

## Development of Purkinje cell axonal torpedoes in the cerebellum of rolling mouse Nagoya

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### Abstract

**The present study examined the development of the axonal torpedoes of the cerebellar Purkinje cells in a Ca<sub>v</sub>2.1 channel mutant, rolling mouse Nagoya. Calbindin D-28k immunostaining revealed a few torpedoes in both the cerebellar white matter and all three subdivisions of the deep cerebellar nuclei of rolling mice on postnatal day (PD) 21, while there was no difference in either number among the age-matched controls. On PD 120, the number of torpedoes drastically increased in the white matter of rolling mice. Their numbers in the deep cerebellar nuclei also increased in rolling mice during PDs 21 to 120 with no change in their distributions. These results suggest that the axonal torpedoes develop progressively with aging in non-specific populations of Purkinje cells in the rolling mouse cerebellum.**

**Key words:** Ca<sup>2+</sup> channelopathy, Neuropathy, Swelling, Cerebellum, Ataxia

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### Introduction

Rolling mouse Nagoya is an ataxic mutant mouse that carries a recessive mutant allele of the *tottering* locus (*tg<sup>rol</sup>*) on chromosome 8 [1] which encodes a gene for the P/Q-type Ca<sup>2+</sup> channel  $\alpha_{1A}$  subunit (Ca<sub>v</sub>2.1) [2], as do tottering, leaner [3], rocker [4] and wobbly mice [5]. Our immunohistochemical study reveals a uniform expression of the mutated Ca<sub>v</sub>2.1 channel throughout all Purkinje cells in rolling mice [6;7], and most likely to other Ca<sub>v</sub>2.1 mutant mice as well [3;8]. The Ca<sub>v</sub>2.1 gene mutation selectively reduces the voltage sensitivity and activity of the P/Q-type Ca<sup>2+</sup> channels in the cerebellar Purkinje cells of Ca<sub>v</sub>2.1 mutants [9-11] including rolling mice [2], and the defects in this gene in humans are responsible for several neurological disorders such as episodic ataxia type-2 and spinocerebellar ataxia type-6 [12].

Axonal torpedoes are recognized as neuropathological signs in neurological diseases [13;14] characterized by local accumulations of malaligned neurofilaments and mitochondria [13-15]. The torpedoes (or abnormal swellings) of Purkinje cell axons in the cerebellar white matter have been reported in rolling mice [7;16;17] and other Ca<sub>v</sub>2.1 mutant mice [18]. Recently, we further revealed a number of Purkinje cell axonal torpedoes in the deep cerebellar nuclei of rolling mice [19]. The present study was

undertaken to clarify at what point Purkinje cell axonal torpedoes developed in rolling mice. We conducted calbindin D-28k immunohistochemistry to detect Purkinje cell axonal torpedoes in either the cerebellar white matter or the deep cerebellar nuclei in immature and adult rolling mice.

### Materials and Methods

#### *Animals*

All experimental procedures were conducted in accordance with the guidelines of the National Institutes of Health (NIH) for the Care and Use of Laboratory Animals (No. 80-23, revised 1996). The Institutional Animal Care and Use Committee of the University of Tokushima approved the procedures, and all efforts were made to minimize the number of animals used and their attendant suffering. Rolling mice were raised on a C3Hf/Nga background. Homozygous rolling mice (*tg<sup>rol</sup>/tg<sup>rol</sup>*), raised by intercrossing heterozygous pairs, were readily identifiable by their ataxic locomotion between postnatal days 10 and 14; wild-type (+/+) mice were used as controls. All mice were provided with a pellet diet (NMF, Oriental Yeast Co., Ltd., Japan) and tap water *ad libitum*, and were kept at 24 ± 1 °C under 12-h artificial illumination.

