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Immunohistochemical study on the expression of calcium binding proteins (calbindin-D28k, calretinin, and parvalbumin) in the cerebellum of the nNOS knock-out(-/-) mice

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Abstract: Nitric Oxide (NO) actively participates in the regulation of neuronal intracellular Ca^{2+} levels by modulating the activity of various channels and receptors. To test the possibility that modulation of Ca^{2+} buffer protein expression level by NO participates in this regulatory effect, we examined expression of calbindin-D28k, calretinin, and parvalbumin in the cerebellum of neuronal NO synthase knock-out ($nNOS^{(-/-)}$) mice using immunohistochemistry. We observed that in the cerebellar cortex of the $nNOS^{(-/-)}$ mice, expression of calbindin-D28k and parvalbumin were significantly increased while expression of calretinin was significantly decreased. These results suggest another mechanism by which NO can participate in the regulation of Ca^{2+} homeostasis.

Key words: Calbindin-D28k, calretinin, parvalbumin, cerebellum, neuronal nitric oxide synthase (nNOS) knock-out (−/−) mice
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Introduction

Nitric Oxide (NO) is not only a neurotransmitter but also a neuromodulator which exerts many functions in the nervous system (Esplugues, 2002; Rhyu *et al.,* 2003; Abbott & Nahm, 2004). Since NO can cross the lipid bilayer freely and has a very short lifespan, neurons cannot sequester NO nor regulate its local concentration (Dawson & Dawson, 1996). Therefore, the key to regulating NO in the brain is to control NO synthesis by regulating the activity of neuronal NO synthase (nNOS). This regulation of NO synthesis is mainly mediated by cytosolic Ca^{2+} levels. The Ca^{2+} influx from extracellular fluid and the release of Ca^{2+} from intracellular stores increase Ca^{2+} concentrations in the neuronal cytoplasm. Increased Ca^{2+} binds calmodulin (CaM) and then the Ca^{2+}CaM complex activates nNOS by direct binding. If the Ca^{2+} concentration falls, it dissociates from CaM, which in turn dissociates from nNOS resulting in nNOS deactivation (Knowles *et al.,* 1989; Sheng *et al.,* 1992).

While the synthesis of NO is regulated by Ca^{2+}, NO can also influence Ca^{2+} levels in neuronal cytoplasm. NO diminishes activity of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) (Lei *et al.,* 2000) and N-methyl-D-aspartate (NMDA)-type glutamate receptor (Lei *et al.,* 1992; Manzoni *et al.,* 1992). NO inhibits voltage-gated $Ca2^{2+}$channels such as L-type (Doerner & Alger, 1988) and N-type Ca^{2+} channels (Yoshimura *et al.,* 2001). The increase of Ca^{2+} concentration through these receptors and channels can be reduced by these means. Not only Ca^{2+} influx from extracellular fluid but also Ca^{2+} release from intracellular Ca^{2+}

