Parasagittal compartmentalization of the metabotropic glutamate receptor mGluR1b in the cerebellar cortex

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Summary

The parasagittal distribution of the splice variant 1b of the metabotropic glutamate receptor 1 (mGluR1b) was studied in the cerebellar cortex by using a specific mGluR1b antiserum combined with immunocytochemical methods. In frontal cerebellar sections, the antibodies revealed alternating bands of immunopositive and immunonegative Purkinje cells in lobules I to X of the vermis. In these regions, the distribution of mGluR1b was complementary to longitudinal bands of Purkinje cells expressing Zebrin II. The width of the mGluR1b-positive bands decreased progressively in the rostro-caudal direction, contrary to the rostro-caudal increase in the width of the Zebrin-positive bands. In the hemispheres, mGluR1b-positive bands alternated with Zebrin II-immunopositive bands in the paravermal portions of Crus I and Crus II. MGluR1b and Zebrin II immunoreactivities often colocalized in other regions of the hemispheral lobules.

The compartmentalization of mGluR1b suggests that adjacent Purkinje cells might respond differently depending on the presence of mGluR1b at parallel fiber-Purkinje cell synapses.

Key Words: Isoforms – Zebrin – Immunocytochemistry – Cerebellum – Purkinje cell

Introduction

Purkinje cells are central elements in the modular organization of the cerebellar cortex. Their dendritic arborizations are oriented parasagittally and receive information from parallel fibers and climbing fibers, and their axons project to the deep cerebellar and vestibular nuclei. Afferent and efferent systems are topographically organized in zones that extend along the anterior-posterior axis across the cerebellar lobules. Histochemical and immunocytochemical studies have identified a number of molecules localized in different parasagittal bands of Purkinje cells (Chan-Palay et al., 1981; Hawkes et al., 1985; Ingram et al., 1985; Boegman et al., 1988; Eiseman and Hawkes, 1989,1993; Leclerc et al., 1989,1990,1992; Brochu et al., 1990; Nair et al., 1993; for review see: Herrup and Kuermele, 1997). Zebrin II, the brain-specific glycolytic isoenzyme aldolase C, has been detected by using a monoclonal anti-Zebrin II antibody (Ahn et al., 1994) in alternating parasagittal bands of immunopositive and immunonegative Purkinje cells (Hawkes et al., 1985; Brochu et al., 1990; Hawkes and Gravel, 1991).

From the neurochemical viewpoint, the cerebellar afferent and efferent systems are of different nature. Whereas mossy, climbing and parallel fibers are excitatory (Somogyi et al., 1986;

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Zhang et al., 1990; Grandes et al., 1994a), Purkinje cells are inhibitory. Glutamate is the main excitatory neurotransmitter in the brain (Hollmann and Heinemann, 1994), and its excitatory actions are mediated by two classes of glutamate receptors: ionotropic, which are ligand-gated cationic channels, and metabotropic, coupled to second messengers via G-proteins. So far, there is no evidence for a preferential distribution of glutamate receptors in cerebellar zones associated with any particular afferent or efferent systems. The GABAB-R1b receptor, however, has been shown to localize in Purkinje cells expressing Zebrin II (Fritschy et al., 1999).

As to the mGluRs family, eight subtypes have been identified and named mGluR1 to mGluR8 (for review: Conn and Pin, 1997). MGluR1 presents alternative splice variants with differences in the length and sequence of their intracellular carboxy-terminal domains. Purkinje cells express high levels of mRNA for distinct mGluR1 isoforms (Berthele et al., 1998). Ultrastructural studies have demonstrated a perisynaptic localization of mGluR1a in dendritic spines of Purkinje cells contacted by parallel fiber synaptic terminals (Baude et al., 1993; Nusser et al., 1994). Furthermore, the distribution of mGluR1b at perisynaptic and extrasynaptic positions has also been demonstrated in Purkinje cell dendritic spines (Mateos et al., 2000). Under the light microscope, however, mGluR1a is localized in all Purkinje cells, while mGluR1b staining intensity varies in the cerebellar lobules (Grandes et al., 1994b). To investigate whether this distinct expression of mGluR1b reflects a compartmentalization of the receptor in certain populations of Purkinje cells expressing or not Zebrin II, we used immunocytochemical methods for the localization of mGluR1b and Zebrin II. The results revealed a compartmentalization of mGluR1b in parasagittal bands of Purkinje cells complementary to the distribution of Zebrin II.

