**DEVELOPMENT OF PINCEAUX FORMATIONS AND DENDRITIC
TRANSLOCATION OF CLIMBING FIBERS DURING THE ACQUISITION OF
THE BALANCE BETWEEN GLUTAMATERGIC AND GABAERGIC INPUTS IN
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Review

DEVELOPMENT OF “PINCEAUX” FORMATIONS AND DENDRITIC TRANSLOCATION OF CLIMBING FIBERS DURING THE ACQUISITION OF THE BALANCE BETWEEN GLUTAMATERGIC AND GABAERGIC INPUTS IN DEVELOPING PURKINJE CELLS.

Constantino Sotelo

Cátedra de Neurobiología del Desarrollo “Remedios Caro Almela”, Instituto de Neurociencias de Alicante, Universidad Miguel Hernández y CSIC, Sant Joan d’Alacant, 03550 Alicante, Spain, and Centre National de la Recherche Scientifique, Unité Mixte de Recherche 7102, Université Paris 6, 75005 Paris, France.

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Corresponding author,

Constantino Sotelo

Cátedra de Neurobiología del Desarrollo “Remedios Caro Almela”, Instituto de Neurociencias de Alicante, Universidad Miguel Hernández y CSIC, Sant Joan d’Alacant, 03550 Alicante, Spain

Phone: 34-965 919556

Fax : 34-965 919555

E-mail: sotelo@umh.es

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ABSTRACT

The question of the acquisition of the dynamic balance between excitation and inhibition, necessary for the proper functioning of developing Purkinje cells, has been specifically addressed. The newborn (P0) mouse cerebellum contains glutamatergic (VGLUT2-IR) and GABAergic (VIAAT-IR) axons. The former prevail and belong to climbing fibers. The latter neither belong to Purkinje cell axon collaterals nor to axons of cortical GABAergic interneurons: During the first postnatal week, the number of VIAAT-IR axons close to Purkinje cells is very low and the first synapses with basket fibers are formed at P7, when climbing fibers have already formed dense pericellular nets. The descending basket fibers reach the Purkinje cell axon initial segment by P9, and immediately establish axo-axonic synapses. The pinceaux exhibit the appearance of a primitive vortex-like arrangement by P12, and by P20 inter-basket fiber septate-like junctions characteristic of full mature pinceaux are still missing. The climbing fibers somato-dendritic translocation occurs later than expected, after the regression of the multiple innervation, and follows the ascending collaterals of the basket axons, apparent optimal substrate for the proper subcellular targeting of the climbing fibers. These results emphasize that chemical transmission in the AIS precedes the electrical inhibition generated by field effects. In addition GABAergic Purkinje cells, as opposed to glutamatergic projection neurons in other cortical structures, do not start to receive their excitation to inhibition balance until the end of the first postnatal week, despite the early presence of potentially functional GABAergic axons provided with the required vesicular transport system.

INTRODUCTION

The proper functioning of developing neural assemblies requires for their own homeostasis, the acquisition of a dynamic balance between excitation and inhibition. Thus, from the beginning of the synaptogenesis, the development of inhibitory synapses should be precisely regulated. It has been established for hippocampal circuits, that neuronal activity is the regulator that controls the number of inhibitory synapses and, therefore, the maintenance of the dynamic balance (Marty et al. 2000). In addition, for many years it has been considered that excitatory synapses, with its essential role in the stabilization of developing immature synapses (Changeux and Danchin, 1976), should be the earliest ones to appear in development. However, during the last 15 years a large amount of evidence has been gathered indicating that in many central structures (cerebral cortex, hippocampus, striatum, spinal cord) the earliest neurons to establish functional synapses are the GABAergic interneurons (Ben-Ari et al. 2004). These two apparently discrepant results lost part of their contradictory significance when it was established that GABA, the major inhibitory transmitter in the adult brain, exerts through GABA(A) receptors an excitatory drive in immature neonatal neurons of some regions of the developing CNS, producing depolarization instead of the adult hyperpolarization (Cherubini et al., 1990; Luhmann and Prince, 1991; Rohrbough and Spitzer, 1996). This paradoxical effect of GABA is the result of an elevated intracellular concentration of chloride $[Cl^-]_i$, due to the functional expression of Cl^- transport-mediated mechanisms and Cl^- channels (Alvarez-Leefmans, 1990; Clayton et al., 1998a, b). In principle, the Cl^- extruding K^+ , Cl^- co-transporter (KCC2, involved in the generation of the hyperpolarizing effects of GABA; Rivera et al., 1999) would be expressed relatively late. In contrast, the Cl^- accumulating Na-K-2Cl co-transporter (NKCC, which seems to be involved in the generation of the depolarizing effects of GABA) would be expressed much earlier (Sun and Murali, 1999; Marty et al., 2002). Therefore, the switch between the depolarizing and the hyperpolarizing effects of GABA can be explained by the maturation of the chloride homeostasis systems, which is dependent on the GABAergic activity, the only one able to modulate the mRNA levels of KCC2, the Cl^- excluding co-transporter (Ganguly et al., 2001).

Most of the information assembled on the developmental sequence of inhibitory and excitatory synapses has been obtained from the hippocampus and the cerebral cortex (Ben-Ari, 2002). The cerebellar cortex provides an ideal system in which to examine such a developmental sequence because of the small number of cell types and the fact that extracerebellar afferents make specific stereotyped connections mostly postnatally (Sotelo, 2004). However, the sequence remains unknown due to the lack of a concomitant temporal study of the onset of excitatory and

inhibitory synapses. In the adult cerebellum, Purkinje cells are the pivotal elements of the cortical circuitry and its unique output, to which excitatory and inhibitory inputs converge. These neurons, in the adult cerebellum, receive excitatory synaptic inputs from climbing and parallel fibers, and inhibitory inputs from stellate and basket cell axons. Moreover, each class of excitatory and inhibitory synapse is targeted to determined cellular compartments. Climbing fibers synapse on the thorns emerging from the proximal dendritic compartment, whereas parallel fibers establish synaptic contacts with dendritic spines arising from the distal spiny branchlets. In contrast, the descending branches of the basket cell axons enwrap completely the perikarya and terminate forming a peculiar structure, the pinceau formation, around the initial segment of the axon (AIS; Palay et al., 1968), and stellate cell axons establish synaptic contacts with the smooth surfaces of the proximal compartment of Purkinje cell dendritic trees (Palay and Chan-Palay, 1974).

During development, basket cell axons are considered to be the first known GABAergic fibers to contact Purkinje cells (Yan and Ribak, 1998; Rosina et al., 1999; Ango et al., 2004; Takayama and Inoue, 2004; 2005). Descending branches of these axons establish their first synaptic contacts on Purkinje cell bodies, and continue their descending course toward the Purkinje cell AIS, to form the pinceaux. The mechanisms controlling the targeting of basket cell axons have been disclosed. This targeting is regulated by genetic programs and seems independent of neuronal activity (Ango et al., 2004). Although nothing is known about the targeting of climbing fibers, the situation is quite different. In his pioneer study of the development of the climbing fibers, Ramón y Cajal (1911) described three stages, the “pericellular nest”, the “capuchon stage” and the “young climbing fiber stage”, which were precisely correlated with the successive stages in Purkinje cell dendritogenesis: the “phase of stellate cell with disoriented dendrons” matched with the “nest stage”, the “phase of orientation and flattening of dendritic trees” correlated with the “capuchon stage” and the “phase of adult-like form” with that of “young climbing fiber stage”. Our own past work has somewhat modified these sequences (Armengol and Sotelo, 1991; Chedotal and Sotelo, 1993) by adding a new early phase of dendritogenesis (“complex-fusiform” Purkinje cells), paralleled by a new initial stage in the climbing fiber-Purkinje cell synaptogenesis (“creeper stage”), characterized by the formation of synaptic contacts on the transient Purkinje cell dendrites. Nevertheless, what remains is that after the perinatal establishment of these few transient dendritic synapses, the targeting of climbing fibers is made first on cell bodies and later on dendrites. Therefore, the somata of the Purkinje cells seem to be the main target of the synaptogenesis during the postnatal period of the development of the cortical cerebellar circuit by the formation of baskets and

pericellular nests. In addition, it is important to mention some functional data on the climbing fiber-Purkinje cell synapse formation. The peak of the “nest stage”, around P5, corresponds to the peak of multiple innervation of Purkinje cells by climbing fibers (Crepel et al., 1976). It is during the somatodendritic translocation of the climbing fibers (P12 to P15, Chedotal and Sotelo, 1992) that an abrupt decline of the multiple innervation occurs and that the adult numerical relationship of one climbing fiber per one Purkinje cell is finally established (Mariani and Changeux, 1981). This important morpho-functional change has a coincidental timing with the development of the pericellular baskets, suggesting some kind of steric competition between the two classes of synaptic inputs, and pointing to the basket axons as responsible for the dendritic translocation of the climbing fibers.

The present study is aimed at establishing the temporal sequence of the formation of inhibitory and excitatory synapses on the cell bodies and AIS of postnatal Purkinje cells. It will also be determined whether and when climbing fibers and basket axons can be concurrent on the same Purkinje cell body, and how progressively the latter will substitute the former. To these aims two complementary approaches are used: i) light microscopy using an immunohistochemical analysis with markers of the glutamatergic climbing fibers (the vesicular glutamate transporter, VGLUT-2) and of the GABAergic basket axons (the plasmalemmal GABA transporter, GAT-1 or the vesicular transporter, VIAAT). ii) An electron microscopic study, which allows the identification of both classes of axons by their specific cytological features (Palay and Chan-Palay, 1974). In addition, since the sequential formation of pinceaux has been till now only analyzed at the light microscopic level (Yan and Ribak, 1998; Rosina et al., 1999; Ango et al., 2004; Lujan et al., 2005; Takayama and Inoue, 2005), the present study will start by reporting the electron microscopic examination of the development of these formations.