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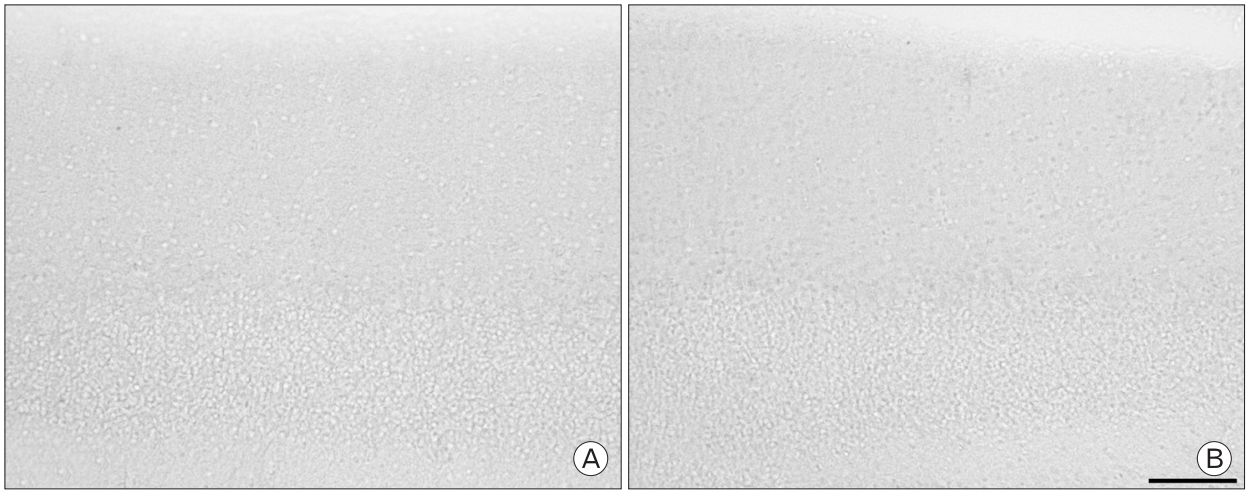

Fig. 1. Tests for specificity of primary antibodies (A, B). A sample of sections reacted without primary antiserum (A), and a different sample exposed to primary antiserum preabsorbed for 24 hours with CB (B) do not exhibit any immunoreactivity. No immunoreactivity is observed in both samples exposed to primary antiserums preabsorbed for 24 hours with CR and PV (data not shown). Scale bar = 150 μm.

stores are modulated by NO. NO induces ryanodine receptor phosphorylation through protein kinase G, which results in increased Ca^{2+} release from the endoplasmic reticulum into the cytoplasm (Clementi *et al.,* 1996). Therefore it can be said that NO actively participates in the regulation of Ca^{2+} homeostasis of neurons.

The entire neuronal Ca^{2+} homeostasis regulation system consists of a Ca^{2+} entry system, intracellular Ca^{2+} store, Ca^{2+} extrusion system, and Ca^{2+} buffer. It can be hypothesized that NO participates in the regulation of Ca^{2+} homeostasis through mechanisms other than modulating the Ca^{2+} entry system and intracellular Ca^{2+} store. Previously it was shown that Ca^{2+} binding proteins (CaBPs) such as calbindin-D28k (CB) (Geula *et al.,* 1993; Bertini *et al.,* 1996) and calretinin (CR) (Arévalo *et al.,* 1993) colocalize with nNOS in some populations of neurons. Similar cerebellar function defects are detected in both nNOS (Nelson *et al.,* 1995) and CaBP knock-out mice (Airaksinen *et al.,* 1997; Cheron *et al.,* 2000). Based upon these findings, Ca^{2+} buffer may be a candidate for Ca^{2+} homeostasis regulation by NO. It is well known that CaBPs such as CB, CR, and parvalbumin (PV) act as Ca^{2+} buffers in neurons (Schwaller *et al.,* 2002) and that nNOS and these proteins are abundantly expressed and exert several functions in the cerebellum (Nelson *et al.,* 1995; Schwaller *et al.,* 2002). Therefore, to test NO's influences on these Ca^{2+} buffer proteins, we examined changes in their expression in the cerebellum of nNOS knock-out mice ($nNOS^{(-/-)}$ mice) (Huang *et al.,* 1993) using immunohistochemistry. We were able to demonstrate specific changes in expression of each Ca^{2+} buffer protein in the cerebellum of the $nNOS^{(-/-)}$ mice.

