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Short communication

Spinocerebellar mossy fiber terminal topography in the NR2C/PKCγ double mutant cerebellum

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Abstract

The spatiotemporal expression patterns of the NR2C subunit of the NMDA receptor and PKCγ isoform during cerebellar development suggests that both proteins are involved in the molecular mechanisms of synaptogenesis. However, the topographic distribution of WGA-HRP labeled spinocerebellar mossy fiber terminals in NR2C/PKCγ double mutants ($n = 4$) appears similar to controls ($n = 3$). While the results do not rule out a role for NR2C receptor subunits and the PKCγ isoform in cerebellar synaptogenesis, they indicate that neither is necessary for the formation or maintenance of normal spinocerebellar mossy fiber afferent maps.

Keywords: Transgenic mutant; Granule cells; Purkinje cells; PKCγ; NR2C; Pattern formation; Synaptogenesis

Mossy fibers project to the cerebellar cortex in distinctive topographic projection patterns. Although mossy fibers ultimately synapse on granule cell dendrites, the available evidence suggests that the topography of mossy fiber projections is initially organized by Purkinje cells [24]. Mossy fibers first appear in the cerebellum by E13–14 [13] and initially segregate into protocolumns that align with clusters of Purkinje cells [3,18]. After granule cells migrate into the internal granule cell layer, mossy fibers begin to synapse on the maturing granule cells [20]. The formation of granule cell-mossy synapses may be important in the stabilization of mossy fiber terminals; if the formation of the internal granule cell layer is disrupted by partial ablation of the external granule cell layer with MAM, mossy fiber terminals become more homogeneously distributed within the vermal lobules [17]. Neuronal activity, and in particular activation of NMDA receptors, may play an important role in synapse stabilization in the cerebellum since both the removal of climbing fiber polyneuronal innervation of Purkinje cells and the refinement of mossy fiber terminal fields may be disrupted by chronic blockade of cortical activity with the competitive NMDA receptor antagonist AP5 [21,25].

To analyze the role of activity-dependent mechanisms for synapse stabilization in the formation and maintenance of mossy fiber topographic projections, we have analyzed the distribution of spinocerebellar mossy fiber terminals in mutant mice with genetic knockouts of the NR2C subunit of the NMDA receptor [11] and the γ subtype of calcium-phospholipid-dependent protein kinase (PKCγ) [1]. Synapse stabilization is thought to involve NMDA receptor stimulation caused by coincident activation of the postsynaptic cell by neighboring afferents [8]. NMDA receptor stimulation increases intracellular calcium levels and activates second messenger systems which lead to synapse stabilization. The mechanisms of synapse stabilization may be similar to the mechanisms proposed for long-term potentiation (LTP). LTP induction is believed to involve the modulation of NMDA receptors by protein kinase C (PKC) [5].

We have chosen to focus our analysis on deletions of NR2C and PKCγ isoforms because their spatiotemporal expression patterns in the cerebellum suggest that they may be involved in cerebellar synaptogenesis [16,23,28]. The NR2C subunit is preferentially expressed in granule cells in the postnatal cerebellum, with levels of NR2C increasing from P11 to P20 concomitant with decreases in

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the levels of NR2B. The physiological properties of granule cells change as they mature, and the postnatal replacement of NR2B subunit expression by NR2C subunit expression may underlie these changes [12]. In the NR2C knockout mutant, the excitatory postsynaptic currents of mature granule cells are similar to those in immature granule cells indicating that NR2C subunit expression is important in granule cell maturation [11]. PKCγ is preferentially expressed in Purkinje cells, but it may also be transiently expressed in cerebellar granule cells during early cerebellar development with increased activity levels in cerebellar synaptosomes [15,16,23]. LTP is greatly diminished in PKCγ knockouts mutants, although not completely blocked, which suggests that PKCγ may be involved in the regulation of LTP but not the actual molecular mechanisms for generating LTP [1]. There is evidence that the removal of climbing fiber-Purkinje cells polyneuronal innervation is impaired in PKCγ knockout mice [19]. The distribution of mossy fibers in NR2C/PKCγ knockout mutants was analyzed in this study to determine if PKCγ activity is necessary for the formation of mossy fiber projection patterns and if the change in the properties of NMDA receptor currents in maturing granule cells is necessary to stabilize mossy fiber terminals.