Materials and methods

The protocols for animal care and use were according to the European Communities Council Directive (85/609/EEC). Three adult albino rats (*Sprague Dawley)* were anaesthetised with sodium pentobarbital (60mg/kg body weight, intraperitoneal injection) and perfused transcardially with phosphate-buffered saline (PBS; pH 7.4, at room temperature) for twenty seconds followed by 1 liter of a fixative containing 4% formaldehyde and 0.2% picric acid in ice-cold 0.1 M phosphate buffer (PB; pH 7.4) for 10-15 minutes. Cerebella were removed from the skulls and transferred first to a 15% (wt/vol) sucrose solution (0.1 M; pH 7.4) and later to a 30% sucrose solution after the tissue had sunk. Frontal 30 µm-thick cerebellar serial sections were cut on a cryotome, rapidly placed in plastic vials containing PBS, and subsequently preincubated in 1.5% normal goat serum in PBS for 1 hour at room temperature.

Sets of sections were incubated with the mGluR1b antiserum (1:500) or with the anti-Zebrin II antibodies (1:1,000; Hawkes et al., 1985) overnight at room temperature. Then, the tissue was processed using a conventional avidin-biotin horseradish peroxidase complex method (ABC Elite; Vector Laboratories, Burlingame, CA, USA). Briefly, sections were incubated sequentially with a biotinylated secondary antibody and with an avidin-biotin complex, each for 1 hour at room temperature. Next, sections were preincubated with 0.05% 3,3'-diaminobenzidine for 5 minutes and then incubated for 5 minutes by adding 0.01% hydrogen peroxide to the same solution. Stained sections were mounted, dried, dehydrated and coverslipped with DPX (Fluka Chemie AG, Switzerland).

For colocalization of mGluR1b and Zebrin II, an immunofluorescence method was used. Frontal cerebellar sections were incubated simultaneously with the polyclonal anti-mGluR1b antibodies (1:250) and the monoclonal anti-Zebrin II antibody (1:50 in 1.5% normal goat serum/PBS) overnight at room temperature. Following this, the tissue was incubated with Cy3-conjugated goat anti-rabbit (1:100; Jackson Immunoresearch Laboratories, Inc. PA, USA) and fluorescein (DTAF)-conjugated goat anti-mouse (1:100; Jackson Immunoresearch Laboratories, Inc. PA, USA) in PBS for 1 hour at room temperature. Then, sections were mounted, dried and coverslipped with Glycergel (Dako Corp., CA, USA).

The mGluR1b antiserum has been extensively characterized, showing a high specificity for mGluR1b on immunoblots of transfected HEK 293 cells (Ferraguti et al., 1998; Mateos et al., 1998) and membrane preparations from rat, wild-type and Knockout-mGluR1 mice, as well as on immunoblots of synaptosomes (Ferraguti et al., 1998). The immunocytochemical pattern yielded by this antiserum in rat and wild-type mouse is absent in cerebellar cortices of Knockout-mGluR1 mice (Mateos et al., 2000).

Results

In the sagittal plane, numerous Purkinje cell bodies and the proximal portions of their main apical dendrites expressed mGluR1b, as described previously (Grandes et al., 1994b; Mateos et al., 2000), with strongly immunoreactive Purkinje cells alternating with unlabeled ones. Abundant small mGluR1b-stained dots were also present throughout the molecular