Materials and Methods

Male mice 3~4 months old were utilized for this study. There were 12 C57BL/6 controls and 10 $nNOS^{(-/-)}$ B6, 129S-$Nos1^{tm1Pih}$ obtained from Dr. Oh (Induced Mutant Resources Program, Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea). All animals were treated in accordance with the 'Principles of Laboratory Animal Care' (NIH publication No. 86~23, revised in 1985). The mice were perfused transcardially with cold phosphate buffered saline (PBS, 0.05M, pH 7.4), followed by ice-cold 4% paraformaldehyde. The brains were cryoprotected in a series of cold sucrose solutions, and cut at 40 μm in the coronal plane. Immunohistochemistry was performed in accordance with the free-floating method described earlier (Chung *et al.,* 2000). Rabbit anti-CB polyclonal antibody, rabbit anti-CR polyclonal antibody, and anti-PV monoclonal antibody (AB1778 and AB5054; Chemicon International, Temecula, CA, USA for CB and CR respectively and P3088; Sigma, Saint Louis, MI, USA for PV) were used as primary antibodies.

A sample of sections was reacted without primary antiserum, and different samples were exposed to primary antiserum that had been preabsorbed for 24 hours with control antigen peptides. Sections from these samples did not exhibit any immunoreactivity as described in this report

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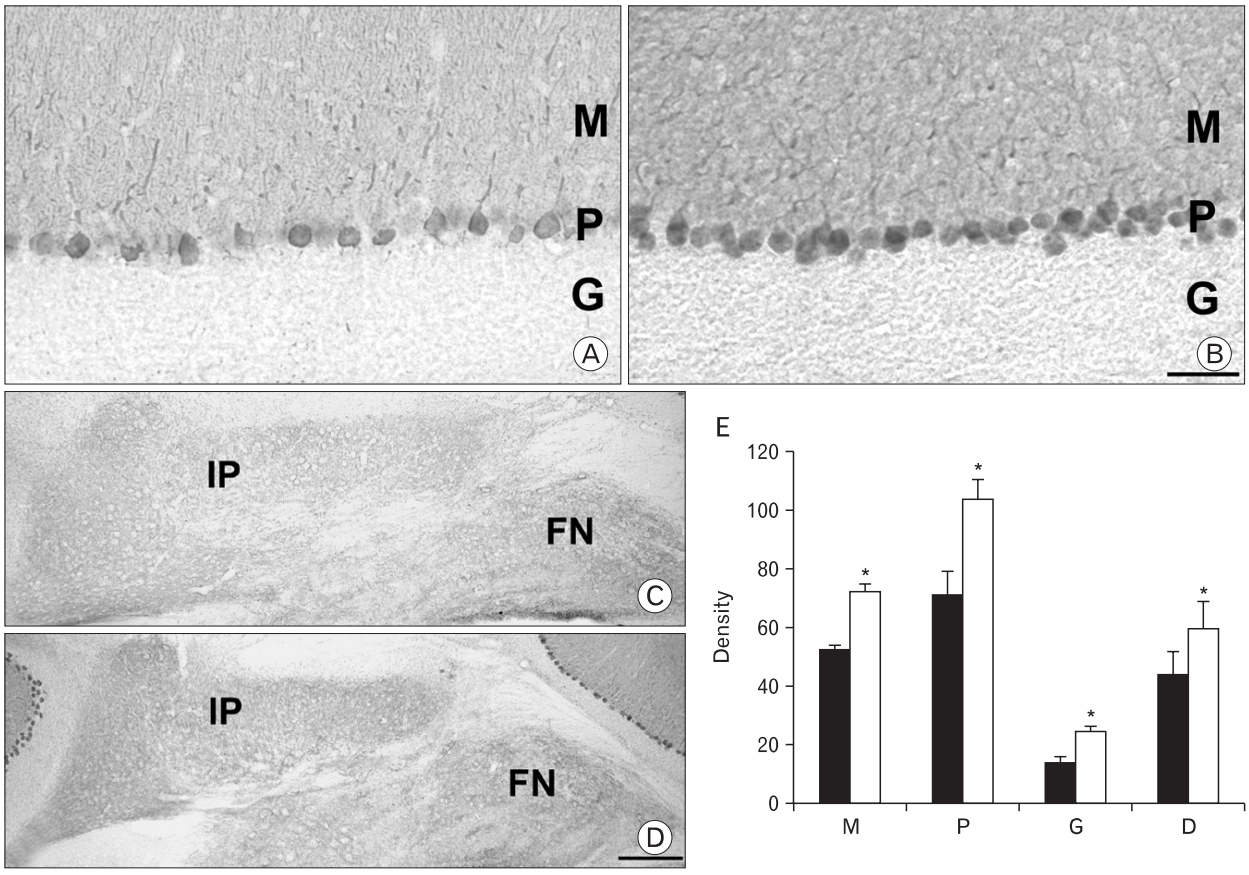