NR2C and PKCγ knockout mutant mice were generated as described previously using standard techniques for homologous recombination with ES cell technology [1,11]. To generate the NR2C null mutant, one of the four putative transmembrane segments was replaced with a neomycin resistance cassette [11]. The PKCγ null mutant was generated by replacing the exon containing the nucleotide-binding domain required for catalytic activity [1]. Mice homozygous for the NR2C and PKCγ mutations were mated to generate NR2C/PKCγ double mutants. The distribution of spinocerebellar mossy fiber terminals was analyzed as described previously [26]. Adult mutant ($n = 4$) and wild type adult mice ($n = 3$) were anesthetized with i.p. injections of Avertin. 0.2–0.5 μl of wheat germ agglutinin conjugated horseradish peroxidase (WGA-HRP; 2% in distilled water) was bilaterally injected into the lower thoracic-upper lumbar spinal cord following a dorsal laminectomy. The animals were killed 18–24 h later by cardiac perfusion under deep Avertin anesthesia with 30–50 ml of 0.9% warm saline followed by 50–80 ml 4% cold paraformaldehyde in 0.1 M phosphate buffer solution (PBS). Cerebella were cryoprotected in 20% sucrose-PBS overnight and then serially sectioned at 32 μm in coronal cryostat sections. Orthogradely labeled mossy fiber terminals were visualized using the deOlmos et al. [10] modification of the TMB incubation procedure.

The NR2C/PKCγ double mutant mice showed no gross ataxia, but behavioral tests have shown that motor coordination and spatial and contextual learning is affected in PKCγ mutants [2,6], so the double mutant mice may have similar defects. NR2C/PKCγ double mutant cerebella were similar in size to wild type cerebella and foliation patterns did not differ between wild type and mutants. Spinocerebellar mossy fibers from the lower thoracic-upper lumbar level of the spinal cord in the mutants terminated in the normal spinocerebellar projection fields (Fig. 1): lobules I–V in the anterior cerebellum and VIII and the dorsal half of lobule IX in the posterior cerebellum [27]. Although the strength and density of the labeling varied from one animal to another, the general topographic distribution pattern of WGA-HRP labeled terminals in the mutants was similar to that in wild type mice. Mossy fiber terminals segregate into parasagittal bands that can be followed through anterior and posterior vermal lobules along the anterior-posterior axis of the cerebellum. Fig. 2 shows columns of mossy fiber terminals in coronal sections at two levels of the anterior cerebellum (A, B, D, E) and one level of the posterior cerebellum (C, F) in both a control cerebellum (left column, A–C) and a double mutant cerebellum (right column, D–F). The mossy fiber columns in both control and mutant cerebella are symmetrical about the midline, with a relatively weakly labeled midline band compared to the more densely labeled medial and lateral bands on either side. The position and width of the bands in mutants are similar to those of the control. In the posterior lobe, lobule VIII is the main projection area for spinocerebellar terminals area. At the anterior-posterior level of the coronal sections shown in Fig. 2C, there are two symmetrical compartments on the right and left sides (indicated by asterisks) of the vermis, flanked by a more lateral patch of terminal labeling in the vermis. In addition to the medial and lateral vermal bands shown in Fig. 2C, there is a midline band in control and mutant lobule VIII that is not detectable at this level. Lobule IX normally receives a smaller projection to its dorsal half that is split at the midline along part of its anterior-posterior extent

