling pattern (Fig. 1D) similar to that present on GCL interneurons. Most of these CAT-301+ DCN neurons were larger than labeled neurons in the cerebellar cortex but occasionally smaller labeled DCN neurons were observed.

The least frequent population was a very large Cat-301+ somas (50–70 μm) which lay in between the axon bundles of the White Matter (WM) within a folium. On these WM neurons, Cat-301 labeling was limited to the soma and a very short segment of initial dendrites. These cells were distinct from the Cat-301+ DCN neurons.

Double Labels of Cat-301 and Glycine

Recently we described the expression of Gly in GCL and PCL interneurons of Macaca cerebellum (Crook et al., 2006). In the GCL Gly immunolabels most but not all Lugaro and Golgi cell somas and processes. Of the total number of these Gly+ interneurons, only 9% were also Cat-301+; however, of the total number of Cat-301+ interneurons, 69% were Gly+ (Fig. 3C,D).

Cat-301+/Gly+ double labeling more clearly shows that Cat-301 labels only cell somas and proximal processes (Fig. 3C,D) as described in other studies (Sahin and Hockfield, 1990). Gly labels much more of the neuronal processes which allowed us to deduce additional details of dendritic and axonal arborizations. In Figure 3A,B, the dendrites of a Gly+/Cat-301+ cell clearly enter the PCL, while a fine axon (arrowheads) emerges from a proximal dendrite and runs into the PCL. Rarely a Gly+ axon from a Cat-301+ neuron in the GCL was seen to run across the WM into the opposite GCL in the same folium. Even with the extra detail given by

Gly immunolabeling, we were unable to show conclusively where Gly+/Cat-301+ axons terminate.

Double Labels of Cat-301 and Glutamic Acid Decarboxylase

The combination of Cat-301 and GAD showed that the Cat-301+ surface coat was a net or meshwork rather than a solid covering (Fig. 4B,F). This mesh varied in intensity for individual cells within the same section, but with no consistent distribution. An earlier report found that a subpopulation of Gly+ GCL interneurons stood out due to their dense somatic investment of large GAD+ synaptic terminals (Crook, et al., 2006). Double labeling for GAD and Cat-301 finds that Cat-301+ cell bodies throughout the GCL always exhibit a very high density of GAD+ synaptic terminals on their soma (Fig. 4A–F, 6A). In fact, Cat-301+ cells could be identified by looking first at GAD staining for the characteristic dense mosaic investment on the soma. As shown above, these cells also were Gly+, indicating that we are dealing with a specific subpopulation of Cat-301+/Gly+ interneurons that receive a large GAD input.

A similar, but much less dense GAD+ investment also coincided with Cat-301+ DCN neurons and with Cat-301 labeling on presumed Basket Cells in the lower ML (Fig. 4C,D,G,H and 6B).

Calbindin-28k Labeling

Similar to other species (Sahin and Hockfield, 1990; Fortin et al., 1998; Laine and Axelrad, 2002), in Macaca cerebellar cortex CalB consistently labeled PC somas, dendrites, axons and collaterals with high specificity and intensity (Fig. 5A,B). CalB+ PC somas formed a monolayer making up the PCL, and in the ML CalB+ dendrites branched profusely filling the layer out to the pia. The PC axon exited from the basal portion of the soma and headed straight across the GCL into WM where it joined other CalB+ PC axons. Prominent CalB+ collaterals branched from various points on the axon and typically ran for long distances below the PCL, up into the ML (Fig. 5G and 6F) or throughout the GCL (Fig. 5B,C). The collaterals (Fig. 5C, arrow) were half the width of an axon (Fig. 5C, arrowhead) and often beaded. Collaterals traveled alone or in groups and formed intermittent small clumps or plexuses. Of direct importance to this study was the observation of a CalB+ plexus formed by many collateral branches which appeared to encase a GCL cell body in a dense mosaic of synaptic contacts (Fig. 5B,C; 6D,F,G). These collaterals wrapped around Cat-301+ somas and dendrites like multiple strings of beads (Fig. 6D,G). Double labeling with CalB and Gly confirmed that the CalB+ PC collateral plexus formed a dense terminal mosaic onto Gly+ somas and primary dendrites in the GCL (Fig. 5C,D). Double labeling with GAD finds that this CalB+ investment is formed by GAD+ synaptic terminals, and that they label for both isoforms GAD65 and GAD67 (Fig. 5E,F,G,H and 6C,F).