MATERIALS AND METHODS

Animals

Pregnant ICR mice, bred in our animal facilities, generated the 24 mouse pups used for the immunocytochemical analysis. Pups were aged postnatal day 0 (P0), P5, P7, P10, P15, P17 and P21 (at least three for each age point). In addition, heterozygous GAD65-GFP transgenic mouse pups on C27BL6 background, originated from the colony obtained at the Department of Gene Technology and Developmental Neurobiology, Institute of Experimental Medicine, Budapest, Hungary by Drs. Ferenc Erdélyi and Gábor Szabo (Erdélyi et al. 2002), were raised by Dr. Guillermina López-Bendito in our Institute (López-Bendito et al. 2004). Transgenic pups aged postnatal day 0 (P0), P5, P7, P10 and P21 (two for each time point) were used. The electron microscopic study was done on 23 rat pups generated by pregnant Wistar rats obtained from IFFA-Credo (Lyon, France). The pups were aged postnatal day 5 (P5), P7, P9, P10, P12, P15 and P20 (at least three for each age point). All animals experiments were carried out under Spanish and EU regulation (Council Directive 86/609/EEC). The Ethics Review Committee of the University Miguel Hernández approved all animal protocols that were used in the present study.

Immunostaining

Antibodies

Polyclonal antibodies against either vesicular glutamate transporter-2 (VGLUT2) or VGLUT1 (raised in guinea pig, 1:3000; Chemicon, Temecula, CA), the GABA transporter GAT-1 (raised in rabbit, 1:3000; AB1570, Chemicon), the anti-vesicular inhibitory amino acid transporter [VIAAT, gift from B. Gasnier, Institut de Biologie Physico-Chimique, Paris, France; raised in rabbit, 1:500 (Marty et al., 2002)], the rabbit anti-Pax2 (1/300; Zymed, San Francisco, CA), and the mouse monoclonal antibody against calbindin D-28K (CaBP, 1/200; Sigma), were used at the indicated dilutions. The rabbit polyclonal antibodies against GAT-1 and VIAAT were visualized with sheep anti-rabbit antibodies coupled to FITC (diluted 1/200, Eurobio, France), and the guinea pig polyclonal antibody against VGLUT1 or VGLUT2 with donkey anti-guinea pig coupled to Cy3 (diluted 1/200, Jackson ImmunoResearch, West Grove, PA). The rabbit polyclonal antibodies against Pax2 were visualized with goat anti-rabbit Cy3 (diluted 1/200, Jackson ImmunoResearch) and the mab CaBP was visualized using FITC conjugated to goat anti-mouse IgG (1:200; Jackson ImmunoResearch).

Immunohistochemistry

Mouse pups were anesthetized by an i.p. injection of ketamine (100 mg/Kg) and xilacine (10 mg/Kg), and fixed by transcardiac perfusion with 4% paraformaldehyde, in 0.12M phosphate buffer (pH 7.3). Brains were dissected out, postfixed in the same fixative for 4 to 12 hours at 4°C, and cryoprotected in 30% sucrose-phosphate buffer saline (PBS; pH 7.3). The lateral hemispheres of the cerebellum were cut down, and vermal regions with their attached brainstems were cut frozen in the parasagittal plane on a sliding microtome at 40 µm. Free-floating sections were rinsed in PBS containing 0.2% gelatin, 0.25% Triton X-100 and 0.1% sodium azide (PBSGTA). Then, they were incubated for 1 hour in PBSGTA containing 0.1 M lysine (blocking solution). Sections were processed for immunocytochemistry as described previously (Morel et al., 2002). No staining was observed when primary antibodies were omitted.

Acquisition of confocal micrographs in double-labeling experiments was performed with a Leica TCS-NT microscope equipped with a Krypton-Argon laser (excitation bands 488 nm and 520 nm). Two channel fluorescence acquisitions were done on serial 1 µm thick sections using x63 or x100 objectives. Images (1024 x 1024 pixels) in each series were analyzed. The overlaid projections were obtained from three to occasionally a maximum of eight single 1 µm thick sections. Leica Control software (Leica), and Adobe Photoshop 8.0.1 (Adobe Systems, San Jose, CA) software were used for image processing. To eliminate as much regional variation as possible, particularly in the chronology of neuronal maturation and protein expression, the morphological analysis was carried out in lobules IV to VII of the vermal and paravermal cortices.

Electron microscopy

The pups were anesthetized with chloral hydrate (400 mg/Kg i.p.; P9-920) and fixed by intracardiac perfusion of a freshly prepared solution of 1 percent paraformaldehyde and 1 percent glutaraldehyde in 0.12 M phosphate buffer (pH 7.3) for 20 minutes at room temperature. After perfusion, the cerebella were carefully dissected out and left in the same fixative overnight at 4°C. Vermal slices about 1 mm-thick were cut in the parasagittal plane. The slices, after 2 hours postfixation in 2 percent osmic acid in 0.1 M phosphate buffer (pH 7.3), were stained “en bloc” with uranyl acetate, dehydrated in graded ethanol solutions and flat-embedded in Araldite. Ultrathin sections (60-70 nm) were cut on a Reichert Ultramicrotome, stained with uranyl acetate and lead citrate, and examined under a Philips CM120 electron microscope at 80 kV.

RESULTS

ELECTRON MICROSCOPIC OBSERVATIONS OF THE DEVELOPMENT OF PINCEAUX FORMATIONS AROUND HILLOCKS AND INITIAL SEGMENTS OF PURKINJE CELL AXONS IN THE POSTNATAL RAT CEREBELLUM:

Previous studies with light microscopy have provided an approximate schedule of the development of the pinceau formation (Rosina et al., 1999; Ango et al., 2004). The horizontally running basket cell axons start to send their descending branches toward the Purkinje cell somata by P7-9, and between P12 and P20, they wrap almost completely the Purkinje cell bodies forming the typical perisomatic baskets, and began to accumulate around the initial segments to form the pinceaux. This timetable, although imprecise, allowed us to better select the ages of the postnatal rats used in our study (P5, P9, P12, P15 and P20), and to focus the analysis between P9 and P15, when most of the process of pinceau formation should occur.

P5 Purkinje cells:

P5 Purkinje cells were almost already aligned in a monolayer at the interface between the nascent molecular and granule cell layers, close to their location in the adult cerebellum. This geometrical disposition of the Purkinje cell bodies facilitated their identification in the thin sections used for electron microscopy. Despite the well established asynchrony in the maturation of Purkinje cells (Inouye and Murakami, 1980), in the vermal cortex at P5, almost all of them already exhibited the features of “stellate cells with disoriented dendrons” of the classical description of Ramón y Cajal (1890), characterized by the occurrence of numerous long and thin processes emerging from the lateral and apical aspects of the soma. At this developmental age, Purkinje cell bodies exhibited immature cytological features. The nucleus had an eccentric basal location, and the cytoplasm between the nucleus and the basal pole of the cell, from which the axon hillock emerged, was mostly occupied by a large aggregate of free ribosomes, the polysomal mass (Larramendi, 1969; Nosal and Radouco-Thomas, 1971; Altman, 1972). Owing to the asynchrony in the maturation of Purkinje cells, a few of them already had some Nissl bodies, particularly close to the nuclear indentations (Fig.1 A), but in the vast majority of the Purkinje cells the granular endoplasmic reticulum appeared like dispersed cisterns separated from each other by numerous free ribosomes. The hypolemmal cistern, first described by Kaiserman-Abramof and Palay (1969), consisted of a shallow cistern of agranular endoplasmic reticulum that in single sections appeared to be interrupted at irregular intervals, and that

followed the contour of the cell bodies and dendrites close beneath the plasmalemma. This cistern, which is a major cytological feature of mature Purkinje cells, was lacking in P5 cerebellum (Fig. 1), another important sign of immaturity.

At P5, the axon left the cell body by a funnel-shaped process that rapidly narrowed to a thin fiber between 1 and 2 μm in diameter. The funneling process corresponded to the axon hillock (AH) (Fig. 1A, B) and the thin fiber to the axon initial segment (AIS). The AH was a small region characterized by the occurrence of fascicles of bound microtubules, together with numerous cisternal profiles of the smooth endoplasmic reticulum suspended in the axoplasmic matrix funneling into the axon. Although it also contained some clusters of ribosomes, there was a clear border abruptly separating the polysomal mass from the beginning of the axon hillock (Fig. 1A, B). The AIS, in addition to the presence of the AH organelles, was characterized by the undercoat of its inner axolemmal surface, that at this age was very tenuous consisting in a thin dense layer of fine granular material (Fig. 1B). Thus, the P5 Purkinje cells already shared some of the ultrastructural features of these two axon compartments (AH and AIS) with those that later on will characterize their adult counterparts (Palay et al., 1968).

Axon terminals were only occasionally apposed to the smooth surface of the lateral aspect of the perikarya. The majority of them were embedded within the numerous somatic protrusions, on which they established mature synaptic junctions (not shown). These axon terminals have been previously described using immunocytochemical markers and belong to climbing fibers (see Fig. 16 in Chedotal and Sotelo, 1993). No other types of axon terminals have been encountered in the close vicinity of Purkinje cell bodies. The AH and AIS were devoid of synaptic investment, and were partially covered by thin astrocytic processes (Fig. 1A, B). Therefore, at this developmental age basket cell axons were completely lacking, and the AIS was covered by a thin astrocytic sheath.

