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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 positive (+) cells. Most Cat-301+ GCL interneurons are glycine+ and all are densely innervated by a meshwork of calbindin+/glutamic acid decarboxylase+ 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.

Key words: 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 CNS 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; Matthews 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 breakdown of PNNs has been shown in studies throughout the brain to be a necessary step to promote CNS and peripheral nervous system repair (Berardi 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 is composed 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; Corvetti ad Rossi, 2005; Carulli et al., 2006, 2007). PNNs are expressed postnatally on Golgi cells in the GCL and on excitatory deep cerebellar nuclei (DCN) neurons (Carulli et al., 2006, 2007). Double label experiments show that Purkinje cells (PC) contact both these neurons and that the break down of PNNs induces 'vigorous outgrowth' of Purkinje collaterals (Corvetti 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; Corvetti ad Rossi, 2005). In our earlier paper (Crook et al., 2006) it was noted that a subset of Gly+ GCL interneurons was coated with a dense layer of large glutamic acid decarboxylase (GAD)+ puncta. This study finds that the Gly+/GAD+ interneurons are those which are Cat-301+ and densely innervated by GAD+/CalB+ PC collaterals. This relationship shows an underappreciated role for local feedback of PC activity in the cerebellar cortex.

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Abbreviations: CalB, calbindin; CalR, calretinin; CSPG, chondroitin sulfate proteoglycan; DAB, diaminobenzidine; DCN, deep cerebellar nuclei; Fd, fetal day; GAD, glutamic acid decarboxylase; GCL, granule cell layer; Gly, glycine; ML, molecular layer; P, postnatal; PB, phosphate buffer; PBS, phosphate-buffered saline; PC, Purkinje cell; PCL, Purkinje cell layer; PNN, perineuronal net; WM, white matter.

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EXPERIMENTAL PROCEDURES

Tissue preparation

Seven *Macaca* monkey brains aged fetal day (Fd) 55, Fd70, Fd80, Fd90, Fd145 (birth Fd170), postnatal (P) 5 days and P3 months were compared with immunolabeling in three adult *Macaca* monkeys between 7.6–13 years of age. All procedures were approved by the University of Washington Animal Care Committee and conformed to the recommendations from the Institute of Laboratory Animal Resources and the American Association for Accreditation of Laboratory Animal Care. We kept the number of animals to the minimum necessary and minimized their suffering with very deep anesthesia immediately prior to perfusion. Fetal monkeys were delivered by cesarian 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 0.1 M phosphate buffer pH 7.4 (PB). Brains were postfixed for 1–4 h 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, USA), sections cut at 12–25 μm in either the parasagittal or coronal plane and mounted on glass slides or held in 30% sucrose.

Immunohistochemistry

Sections were blocked for 1 h in 10% Chemiblocker (Chemicon, Temecula, CA) diluted in standard medium (0.01 M 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, SUNY Upstate Medical University, Syracuse, NY, USA), 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, USA), CalB (1/5000; SWant); and calretinin (CalR) (1/2000; SWant); and rat polyclonal antibody to Gly (1:2000; gift of D. Pow, University of Newcastle, NSW, Australia). Sections were then washed in PBS, followed by a 1 h incubation with species-specific IgG conjugated to either Alexafluor 594 (mouse or rat), or Alexafluor 488 (mouse or rabbit; Molecular Probes, Eugene, OR, USA) diluted 1/500 in the standard medium. Sections were then washed in PBS and cover slipped with Aqua Polymount (Polysciences Inc., Warrington, PA, USA).

Free floating 25 μm sections were incubated in tissue culture wells using the same protocol as above but for 72 h in the primary antisera, 36 h in wash, and 2 h in Alexafluors. For ABC Vectastain labeling, sections were incubated free floating in a single primary antiserum for 72 h, washed overnight, and then incubated sequentially in species-specific biotinylated IgG (1/200) for 2 h, washed for 2 h and then incubated in ABC reagent (VECTAstain Elite kit) for 1 h. After a 1 h wash they were preincubated in 2% diaminobenzidine (DAB) in 0.1 M PB pH 7.4 for 10 min followed by a 5–15 min 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 adult monkey brains. Regardless of antiserum type or in its absence, a nonspecific immunofluorescence which resembled puncta was present between PC 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. Fig. 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/cm. 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 to 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 to 6 visible dendrites (Figs. 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 Labels of Cat-301 and Gly 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° 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