Fig. 2. Changes of CB-immunoreactivity in the cerebellum of nNOS$^{(-/-)}$ mice (B, D), compared with that of the control mice (A, C). In control mice, CB-immunoreactivity is observed in Purkinje cells, neuropil of the molecular layer of the cerebellar cortex (A) and neuropil of the deep cerebellar nuclei (C). This expression pattern is preserved in the nNOS$^{(-/-)}$ mice (B, D) but in all cerebellar cortical layers (B) and deep cerebellar nuclei (D), CB-immunoreactivity is increased. In image analysis (E), these differences are statistically significant. Mean density is the sum of the white values of all the pixels in the selection divided by the number of pixels within the selection. Values are the mean±standard deviations. A t-test was performed (*P<0.01). D, deep cerebellar nuclei; FN, fastigial nucleus; G, granular layer; IP, interposed nucleus; M, molecular layer; P, Purkinje cell layer. Scale bar (A, B) = 100 μm; (C, D) = 400 μm.

(Fig. 1). We randomly selected 5 unit areas at each region in the cerebellum from control (n=12) and nNOS$^{(-/-)}$ mice (n=10) and calculated the numbers of CB, CR, and PV-ir per unit area. Sections from each control and nNOS$^{(-/-)}$ group were stained together eliminating the variable of different experimental conditions. Visual assessment and densitometric measurements using an NIH image program (Scion Image) were evaluated to determine staining density. The t-test was used to investigate whether changes in CaBPs expression were statistically significant (*P<0.01).

Immunohistochemistry images were taken using a ProgRes C14 digital camera with ProgRes C14 software (JENOPTIC Laser, Optic, System, Munich, Germany). Image-editing software (Adobe Photoshop) was used to adjust size and contrast and combine the images obtained.

Results

In the cerebellar cortex, each CaBP showed a characteristic expression pattern. CB-immunoreactivity was observed in the neuropil of the molecular layer and cell bodies of Purkinje cells (Fig. 2A) whereas CR-immunoreactivity was confined to neuropil of the molecular layer and granular cells of the granular layer (Fig. 3A). PV was expressed by the neuropil, cells of the molecular layer, and cell bodies of Purkinje cells (Fig. 4A). These expression patterns of CB, CR, and PV were well preserved in the nNOS$^{(-/-)}$ mice (Fig. 2A, B for CB; Fig. 3A, B for CR; Fig. 4A, B for PV). However, the immunoreactivity of each CaBP was significantly changed. In all layers of the cerebellar cortex, CB-immunoreactivity was significantly increased (Fig. 2A, B, E) and in contrast to CB, CR-immunoreactivity was significantly decreased (Fig. 3A,

