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Immunohistochemical localization of calbindin D28-k, parvalbumin, and calretinin in the cerebellar cortex of the circling mouse

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ABSTRACT

The spontaneous mutant circling mouse has an autosomal recessive pattern of inheritance and is an animal model for deafness, which is characterized by circling, head tossing, and hyperactivity. Since the main pathology in circling mice lies in the organ of Corti, most studies on deaf mice have focused on auditory brain stem nuclei. No studies regarding behavior-related CNS changes in circling mice have been reported. The major center of sensory input for modulation of motor activity is best-studied in the cerebellum. Considering the importance of calcium homeostasis in numerous processes, calcium-binding proteins (CaBPs), such as calbindin D-28k (CB), parvalbumin (PV), and calretinin (CR), may play crucial roles in preserving cerebellar coordinated motor function. Thus, the distribution of CB, PV, and CR was determined in the cerebellum using immunohistochemical methods to compare immunoreactivity (IR) of CaBPs between wild-type (+/+), heterozygous (+/cir), and homozygous (cir/cir) mice. The IR of CB and PV was predominantly observed in the Purkinje cell layer of all three genotypes. Compared with the +/+ genotype, the relative mean density of CB and PV IR in the Purkinje cell layer and CR IR in the granular layer was significantly decreased in the cir/cir genotype. Changes in calcium homeostasis in parallel fiber/Purkinje cell synapses could diminish cerebellar control of motor coordination. A number of deficiencies among the CaBPs lead to distinct alterations in brain physiology, which may affect normal behavior.

The circling (cir) mouse is a recently developed animal model for autosomal recessive deafness [19]. In cir mice, the deafness is inherited with 100% penetrance [6,7]. The main pathology in cir mice lies in the organ of Corti, showing degeneration of hair cells and spiral ganglion cells [19]. Apart from hearing loss, the cir mouse also exhibits circling behavior, head tossing, and hyperactivity. Such characteristic features of cir mice is displayed by homozygous (cir/cir), but not by heterozygous (+/cir) mice. Recently, neurotransmitter alterations in the auditory brain stem nuclei have also been observed [16]. In the medial nucleus of the trapezoid body (MNTB), lateral superior olive nucleus (LSO) synapses, which are known as inhibitory glycinergic synapses in adult rodents [30], release glutamate as the main neurotransmitter from birth [14]; however, release of glutamate in cir mice is sustained at a later period of development [16]. These pathologic changes are consistent with transient and persistent alterations in the regulation of neurotransmitter release from glutamatergic [23], glycinergic [24,30], and GABAergic pathways [24] by unilateral cochlear ablation and/or artificial removal of the middle ear ossicle. The alterations in developmental changes of neurotransmitter release in cir mice suggest the possibility that such pathologic changes might not be limited in auditory brain stem circuits. The circling behavior might be correlated with the motor system in the central nervous system (CNS); however, no studies about behavior-related CNS changes in cir mice have been reported.

The major center of sensory input for modulation of motor activity is best-studied in the cerebellum [11], in which the gross- and micro-anatomic connections with the rest of the brain are well-understood. In some types of single gene-manipulated mice [18,31], the sensory inputs of the auditory brainstem have been suggested to have an intimate connection with the cerebellar afferent system, which is involved in motor coordination. For example, similar to the pathologic traits in cir/cir mice, targeted disruption of the *frizzled*-4 gene, which is involved in transducing *Wnt* signals and is widely expressed in cerebellar Purkinje cells (PCs), results in characteristic cerebellar ataxia and auditory dysfunction in mice [31]. The plasma membrane Ca^{2+}-ATPase isoform 2 (PMCA2) null mouse also exhibits similar abnormalities involving the organ of

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Corti and an absence of otoconia in the vestibular system [18]. Likewise, double mutant mice for PMCA2 and the transient receptor potential channel, TRPML3, have severe circling behavior, which is not observed in mice with single gene defects, and has inner ear sensory hair cell pathology as well [15]. These studies revealed that a single defective gene causes not only a single organ abnormality, such as deafness, but also other organ dysfunction (e.g., Ca^{2+} concentration of PCs).

Considering the importance of calcium homeostasis in numerous processes for cell viability, including neurons, modulation of intracellular calcium concentration by calcium-binding proteins (CaBPs), such as calbindin D-28k (CB), parvalbumin (PV), and calretinin (CR), could play crucial roles in preserving cerebellar coordinated motor function. Alteration of CaBP expression has been suggested to be involved in a number of neuronal disorders [12,27,29]. The precise functions of CaBPs are unknown, but the neuroprotective role of these CaBPs has been proposed, which is controversial [2].

However, on the basis of the circling behavior in cir/cir mice, there has been no trial to investigate the involvement of CaBPs in the cerebellum. Therefore, for the first time, we investigated the distribution of CB, PV, and CR in the cerebellar cortex by using immunohistochemistry to compare CB, PV, and CR immunoreactivity (IR) between wild-type (+/+), heterozygous (+/cir), and homozygous (cir/cir) mice.