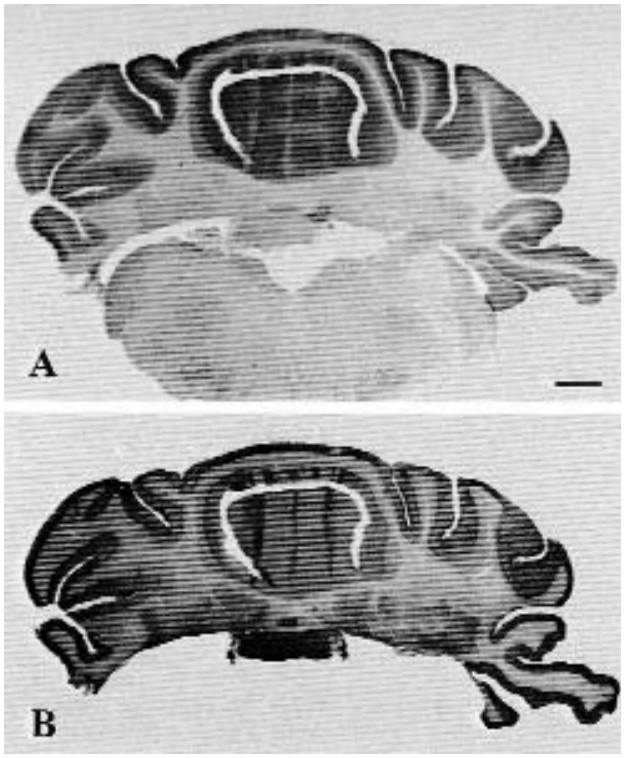

Fig. 1.- Low power micrograph of immunoreactivities for mGluR1b (**A**) and Zebrin II (**B**) in adjacent frontal cerebellar sections, as revealed by an immunoperoxidase technique. Both antigens are distributed in well-delimited parasagittal bands with complementary patterns in vermis, whereas complementarity is less evident in the hemispheres. Scale bar: 1,000 μm.

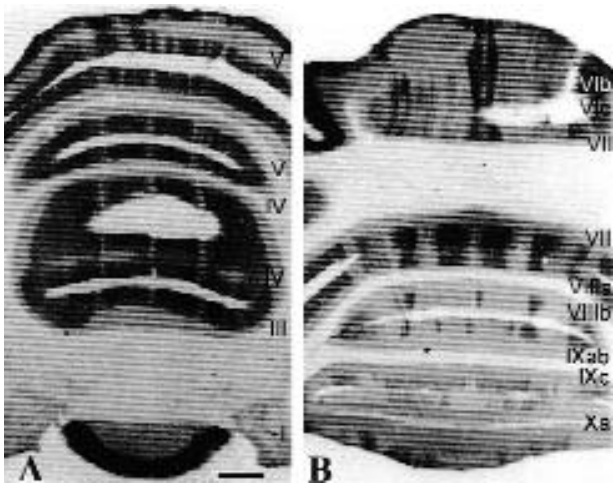

Fig. 2.- Micrographs of frontal cerebellar sections from rostral (**A**) and caudal (**B**) levels, illustrating the rostrocaudal gradient in the thickness of mGluR1b-immunoreactive bands. Roman numerals indicate vermal lobules. Purkinje cells contained in mGluR1b-immunoreactive bands are more numerous in rostral lobules I-V (A) than in caudal lobules VI-X (B). Scale bar: 500 μm.

layer, and Golgi cells in the granule cell layer were positive for mGluR1b.

In the frontal plane, mGluR1b displayed a zonal distribution in the cerebellar lobules (Fig. 1A). The main feature of the immunostaining was the presence of mGluR1b in groups of Purkinje cell bodies and their dendritic ramifications in the molecular layer, which were adjacent to non-immunoreactive Purkinje cell groups. As observed in serial sections stained with antibodies to mGluR1b and Zebrin II, the distribution of mGluR1b in the vermis was complementary to the Zebrin II-positive areas. MGluR1b was expressed in vermal Purkinje cells of lobules I to X, and was organized in longitudinal bands negative for Zebrin II (Fig. 1B). Another feature of the vermal distribution of mGluR1b was the progressive rostro-caudal reduction in the width of mGluR1b-positive bands, which correlated with an enlargement of that of the adjacent Zebrin-II bands. Thus, immunoreactive bands were composed of a higher number of mGluR1b-expressing Purkinje cells in rostral sections. In fact, the caudal portions of the mGluR1b bands were made up of just a few positive cells (Fig. 2). In the hemispheres, mGluR1b-positive bands were observed exclusively in the Zebrin II-immunonegative paravermal regions of Crus I and Crus II. Beyond these regions, Zebrin II and mGluR1b immunoreactivities were found to coincide in the same hemisphere portions.

The use of double immunofluorescence techniques confirmed the non-overlapping patterns of mGluR1b-immunoreactive Purkinje cells and the neighbouring cells labeled for Zebrin II but not for mGluR1b (Fig. 3). The alternation of mGluR1b immunopositive and immunonegative Purkinje cell bands was remarkable in the vermal lobules, but in the hemispheres it was apparent only in Crus I and Crus II (Fig. 4). MGluR1b immunofluorescence was also observed in Golgi cells (Fig. 3).