P9 to P15 Purkinje cells:

By P9, the polysomal masses (Fig. 2A, B) were less prominent, and gradually disappeared during the following days. Thus, Purkinje cells devoid of polysomal mass did occur from P10 onward (Fig. 3A). This important change was accompanied by the displacement of the nucleus upwards, the appearance of the hypolemmal cistern and of numerous profiles of granular endoplasmic reticulum sparsely dispersed throughout the cytoplasm (Figs. 3A and 5A-D), as in mature Purkinje cells. The undercoating of the AIS was now somewhat thicker and denser, clearly demarcating the end of the AH and the initiation of the AIS (Fig. 3B-D). It was during this developmental phase that basket cell axons, which have started by P7 to establish the first

axo-somatic synapses with Purkinje cell bodies, followed their descending outgrowth to reach the basal pole of these neurons and enter in the upper granule cell layer, the region containing the AH and AIS. The neuropil enwrapping these axonal compartments was no longer solely formed by thin astrocytic processes, and axon profiles were frequently intermingled with them (Fig. 2A). Most of these axons were long, thin and contained microtubules, cisterns of the agranular endoplasmic reticulum and scanty mitochondria suspended in a light axoplasmic matrix (Figs. 2A; 3B, C). These basket cell axons exhibited irregularly spaced slight dilatations, the “boutons en passant”, filled with elongated synaptic vesicles and mitochondria (Figs. 2B; 3A-D). Between P9 and P15 the pinceaux around the Purkinje cell AISs were developed following a precise sequential order: the basket axons entering the future AIS region, first established a synaptic contact on the AH (Fig. 3A), or at the border between the AH and the AIS (Fig. 2B), and it was only by P15 that basket fibers synapsed on the proximal third of the axolemma of the AIS (Fig. 3D). These early synaptic junctions were rather long and of asymmetrical type (Figs. 2C; 3D). Thereafter, basket fibers progressively increased their concentration around the Purkinje cell axon, and by P12, in cross or oblique sections of the AIS (Fig. 3B), it was possible to identify a primitive vortex-like arrangement of basket fibers intermingled with astrocytic processes, smaller than but of similar complexity as adult Purkinje cells. Like in the latter, the AIS was almost completely enwrapped by astrocytic processes, and only occasionally intervaricose segments of basket fibers (identified by their high content in microtubules) were directly apposed to it.

P20 Purkinje cells:

At this age, Purkinje cells have acquired most of their adult features. Somatic protrusions have almost completely resolved (see below). In addition, somatic and dendritic plasmalemae were outlined by the hypolemmal cistern that, in addition, was prolonged under the axolemma of the AH (Fig. 4A). This cistern disappeared, however, with the appearance of the undercoating at the level of the AIS (Fig. 4B).

Concerning the synaptic investment by basket fibers, the pericellular baskets and their corresponding pinceaux were almost completely developed. The base of the perikaryon and the AH were covered by astrocytic processes, surrounded by a compact neuropil mostly composed of small axonal profiles filled with elongated vesicles and larger intervaricose segments of the basket fibers. One of the “boutons en passant” was frequently observed in synaptic contact with the surface of the AH (Fig. 4A) or with the axolemma of the proximal third of the AIS. Small attachment plates could, occasionally, link axonal varicosities to the AH (Fig. 4A). In cross

section, the AIS was covered by numerous thin astrocytic processes, which maintained the AIS almost completely isolated from the large mass of encircling elements that constituted the pinceau formation (Fig. 4B). This encircling mass consisted of tightly packed axonal profiles with variations in diameter from 0.1 to 2 μm , intermingled with astrocytic processes. The smaller axonal profiles contained abundant synaptic vesicles, and appeared as fingerlike protrusions emerging from the larger basket fibers. Thus, the synaptic investment of Purkinje cell AH and AIS has acquired already its full ultrastructural maturity, with the exception of the septate-like junctions linking the plasmamembranes of the basket axon profiles encountered in adult pinceaux formations (Sotelo and Llinas, 1972), that were still missing.

The electron microscopic analysis of the development of pericellular baskets and pinceaux allows us to make a number of conclusions. From the beginning of the formation of the pinceau, the descending basket fibers establish one early synaptic contact with the axolemma of the AH and the AIS. Therefore, the establishment of the few synaptic contacts between basket fibers and Purkinje cell AIS, which characterize the pinceaux formations of adult animals (no more than two synapses per pinceau, Somogyi and Hamori, 1976), is a precocious event. In contrast, the appearance of inter-basket axon septate-like junctions should be formed after P21, because they are still lacking at this age. AISs are, from the beginning of the synaptogenic period, enwrapped by thin astrocytic processes, which isolate almost entirely this axon compartment from the descending basket fibers.

SOMATODENDRITIC TRANSLOCATION OF CLIMBING FIBERS AND BASKET AXONS INVESTMENT OF PURKINJE CELLS:

Immunofluorescence study:

GAD65-GFP transgenic mouse strain.

For the analysis of the spatio-temporal pattern of the synaptic investment of Purkinje cell dendrites, cell bodies and AIS, the GAD65/GFP transgenic mouse line can be considered an optimal tool to directly visualize these cells and their GABAergic presynaptic inputs by the endogenous expression of GFP. This, unfortunately, was not the case because the expression of the transgene was spatio-temporally regulated. In the newborn transgenic cerebellum, the vast majority if not all Purkinje cells expressed GFP (Fig. 5 A, C). However, there was a progressive and rapid extinction of this expression, and between P5 and P7 more than 90% of the Purkinje cells lost their GFP content. In contrast, GABAergic interneurons, particularly basket and stellate cells, maintained the GFP expression at least beyond P21, the oldest age examined. Therefore,

the rapid loss of GFP expression by Purkinje cells reduced the utility of these transgenic mice, and most of the analysis of the synaptic covering of Purkinje cell bodies was done on ICR mouse material double labeled with markers of the glutamatergic climbing fibers (the vesicular glutamate transporter, VGLUT2), and of the GABAergic basket axons (the plasmalemmal GABA transporter, GAT-1 or the vesicular transporter, VIAAT). The obtained results with ICR wild-type mice or GAD65-GFP transgenic mice were similar, and were pooled together, although mostly transgenic material was selected to illustrate the early stages (P0 to P7) of the development of the Purkinje cell synaptogenesis.

Newborn Purkinje cells and their innervation.

The cortex of the newborn mouse cerebellum is characterized by an external granular four to six cell-deep layer (EGL), underlain by a much wider cellular stratum mainly composed by haphazardly distributed Purkinje cells. In the vermal cortex of the GAD65-GFP transgenic mice, Purkinje cells exhibited different levels of spontaneous green fluorescence (from high to low fluorescence) and had smooth ovoid cell bodies located at several depths (Fig. 5A, C, G, I). The asynchrony in their dendritic differentiation reported before (Armengol and Sotelo, 1991) was evident. The majority of the deeply located Purkinje cells had a single and long stem dendrite, which emerged from the apical pole and ascended directly to the EGL. The superficial thirds of these ascending segments branched profusely and were provided with long appendages resembling dendritic filopodia. A few of these Purkinje cells had, in addition, short, tortuous and thin dendrites emerging from the lateral aspect of their cell bodies, particularly from the basal regions. The superficially located Purkinje cells were devoid of long apical dendrites but, in contrast, they could have two or more stem dendrites, branching into several much thinner segments, which contributed to the formation of a dendritic plexus distributed below the EGL, the nascent molecular layer (Figs. 5A, G). The axons emerged from the basal poles of the Purkinje cell bodies, and ran parallel to the long axes of the folia in the prospective white matter (Fig. 5A). Their morphology, therefore, corresponds to those termed “complex-fusiform” Purkinje cells (Armengol and Sotelo, 1991).

GFP-positive cells lacking the morphological features of Purkinje cells did occur but were much less numerous and unevenly distributed throughout the P0 cerebella. They were located in the deep central region containing the medial nucleus and in the superficial cortex. In the latter, these GFP-positive cells either occupied the central axes of the forming lobules or were intermixed with Purkinje cells in the developing gray cortical layers, but they were practically absent from the nascent molecular layer. In order to identify these cells as

GABAergic interneurons, some sections of the P0 cerebellum were immunostained with anti-Pax2 antibody, a transcription factor that in the cerebellum is considered to be specific for GABAergic interneurons (Maricich and Herrup, 1999). Surprisingly, the vast majority of Pax2 positive nuclei did not belong to GFP-positive cells, but many of the GFP-positive cells with non-Purkinje cell morphology exhibited Pax2 positive nuclei, although in some others the nuclei were Pax2-negative (Fig. 5A-C). These results suggest that either GABAergic interneurons in the transgenic cerebella express GFP much later than Pax2, or that not all Pax2-positive cells are GABAergic interneurons.

In the P0 wild type mouse cerebella the identification of Purkinje cells was performed with anti-CaBP antibodies, and they were double labeled with either anti-VGLUT2 or anti-VIAAT antibodies (Fig. 5D). Despite the much better identification of individual Purkinje cells done in the transgenic mice, in both instances it was possible to clearly visualize their cell bodies and the superficial dendritic plexus, indicator of the localization of the nascent molecular layer (Fig. 5D). The results with transgenic and wild mice were similar, and they have been pooled in the following description.

The two classes of glutamatergic inputs impinging on Purkinje cells in the adult cerebellum express differentially two of the subtypes of the vesicular glutamate transporter, VGLUT1 in parallel fibers and VGLUT2 in climbing fibers (Fremeau et al., 2001; Kaneko et al., 2002). In contrast, both mRNAs are co-expressed in postmitotic granule cells of the external and internal granular layers from P1 to P10 (Miyazaki et al., 2003). Thus, to avoid possible interferences at the protein level, the expression pattern of VGLUT1 was analyzed in P0 cerebellum. At this age, the cerebellar plate was completely devoid of VGLUT1 immunoreactive (VGLUT1-IR) elements (data not shown), whereas abundant labeling was observed with anti-VGLUT2 particularly in areas corresponding to the future white matter (Fig. 5F). Concerning the cortical gray matter, VGLUT2-IR fibers entered the Purkinje plate and, following the contours of the Purkinje cell perikarya, ascended toward the nascent molecular layer, although only few of them reached the plexus formed by Purkinje cell distal dendritic branches (Fig. 5H, I). In addition, the majority of the immunofluorescence encountered in the Purkinje cell plate appeared as coarse puncta (Fig. 5H). The disposition of the labeled fibers corresponds to that followed by climbing fibers in their “creeper stage”, before the formation of “pericellular nests” (Chedotal and Sotelo, 1993). As opposed to the occurrence of climbing fibers in the newborn cerebellar cortex, GAT-1 immunostaining fibers were not observed in the molecular or Purkinje cell layers (not shown), corroborating previous results in rats (Rosina et al., 1999). In contrast, VIAAT labeling was extensive in the deep cerebellar mass/nascent white matter but scarce in the

Purkinje cell plate, and the density of immunostained puncta in this last location was lower than in VGLUT2-IR preparations (compare Fig. 5D with 5I). Surprisingly, the fibers delineated by the VIAAT-IR puncta did not belong to CaBP-IR axons and, therefore, they were not recurrent collaterals of Purkinje cells, which were already numerous in the newborn cerebellum (Fig. 5D). To corroborate this result, the double staining of Purkinje cell axons was followed until P3 when the branching of their recurrent collaterals formed a very dense intracortical plexus in the nascent internal granular layer (Gianola et al., 2003). Despite the high density of Purkinje cell axonal profiles and the increasing amount of VIAAT-IR elements, we were unable to find double-labeled (calbindin and VIAAT) axons (Fig. 5E).