The large Cat-301+ cell bodies in the WM (Fig. 5J,K,L,M) and the DCN neurons also received a CalB+/GAD+ synaptic investment (Fig. 6E). It could not be determined whether the Cat-301+ Basket Cells were innervated by PC CalB+ collaterals due to the intense PC dendritic labeling in the ML.

Double Labels of Cat-301 with Calretinin

Calretinin (CalR) is a calcium binding protein that labels numerous neuronal elements in Macaca monkey cerebellum including subsets of GCL interneurons and Basket Cells (Dino et al., 1999; Crook et al., 2006). Double labeling with CalR and Cat-301 finds that Cat-301 immunoreactivity outlines both CalR+ and CalR- Basket Cells (Fig. 7A–D). In the GCL, heavily CalR+ Uni-Polar Brush and lightly CalR+ Granule Cells (Dino et al., 1999) were never observed to double label with Cat-301 (Fig. 7C,D). CalR+ classic fusiform Lugaro and Golgi cells were rarely Cat-301+ (Fig. 7E–F).

Development of Cat-301 Extracellular Labeling

The first convincing appearance of Cat-301 staining was on postnatal (P) day 5 on large neurons in the foliar WM (Fig. 6H). Very few neurons were labeled at this age and none were seen in the GCL. Cat-301+ WM neurons co-localized with CalB+ PC axon collaterals at P5 days (Fig. 6H), but the CalB+ collateral baskets so characteristic of adult GCL were not present. At P3 months of age in DCN numerous cells were labeled with a Cat-301 intensity similar to that of the adult and received a CalB+ synaptic investment. In the GCL, Cat-301 staining still was sparse compared to adult and CalB collateral labeling was present but faint. Cat-301+ cells in the lower ML were not observed in any fetal or infant cerebellum.

DISCUSSION

This study reports for the first time the distribution of neurons in primate cerebellum that have PNNs as identified with Cat-301 that recognizes a CSPG (a major component of the PNN). Cat-301 was expressed on four types of neurons and three of these were innervated by GAD +/CalB+ PC processes. Our findings are summarized diagrammatically in Figure 8. Moreover, we find that the Cat-301+ PNNs did not appear until after birth, and still were not at adult density at P3 months. These results replicate studies in mouse, rat and cat (Sahin and Hockfield, 1990; Corvetti and Rossi, 2005; Carulli et al., 2006; Carulli et al., 2007) but also describe additional unique features in primate cerebellum.

In mouse and rat cerebellum, there is a correlation between the postnatal appearance of PNNs and the formation of PC synapses. PC do not begin to form synapses on DCN neurons until after birth (Gardette et al., 1985; Garin and Escher, 2001). At P3 day neurons in the DCN first show PNNs, and at P14 days Golgi cells in the GCL have PNNs with both numbers of Cat-301 + neurons and density of PNNs progressively increasing into adulthood (Carulli et al., 2007).

Cat-301 labeling was only observed in postnatal primate infant brains, not in fetal brains. At P5 days Cat-301+ labeling was confined to the large neurons in the foliar WM; these also were surrounded by CalB+ fibers at this age. This folia WM cell type was not observed in mouse or rat. By P3 months DCN neurons were also Cat-301+ and encased with CalB+ processes. However, at P3 months, Cat-301+ GCL interneurons were rare, lightly labeled, and only weakly invested with CalB+ PC collaterals. Older infant and juvenile brains were not available so the exact time when Cat-301+ PNNs were formed in monkey remains to be identified. Because primate cerebellum develops over a long time period (Rakic, 1971; Zecevic and Rakic, 1976), these PNNs may not be fully mature until monkeys reach puberty at 3 years or even young adulthood at 5 years.

