Changes in Nitric Oxide Synthase Activities in the Cerebellum during Development and Aging of C57BL/6 Mice

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Tsukada, M., Yamazaki, Y. and Koizumi, A. Changes in Nitric Oxide Synthase Activities in the Cerebellum during Development and Aging of C57BL/6 Mice. Tohoku J. Exp. Med., 1995, 176 (2), 69-74 — Nitric oxide (NO) is one of the neurotransmitters in the cerebellum. Activities of NO synthase and densities of granule cells were determined in the cerebellum in an attempt to elucidate a mechanism of biochemical changes on the anatomical basis during aging. Activities of Ca²⁺/calmoduline dependent NO synthase were measured in the cerebellum in male and female mice, from 18 gestation days to 28 months of age. There were significant differences in NO synthase activities among the groups of prenatal (18 gestation days), middle aged (5-18 months) and old aged (21-28 months) mice. Between groups of old and middle ages, a significant decrease with aging was found in the mean density of granular cells, which express NO synthase. Changes in NO synthase activities and granule cell densities occurred to a similar degree between old and middle ages. The present data suggest that an age-associated rise and fall of its activities likely occur in parallel with those of granule cell densities, and thus may represent impairment of cerebellar function during aging. ——— NO synthase; cerebellum; development; aging

Nitric oxide (NO) is a messenger molecule in many organs and systems (Bredt and Snyder 1992; Lowenstein and Snyder 1992; Snyder 1992). NO synthase generates NO and citrulline from arginine. Since NO has a very short half-life and is consumed immediately in situ after production, existence of NO synthase in a given micro environment is a predictor as to whether NO plays an important physiological role in it.

Immunohistochemical and in situ hybridization studies have revealed that in the cerebellum, NO synthase localizes in granule and basket cells (Bredt et al. 1990). Its activity in the cerebellar cytosolic fraction in rats increased between 2 and 3 weeks after birth when granule cells migrate in the cerebellum (Matsumoto et al. 1993), suggesting that NO synthase activities in the cerebellum is a good marker of both anatomical and functional maturity of granule cells.

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The abilities of motor coordination, balance and learning novel motor tasks are gradually lost with aging (Bickford 1993). Cellularity changes underlie such functional impairment of the cerebellum (Ratz et al. 1992), such as loss of Purkinje and granule cells during aging (Ellis 1920; Hall et al. 1975; Torvik et al. 1986).

NO synthase activity is regulated by N-methyl-D-aspartate (NMDA) receptors. NMDA receptors have a variety of functions in the central nervous system including synaptic plasticity during development, learning motor tasks, or neurodegeneration (Cotman and Iversen 1987). These lines of evidence are suggestive of a mechanism of decline of cerebellar functions during aging. The decreases in numbers of granule cells may occur and may thereby lower NO production in the cerebellum. The aim of the present study is to investigate this possibility.

We herein report that cellular density of granule cells and NO synthase decrease in parallel during aging. Such changes may explain age-related decline of cerebellar function on pathological basis.

**Materials and Methods**

**Chemicals**

Calmoduline was purchased from Biomedical Technologies Inc. (Stoughton, MA, USA). Dowx AG50WX-8 (Na+ form) was purchased from Bio Rad Laboratories (Richmond, CA, USA), NADPH from Oriental Yeast Co. Ltd. (Tokyo), and [3H] Arginine (2.85 TBq/mmol) from Amersham International Plc (Amersham, UK), respectively. All other reagents were obtained from Sigma Chemical (St. Louis, MO, USA).

**Mice**

Several pairs of C57BL/6J female and male mice were purchased from Clea, Japan, Inc. (Tokyo). Progenies were obtained from those pairs of mice in our animal facility. After weaning, mice were numbered individually for identification by cutting the tip of the paw. Their birth dates were recorded. After weaning at 4 weeks of age, female and male mice were separately housed in cages with wood shavings in a room at temperature 23°C, a relative humidity of about 50% and a 12/12 (light/dark) photocycle. Mice were fed with commercial available chow (Clea Japan, Inc.) ad libitum.