### Immunohistochemistry

The animals were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (25 µg/10 g body weight), and were perfused with 0.9% NaCl followed by Bouin's solution without acetic acid on postnatal days (PDs) 21 (rolling, n=4; control, n=3) and 120 (rolling, n=4; control, n=3). Cerebella were immersed in the same fixative, embedded in paraffin and sectioned serially along the frontal plane at 3 µm. Deparaffinized sections were irradiated with microwaves for 5 min in 10 mM citrate buffer, pH 6.0, and reacted with a rabbit anti-calbindin D-28k polyclonal antibody (1:20,000, Swant, Switzerland) containing 10% normal goat serum at 4 °C overnight. After incubation, sections were rinsed with PBS and reacted with biotinylated anti-rabbit IgG. The immunoreactive products were visualized by a Vectastain ABC elite kit (Vector Lab., Inc.) using 0.01% 3,3'-diaminobenzidine tetrachloride (Sigma) in 0.03% H<sub>2</sub>O<sub>2</sub> as a chromogen.

### Estimation of the number of Purkinje cell axonal torpedoes

Serial coronal sections of the cerebellum spaced at 150-200 µm, which included all cerebellar lobules, were observed under light microscopy. Calbindin D-28 k immunopositive torpedoes of Purkinje cell axons more than 5 µm in diameter were counted in the cerebellar white matter, and data were expressed as the number of torpedoes per section. Two-way analysis of variance (ANOVA) was carried out with groups (rolling and control mice) and ages (postnatal days 21 and 120) as factors. Calbindin D-28 k immunopositive torpedoes more than 5 µm in diameter were also counted in all three basic subdivisions of the deep cerebellar nuclei, and data were expressed as the number of torpedoes per sectional profile. The right and left sides of each subdivision were separately counted, with each side considered to be "n=1." Three way ANOVA was performed with groups (rolling and control mice), regions (the medial, interpose, and lateral nuclei of the deep cerebellar nuclei) and ages (postnatal days 21 and 120) as factors. After two- or three-way ANOVA, Bonferroni's multiple comparison tests were performed as post-hoc testing for a comparison between rolling and control mice.