In adult primate cerebellum, as in mouse and rat, at least three of the four cell types with PNNs were innervated by PC processes (Fig. 8). Double label experiments in mouse and rat cerebellum showed CalB or parvalbumin+ PC terminals colocalized with cells that have PNNs (Corvetti and Rossi, 2005; Carulli et al., 2007). In this study Cat-301+ neurons in the GCL, WM, and DCN were all specifically and uniquely encased by dense CalB+/GAD+ PC synapses and collaterals. However, intrinsic cell type markers did not seem to be determinants of Cat-301 labeling in that only a few Gly+ presumed Lugaro cells were labeled and only a subset of CalR + Basket cells. The degree of PC axon investment ranged from relatively light on Basket cells to a dense coil of beaded GAD+/CalB+ collaterals encircling Gly+ GCL interneuron somas and proximal processes multiple times. These interneurons had GAD+ puncta limited to the soma and proximal dendrites which are invested by Cat-301 but the puncta did not extend to distal dendrites free of Cat-301.

The Cat-301+ Basket cells in the lower ML have only been described in primates. Because CalB+ PC dendrites pack the ML, it was difficult to determine the extent of PC innervation.

Other studies show PC collaterals contact Basket cells (O'Donoghue et al., 1989) and this study found both PC collaterals entering the ML and Cat-301+ Basket cells surrounded by GAD+ inputs within the Cat-301+ meshwork. However compared to the other Cat-301+/GAD+ neurons, Basket cells were not as densely innervated with GAD or Cat-301.

The function of PNNs has been thought to restrict the structural plasticity of PC processes in adult cerebellum (Corvetto and Rossi, 2005; Carulli et al., 2007) because degradation of the PNN experimentally with Chondroitinase ABC induces vigorous PC sprouting (Corvetto and Rossi, 2005). The functional consequences of 'locking' PC synapses to a neuron at an early postnatal age would be reflected in the neuron's post-synaptic contacts. Elsewhere in the brain, neurons with PNNs have been shown to contribute to the demarcation of specific cortical maps (Hockfield et al., 1990; Bruckner et al., 1999; Adams et al., 2001; Horn et al., 2003). For example, in human primary and secondary visual cortex Cat-301 labeling correlates with the organization of ocular dominance columns and the magnocellular pathway (Hockfield et al., 1990). Thus, DCN could utilize Cat-301+ PNNs to stabilize PC axon input. This in turn would organize the different functions of tracts projecting from these nuclei to the vestibular, oculomotor and voluntary motor systems. The putative role of the PNN in cerebellar cortex is particularly interesting. The cerebellar cortex is often considered to be very flexible (Jorntell and Hansel, 2006) but the PC collaterals associated with PNN+ interneurons should be relatively cemented in place. In the cerebellar cortex, connections between PCs and cells with PNNs may be a mechanism to organize functional compartments in different cortical domains and to create feedback loops within those domains. In particular the Gly+/Cat-301+ Lugaro cell which is so heavily invested by CalB+/GAD+ PC collaterals may play a critical feedback role in one or more of these domains.

The rare large Cat-301+ interneurons in the foliar WM have not previously been described. Neither the dendrites nor its axon could be traced so the extent of this cell remains unknown, although, given its soma size, it may have long and/or extensive processes. All Cat-301+ folia WM interneurons co-localized with GAD+ PC collaterals and were suitably located to receive PC collaterals from either side of the folium. Interestingly, despite its rarity, this cell was the first Cat-301+ neuron observed in postnatal cerebellum.

Cat-301 labeling supports the idea that the primate cerebellum has a multitude of unrecognized cell types or subtypes (Geurts et al., 2001,2003; Laine and Axelrad, 2002; Crook et al., 2006). In the monkey GCL, Cat-301 labeled triangular, round and fusiform cells that had different numbers of processes and orientations, and could be Gly+, Gly- or rarely CalR+, but all had the unifying characteristic that they received a massive input from GAD+ PC collaterals. In mouse and rat, GCL interneurons with PNNs were identified as Golgi cells (Carulli et al., 2007) and in the adult cat GCL as Lugaro cells (Sahin and Hockfield, 1990). Other studies have referred to GCL interneurons innervated by PC collaterals as Lugaro cells (Palay and Chan-Palay, 1974) or 'globular' Lugaro cells (Laine and Axelrad, 2002). Classically, Lugaro cells are fusiform in shape, located below the PC layer and their axons branch and terminate in the ML (Laine and Axelrad, 1998). Although the complete axon could not be traced in this study, the initial portion of Cat-301+/Gly+ axons projected either directly into the ML, or toward the lower GCL or WM where it possibly crossed to the other side of the folia (Crook et al., 2006). Thus in primates, Cat-301+ GCL interneurons could be a subgroup of Lugaro cells or a new type of GCL interneuron. Stimulation of these Gly+/GAD+ cells (Crook et al., 2006) by their massive PC investment would drive a previously unrecognized feedback pathway whose function deserves more investigation.