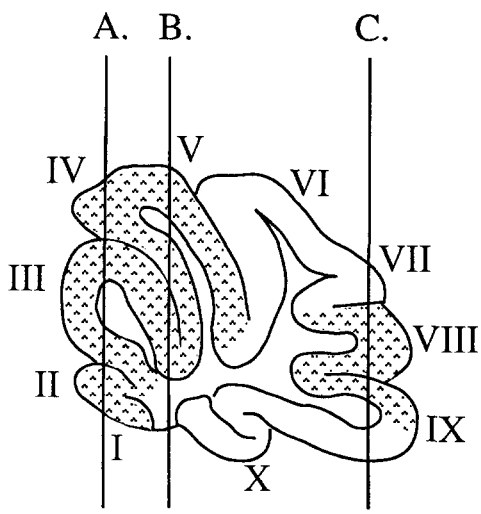

Fig. 1. Diagrammatic representation of the lobular distribution of spinocerebellar mossy fiber terminals in the vermis of wild type cerebella. Mossy fiber terminals are found in lobules I–V, VIII, and the dorsal half of IX. The bars labeled A, B, and C show the planes of section for the coronal views of mossy fiber terminal labeling in Fig. 2.

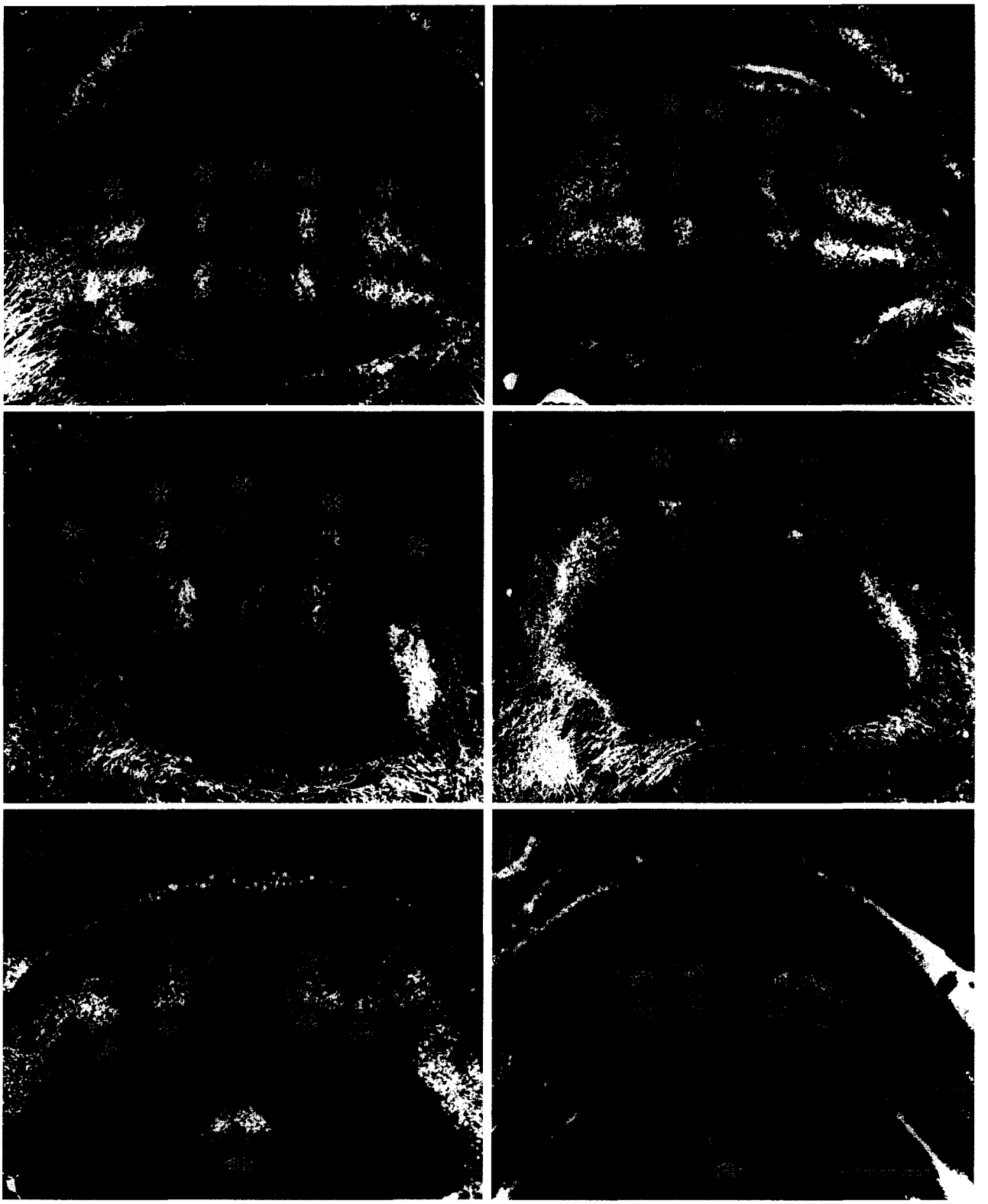

Fig. 2. Spinocerebellar mossy fiber topographic patterns compared between a control cerebellum (left column) and a NR2C/PKCγ mutant (right column) in darkfield photomicrographs. The asterisks indicate columns of WGA-HRP labeled spinocerebellar mossy fiber terminals in the granule cell layer. The arrows in the posterior sections (C) indicate the single, midline column of labeled terminals characteristic of the dorsal aspect of lobule IX. Coronal sections were selected from levels A and B of the anterior cerebella and C of the posterior cerebella as shown in Fig. 1. Scale bar, 500 μm.

[27]. A similar pattern of mossy fiber terminal distribution was observed in the mutant lobule IX.

The results show that the topography of mossy fiber terminal distribution is maintained despite the deletion of the genes for the NR2C receptor subunit and PKCγ. The results argue that neither NR2C nor PKCγ is required for the formation and maintenance of anatomically normal mossy fiber topographic maps. Two possible interpretations are either that: (1) activity-dependent mechanisms are not necessary for the segregation of mossy fiber terminals, or (2) activity-dependent mechanisms participate in the segregation of mossy fiber terminals, but NR2C and PKCγ are either not involved or there are sufficient redundancies in the molecular mechanisms for synapse stabilization to compensate for the lack of NR2C and PKCγ. We favor the second interpretation. Previous studies have shown that NMDA receptor blockade delays the removal of climbing fiber polyneuronal innervation [21], and interferes with the refinement of spinocerebellar mossy fiber projections [25]. In the NR2C/PKCγ double mutants, it is unlikely that NMDA receptor activation or PKC activity is completely blocked. The failure to express