Innervation of Purkinje cells aged 5 to 7 days.

As reported above, in the P5 and P7 GAD65-GFP cerebella most of the Purkinje cells had lost their GFP expression. The remaining GFP-positive Purkinje cells appeared randomly dispersed within the cortical ganglionic layer either isolated (Fig. 6A) or, more often, forming small clusters of up to 10 cells (Figs. 6E, F). At P5, the observed Purkinje cells were already almost perfectly aligned into a monolayer, and had lost the apical, lateral and basal dendrites (“Purkinje cells with regressive-atrophic dendrites”), beginning their phase of “stellate cells with disoriented dendrites” (Fig. 6A, C). This phase was characterized by the rapid and progressive outgrowth of numerous thin and irregular somatic appendages providing the cells with their typical radiated appearance, which paralleled the formation of “pericellular nests” by climbing fibers. In agreement, the VGLUT2-IR coarse puncta, belonging to axon terminals of the climbing fibers, formed nests which ensheathed the lateral and basal aspects of the Purkinje cell perikarya, that began to reach the apical poles (Fig. 6B, C). Some labeled puncta were present in the granule cell layer, corresponding to mossy fibers that normally express VGLUT1 and VGLUT2. In addition, a dispersed, finely granulate labeling and some smaller puncta were also observed throughout the nascent molecular layer (Fig. 6B, C), probably belonging to parallel fiber varicosities that at this stage of development co-express both VGLUT1 and VGLUT2 (Miyazaki et al., 2003). The GAT-1 visualization disclosed neither labeled fibers nor terminals around the Purkinje cell bodies or in the molecular layer. In contrast, a few VIAAT-positive puncta were present in the deeper regions of the molecular layer (not shown), where some basket cells were already present.

In the cerebellum of the P7 transgenic mouse, the few remaining GFP-positive Purkinje cells were in a much more advanced developmental stage, corresponding to that of the

“orientation and flattening of dendritic trees”. The latter stemmed from the apical pole of the cell bodies, commonly as a single primary dendrite, although occasionally two main branches emerged from the apical pole. The primary dendrites ramified into multiple branches, which gave origin to tertiary and distal branches already studded with filopodia and spines respectively (Fig. 6E, F, H). The cell bodies remained covered with irregular appendages as in the previous stage. The pattern of distribution of VGLUT2-positive puncta was almost similar to that described above for P5 Purkinje cells. The immunostained puncta were forming dense “pericellular nests” of climbing fibers (Fig. 6D, E, G, H). The main difference in the VGLUT2-IR between P5 and P7 cerebella was the occurrence of numerous labeled small puncta in the deeper half of the molecular layer of the P7 cerebella, corresponding to presumptive parallel fibers (Fig. 6D, E, G, H). In the more numerous molecular layer regions devoid of Purkinje cells expressing GFP, the VGLUT2-positive fibers were more clearly visualized, and although some of the stained puncta had larger diameters than usual parallel fiber varicosities, they did not climb over the Purkinje dendrites (Fig. 6E, H), and were dispersed throughout the lower half of the molecular layer, which contained rather differentiated basket cells and still migrating stellate cells (Fig. 6D). For the purpose of this study, the most important finding was the presence of some GFP-positive fibers, emerging from the horizontally located axons of the basket cells, which descended toward the Purkinje cell layer and terminated in elongated bouton-like structures, apposed to the lateral aspects of the Purkinje cell bodies (Fig. 6F, H). These GFP-positive boutons intermingled with the numerous VGLUT2-positive puncta of the climbing fiber “pericellular nets”. Thus, synaptogenesis between basket cell axons and Purkinje cells has already started by P7 and, from this age on, the synaptic investment of Purkinje cell perikarya consists in an intermingling of climbing and basket cell fibers.

Innervation of P10 to P17 Purkinje cells.

From P10 on, Purkinje cells in the transgenic cerebella have lost most of their GFP expression, and only a faint and uneven fluorescence could be observed in some of their cell bodies without a clear delineation of their contours. The reported observations were, therefore, obtained from ICR mice. At P10, the VGLUT2 labeling around Purkinje cells differed to that observed at P7 because the coarse immunostained puncta not only covered the cell bodies but also their apical poles, at the origin of the stem dendrite, and some of them even penetrated the deep third of the molecular layer (Fig. 7B, C). Thus, at this age the “pericellular nest stage” starts to switch into the “capuchon stage”. In addition, finely granulate labeling and some puncta (larger than the granulate labeling but smaller than the pericellular puncta) were also dispersed

throughout the molecular layer, indicating that parallel fibers still coexpress VGLUT2 (Fig. 7B, C). The predominant observed changes occurred in the granule cell layer, where the labeling was concentrated into relatively large irregular structures corresponding to more mature mossy fibers (Fig. 7B, C). In contrast, the pattern of VIAAT immunostaining was completely changed since the labeled puncta reached the basal poles of the Purkinje cells, close to the axon hillocks and, in addition, many others were scattered in the deeper third of the molecular layer where they followed the main branches forming the proximal compartment of the Purkinje cell dendrites (Fig. 7A, C). VIAAT-positive puncta were also present in the granule cell layer, distributed around the VGLUT2-positive mossy fibers (Fig. 7A, C). These GABAergic axon terminals belong to Golgi cell axons and contribute to the formation of the glomeruli.

By P15, VGLUT2-labeled climbing fibers had accomplished most of their somato-dendritic translocation because, although they still covered a great part of the Purkinje cell perikarya, the vast majority of them climbed into the two deeper thirds of the molecular layer over the primary and secondary dendrites (Fig. 7, D, E). During their translocation VGLUT2-positive climbing fibers seem to follow VIAAT-positive basket cell axons, and run along parallel with each other as if they ascend over the surface of the proximal segments of the Purkinje cell dendrites (Fig. 7E). Therefore, from the beginning of the translocation, climbing fibers occupied already similar locations as the elaborated plexus of climbing fibers and basket cell axons of the adult cerebellum (Chan-Palay and Palay, 1970). Whereas, the VGLUT2 labeling dispersed within the molecular layer began to disappear (Fig. 7D, E), suggesting that at this developmental stage parallel fibers start to lose their VGLUT2 expression, switching to only the VGLUT1 subtype as in mature cerebellum. In addition, somewhat smaller VIAAT-positive puncta than those belonging to basket axons were dispersed throughout the molecular layer without any apparent correlation with the climbing fibers. In contrast, few if any of the VIAAT labeled puncta were found in the pinceau location, around the Purkinje cell AIS (Fig. 7E). The pinceaux formations were, however, easily visualized in the preparations in which the GABAergic basket fibers were immunostained with anti-GAT-1 antibodies. They were present around all Purkinje cell AIS (Fig. 7D), and exhibited an almost adult appearance, quite similar to that revealed in the classical pictures obtained with reduced silver impregnation: GAT1 labeled basket axons wrapped around Purkinje cell perikarya, descending towards the AIS, and terminating around the latter like small paintbrushes, with their characteristic inverted cone-shaped morphology (Fig. 7F). Therefore by P15, despite the peridendritic translocation of the climbing fibers and the appearance of the pinceaux, many axon terminals belonging to climbing fibers still maintain a perisomatic location.

About one day later (P16), the remaining small VGLUT2-IR puncta dispersed within the molecular layer were no longer present, and the larger labeled puncta delineated mature-like climbing fibers, which covered the whole proximal compartment of the Purkinje cell dendrites, already bearing thin varicose collaterals forming the typical short tendrils (Fig. 7H, I). Nevertheless, in contrast to the mature cerebellum, the majority of Purkinje cell perikarya remained covered by VGLUT2-positive puncta (Fig. 7H, I). The VIAAT labeling failed to show clear pinceau formations, although numerous positive puncta were intermingled with VGLUT2 puncta at the pericellular nests (Fig. 7G, I). The typical interrelations of basket cell axons and climbing fibers running in parallel over the primary and secondary branches of the Purkinje cell dendrites, which started to appear by P15, had acquired by P16 an almost mature appearance (Fig. 7I). More mature forms of glomeruli, with a central core VGLUT2-positive surrounded by VIAAT-positive elements were abundant in the granule cell layer (Fig. 7G, H, I).

Innervation of P21 Purkinje cells.

At this age (the older mice analyzed in this study), the external granular layer had disappeared, and the patterns of VGLUT2 and VGLUT1 labeling were already mature. Climbing and mossy fibers were VGLUT2 positive, whereas parallel and mossy fibers were VGLUT1-positive (Fig. 8 A, B). Climbing fibers occupied most of the molecular layer, but did not reach the subpial position because a small superficial zone (less than one fifth of the layer) remained free of VGLUT2 labeling (Fig. 8A). VGLUT1-positive parallel fibers occupied the whole extent of the molecular layer, which appeared filled by fine granulate fluorescent material with some empty zones corresponding to proximal branches of the Purkinje cell dendrites and blood vessels (Fig. 8B). In double-labeled preparations (VGLUT2 and GAT1), it was possible to disclose the synaptic investment of Purkinje cell bodies and AIS. Often, VGLUT2-positive puncta were found at the apical pole of the somata, whereas GAT1-positive fibers completely enwrapped them and terminated into mature-like pinceau formations (Fig. 8 C, E). Occasionally, apical poles and lateral aspects of some Purkinje cell bodies were still apposed to VGLUT2-positive puncta, which shared the cell surface with GAT1-positive basket cell axons (Fig. 8D, E). Thus, even at these advanced stages of synaptogenesis, climbing fibers have not totally translocated from their perisomatic to their peridendritic locations.