Unlike in any other species, Cat-301 labeled a subset of Basket cells in the lower ML. Although CalR labels many Basket Cells, double labeled Cat-301+/CalR+ Basket Cells were uncommon. Previously subtypes of Basket cells have been observed based on differences in morphology

and staining (Bishop et al., 1993; Dino et al., 1999). Cat-301+ Basket Cells could represent some or all of these classes.

This study has shown that at least three types of cerebellar interneurons and the DCN neurons have Cat-301+ PNNs. Because these PNNs do not mature until well after P3 months, these circuits probably continue to change as the cerebellum matures. Of particular interest are a type of Lugaro interneuron and the large foliar WM neurons which receive a dense PC innervation and may form feedback circuits. Currently these neurons are not included in any models of cerebellar function. The mab Cat-301 antibody could be a helpful tool for physiological studies of these intriguing neurons to determine their function and significance.

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Abbreviations

CalB	Calbindin
CalR	Calretinin
DCN	deep cerebellar nuclei
E	embryonic day
GAD	glutamic acid decarboxylase
GCL	granule cell layer
Gly	Glycine
ML	molecular layer
PC	Purkinje cell
PCL	Purkinje cell layer
P	postnatal
PNN	perineuronal net
WM	White Matter

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Figure 1.

Abbreviations in all figures: Molecular layer (ML); Purkinje Cell Layer (PCL); Granule Cell Layer (GCL); White Matter (WM); deep cerebellar nuclei (DCN); glycine (Gly); calbindin (CalB); glutamic acid decarboxylase (GAD); calretinin (CalR). **A.** A parasagittal slice through Macaca monkey cerebellum stained with cresyl violet. The black dots indicate the location of a Cat-301+ GCL interneurons. These are present in low density and have no obvious regular labeling pattern. **B.** In the GCL two triangular shaped somas (arrows) and their proximal dendrites are Cat-301+. In the ML, two small Basket Cells (arrowheads) are also labeled. **C.** A Cat-301+ cell body (arrow) lying under the PCL sends a dendrite deep into the GCL. Just above the PCL is a large Cat-301+ Basket Cell (arrowhead). **D.** The DCN contain many Cat-301+ neurons (arrow). **E, F.** Cat-301+ labeling shows the dendritic pattern of a vertical (E) and a horizontal (F) fusiform GCL interneuron.

Figure 2.

A graph depiction of Cat-301+ GCL interneuron soma morphology (A) and process numbers (B). Percentages are taken from counts of all Cat-301+ GCL interneurons from seven parasagittal hemisphere sections from three Macaca adult monkeys.

Figure 3.

A, B. A Gly+ (A) Cat-301+ (B) GCL interneuron with a fusiform soma, vertical orientation and axon heading toward the PCL (arrowheads). Cat-301 labeling is limited to the soma and proximal processes of the cell while Gly labeling can be traced into much finer processes. In B, note the meshlike appearance of the Cat-301 surface coat on the cell body and primary dendrites. **C, D.** A low power view of Gly+ GCL interneurons (C) showing that Gly+ interneurons are numerous throughout the GCL. Only one large Gly+ cell (C, arrow) is double labeled for Cat-301 (D, arrow). This Cat-301+/Gly+ interneuron is fusiform in shape with a large soma and dendrites extending into the lower PCL and GCL.

Figure 4.