The zero day of the gestation is the day when vaginal plugs were found in the female mice. On gestation day 18, a dam mouse was killed and fetuses were collected. Five randomly selected fetuses from the litter mates were sacrificed for assays of NO synthase activities. Gender of individual fetuses was not determined.

**Assay of Ca^{2+}/calmoduline dependent NO synthase activity**

Mice were killed by cervical dislocation and checked carefully whether they
have gross pathological changes in the brain by autopsy. Then, half of the
cerebellum was removed, weighed, and homogenized in 4 volumes homogenizing
buffer (50 mM Hepes pH 7.4, 1 mM EDTA, 1 mM DTT, 1 mM CaCl₂) at 4°C. The
homogenate was centrifuged at 9,000 × g for 15 min. The supernatant solution
was used as an enzyme source after its protein concentration was measured by the
Coomassie Blue method described by Sedmak and Grossberg (1977).

NO synthase activity was measured by measuring the conversion of [³H]
arginine to [³H] citrulline by the method of Bredt and Snyder (1989). The assay
mixture containing 20 μg of protein, 100 nM [³H] arginine, 50 mM Hepes (pH 7.4),
1 mM NADPH, 1 mM EDTA, 1 mM DTT, 1.25 mM CaCl₂ and 1.5 μg calmoduline
in total 150 μl was incubated at 22°C for 5 min. The reaction was stopped by
adding 2 ml of 20 mM Hepes pH 5.5/ 2 mM EDTA. After removing [³H] ar-
ginine with 1-ml columns of Dowx AG 50WX-8 (Na⁺ form), [³H] citrulline was
eluted with 2 ml of water and quantified by liquid scintillation spectroscopy.
Our preliminary study revealed the rate of conversion of arginine to citrulline in
assay mixtures without Ca²⁺ and calmoduline was equal to that of non-enzymatic
conversion. Therefore Ca²⁺/calmoduline dependent NO synthase activities were
calculated by subtracting the values of non-enzymatic conversion from those in
assay mixtures including Ca²⁺ and calmoduline. Non-enzymatic conversion of
arginine to citrulline was determined by the assay mixture of which enzyme
source was replaced by water at least three samples independently in each
experiment. We confirmed that those values of enzyme activities were propor-
tional up to 60 μg protein amounts (Yamazaki et al. 1994).

Evaluation of granule cell density in cerebellar vermis

Five mice were randomly chosen from groups of 5–12 and 21–28 months of
age. After removing halves of cerebellum for enzyme assay, other halves from
these ten mice were fixed in buffered formalin solution for one week, then
processed as paraffin blocks. Tissues were prepared as midsagittal serial sections
in 5 μm thickness, and then stained with hematoxylin eosin.

Cell density in the granular layer was determined. Within several folia of
the cerebellum, one folium was chosen from each mouse. Numbers of granule
cells contained in a whole folium were counted and areas occupied by cells were
measured. Granule cell density was expressed as cell numbers of every 10⁻³ cm²
of granular cell layer.

Statistics

Ca²⁺/calmoduline dependent NO synthase activities were compared among
the groups classified by animal's age by the use of one-way ANOVA, followed by
Shiffe's test (Gad and Weil 1989). A p value less than 5% was considered
significant throughout this study.
RESULTS AND DISCUSSION

In total 45 male and female mice were used for the present experiments. Microscopic observation of the cerebellum did not suggest any tumors, malformation or other pathological lesions. Fig. 1 shows that Ca\(^{2+}\)/calmoduline dependent NO synthase activities in the cerebellum changed with age. Among four groups, prenatal (18 days of fertilization), neonatal (9 days-1 month), middle-aged (5-18 months) and old-aged (21-28 months) ones, there are significant differences between prenatal and middle age groups and between middle and old age groups (p<0.05) (Table 1). However, no significant differences were detected between prenatal and neonatal mice or between neonatal and middle age ones. No significant differences were observed between genders either. From these observations, it was concluded that Ca\(^{2+}\)/calmoduline dependent NO synthase activities in cerebellum increased during development but decreased again with aging after maturity.