Electron microscopic analysis of the Purkinje cell bodies' synaptic investment between P5 and P20:

Our ultrastructural observations have corroborated those obtained with confocal microscopy. At P5 the only type of axon terminal in close apposition to basal and lateral aspects of Purkinje cell bodies consisted of rounded to ovoid varicosities containing numerous agranular spherical synaptic vesicles suspended within a somewhat dense axoplasm. The crowded vesicles together with the density of the axoplasm provided the terminals with a characteristic dense appearance that, in addition to the occurrence of some few large granular vesicles, allowed us to identify them as belonging to climbing fibers. These synaptic varicosities, although often apposed to Purkinje cell perikarya, established asymmetric synaptic contacts on numerous somatic protrusions (see Armengol and Sotelo, 1991; Chedotal and Sotelo, 1993). From P7 on, the climbing fiber varicosities were not the unique type of axon terminal encountered in a perisomatic location. The second type was much less abundant and its rare axon terminals were located mostly on the lateral aspects of the Purkinje cell perikarya. These axon terminals were “boutons en passant” of the slender basket axons. By P10, climbing fiber varicosities linked to the Purkinje cell perikarya by attachment plaques (Fig. 9A), were still numerous. Basket axon terminals increased in number and, as described above, exhibited a light axoplasm filled with irregularly shaped synaptic vesicles, some microtubules and occasional mitochondria (Fig. 9B). These “boutons en passant” established symmetrical synaptic contacts with the smooth membrane of the Purkinje cell bodies (Fig. 9B). At P12-P15 there was a progressive increase in the number of basket axon terminals and a decrease of climbing fiber varicosities, concomitant with the starting of their dendritic translocation. However, at the latero-apical aspects of the Purkinje cell bodies, it was still frequent to find side-by-side basket axon terminals (synapsing on the smooth perikaryal surface) and climbing fiber varicosities (synapsing on somatic protrusions) (Fig. 9C). This apparent association between basket axons and climbing fibers is a common feature during the period of early dendritic translocation of the climbing fibers, when the latter seem to follow basket axons, particularly ascending collaterals, on their way to their peridendritic location. As a consequence of the parallel routes of both fiber systems, climbing fiber varicosities synapsing on dendritic thorns are commonly associated with basket axon terminals synapsing on the smooth membrane of the proximal branches of Purkinje cell dendrites (Fig. 9D), as depicted in the confocal microscope study.

The most interesting results were those encountered in P20 rats, when the basket axons enwrapped the Purkinje cell bodies forming adult-like baskets, and when climbing fibers were supposed to have finished their translocation period. However, at this stage of development, climbing fiber varicosities still maintained some of their perisomatic synaptic contacts, either at the apical pole of the cell body, close to the initiation of the stem dendrite (Fig. 10A) or, more

importantly, at the upper half of their lateral aspects (Fig. 10B, C). In both instances, as well as at their peridendritic location (Fig. 10D), climbing fiber varicosities remained associated to their respective basket axon terminals, each one preserving their respective postsynaptic elements, dendritic thorns or smooth surface of the dendrite (Fig. 10A, B, D).

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DISCUSSION

The development of synapses in the molecular and Purkinje cell layers of the cerebellum has been amply studied with electron microscopy (Altman, 1972; Mason and Gregory, 1984; Landis, 1987; Armengol and Sotelo, 1991; Chedotal and Sotelo, 1993; Morara et al., 2001; Takayama and Inoue, 2005), yet relatively few studies have addressed the specific search of the acquisition of the spatio-temporal balance between excitatory and inhibitory inputs on Purkinje cells. Using immunohistochemistry, it has been disclosed that both glutamatergic (VGLUT2-IR) and GABAergic immunoreactive fibers (VIAAT-IR), although the latter occurred at much lesser density, were present in the Purkinje cell plate of the newborn mouse cerebellum, which in contrast was devoid of VGLUT1-IR fibers. Therefore, in the newborn cerebellum coarse VGLUT2-positive puncta should belong to axon terminals of the climbing fibers, which are by far the most frequent presynaptic elements of the synaptic investment of Purkinje cells (Chedotal and Sotelo, 1993; Morara et al., 2001). By P5, the “Purkinje cells with regressive-atrophic dendrites” (Armengol and Sotelo, 1991), converted to pear-shaped cell bodies provided with somatic spines, were apposed to VGLUT2 coarse puncta. No VIAAT-IR fibers were found in the neighborhood of the Purkinje cell bodies until P7, when the first GABAergic boutons (GAD65/GFP or VIAAT-positive basket cell axons) contacted Purkinje cell somata, and followed their descending pathway to their axon hillocks and AIS, which were reached by P9, a day before the initiation of the somatodendritic translocation of the climbing fibers. This translocation was not finished by P21, when some of the climbing fibers varicosities were systematically apposed to the Purkinje cell bodies, still establishing synaptic contacts on short perisomatic protrusions. The electron microscopic study of the development of pinceaux revealed that the rare axo-axonic synaptic contacts occurring in the adult pinceaux (Somogyi and Hamori, 1976) were established very early, once the descending branches of the basket cell axons reached the AIS by P9. It was only by P12 that the structure of an immature pinceau could be identified as a primitive vortex-like arrangement of basket fibers and their intermingled astrocytic processes around the AIS. These results emphasize that the synaptic covering of the somatic surface of Purkinje cell bodies by climbing fibers does not hinder the later synaptic covering by basket axons and the establishment of pinceaux formations. In addition, the somatodendritic translocation of the climbing fibers and the development of pericellular baskets and pinceaux formations are two asynchronous and independent processes.

Onset of the innervation of Purkinje cells

Climbing fibers are supposed to be, from all known afferent inputs to Purkinje cells, the first ones to establish synaptic contacts. These fibers reach the border of the cerebellum at E16, and progressively (E16 to E19) build up a dense plexus that fills most of the cerebellar plate parenchyma (Wassef et al., 1992), and that starts synaptogenesis before E19 because at this age climbing fiber-Purkinje cell synapses do already exist (Morara et al., 2001). The vast majority of these newly formed synaptic contacts occupy dendritic locations, and only some rare axo-somatic synapses occur. In agreement with this observation, climbing fibers in their “creeper stage” were the unique identified input to the Purkinje cells in the newborn rodent cerebellum (Chedotal and Sotelo, 1993, Morara et al., 2001). However, it is known that mossy fibers co-express both classes of vesicular glutamate transporters (Kaneko et al., 2002; Hioki et al., 2003), and are able to establish transient synaptic contacts with Purkinje cells (Mason and Gregory, 1984). In addition, VGLUT2 is transiently expressed by parallel fibers during the first 10 days of postnatal life (Miyazaki et al., 2003). Therefore, some of the VGLUT2-positive puncta encountered in the P0 mouse cerebellum might not belong to climbing fibers. However, this possibility is untenable because: at birth mossy fibers are concentrated in the prospective white matter at the central axes of the folia (Arsenio Nunes and Sotelo, 1985), the contacts between mossy fibers and Purkinje cells mostly occur during the second postnatal week (Mason and Gregory, 1984) and, although more indirect evidence, the total lack of VGLT1 labeling and the coarse appearance of the VGLUT2 puncta observed in the P0 cerebellum strongly indicate that parallel fibers cannot be in synaptic contact with Purkinje cells in newborn mice.

An unexpected result has been the discovery of abundant VIAAT-IR fibers in the P0 mouse cerebellum, particularly in non-cortical central areas, suggesting that they are axons of projecting neurons. To corroborate this suggestion, we were first obliged to discard GABAergic interneurons as the origin of these fibers. For that, we used the Erdélyi and Szabo transgenic mouse line (GAD65/GFP, Erdélyi et al., 2004) and observed that GABAergic interneurons at P0 are still migrating through the central cerebellar parenchyma (prospective white matter) toward their ultimate locations in the molecular layer, and only some rare immature GAD65/GFP have already reached the cortical gray matter (see Fig. 5C). The absence of GABAergic interneurons in the P0 mouse cerebellum has been corroborated with another transgenic mouse line, the one expressing GFP under the Pax2 promoter (Maricich and Herrup, 1999), where the cortical arrival of the GABAergic interneurons occurs even later (Weisheit et al., 2006). Therefore, VIAAT-positive fibers do not belong to cortical GABAergic interneurons in the P0 cerebellar cortex.

Among projecting neurons, the known extracerebellar afferent fibers, mossy, climbing and monoaminergic fibers, are not GABAergic. Thus the best candidates for these early VIAAT-IR fibers would be the efferent axons of the Purkinje cells and their recurrent collaterals, which between P0 and P4 establish a dense fibrous plexus in the nascent internal granular layer (Gianola et al., 2003). However, in our double-labeled CaBP-VIAAT preparations of P0 to P3 cerebella, colocalization of both markers has never been observed, wiping out this possibility. Therefore, the origin of the VIAAT-positive fibers in early postnatal cerebella remains unidentified. In any case, it is of interest to remark that Takayama and Inoue (2004) have reported a low density of VIAAT-IR puncta (badly named VGAT-IF dots, because this transporter is also functional in glycinergic neurons, Chaudhry et al., 1998) in the Purkinje cell plate of the P3 mouse cerebellar cortex but, despite a thorough electron microscopic examination, no synaptic contacts have been encountered. These results suggest that the few GABAergic fibers of the neonatal cerebellum do not establish synaptic contacts with Purkinje cells. However, they are equipped (presence of the appropriated vesicular transporter) to release GABA in a paracrine manner, and since the nearby Purkinje cells have already expressed GABA-A and GABA-B receptors (Laurie et al., 1992; Fritschy et al., 1994; Lujan and Shigemoto, 2006), paracrine released GABA might elicit a functional response in newborn Purkinje cells. Thus, postnatal Purkinje cells may respond to GABA release far before the formation of the first synapses with basket fibers (P7, Ango et al., 2004; present study), and the appearance of immunostained puncta visualized with other GABAergic markers than VIAAT (McLaughlin et al., 1975; Greif et al., 1991; Yan and Ribak, 1998; Rosina et al., 1999; Takayama and Inoue, 2005).