Double labeling for GAD (A, C, E, G) and Cat-301 (B,D,F,H). **A, B.** Two triangular shaped GCL interneurons with processes extending below the PCL and into the GCL are heavily innervated with GAD+ puncta. The soma of the interneuron on the left (arrow) was sliced along its surface and the one on the right (arrowhead) through its center. Both show the dense GAD + somatic puncta (A) and the surface meshwork of Cat-301 (B). **C, D.** The dense labeling for GAD+ (C) and Cat-301+ (D) on a fusiform GCL interneuron should be compared to the light labeling on small Basket cells (B) in the lower ML. **E, F.** A GAD+ (E) and Cat-301+ (F) GCL interneuron has a round soma and three processes. The upper dendrite heads into the PCL past a PC. **G, H.** Two GAD+ (G) and Cat-301+ (H) Basket cells in the lower ML.

Figure 5.

Double labeling for CalB (A,B,C,E,G,J,L) compared to Gly (D), GAD67 (F) and GAD65 (H) and Cat-301 (K,M). **A.** A low power view of a primate folium showing that all PC are CalB+. Their dendrites fill the ML, cell bodies form the PCL and axons cross the GCL to form bundles in the WM. **B.** A higher power view of CalB+ PC soma, dendrites, axons and axon collaterals. Many CalB+ PC collaterals join to form a bundle which ensheaths an unstained GCL interneuron. **C, D.** CalB+ collaterals encase a Gly+ GCL interneuron and its dendrites (arrow). A neighboring PC axon (C, arrowhead) shows the small size of the collaterals. **E, F, G, H.** CalB+ PC terminals on unstained GCL interneurons (E,G) double label for GAD67 (F) and GAD65 (H). A PC collateral branch enters the PC layer (G,H, arrowhead). **J, K, L, M.** A large neuron in the foliar WM (J,K, arrow) is Cat-301+ and is innervated by dense CalB+ processes. This sheath can be seen more clearly in the higher magnification view (L,M). Surrounding CalB+ axons are indicated by an arrowhead (L).

Figure 6.

A, B. Co-localization of Cat-301 (red) and GAD-67 (green) on GCL interneuron (A) and Basket cells (B). **C.** Co-localization of CalB+ (red) with GAD 67 (green) is shown by the yellow collateral processes and puncta surrounding this GCL interneuron which lies just below the CalB+ PC (P). **D.** A Cat-301+ (red) GCL interneuron is cloaked with fine CalB+ collaterals (green). **E.** A large WM neuron (arrow) is double labeled for GAD65 (red) and CalB+ collaterals (green). **F.** CalB+ (green) PC collateral terminals onto a GCL interneuron just below the PC (P) colocalize with GAD65 (red). Some collaterals enter the PCL (arrowheads). **G.** A Cat-301+ (red) GCL interneuron soma is encircled by a dense meshwork of CalB+ PC

collaterals (green) which form large beaded puncta on the soma and dendrites. **H.** A large WM neuron from P5 day brain is Cat-301+ (red). It is contacted by CalB+ (green) PC processes.

Figure 7.

A, B. CalR (A) and Cat-301 (B) double label the soma and processes (arrows) of a Basket cell in the lower ML. **C, D.** Basket cells which are Cat-301+ (D, black arrows) do not contain CalR (C, black arrows), whereas a CalR+ Basket cell (C, arrowhead) is not labeled by Cat-301 (D, arrowhead). In the GCL, Uni-Polar Brush cells are intensely single labeled for CalR+ (C, asterisks). Cat-301+ GCL interneurons (D, white arrow) very rarely colocalize Cat-301. **E, F.** Characteristic CalR+ small fusiform Lugaro cells (arrowheads) are negative for Cat-301.

Figure 8.

The localization of four types of Cat-301+ neurons in Macaca monkey cerebellum. Most of the DCN neurons are labeled. Interneurons in the GCL are mainly found in the upper half. Some of these are Gly+ and a very few are CalR+. A few large Cat-301+ neurons lie in the WM of the folia. All of these types receive a prominent input from GAD+/CalB+ PC axon collaterals. Many Basket cells in the lower third of the ML are also Cat-301+, but their investment by PC collaterals is less clear.