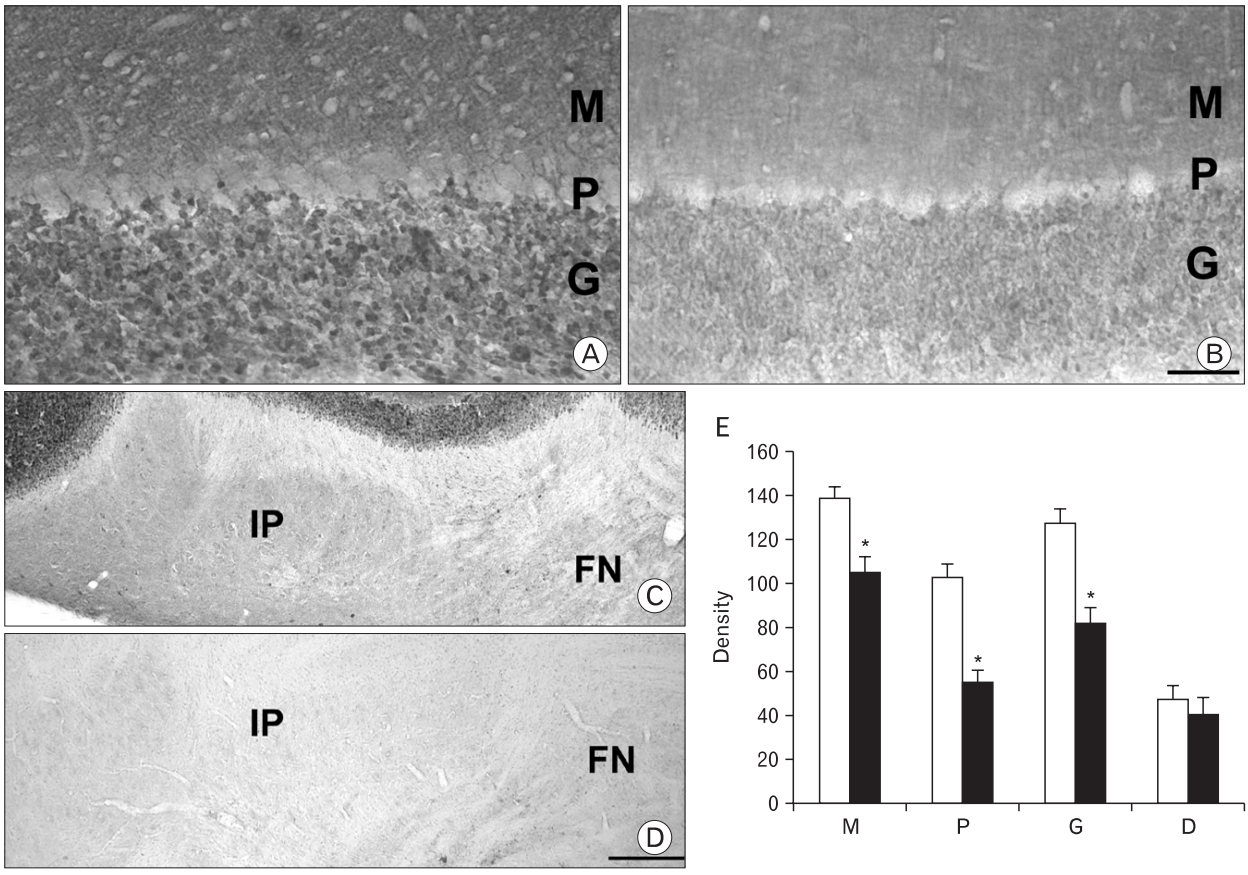

Fig. 3. Decreased CR expression in the cerebellum of nNOS$^{(-/-)}$ mice (A, C for the control mice and B, D for the nNOS$^{(-/-)}$ mice). In the cerebellar cortex of both group, granule cells and neuropil of the molecular layer express CR but Purkinje cells express little CR (A, B). Compared with control mice (A), CR-immunoreactivity is significantly decreased in all cerebellar cortical layers of nNOS$^{(-/-)}$ mice (B, E). In contrast to the cerebellar cortex, CR-immunoreactivity of the deep cerebellar nuclei is very low (C, D). In many animals CR expression seems to be decreased in these regions in the nNOS$^{(-/-)}$ mice although their differences are not statistically significant (E). Mean density is the sum of the white values of all the pixels in the selection divided by the number of pixels within the selection. Values are the mean±standard deviations. A t-test was performed (*P<0.01). The graph in E shows results from image analysis. D, deep cerebellar nuclei; FN, fastigial nucleus; G, granular layer; IP, interposed nucleus; M, molecular layer; P, Purkinje cell layer. Scale bar (A, B) = 100 μm; (C, D) = 400 μm.