The functionality of the GABA system in postnatal Purkinje cells has been proven by Eilers et al. (2001), who with Ca^{2+} imaging and perforated-patch recordings, have shown that in P3 to P6 rat cerebellum, before the synaptogenesis between basket fibers and Purkinje cells, the latter are able to evoke transient increases in $[\text{Ca}^{2+}]_i$ when exposed to either GABA or muscimol, whereas this GABA-mediated Ca^{2+} signaling disappeared in rats older than one week. They have also shown that during the period of time of GABA-mediated Ca^{2+} transients, focal application of GABA evoked Purkinje cell depolarization, instead of the expected hyperpolarization. This ontogenetic shift from depolarizing to hyperpolarizing GABA action in Purkinje cells (Eilers et al. 2001), indicates that in these cells, as it has been reported for other immature neurons (Rivera et al. 1999; Yamada et al. 2004; Lee et al. 2005; Marty et al., 2002), a concomitant developmental down-regulation of chloride accumulating NKCC1 co-transporter and an up-regulation of the chloride extruding KCC2 co-transporter should occur. The

developmental changes of the cerebellar expression patterns of the mRNAs and proteins of these two co-transporters have been analyzed with *in situ* hybridization, immunocytochemistry, Northern and Westerns blots (Plotkin et al., 1997; Lu et al., 1999; Mikawa et al., 2002). Despite the reported up-regulation of the KCC2 expression, particularly between P14 and adulthood (Lu et al., 1999), and down-regulation of the NKCC1 expression also between P14 and P28 (Plotkin et al., 1997), the NKCC1 is expressed too late in Purkinje cells (around P11) to account for the depolarizing action of GABA (P2-P6, Eilers et al., 2001). In addition to this temporal mismatching, it seems that in Purkinje cells KCC2 appears earlier, and with a much higher level of expression than NKCC1 (Mikawa et al., 2002), data which do not support the hypothesis that the developmental switch in GABA effect involves the maturation of the chloride homeostasis systems, maturation which depends on the GABAergic activity and its modulation of the mRNA levels of the Cl⁻ extruding co-transporter (KCC2, Ganguly et al., 2001). In any case, the temporal correlation between the first synaptic contacts of the basket fibers with Purkinje cells and the developmental switch in the GABAergic transmission from depolarizing to hyperpolarizing is of interest, because it emphasizes that basket cells exert their inhibitory function on Purkinje cells from the very beginning of their synaptogenesis.

In conclusion, although the possibility of some synaptic contacts between not-yet identified VIAAT-IR axons and Purkinje cells remains open, glutamatergic fibers are the first to start the establishment of synaptic contacts on Purkinje cells. Therefore, the cerebellum seems to be the only of the already studied centers where GABAergic synapses are not established before the glutamatergic synapses (Ben-Ari, 2002, Owens and Kriegstein 2002). However, the lack of early GABAergic synapses does not preclude the occurrence of early VIAAT-expressing fibers able to release GABA and to contribute to the depolarization of neonatal Purkinje cells.

Subcellular compartments of Purkinje cells: selective targeting of afferent synaptic inputs

During synaptogenesis and the building up of the central neuronal circuits, afferent fibers are required to single out their appropriate postsynaptic partners. A more elaborated choice is later imposed, since they should also select in which cell compartment they should impinge the postsynaptic neuronal membrane. In other words, each kind of afferent input should synapse on a particular subcellular compartment of the Purkinje cell for the proper regulation of the synaptic integration and neuronal excitability. These choices are very selective for developing Purkinje cells. Thus among excitatory inputs, the early forming climbing fibers, after some transient dendritic contacts (Chedotal and Sotelo, 1993), will start their synapse formation on somatic

filopodia to ultimately end by establishing synaptic contacts on the thorns arising from the thick branches of the proximal dendritic compartment (Larramendi and Victor, 1967). Whereas, parallel fibers, the other and latterly developed excitatory system, impinge from the beginning upon the thinner and longer spines arising from the distal dendritic compartment. Inhibitory axons contacting Purkinje cells are also compartmentalized and the ascending collaterals of the basket and stellate axons will contact the smooth membrane of the dendritic shafts in the proximal compartment (Larramendi, 1969). In contrast, the descending branches of the basket cell axons will build up the pericellular baskets surrounding the Purkinje cell bodies, and will end around the AIS as pinceaux formations. Therefore, specific types of presynaptic fibers will contact, in a precise temporal sequence, each Purkinje cell subcellular domain. Despite many common synaptogenetic organizational principles shared with other projecting neurons, Purkinje cells exhibit two rather specific features: i) the targeting of the descending branches of the basket axons onto the AIS, to build up the pinceau arrangement a unique structure of the mammalian brain, and ii) the initial targeting of climbing fibers onto Purkinje cell somata and their later somatodendritic translocation.

The development of pinceaux formations

Basket cell fibers in adult rats and mice can be identified with a long series of antibodies or in some transgenic mouse lines that allow the direct visualization of these fibers surrounding Purkinje cell perikarya and establishing pinceaux arrangements around their AISs. Only some of these tools have exploited for developmental studies. For instance, GABAergic markers (GAT1, Yan and Ribak, 1998; Rosina et al., 1999; GAD65, Greif et al., 1991; Ango et al., 2004; Lujan et al., 2005; VIAAT, Takayama, 2005), GAD67/GFP mice (Ango et al., 2004); GAD65/GFP mice (present study), particular types of potassium channels (HCN1, a subunit of a cyclic nucleotide-gated cation channel, Lujan et al., 2005), and proteins commonly associated with synapses (PSD-95 also named SAP-90, Kistner et al., 1993). Particular attention should be paid to a remarkable series of four monoclonal antibodies (IgM) obtained by using *Xenopus* oocytes with rat brain mRNA for immunization, which specifically label the basket fibers at the level of the pinceaux; particularly mAb 2G6, the antigens of these mAbs remain unknown (Tigyi et al., 1990). The results obtained with all these tools have disclosed that, in spite of the general precocity in mouse compared to rat development, pinceaux are developed almost simultaneously in the two species, in rats (Yan and Ribak, 1998; Rosina et al., 1999, Lujan et al., 2005) and mice (Ango et al., 2004; Takayama and Inoue, 2005). The earliest visualized descending branches of the basket

axons reach the AH and the most proximal part of the AIS by P9 (in the GFP transgenic mice and when VIAAT is used as a marker), between P9 and P15 these descending branches completely entwine Purkinje cell bodies, and by P15 pinceaux exhibit apparently mature features with the majority of the markers (with exception of those related to septate-like junctions). However, we have shown here that in P20 rat cerebellum the pinceaux have not reached full maturity because the septate-like junctions between basket axons have not yet appeared, and that such junctions have a presumably important functional role (Sotelo and Llinas, 1972).

Basket axons forming the tightly packed meshwork around the AIS contain abundant synaptic vesicles, but paradoxically they establish very few axo-axonic synaptic contacts (no more than two per pinceau, Somogyi and Hamori, 1976). In contrast, the descending basket fibers are bound to each other by numerous septate-like junctions with its associated proteins, particularly PSD-95 (Kistner et al., 1993; Hunt et al., 1996), and are almost completely separated from the AIS by intervening astrocytic processes, which in addition encapsulated the entire pinceau arrangement. The occurrence of septate-like junctions between basket axon terminals (Sotelo and Llinas, 1972) at the pinceau and its glial encapsulation support the electrophysiological suggestion that this unique structure is involved in the generation of an inhibitory electrical field imposing a passive hyperpolarizing potential on the AIS (Korn and Axelrad, 1980). Thus, there is some functional and structural resemblance between Mauthner cell caps and Purkinje cell pinceaux, because basket cells also mediate electrical (electric field inhibition) and chemical inhibition, which could contribute to the synchronization of Purkinje cell discharges (Korn and Axelrad, 1980). The present electron microscope study has allowed us to determine that from the very beginning of the descending outgrowth of basket axons, once they reach by P9 the basal pole of Purkinje cells, and enter the region containing the AH and AIS, they establish synaptic junctions with these axonal compartments. In contrast, even in our older analyzed Purkinje cells (P20), septate-like junctions were still not present. The late appearance of septate-like junctions has been correlated in time to the late appearance of some proteins such as PSD-95, mRNA of which peaks at P15 (Kistner et al., 1993) and protein should be expressed at the pinceaux after P21 (Castejon et al., 2004). This correlation could be explained by the structural similarities of PSD-95 and the *Drosophila* gene *dlg* (*lethal(l)discs large-1*), a tumor suppressor gene with its protein product located at the septate junctions of the imaginal disc (Woods and Bryant 1991). The uniqueness of septate-like junctions at the pinceau, the late expression of the antigen recognized by the mAb 2G6, which does not begin to detect immunoreactivity until P20 and attains its adult pattern by P30, and its extreme specificity (Tigyi et al., 1990), let us suppose that this mAb also recognizes proteins located at the septate-like

junctions. Therefore, the electron microscopic study reported here correlates to previous immunohistochemistry analysis, and allows us to conclude that, as it was the case for electrotonic coupling through gap junctions between neurons in the inferior olive (Bourrat and Sotelo, 1983) and lateral vestibular nucleus (unpublished observation), where gap junctions appear a long time after the establishment of chemical synaptic junctions, the morphological specializations at the AIS of developing Purkinje cells, which might be correlated with electrical inhibitory interactions mediated by extracellular current or field effects, also appeared a long time after the chemical synapses at least after P21. These results suggest that in the rodent CNS chemical transmission precedes not only excitatory electrotonic coupling but also electrical inhibition generated by field effects, as if the phylogenically considered more primitive mode of synaptic transmission was ontogenetically the latest to develop.

A few years ago, the molecular mechanisms which oriented the descending branches of the basket fibers towards the AH and AIS of Purkinje cells were elucidated (Ango et al., 2004). The GABAergic fibers are targeted to their axonic subcellular compartments by the signaling of an immunoglobulin protein of the L1 family (neurofascin, particularly neurofascin-186, NF186). This L1-CAM molecule is recruited by ankyrin-G, which plays the role of a specialized based-membrane adaptor protein with NF186 high affinity binding, and expression of which is confined to the AIS. The NF186 is distributed in the form of an AIS to dendrite gradient, which is laid down by P7, when the first descending basket fibers reach the Purkinje cell body. Ango et al. (2004) have clearly shown that this gradient is essential for the proper formation of pinceaux and that ankyrin-G is required for the laying down of the NF186 gradient. The observation reported here of early synaptogenesis between basket fibers and the AIS validates the above mentioned mechanism as the molecular mechanism for synapse targeting to the Purkinje cell AIS. It is supposed that the gradient provides an optimal substrate for basket fibers outgrowth, it becomes urgent to identify in the basket fibers the partner adhesion molecule of the NF186 gradient, in particular when abundant astrocytic processes are, from the beginning of the development of pinceaux, separating the AIS axolemma from the basket fibers.