the NR2C subunit in granule cells of −/− NR2C mutants results in the loss of low conductance NMDA receptors [11]. Granule cells are still activated by mossy fiber inputs in adult NR2C −/− mutants, but the composite excitatory postsynaptic currents (EPSCs) from NMDA and AMPA receptors resemble those found in immature granule cells. Regardless of whether or not NMDA receptor activation is critical for in the initial refinement of mossy fiber projection maps, our results indicate that NR2C receptor expression is not necessary to maintain normal projection patterns.

With respect to PKC activity, other PKC isomers are expressed in the cerebellum. Previous studies of knockout mice have shown that it is important to consider the possibility that a related enzyme can compensate for the missing gene [9]. As assayed in whole rat brain homogenates, PKC type I activity (γ sequence) is initially detected only during the first week of postnatal development, but PKC type II activity (from βI and βII sequences) is present in the CNS at birth, and increases rapidly to plateau levels by P14–28 [15]. PKC type II activity in rat cerebellar tissue and synaptosomal extracts is higher than PKC type I activity levels at P7, but activity levels of PKC type II decline with age as tissue extract levels of PKC type I activity rise [23]. In the adult cerebellum, PKCγ expression is restricted to Purkinje cells, but enzyme activity measurements [23] and mRNA expression studies [16] show that PKCγ is transiently expressed at higher levels in presynaptic terminals and cerebellar granule cells during the period of cerebellar synaptogenesis, reaching a peak in activity and expression levels around P15. PKCβI is also expressed in granule cells and PKCβII is preferentially localized to the molecular layer, possibly in the nerve terminals presynaptic to Purkinje cell dendrites [4]. Although the deletion of PKCγ does not change expression levels of the α, βI, or βII isoforms of PKC in the adult [14], if these or other isoforms can substitute for the absent PKCγ activity, then activity-dependent mechanisms of synapse stabilization may still function in the establishment or refinement of appropriate mossy fiber projections.