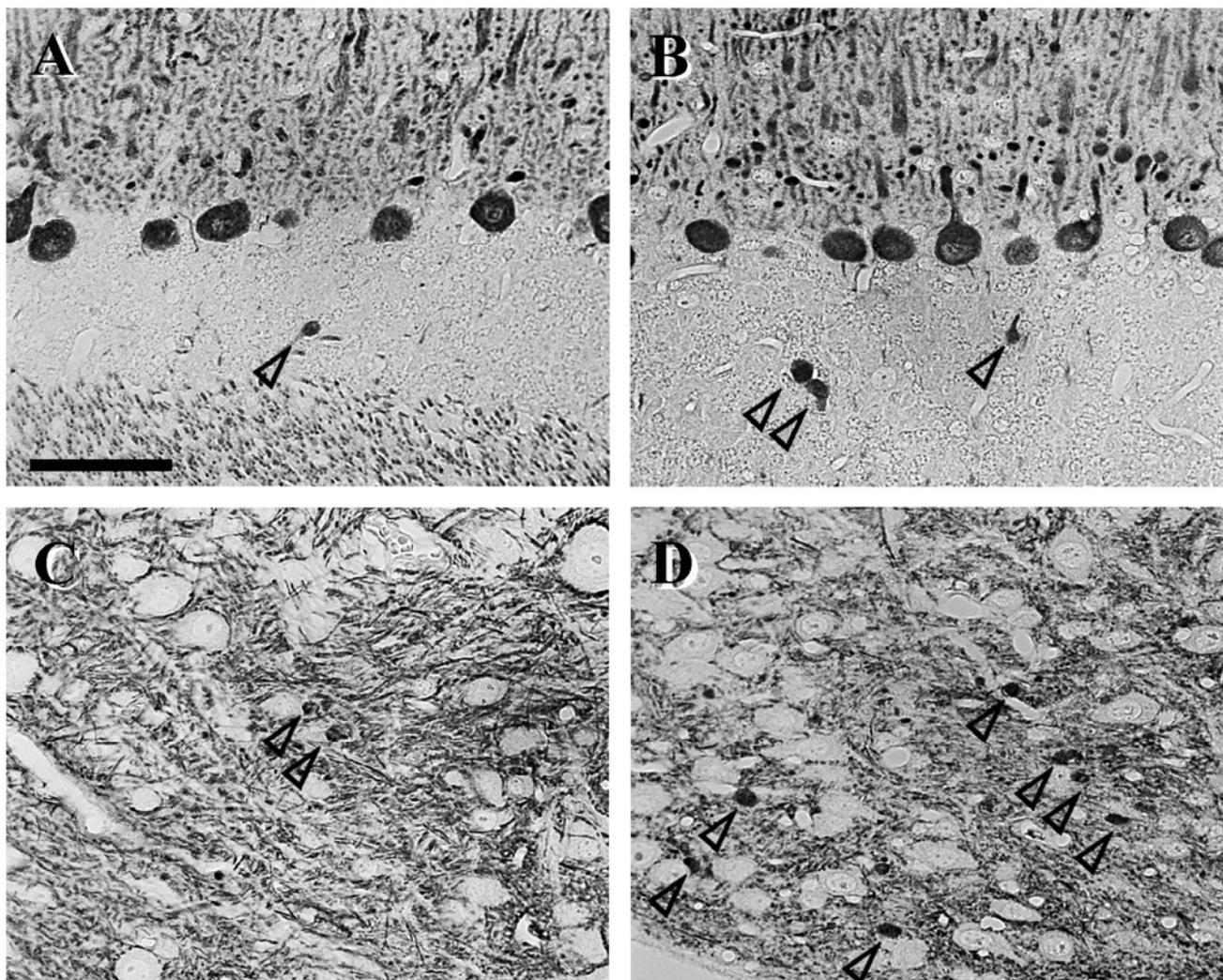
### Results

As reported previously [7,16,19], anti-calbindin D-28 k clearly labeled the torpedoes of Purkinje cell axons either in the cerebellar white matter or the deep cerebellar nuclei of rolling and control mice. Calbindin D-28 k immunopositive torpedoes were very sparse in the white

matter of rolling mice on PD 21 (Fig. 1A) as well as in age-matched control mice. In contrast, the torpedoes were frequently found in rolling mice (Fig. 1B), but rarely in control mice on PD 120. Likewise, the torpedoes in all three subdivisions of the deep cerebellar nuclei were very few in either rolling (Fig. 1C) or control mice on PD 21. On PD 120, a large number of torpedoes was observed in each deep cerebellar subdivision of rolling mice (Fig. 1D), while they were denser in the lateral nuclei than in other deep cerebellar subdivisions. In control mice, only a few torpedoes were observed throughout the deep cerebellar subdivisions by PD 120.

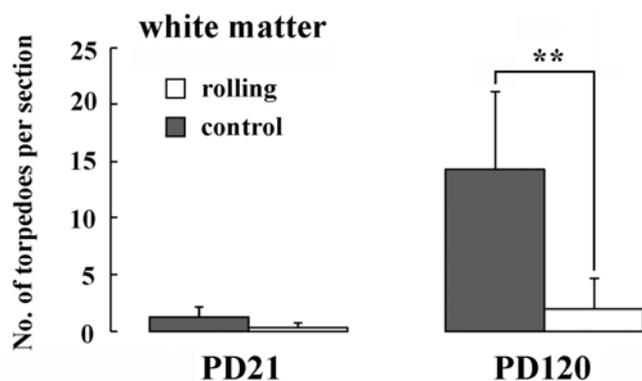
The number of the torpedoes in either the cerebellar white matter or the deep cerebellar subdivisions was estimated. Consistent with histopathological observations, that number in the white matter was low in both rolling and control mice on PD 21, and increased drastically in rolling mice, but was sustained at a lower number in control mice on PD 120 (Fig. 2). Two-way ANOVA revealed significant effects on both groups (rolling and control;  $F_{1,10}=9.881$ ,  $P<0.05$ ), ages ( $F_{1,10}=15.157$ ,  $P<0.01$ ), and their two interactions ( $F_{1,10}=7.348$ ,  $P<0.05$ ). Post-hoc testing indicated that the number of torpedoes in the white matter was significantly higher in rolling mice than in control mice (Fig. 2).