The development of perisomatic nets and the somatodendritic translocation of the climbing fibers

Due to the asynchrony in the maturation of Purkinje cells (Inouye and Murakami, 1980), Golgi impregnation (Ramon y Cajal, 1911; O'Leary et al., 1971; Palay and Chan-Palay, 1974; Sotelo, 1991) and the electron microscope studies (Larramendi, 1969; Chedotal and Sotelo,

1993; Morara et al., 2001) have only provided an approximate timetable of the somatodendritic translocation of the climbing fibers during synaptogenesis. When with immunohistochemical staining the simultaneous visualization of large numbers of climbing fibers was possible, we were able to establish that the climbing fiber translocation starts by P10 and is almost completed by P16 (Chedotal and Sotelo, 1992). In the present study, the date of the onset of the translocation was confirmed but its duration was extended because by P21 the translocation was not finished in spite of an extensive pericellular basket covering. The coexistence of a complete basket covering and some perisomatic climbing fibers, remnants of the pericellular nets, provides evidence that the somatodendritic translocation of the climbing fibers does not result from a direct competition between the two fiber systems by “steric hindrance” for a limited postsynaptic space. Electrophysiological studies have supported the existence of a transient phase of multiple innervation of Purkinje cells by climbing fibers, which lasts until P15 in the rat, when the adult numerical matching of one climbing fiber per one PC is achieved (Crepel et al., 1976; Mariani and Changeux, 1981). The partial temporal overlap between the regression of the multiple innervation and the climbing fiber translocation has been interpreted as if both processes were interrelated (Heckroth et al., 1990). However, the study of some cerebellar mutations, such as *weaver*, *staggerer*, *hyperspiny Purkinje cell* (Sotelo, 1990) has disclosed that the translocation is not a key factor in attaining mono-innervation. On the other hand, a number of electrophysiological studies in adult X-irradiated rat cerebella (Crepel et al., 1981) have revealed a retention of the immature, transient stage of multiple innervation, indicating that granule cells and their parallel fiber/Purkinje cell synapses promote the regression of the PC multiple innervation. Therefore, the transition from multiple to single innervation is caused by a modification of the state of activity of the Purkinje cells due to the absence of the parallel fibers input, but independent of climbing fiber translocation.

Finally, while descending basket fibers are targeted to the AH and AIS, the ascending collaterals of the same axons are targeted to the smooth dendritic shafts of the proximal compartment. The synaptic covering of these dendrites by ascending basket axon collaterals shortly precedes the movement of the translocating climbing fibers. Therefore, both kinds of fibers climb along with each other as they ascend over the surface of the Purkinje cell stem dendrites, building up the elaborated axonal plexus that characterizes the interrelations of climbing fibers, basket cell axons, and Purkinje cell dendrites in the adult cerebellum (Chan-Palay and Palay, 1970). The parallel pathways followed by the ascending collaterals of the basket axons and the climbing fibers suggest that the latter should be involved in the targeting of

the climbing fibers toward their ultimate subcellular targets. In conclusion, in contrast to the old concept considering that the somatodendritic translocation of the climbing fibers was required for the instauration of the pericellular baskets and their pinceaux, the present study supports the opposite concept: it is only after the appearance of the ascending collaterals of the basket fibers and their initial synaptic covering of the proximal compartment of the Purkinje cell dendrites that the climbing fiber translocation starts, as if the ascending collaterals of the basket fibers were the required substrate for the climbing fiber targeting its ultimate postsynaptic domains.

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For Peer Review

LEGENDS FOR FIGURES

Figure 1: Electron micrographs of Purkinje cell axon hillock (AH) and initial segment (AIS) in early postnatal rats (P5).

A) The basal pole of the cell body, between the nucleus and the AH, contains the polysomal mass (asterisk), a large region of the cytoplasm filled with large amounts of free ribosomes, which characterize these early postnatal Purkinje cells. The hypolemmal cistern is lacking in these juvenile neurons, as opposed to their more mature counterpart. The AH is already identifiable by the presence of abundant bundles of microtubules (arrowheads). Note that the basal region of the cell body as well as the AH are covered by one to four layers of thin astrocytic processes (g).

B) At this somewhat higher magnification, the transition between the region containing the polysomal mass (asterisk) and the AH is characterized by a rapid and progressive reduction in the density of free ribosomes and the appearance of the microtubular bundles. The AH continues with the AIS, identified by the occurrence of an inner cytoplasmic undercoating of dense material (small arrows). Despite the fast and progressive reduction in the density of ribosomes, some clusters persist in the AIS (r). Astrocytic processes (g) are apposed to the axolemma.

A, B scale bars = 1 μ m.

Figure 2: Electron micrographs of axon hillocks (AH) and initial segments of Purkinje cells in P9 rat cerebellum.

A) At this age, the polysomal mass (asterisk), although regressive, still extends considerably and the hypolemmal cistern has not yet appeared. The AIS exhibits its three distinct features: the presence of some small aggregates of free ribosomes, bundles of microtubules (small arrows), and the dense undercoating of the axolemma (arrowheads). The main maturational changes visible surrounding the AIS consist in an increase in the amount of astrocytic processes (g) and, particularly, the appearance of axonal profiles (ax) probably belonging to basket cell axons. In the vicinity of the AIS there is also the cellular perikaryon of a Golgi epithelial cell (GEC), characterized by the presence of a centriole (arrowhead). Note the presence of a mature looking synapse (large arrow) between a small axon and the shaft of a small dendritic profile.

B) This AIS, containing some free ribosomes (r), receives close to its emergence from the AH a synaptic contact (arrow) from a basket axon (BF). Polysomal mass (asterisk).

C) This high magnification illustrates the typical axo-axonic asymmetrical synapse encountered in the pinceau formation, and illustrated in B.

A, B scale bars = 1 μm . C = 0.15 μm .

Figure 3: Electron micrographs illustrating the maturation of pinceaux formations in the cerebellum of rats aged P10 (A), P12 (B) and P15 (C, D).

A) By P10 the polysomal mass is very much reduced, and the Purkinje cell soma has attained almost adult features, particularly, the presence of the hypolemmal cistern (arrows). Note a basket axon (BF) on axo-axonic contact with the axolemma of the axon hillock (AH). This synaptic bouton is filled with small irregularly shaped vesicles and establishes type II or symmetrical synaptic junctions (small arrows) with the AH. (r) free ribosomes in the AH.

B) By P12, the distal third of the AIS, with its free ribosomes (r), bundles of microtubules (arrowhead) and axolemmal undercoating (small arrows), is surrounded by a vortex-like arrangement of basket axons (BF), intermingled with numerous thin processes of astrocytes (g). Note that this distal part of the AIS is almost completely enwrapped by glial processes, and that direct appositions between basket axons and the AIS membrane only occur very occasionally.

C, D) By P15, the features of the AIS are adult-like (C, D), particularly the axolemmal undercoating that is already well marked (C insert). Intertwined astrocytic processes (g) and basket axons (BF) are forming the cellular environment of the AIS. The latter can occasionally be apposed to the AIS, and even establish synaptic contact with it (C arrow). Note that the varicosities of the basket axons are filled with pleomorphic synaptic vesicles (C, D).

A – D, scale bars = 1 μm

Insert in C, scale = 0.1 μm

Figure 4: The pinceaux formations of P20 rat cerebellum have not yet reached complete maturity.

A) Axon hillock (AH) and initial segment (AIS) exhibiting adult-like features. In this electron micrograph, numerous open arrowheads point to the hypolemmal cistern. The environment of the Purkinje cells is formed by astrocytic processes (asterisks) and axonal profiles (ax); some of the latter are elongated and filled with pleomorphic vesicles, others are larger fibers with light axoplasmic contents and neurofilaments; in both instances these profiles have been identified as belonging to basket axons (BF). Note the occurrence of a synaptic contact (arrowhead) between a basket axon and the most proximal part of the AIS. Finally, a small axonal profile (ax) apposed to the AIS is linked to the latter by a small attachment plate (arrow).

B) Cross section of the AIS illustrating the complexity of the pinceau and its lack of maturity. The AIS is completely enwrapped by astrocytic processes (asterisks). The left three quarters of

the micrograph illustrate the almost compact mass of large basket axons (BF), filled with synaptic vesicles, intermingled with smaller axonal profiles containing pleomorphic synaptic vesicles, belonging to the small collaterals of the basket axons. Note the absence of septate-like junctions, which would appear as small Greek-like crosses of electron-opaque densities perpendicular to the plasmamembranes of directly apposed basket axons.

A, B scale bars = 1 μ m.

Figure 5: Laser scanning confocal double immunofluorescence of GFP and Pax2 (A, C), GFP and VGLUT2 (F, G, I), CaBP and VIAAT (D, E) of newborn transgenic (GAD65/GFP) and wild-type P0-P3 mouse cerebellum. Each micrograph corresponds to a z-stack of 6 sections taken at 0.5 μ m intervals. In the sections of the transgenic cerebellum, the green autofluorescence mainly visualizes the Purkinje cells (A, C, and G, I), exhibiting a large variety of shapes, although the elongated shapes of the complex-fusiform type prevails (large arrow in A, C). In addition, a few GABAergic interneurons, identified by either the Pax2 (red) colabeling of their nuclei (small arrows in C) or their smaller sizes and simple shapes (arrowheads in C), are already present in the cortical region. Note that the vast majority of Pax2 expressing nuclei do not belong to GFP-positive cells (B, C). CaBP immunolabeling in wild-type mice also allows the identification of the Purkinje cell plate (D), although individual Purkinje cells are less Golgi-like than in the transgenic cerebella. Surprisingly, the VIAAT- immunofluorescence is dense in the prospective white matter and very light at the Purkinje cell plate (D). The density of VIAAT innervation, although increased in the white matter of the P3 cerebellum, does not vary in the Purkinje cell plate (compare D and E). Note that the VIAAT-labeling does not colocalize with Purkinje cell axons, which at this age form a dense plexus in the prospective granular layer (D, E). When sections of the GAD/GFP transgenic cerebellum are immunolabeled with anti VGLUT2 antibody (red fluorescence in F and H, I), the deep central white matter is highly fluorescent (F), and the density of immunopositive puncta within the Purkinje cell plate (PCP) is higher than in the case of VIAAT labeling (compare D with H, I). The rectangle in F encloses part of the lobules IV and V, area illustrated at higher magnification in G-I.