Discussion

Compartmentalization of the cerebellar cortex

In this study, we have demonstrated a topographical distribution of mGluR1b in the cerebellar cortex. Thus, groups of mGluR1b-positive Purkinje cells organized parasagittally within rostro-caudal longitudinal bands alternated with mGluR1b-negative/Zebrin II-positive Purkinje cell strips. Early studies had demonstrated Zebrin-negative Purkinje cells containing different molecules, such as the P-path antigen, an epitope of 9-*O*-acetylated glycolipids (Leclerc et al., 1992), the microtubule-associated protein MAP-1 (Touri et al., 1996), and dystrophin, the protein product of the Duchenne muscular dystrophy gene found in postsynaptic densities of Purkinje cell dendritic spines (Blake et al., 1999). In contrast, acetylcholinesterase (Boegman et al.,

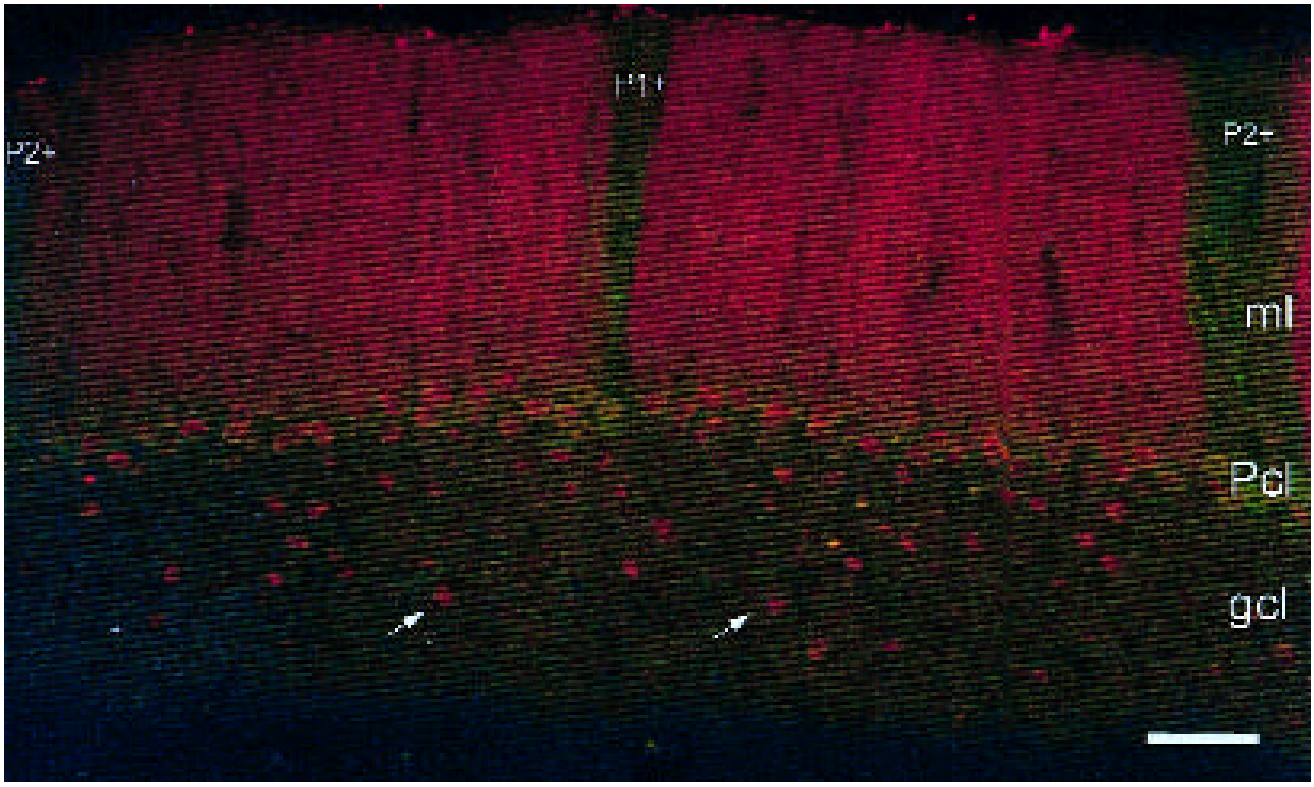

Fig. 3.- Double fluorescent immunolabeling showing the complementary distribution patterns for mGluR1b (red) and Zebrin II (green) in a frontal section from the vermal cerebellar cortex. MGluR1b is expressed in the Purkinje cell layer (Pcl), as well as in the molecular layer (ml) and in numerous Golgi cells (arrows) in the granule cell layer (gcl). In contrast, cell bodies and dendritic arborizations of Purkinje cells and the molecular layer neuropil contained in mGluR1b-negative bands are reactive to the antiserum against Zebrin II (P1+ and P2+). Scale bar: 50 μm.