In the present study, 15 C57Bl/6J mice (+/+, $n = 5$; +/cir, $n = 5$; and cir/cir, $n = 5$) were used. All animal procedures were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised in 1996). Postnatal day 16 (P16) +/cir and cir/cir mice were obtained from mating cir/cir males with +/cir females. To prevent genetic mixing, both +/cir and cir/cir mice were completely separated from +/+ mice, which were purchased from Samtako (Osan, Korea). Polymerized chain reaction (PCR) analysis was performed to differentiate +/cir and cir/cir mice using genomic DNA obtained from the tails of mice. The genomic DNA was isolated according to the manufacturer's instructions (Bioneer, Daejeon, Korea). Identification of cir/cir mice was assured by absence of the *tmie* gene [10]. PCR was performed with primers designed to amplify the exon 1 coding region of the *tmie* gene (forward, 5′ AGCTGTAGCTCTGAAATCT 3′; reverse, 5′ TCTGGCAGAATGCATGGAGGCT 3′) [9]. One hundred nanograms of template DNA was used in a final reaction volume of 20 μl containing 10 mM Tris–HCl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, 250 mM dNTP, 20 pmol of each primer, and 1 U Taq DNA polymerase (Bioneer Corporation, CA, USA). PCR was carried out in a thermal cycler (C1000TM; Bio-Rad, Singapore). The PCR was performed in two steps. The first step consisted of denaturation at 96 °C for 5 min, annealing at 59 °C for 1 min, and extension at 72 °C for 1 min at the end of 4 cycles. The second step included denaturation at 96 °C for 1 min, annealing at 59 °C for 1 min, extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min at the end of 30 cycles. Electrophoresis was performed at 93 V for 1 h at 25 °C to identify the amplification fragments (supplementary data).

For immunohistochemistry, the mice were deeply anesthetized and perfused transcardially with 0.1 M phosphate buffered saline (PBS), followed by cold 4% paraformaldehyde PBS. The brains were cryoprotected in a series of sucrose solutions, and then cut at 40 μm thicknesses in the coronal plane. Briefly, the previous free-floating method [16] was used for staining with polyclonal anti-rabbit CB

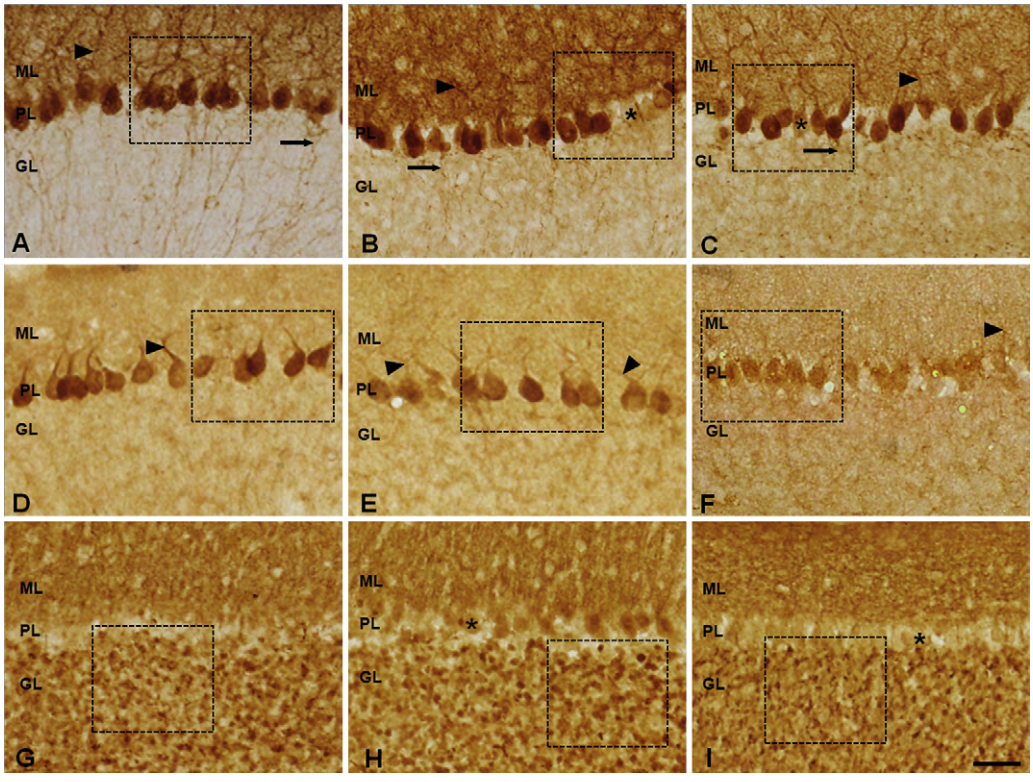

Fig. 1. Distribution of calbindin D28-k (A–C), parvalbumin (D–F), and calretinin (G–I) in the cerebellar cortex of +/+, +/cir, and cir/cir mice. CB IR in the cerebellar cortex of +/+ (A), +/cir, (B) and cir/cir mice (C). CB IR Purkinje cells with immunoreactive dendrites (arrow head) show a high degree of arborization into the molecular layer. Axons of the Purkinje cells (arrow) descending into the granular layer. Unstained or lesser CB IR PCs (asterisk) observed in +/cir and cir/cir mice. PV IR in the cerebellar cortex of wild-type (D), +/cir (E), and cir/cir mice (F). The majority of Purkinje cells exhibit PV immunostaining and are arranged in a monocellular layer. Prominent dendrites ascending vertically in the molecular layer (arrow head). CR IR in the cerebellar cortex of +/+ (G), +/cir (H), and cir/cir mice (I). Immunostained horizontally coursing fibers (arrow head) in the molecular layer close to the Purkinje cell layer (G–I). Absence of CR IR in the Purkinje layer in +/+, while faintly stained PCs in the Purkinje layer of +/cir and cir/cir mice (asterisk). Abr: ML = molecular layer, PL = Purkinje layer, GL = granular layer. Scale bar = 100 μm.