B, E). Regarding PV, PV-immunoreactivity of the molecular layer and the Purkinje cell layer were significantly increased whereas that of the granular layer was significantly decreased (Fig. 4A, B, E).

Immunoreactivity of CB and CR in the deep cerebellar nuclei was much lower than that in the cerebellar cortex and most were confined in neuropil (Fig. 2C, 3C). Similar to changes in the cerebellar cortex, CB-immunoreactivity of the nNOS$^{(-/-)}$ mice was significantly increased (Fig. 2D, E) and CR-immunoreactivity of the nNOS$^{(-/-)}$ mice was decreased, although this change was not statistically significant (Fig. 3D, E). In contrast to CB and CR, relatively high levels of PV were observed in the deep cerebellar nuclei and expressed not only in neuropil but also in cell bodies (Fig. 4C). Immunoreactivity was similar between control and nNOS$^{(-/-)}$ groups (Fig. 4C, D, E) but cell bodies of nNOS$^{(-/-)}$ mice appeared to express higher PV. (Fig. 4C, D).

Previously the distribution of CB, CR, and PV in the cerebellum was carefully examined and their characteristic localizations reported (Celio, 1990; Résibois & Rogers, 1992). In the molecular layer, stellate cells and basket cells expressed PV, parallel fibers of granule cells expressed CR, and dendrites of Purkinje cells expressed CB and PV. In the Purkinje cell layer the cell bodies of Purkinje cells showed CB and PV-immunoreactivity. In the granular layer only CR was expressed by granular cells. These characteristic expressions of CB, CR, and PV match well with our results (Fig. 2~4). Therefore CB and CR-immunoreactivity in neuropil of the molecular layer are thought to be due to dendrites of Purkinje cells and parallel fibers of granule cells respectively. PV-

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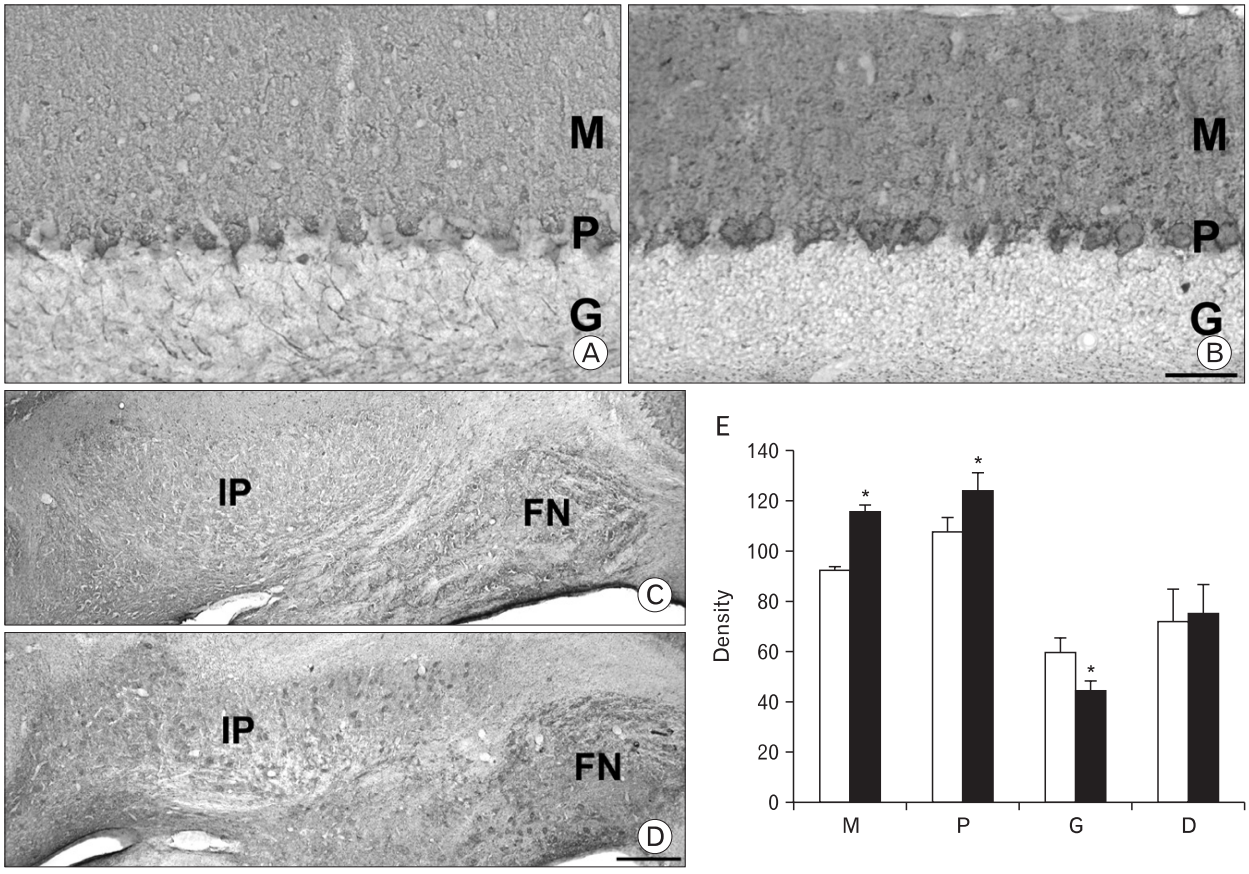