There may be at least two possibilities to explain such changes. The first one is that decrease in transcriptional activities of NO synthase gene may be primarily involved in the decrease. Alternatively, a relative decrease in the cellular density of granule cells may be responsible. Matsumoto et al. (1993) demonstrated that relative increase in NO synthase activity in rats during the development is accompanied by an increase in granule cell numbers in the cerebellum. Given that the reverse is true, decreases in the granule cell number should decrease NO synthase activities.

![Fig. 1. Changes in Ca\(^{2+}\)/calmoduline dependent NO synthase activities in cerebellum with development and age. Specific activities of NO synthase in 5 prenatal mice (□), 30 male mice (○) and 10 female mice (●) were plotted against age (months). Values of male and female mice were not statistically different by one-way ANOVA followed by Shifte’s test. See details in Materials and Methods with respect to the method of measuring enzyme activities.](image-url)
synthase activity.

We thus determined the density of granule cells between mice of middle and old ages to answer whether decrease in its density may occur during aging. The mean density of the granule cell density of old age group was about 80% of that of the middle age group (Table 2). The change shows a rough agreement with 23% decrease in NO synthase activity between two age groups. These observations suggest that the changes of numbers of granule cells underlie the decrease in NO synthase activity.

Recent experiments have revealed that release of NO is essential for long-term synaptic depression (LTD) in the cerebellum (Shibuki and Okada 1991). LTD blocks transmissions from the parallel fiber to Purkinje cells, and is speculated to be a memory element for cerebellar motor learning (Ito 1989). Such loss of granule cells and the associated decrease in NO synthase activity would affect minute control of locomotor behaviors of old animals. Lowered NO synthase activity and decrease in granule cell density might give a primary momentum to sequential decline of cerebellar function during senescence. At any rate, further

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**Table 1.** An age-associated profile of NO synthase activities in the cerebellum

<table>
<thead>
<tr>
<th>Age of groups</th>
<th>Number of animals</th>
<th>NO synthase specific activities (fmol/μg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation day 18</td>
<td>5</td>
<td>$19.5 \pm 7.61^b$</td>
</tr>
<tr>
<td>9 days-1 month</td>
<td>7</td>
<td>$30.4 \pm 8.73^{ab}$</td>
</tr>
<tr>
<td>5-18 months</td>
<td>21</td>
<td>$38.4 \pm 8.27^a$</td>
</tr>
<tr>
<td>21-28 months</td>
<td>12</td>
<td>$29.5 \pm 6.17^c$</td>
</tr>
</tbody>
</table>

Values are Means±S.D.

Comparisons were made by one-way ANOVA followed by Shiffle's test. Values X and Y are statistically different ($p < 0.05$) when they carry the same superscript (e.g., $X^a$ and $Y^a$ or $X^b$ and $Y^b$), but are not when they carry different ones (e.g., $X^a$ and $Y^b$) (Gad and Weil 1989).

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**Table 2.** Comparison of granule cell densities in cerebellum between middle and old-aged groups

<table>
<thead>
<tr>
<th>Age of groups</th>
<th>Number of animals</th>
<th>No. of granule cells/ $10^{-3}$ cm$^2$ cell layer$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-12 months</td>
<td>5</td>
<td>$2535 \pm 201.0$</td>
</tr>
<tr>
<td>21-28 months</td>
<td>5</td>
<td>$2029 \pm 350.2$</td>
</tr>
</tbody>
</table>

Values are Means±S.D.

Counted areas were greater than $2.5 \times 10^{-3}$ cm$^2$ in all cases.

$^a$Comparison was made by one-way ANOVA followed by Shiffle's test. Significant difference exists at $p < 0.05$. 
studies need to be done to investigate mechanisms of age-associated decreases in NO synthase activity and granule cell density.

References