Similar to the case of cerebellar white matter, the number of torpedoes in all three subdivisions of the deep cerebellar nuclei was low in both rolling and control mice on PD 21. The number of torpedoes in each deep cerebellar subdivision increased in rolling mice but not in control mice on PD 120. Three-way ANOVA revealed significant effects on both groups (rolling and control;  $F_{1,72}=26.098$ ,  $P<0.001$ ), regions (deep cerebellar subdivisions;  $F_{2,72}=10.121$ ,  $P<0.001$ ), ages ( $F_{1,72}=69.180$ ,  $P<0.001$ ), group x age interactions ( $F_{1,72}=26.491$ ,  $P<0.001$ ), and region x age interactions ( $F_{2,72}=9.766$ ,  $P<0.001$ ). Post-hoc testing indicated that the number of torpedoes was significantly higher in all three deep cerebellar subdivisions of rolling mice than in control mice (Fig. 3). A regional difference in the number of torpedoes among the deep cerebellar subdivisions was detected only in 120-day-old rolling mice, with a significantly higher number noted in the lateral nuclei than in the medial ( $P<0.001$ ) and interposed nuclei ( $P<0.001$ ). However, it should be noted that no significant effect was observed on either group x region interactions ( $F_{2,72}=1.870$ ,  $P=0.1616$ ) or group x region x age interactions ( $F_{2,72}=1.708$ ,  $P=0.1616$ ). These results indicate that the torpedoes in the deep cerebellar nuclei of rolling mice increased with aging, while their distributions were not altered.

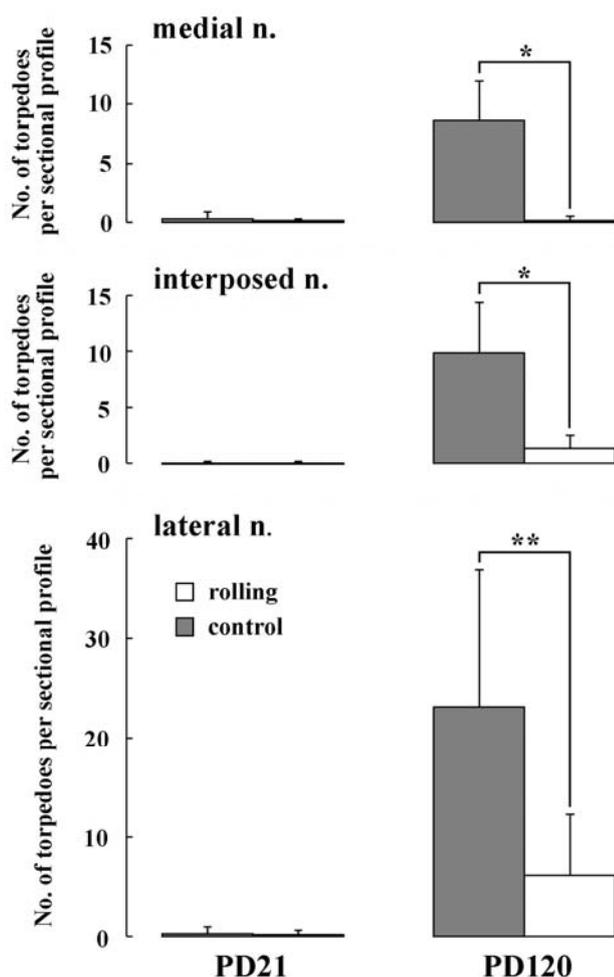


**Figure 1.** Calbindin D-28 k immunostaining in the cerebellar cortex and deep cerebellar nuclei of rolling mice. A: Cerebellar cortex on postnatal day (PD) 21. B: Cerebellar cortex on PD 120. C: Interposed nucleus of the deep cerebellar nuclei on PD 21. D: Interposed nucleus of the deep cerebellar nuclei on PD 120. Open arrowheads indicate Calbindin D-28 k immunopositive torpedoes. Scale Bar = 50  $\mu$ m.