Fig. 4. Changes of PV-immunoreactivity in the cerebellum of the nNOS$^{(-/-)}$ mice (B, D) compared with that of control mice (A, C). In the cerebellar cortex of the control mice, the Purkinje cells, neuropil, and cells of the molecular layer express PV (A). Although PV expression patterns of nNOS$^{(-/-)}$ mice are similar to that of control mice, PV-immunoreactivity of Purkinje cells and the molecular layer is significantly increased (A, B, E) and that of the granular layer is significantly decreased (A, B, E) in nNOS$^{(-/-)}$ mice. In the deep cerebellar nuclei of the control and the nNOS$^{(-/-)}$ mice, cell bodies as well as neuropil express PV (C, E). The change of immunoreactivity was minor but cell bodies of the nNOS$^{(-/-)}$ mice seemed to express higher PV-immunoreactivity (C~E). The graph in E shows results from image analysis. Mean density is the sum of the white values of all pixels in the selection divided by the number of pixels within the selection. Values are the mean±standard deviations. A t-test was performed (*P<0.01). D, deep cerebellar nuclei; FN, fastigial nucleus; G, granular layer; IP, interposed nucleus; M, molecular layer; P, Purkinje cell layer. Scale bar (A, B) = 100 μm; (C, D) = 400 μm.

immunoreactivity of cells and neuropil of the molecular layer may be due to stellate cells, basket cells, and dendrites of Purkinje cells.

The CB, CR, and PV actively participate in the regulation of neuronal intracellular Ca^{2+} levels by binding Ca^{2+} and lowering its concentration. The intracellular steady state Ca^{2+} level of neurons is not affected by these CaBPs (Chard *et al.,* 1993; Schwaller *et al.,* 2002), because it is determined by Ca^{2+} uptake and extrusion system which remains functional until Ca^{2+} has attained its steady state level. Instead, these calcium buffer proteins modulate the temporal and spatial properties of intracellular Ca^{2+} distribution. For example, CB significantly reduces the amplitude of the Ca^{2+} transient and slows down the decay of Ca^{2+} levels in cerebellar Purkinje cells (Airaksinen *et al.,* 1997). PV, which has slower Ca^{2+} binding properties than CB, increases the initial rate of decay of Ca^{2+} and subsequently prolongs its late phase in bovine chromaffin cells (Lee *et al.,* 2000). Therefore, it is possible that NO influences intracellular Ca^{2+} levels of the cerebellar neurons by modulating CaBPs expression levels.