The normal appearance of mossy fiber topography in the NR2C/PKCγ double mutants cannot rule out the possibility that the NR2C receptor subunit and PKCγ are involved in other types of synapse stabilization in cerebellar development. For example, PKCγ may be involved in regulating the removal of climbing fiber polyneuronal innervation of Purkinje cells [19]. There may also be subtle changes in the functional organization of mossy fiber projections or synaptic ultrastructure that are not detected by the anatomical techniques used in this study. Inhibition of NMDA receptors and/or PKC activity has been shown to affect synapse stabilization in topographic map formation in a variety of other experimental systems [7,8,22]. While activity-dependent mechanisms may be important in the development of afferent projections in the cerebellum, the results of this study argue against specific roles for NR2C NMDA receptor subunit or PKCγ expression in the establishment and maintenance of spinocerebellar mossy fiber topographic projections.

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References

[1] Abeliovich, A., Chen, C., Goda, Y., Silva, A.J., Stevens, C.F. and Tonegawa, S., Modified hippocampal long-term potentiation in PKCγ-mutant mice, *Cell*, 75 (1993) 1253–1262.

[2] Abeliovich, A., Paylor, R., Chen, C., Kim, J., Wehner, J. and Tonegawa, S., PKCγ mutant mice exhibit mild deficits in spatial and contextual learning, *Cell*, 75 (1993) 1263–1271.

[3] Arsénio Nunes, M.L. and Sotelo, C., Development of the spinocerebellar system in the postnatal rat, *J. Comp. Neurol.*, 237 (1985) 291–306.

[4] Ase, K., Saito, N., Shearman, M.S., Kikkawa, U., Ono, Y., Igarashi, K., Tanaka, C. and Nishizuka, Y., Distinct cellular expression of βI- and βII-subspecies of protein kinase C in rat cerebellum, *J. Neurosci.*, 8 (1988) 3850–3856.

[5] Ben-Ari, Y., Aniksztejn, L. and Bregestovski, P., Protein kinase C modulation of NMDA currents: an important link for LTP induction, *Trends Neurosci.*, 15 (1992) 333–339.

[6] Chen, C., Kano, M., Abeliovich, A., Chen, L., Bao, S., Kim, J.J., Hashimoto, K., Thompson, R.F. and Tonegawa, S., Impaired motor coordination correlates with persistent multiple climbing fiber innervation in PKC gamma mutant mice, *Cell*, 83 (1995) 1233–1242.

[7] Cline, H.T. and Constantine-Paton, M., The differential influence of protein kinase inhibitors on retinal arbor morphology and eye-specific stripes in the frog retinotectal system, *Neuron*, 4 (1990) 899–908.

[8] Constantine-Paton, M., Cline, H.T. and Debski, E., Patterned activity, synaptic convergence, and the NMDA receptor in developing visual pathways, *Annu. Rev. Neurosci.*, 13 (1990) 129–154.