1988), 5´-nucleotidase (Eiseman and Hawkes, 1989), cytochrome oxidase (Leclerc et al., 1990) and nitric oxide synthase (Hawkes and Turner, 1994) have been found in Zebrin-immunopositive zones. More recently, parasagittal bands of rodent Purkinje cells have been shown to contain the 25-kDa heat shock protein Hsp25 (Armstrong et al., 2000) with a different expression pattern during development (Armstrong et al., 2001), and protein kinase C-δ has been reported to be restricted to Purkinje cells in the posterior cerebellar cortex (Barmack et al., 2000). Furthermore, a longitudinal zonal distribution of mossy and climbing fibers as well as corticonuclear and corticovestibular efferent projections have been observed in the cerebellum. Interestingly, there are some indications of a relationship between Zebrin-positive bands and patterns of afferent and efferent projections. For instance, sensory mossy fibers carrying information from the rat upper lip preferentially end in Zebrin-positive regions in Crus II of the ansiform lobule (Hallem et al., 1999), and spinocerebellar mossy fibers coincide with Zebrin distribution in lobule IV (Gravel and Hawkes, 1990). Climbing fibers from different nuclei of the inferior olivary complex terminate on Purkinje cell dendrites within Zebrin-positive or Zebrin-negative bands (Gravel et al., 1987). Also, cerebellar projections to the deep cerebellar and vestibular nuclei are organized parasagittally in longitudinal strips extending from the vermis to the hemispheres (for review: Voogd, 1995).

Neurotransmitter localization in the cerebellar cortex

The existence of a topographical relationship between these afferent projections and the neurochemical compartmentalization in the cerebellar cortex is probable. Climbing and mossy fibers exert an excitatory effect on their postsynaptic targets. However, the identity of their neurotransmitters has been a matter of debate for a long time (for review: Cuénod et al., 1989). Whereas aspartate was the neurotransmitter candidate for climbing fibers (Wiklund et al., 1982), acetylcholine was the one for at least some mossy fibers (Garthwaite and Garthwaite, 1988). Currently, much of the evidence favours a role for glutamate as a neurotransmitter in both afferent fibers (Somogyi et al., 1986; Liu et al., 1989; Ottersen et al., 1990, 1992; Zhang et al., 1990; Grandes et al., 1994a).

In some cases, the distribution of neuroactive substances in climbing and mossy fibers is compartmentalized. Thus, glutamate-like immunoreactive climbing fibers have been reported to be localized in vermal lobule VI and in Crus I and

Crus II of the ansiform lobule (Grandes et al., 1994a), and cholinergic mossy fibers have been detected in Zebrin-immunoreactive bands (Boegman et al., 1988).

Subsynaptic distribution of receptors in Purkinje cell dendritic spines

The hodological and neurochemical compartmentalization in the cerebellar cortex prompts one to speculate on the existence of a zonal distribution of neurotransmitter receptors. There is only one report available demonstrating an extrasynaptic localization of the GABAB-R1b receptor in the dendritic spines of Zebrin-immunopositive Purkinje cells (Fritschy et al., 1999). Regarding glutamate receptors, the present results provide the first evidence for a zonal distribution in the cerebellar cortex. We have previously demonstrated a perisynaptic and extrasynaptic localization of mGluR1b in dendritic spines of Purkinje cells receiving synapses from parallel fibers (Mateos et al., 2000). By contrast, mGluR1a is placed perisynaptically on the same synapses (Baude et al., 1993; Nusser et al., 1994; Mateos et al., 2000). While mGluR1a is found in Purkinje cells throughout the cerebellar lobules, mGluR1b is restricted to longitudinal bands that are immunonegative for Zebrin, as shown here. In the vermal lobule VIII, the Zebrin-negative Purkinje cells receiving olivocerebellar fibers from the caudal part of the medial accesory, dorsal accesory and principal olive (Gravel et al., 1987) express mGluR1b. These olivary neurons in turn receive information from the spinal cord, dorsal column nuclei and higher centers, and hence mGluR1b may be critically positioned with respect to these particular olivocerebellar fibers, suggesting a role for mGluR1b in the modulation of specific cerebellar information.