A-C) A: green autofluorescence of the GFP expressed by GABAergic neurons in transgenic mice. B: red Pax2 nuclear labeling. C: merged image of the green and red fluorescence. Scale bar (in I) = 58 μ m. D and E) Merged images of green (CaBP fluorescence) and red (VIAAT fluorescence). Scale bar (in I) = 43 μ m. F) Low magnification of a merged image of GFP (green) and VGLUT (red). Scale bar (in I) = 333 μ m. G-I) G: green autofluorescence of the GFP

expressed by GABAergic neurons. H: red VGLUT2 labeling. I: merged image of the green and red fluorescence. Scale = 33 μ m.

Figure 6: Laser scanning confocal double immunofluorescence of GFP and VGLUT2 (A-H) of P5-P7 transgenic (GAD65/GFP) mice cerebellum. Each micrograph corresponds to a z-stack of 6 sections taken at 0.5 μ m intervals. In the sections of the transgenic cerebellum, the green autofluorescence of Purkinje cells has almost disappeared, since only very few of these neurons still express GFP. The few remaining Purkinje cells are either isolated (A, C) or in small clusters (E). At P5, Purkinje cells are aligned in a monolayer and have lost their long dendrites. Their pear-shaped cell bodies are not smooth but studded with short filopodia (A, C). The nascent molecular layer contains few GFP interneurons, and in many regions appears free of GAD65/GFP elements (A, C). In contrast, VGLUT2 immunopositive puncta are abundant around the Purkinje cell bodies, forming the climbing fiber pericellular nests (B, C). In addition, some much thinner puncta are dispersed throughout the deeper region of the forming molecular layer. By P7, Purkinje cells are provided with oriented and flattened dendritic trees, the distal segments of which are already studded with spines (E, F, H). The molecular layer contains numerous GFP-expressing interneurons, some of them resembling basket cells in the last phase of their migration (arrowhead in D) or in their initial phases of their differentiation (arrow in D). More importantly, some of the differentiating basket cells send descending axonal segments that reach the Purkinje cell bodies and establish contacts with them (arrowheads in F, arrows in H). At P7, VGLUT2-positive puncta of large size surround Purkinje cell bodies, encircling them as peripheral rings (see asterisks in D, E and G). At this age, the synaptic investment of the developing Purkinje cells is, therefore, formed by numerous climbing fiber boutons intermingled with a few basket cell axons (H). Abundant VGLUT2-immunopositive puncta, many of them of smaller size, are dispersed throughout the molecular layer (ML in D and G). Note that mossy fibers in the granule cell layer are numerous (D, E).

The scale bar in C corresponds to the following magnifications: A-C = 26 μ m; C is the merged image of A plus B. D = 43 μ m. E = 22 μ m. F-H = 12 μ m; H is the merged image of F and G.

Figure 7: Laser scanning confocal double immunofluorescence of VIAAT (green) and VGLUT2 (A-C, E, G-I) and GAT-1 (green) and VGLUT2 (D, F) of P10-P16 mice cerebellum. Each micrograph corresponds to a z-stack of 6 sections taken at 0.5 μ m intervals. By P10 VIAAT and VGLUT2 positive puncta surrounding Purkinje cell bodies (asterisks in A-C) are intermingled (C). Note that VGLUT2-IR elements are more numerous than VIAAT-IR ones (compare B and

A). Pinceaux are not evident (arrowheads in A). The VGLUT2 fine and diffuse labeling of the molecular layer, although decreased, still remains (B). The large puncta, belonging to climbing fiber varicosities, begin to cover the region of the Purkinje cell stem dendrite (arrow in B, C), where they are interlaced with VIAAT-IR puncta belonging to ascending collaterals of the basket axons. By P15, GAT-1 immunolabels the descending branches of the basket axons (pericellular baskets and pinceaux, arrows in D, F), and their ascending branches (D, F). Axonal varicosities of the basket axons are better visualized with VIAAT-IR, although pinceaux formations are not shown (E). VIAAT-IR puncta intermingled with VGLUT2-positive climbing fibers cover the Purkinje cell perikaryon (asterisk in E) almost in an equally quantitative manner, and delineate the primary and secondary branches of its proximal dendritic compartment (arrows in E), revealing an important somatodendritic translocation of the climbing fibers. One day later, the translocation appears almost complete, and the climbing fibers reach the distal segments of the proximal compartment of the Purkinje cell dendrites (arrows in H), where they are interlaced with VIAAT-positive fibers (I). Note that the fine and diffuse VGLUT2 immunolabeling has disappeared (H, I), and that the somatic covering ratio by VIAAT-IR puncta is now higher than that covered by VGLUT2-positive puncta (compare G and H).

The scale bar in F corresponds to the following magnifications: A-C = 19 μ m; C is the merged image of A and B. D = 81 μ m. E-F = 9 μ m. G-I = 16 μ m; I is the merged image of G and H.

Figure 8: Laser scanning confocal immunofluorescence of VGLUT2 (A) and VGLUT1 (B), and double immunofluorescence of GAT-1 (green) and VGLUT2 (red) (C, E) of P21 wild-type mice cerebellum. Each micrograph corresponds to a z-stack of 4 sections taken at 0.5 μ m intervals. At this age, VGLUT2 immunolabeling visualizes mature-like climbing fibers spanning the molecular layer (CF in A), and large mossy fibers dispersed through the granule cell layer (MF in A). VGLUT1 also visualizes mossy fiber in the granule cell layer (MF in B) but, in contrast, in the molecular layer only parallel fibers are labeled. In these young mice, GAT-1-positive pericellular baskets and pinceaux (arrow in C and E) exhibit a mature-like appearance, but still some VGLUT2 positive climbing fibers are apposed to Purkinje cell bodies (arrowhead in D and E). As illustrated in (E) the synaptic covering of the somata of these neurons still remains composed by the intermingling of both categories of pericellular axons.

The scale bar in B corresponds to the following magnifications: A = 28 μ m. B = 25 μ m. C-E = 7 μ m. E is the merged image of C and D.

Figure 9: Electron micrographs of Purkinje cell bodies illustrating their synaptic investment in P10 (A, B) and P12 (C, D) rat cerebellum.

A) By P10, Purkinje cell bodies are surrounded by climbing fiber varicosities (CF), which contain numerous compacted spherical synaptic vesicles and some large granular vesicles (open arrowhead). Note that this climbing fiber varicosity is fastened to the smooth somatic surface by attachment plates (arrows), and apposed to a somatic filopodia (asterisk), its current postsynaptic targets.

B) In addition to the numerous perisomatic climbing fibers, the smooth surface of the Purkinje cell body begins, at this age, to be contacted by large elongated or ovoid-shaped axon terminals corresponding to the boutons en passant established by descending basket axons (BF), and connected by intervaricose segments with much lower density of pleomorphic synaptic vesicles. The arrow points to the synaptic complex between the BF and the Purkinje cell soma.

C) By P12, basket axonal varicosities (BF) and climbing fiber varicosities (CF) are frequently encountered side by side, particularly at the apical half of the Purkinje cell body, the basket fiber (BF) synapsing on the smooth somatic surface (arrowhead), whereas the climbing fiber (CF) synapses on somatic spines (asterisk).

D) This electron micrograph illustrates the close relationship between ascending collaterals of the basket fibers and climbing fibers in their somatodendritic translocation by P15. Note that the basket fiber varicosity (BF) is very close, and at the front of a smaller climbing fiber varicosity (CF), which synapses on a dendritic spine (asterisk), probably belonging to the same dendritic profile giving rise to another large spine (large arrow). The small arrows point to parallel fiber varicosities synapsing on distal spines of Purkinje cell dendrites.

A-D, Scale bars = 1 μ m.

Figure 10: Electron micrographs of P20 rat Purkinje cells illustrating that the somatodendritic translocation of climbing fibers is still unfinished at this age, and that basket and climbing fibers are interlaced during this translocation.

A) Apical half of a Purkinje cell body, giving rise to a small spine (asterisk) contacted by a climbing fiber varicosity (CF), characterized by its content of densely packed spherical synaptic vesicles together with a few large granulated vesicles. The synaptic complex is of the asymmetrical type. At the bottom left of the micrograph, a basket axon varicosity (BF) synapses on the smooth surface of the cell body. The symmetrical synaptic complex is marked by an arrow. Thin astrocytic processes (g) cover a large area of the smooth surface of the Purkinje cell body.

B) Two axonal varicosities belonging to the same climbing fiber are synapsing on somatic spines (arrows). The smooth surface of the Purkinje cell perikaryon is covered by astrocytic processes (g). Thus, although perisomatic climbing fiber varicosities are scarce at this age, their presence testifies that the somatodendritic translocation of these fibers is unfinished by P20.

C) Note that at the apical pole of the Purkinje cell body, a border region between perikaryon and dendrite, two basket axon varicosities (BF) are flanking a climbing fiber varicosity (CF), which is synapsing on a spine (asterisk).

D) The correlation between basket fibers (BF) and climbing fibers (CF) persists even during their covering of the main branches of the Purkinje cell dendrite (Den). The climbing fiber varicosity synapses on a dendritic thorn (asterisk), and is linked to the smooth surface of the dendrite by two attachment plates (arrowheads), while the basket fiber varicosity establishes a symmetrical synapse (arrow) with the smooth surface of the dendrite.

A – D Scale bars = 1µm.