Discussion

The nNOS$^{(-/-)}$ mice have no evident defects in usual locomotor activity (Huang *et al.,* 1993) but show discrete abnormalities in balance and motor coordination selectively at night (Kriegsfeld *et al.,* 1999). Similarly, CB knock-out mice

(Airaksinen *et al.,* 1997) and Purkinje cell-specific conditional CB knock-out mice (Barski *et al.,* 2000) show normal motor functions in usual environments but slip when transversing narrow runways, which force them to change and adapt their stride. Also in CR knock-out mice, abnormal Purkinje cell activity in the cerebellum and abnormal wheel running test results are observed whereas other motor functions remain normal (Schanne *et al.,* 1979; Cheron *et al.,* 2000). Regarding these findings, the nNOS$^{(-/-)}$ mice, the CB knock-out mice, and the CR knock-out mice seem to have very similar cerebellar functional defects, although motor function tests used are not exactly the same. This suggests that there may be a common underlying mechanism. Since altered CB and CR expression in the nNOS$^{(-/-)}$ mice was observed in this study, altered expression of these proteins and resulting temporal and spatial intracellular Ca^{2+} distribution property changes may be good candidates for that common mechanism.

It is well known that intracellular concentration of Ca^{2+} and the Ca^{2+}-dependent signaling system including NOS are closely related to neuronal degenerations (Schanne *et al.,* 1979; Lipton *et al.,* 1993). The Ca^{2+} buffering property of CB, CR, and PV allows for the hypothesis that these buffers may have neuroprotective effects. There is some in vitro data supporting this hypothesis (D'Orlando *et al.,* 2001; D'Orlando *et al.,* 2002). In spinocerebellar ataxia type I, it is suggested that down-regulation of CB and PV leads to cerebellar Purkinje cell death (Vig *et al.,* 2000). Many studies have also shown results against this hypothesis (Kuźnicki *et al.,* 1996; Airaksinen *et al.,* 1997; Bouilleret *et al.,* 2000). In CB, CR, and PV knock-out mice there is no evidence of abnormal neuronal loss (Schwaller *et al.,* 2002), resulting in doubts as to a generalized neuroprotective role for these proteins. Like the Ca^{2+} buffer proteins, no unusual neuronal death is observed in the nNOS$^{(-/-)}$ mice (Huang *et al.,* 1993; Nelson *et al.,* 1995); therefore, it is not obvious that Ca^{2+} buffer proteins such as CB, CR, and PV have generalized neuroprotective effects. Their modulated expression by NO seems to have minor effects on neuronal survival.

In the present study, we demonstrated that expression levels of CB, CR, and PV are characteristically and significantly altered in the cerebellum of nNOS$^{(-/-)}$ mice using immunohistochemistry techniques (Figs. 2~4). These changing patterns were preserved in all animals studied. Regarding these findings, it can be concluded that NO modulates CaBP expression in neurons of the cerebellum, and by this means, NO participates in Ca^{2+} homeostasis and regulation of neurons. As expression patterns of these proteins are not changed, it appears that NO influences only the expression level, not the expression pattern in the cerebellum.

For the first time, we demonstrated that NO specifically modulates the expression of Ca^{2+} buffer proteins such as CB, CR, and PV in the cerebellum. This result suggests another mechanism by which NO participates in the regulation of Ca^{2+} homeostasis. Since modulation of expression levels of Ca^{2+} buffer proteins can influences temporal and spatial properties of intracellular Ca^{2+} distribution, it appears that NO can exerts its various functions not only in the cerebellum but also in the other parts of the brain. The exact mechanism of this regulation and its functional significance requires further elucidation.

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