**Figure 2.** Number of Calbindin D-28 k immunopositive torpedoes in cerebellar white matter and three basic subdivisions of deep cerebellar nuclei of rolling (closed column) and control (open column) mice.



Results are presented as the mean  $\pm$  SD. Two-way ANOVA revealed a significant effect on both groups (rolling and control;  $F_{1,10}=9.881$ ,  $P<0.05$ ), age ( $F_{1,10}=15.157$ ,  $P<0.01$ ), and their two interactions ( $F_{1,10}=7.348$ ,  $P<0.05$ ). \*:  $P<0.001$  (Bonferroni's multiple comparison test).



**Figure 3.** Number of Calbindin D-28k immunopositive torpedoes in deep cerebellar subdivisions of rolling (closed column) and control (open column) mice. Results are presented as the mean  $\pm$  SD. Three-way ANOVA revealed a significant effect on both groups (rolling and control;  $F_{1,72}=26.098$ ,  $P<0.001$ ), regions (deep cerebellar subdivisions;  $F_{2,72}=10.121$ ,  $P<0.001$ ), age ( $F_{1,72}=69.180$ ,  $P<0.001$ ), group  $\times$  age interactions ( $F_{1,72}=26.491$ ,  $P<0.001$ ), and region  $\times$  age interactions ( $F_{2,72}=9.766$ ,  $P<0.001$ ). \*:  $P<0.05$ , \*\*:  $P<0.001$  (Bonferroni's multiple comparison test).

## Discussion

Consistent with our previous studies [7;16;17], a number of calbindin D-28 k immunopositive torpedoes of Purkinje cell axons were observed in the cerebellar white matter in adult rolling mice in the present study. Our previous [19] and present studies further revealed Purkinje cell axonal torpedoes in the deep cerebellar nuclei of adult rolling mice. Since such Purkinje cell axonal torpedoes were also found in other  $Ca_v2.1$  mutant mice [18], they may constitute one of the neuropathological characteristics of  $Ca_v2.1$  mutants. Interestingly, there were very few Purkinje cell axonal torpedoes in either the cerebellar

white matter or the deep cerebellar nuclei in 21-day-old rolling mice compared to those in age-matched control mice. Such results indicate that the Purkinje cell axonal torpedoes in the rolling cerebellum developed after PD 21. Axonal torpedoes are recognized as neuropathological factors in neurological diseases [13;14] characterized by local accumulations of malaligned neurofilaments and mitochondria [13-15]. Traces of mutated  $Ca_v2.1$  channel immunostaining appeared in the torpedoes of Purkinje cell terminals in the cerebellum of rolling mice [19]. This suggests that the altered functions of the  $Ca_v2.1$  channel in rolling mice are involved in the accumulations of malaligned neurofilaments and mitochondria in the Purkinje cell axons, resulting in the development of torpedoes with aging.

In our previous study, the density of the torpedoes differed regionally in adult rolling mice, as evidenced by a more pronounced density in the lateral than in the medial and intermediate nuclei [19]. However, current three-way ANOVA analysis and post-hoc testing concluded that torpedoes in the deep cerebellar nuclei of rolling mice increased with aging without any change in their distributions. These results suggest that the axonal torpedoes appeared in non-specific populations of Purkinje cells in rolling mice. Since mutated  $Ca_v2.1$  immunostaining appeared throughout all Purkinje cells at uniform levels in  $Ca_v2.1$  mutant mice [6;8], the development of torpedoes may be associated with  $Ca_v2.1$  channel dysfunction.

Motor deficits of rolling mice are characterized by frequent lurching and abnormal cyclical movements of the hindlimbs when walking [20;21]. These are readily identifiable by their ataxic locomotion between PDs 10 and 14 [22]. As mentioned above, however, the Purkinje cell axonal torpedoes in either the cerebellar white matter or the deep cerebellar nuclei developed after PD 21. Therefore, the development of the torpedoes may not be involved primarily in the motor deficits of rolling mice, but may rather indicate a neuropathological change in the Purkinje cell axons caused by an interaction between the  $Ca_v2.1$  channel dysfunctions and aging in rolling mice.

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