[9] Crawley, J., Unusual behavioral phenotypes of inbred mouse strains, *Trends Neurosci.*, 19 (1996) 181–182.
[10] DeOlmos, J., Hardy, H. and Heimer, L., The afferent connections of the main and the accessory olfactory bulb formations in the rat: An experimental HRP study, *J. Comp. Neurol.*, 181 (1978) 213–244.
[11] Ebralidze, A.K., Rossi, D.J., Tonegawa, S. and Slater, N.T., The role of the NR2C subunit in NMDA receptor-gated channels in mouse cerebellar granule cells, *Soc. Neurosci. Abstr.*, 21 (1995) 592.
[12] Farrant, M., Feldmeyer, D., Takahashi, T. and Cull-Candy, S.G., NMDA-receptor channel diversity in the developing cerebellum, *Nature*, 368 (1994) 335–339.
[13] Grishkat, H.L. and Eisenman, L.M., Anterograde labeling of spinocerebellar fibers in fetal mouse cerebellum, *Soc. Neurosci. Abstr.*, 20 (1994) 1749.
[14] Harris, R.A., McQuilkin, S.J., Paylor, R., Abeliovich, A., Tonegawa, S. and Wehner, J.M., Mutant mice lacking the γ isoform of protein kinase C show decreased behavioral actions of ethanol and altered function of γ-aminoburyrate type A receptors, *Proc. Natl. Acad. Sci. USA*, 92 (1995) 3658–3662.
[15] Hashimoto, T., Ase, K., Sawamura, S., Kikkawa, U., Saito, N., Tanaka, C. and Nishizuka, Y., Postnatal development of a brain-specific subspecies of protein kinase C in rat, *J. Neurosci.*, 8 (1988) 1678–1683.
[16] Herms, J., Zurmohle, U., Schlingensiepen, K.-H. and Brysch, W., Transient expression of PKCγ mRNA in cerebellar granule cells during rat brain development, *NeuroReport*, 4 (1993) 899–902.
[17] Ji, J. and Hawkes, R., Partial ablation of the neonatal external granular layer disrupts mossy fiber topography in adult the rat cerebellum, *J. Comp. Neurol.*, in press (1996).
[18] Ji, Z. and Hawkes, R., Developing mossy fiber terminal fields in the rat cerebellar cortex may segregate because of Purkinje cell compartmentation and not competition, *J. Comp. Neurol.*, 359 (1995) 197–212.
[19] Kano, M., Hashimoto, K., Chen, C., Abeliovich, A., Aiba, A., Kurihara, H., Watanabe, M., Inoue, Y. and Tonegawa, S., Impaired synapse elimination during cerebellar development in PKC gamma mutant mice, *Cell*, 83 (1995) 1223–1231.
[20] Mason, C.A. and Gregory, E., Postnatal maturation of cerebellar mossy and climbing fibers: transient expression of dual features on single axons, *J. Neurosci.*, 4 (1984) 1715–1735.
[21] Rabacchi, S., Bailly, Y., Delhaye-Bouchaud, N. and Mariani, J., Involvement of the *N*-methyl-D-aspartate (NMDA) receptor in synapse elimination during cerebellar development, *Science*, 256 (1992) 1823–1825.
[22] Schmidt, J.T., C-kinase manipulations disrupt activity-driven retinotopic sharpening in regenerating goldfish retinotectal projection, *J. Neurobiol.*, 25 (1994) 555–570.
[23] Shearman, M.S., Shinomura, T., Oda, T. and Nishizuka, Y., Synaptosomal protein kinase C subspecies: A. Dynamic changes in the hippocampus and cerebellar cortex concomitant with synaptogenesis, *J. Neurochem.*, 56 (1991) 1255–1262.
[24] Sotelo, C. and Wassef, M., Cerebellar development: afferent organization and Purkinje cell heterogeneity, *Phil. Trans. R. Soc. Lond. B*, 331 (1991) 307–313.
[25] Tolbert, D.L., Pittman, T., Alisky, J.M. and Clark, B.R., Chronic NMDA receptor blockade or muscimol inhibition of cerebellar cortical neuronal activity alters the development of spinocerebellar afferent topography, *Dev. Brain Res.*, 80 (1994) 268–274.
[26] Vogel, M.W. and Prittie, J., Segregation of spinocerebellar mossy fiber terminals in *+/Lc* mutant mice, *Soc. Neurosci. Abstr.*, 18 (1992) 947.
[27] Vogel, M.W. and Prittie, J., Topographic spinocerebellar mossy fiber projections are maintained in the *Lurcher* mutant, *J. Comp. Neurol.*, 343 (1994) 341–351.
[28] Zhong, J., Carrozza, D.P., Williams, K., Pritchett, D.B. and Molinoff, P.B., Expression of mRNAs encoding subunits of the NMDA receptor in developing rat brain, *J. Neurochem.*, 64 (1995) 531–539.