Possible functional aspects of the zonal distribution of mGluR1b

Climbing fiber activity evokes a powerful excitation in Purkinje cells. Activation of voltage-dependent calcium channels by climbing fibers results in a prolonged potentiation of the mGluR-mediated EPSP in Purkinje cells elicited by parallel fiber activation (Batchelor and Gartwaite, 1997). MGluR1b present perisynaptically and extrasynaptically in certain longitudinal strips of parallel fiber-Purkinje cell synapses (Mateos et al., 2000) might be a candidate participant in the integration of non-coincident signals through the transient rise in calcium concentration elicited by climbing fibers in Purkinje cells (Batchelor and Gartwaite, 1997).

These interactions between climbing and parallel fibers might have consequences in the induction of long-term depression (LTD) of the excitatory synaptic transmission at the parallel

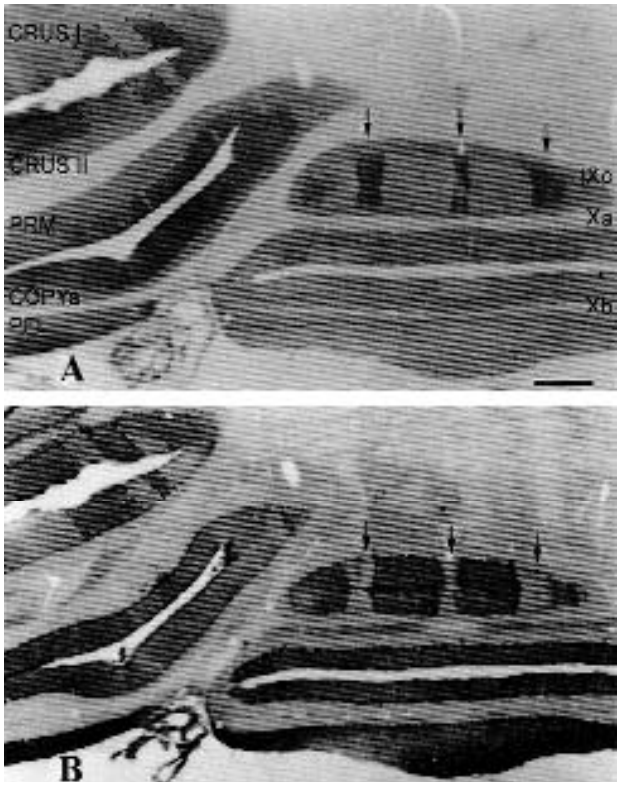

Fig. 4.- Complementarity of immunoreaction patterns for mGluR1b (**A**) and Zebrin II (**B**) in adjacent frontal sections comprising vermal and hemisphere areas. MGluR1b and Zebrin II are expressed in well-delimited, complementary bands (arrows) in vermal lobules. Although band boundaries are not clear-cut in the hemispheres, immunoreactive bands still exhibit an alternating pattern (dashed lines in CRUS I and CRUS II). PRM: paramedian lobule; COPYa: copula pyramidis, sublobule a; PFL: paraflocculus. Scale bar: 500 µm.

fiber-Purkinje cell synapses. This phenomenon, which is thought to be the cellular basis of motor learning (Ito, 1989), involves the activation of AMPA and mGluR1 receptors at parallel fiber-Purkinje cell synapses, and activation of voltage-dependent calcium channels by climbing fibers. Furthermore, the expression of LTD requires internalization of postsynaptic AMPA receptors (Wang and Linden, 2000). MGluR1b exhibits a rapid internalization and distinct functional properties (Flor et al., 1996; Prézeau et al., 1996, Ciruela and McIlhinney, 1997). At present, it is not known whether the presence or absence of mGluR1b in certain populations of Purkinje cells might be related to the synaptic influence exerted by particular climbing fiber inputs on the parallel fiber-Purkinje cell synaptic transmission. A recent study has demonstrated the distribution of the high affinity excitatory amino acid transporter EAAT4 in dendritic spines of Purkinje cells at extrasynaptic sites of parallel fiber synapses. Interestingly, EAAT4 is present in Purkinje cells expressing Zebrin II (Dehnes et al., 1998). It is possible that the release of glutamate by high

frequency stimulation of parallel fibers would be able to activate the extrasynaptic mGluR1b distributed in Zebrin II-negative Purkinje cells where EAAT4 is not present.

In conclusion, the presence of mGluR1b in Zebrin-negative Purkinje cells adds a new piece to the puzzle of the zonal compartmentalization of Purkinje cell phenotypes and connectivity in the cerebellar cortex.

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