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Laminar cytoarchitecture of cerebral cortex in $Ca_v2.1$ mutant, rolling mouse Nagoya

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Running title: Cortical cytoarchitecture in rolling mice

Abstract

The present study quantitatively examined the laminarcytoarchitecture of functional cortical areas of the cerebrum in $Ca_v2.1$ mutant, rolling mouse Nagoya. Brain weights were not significantly different between rolling and wild-type mice. Klüver Barrera's staining of the cerebral cortex revealed no obvious changes in ctytoarchitecture of three functional cortical areas, i.e.,primary motor (M1), somatosensory (S1) and primary visual (V1) areas,in rolling mice.The cortical thickness and the thickness of the cortical layers (I-VI) of those three areas were not significantly different between rolling and wild-type mice.The results suggest that the cytoarchitectural organization in the functional cortical areas of the cerebral cortex is not altered by a $Ca_v2.1$ gene mutation.

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Introduction

Many neuronal processes such as membrane excitability and neurotransmitter release are regulated by calcium influx through the voltage-gated Ca^{2+}channels, which areclassified intoT-, L-, N-, P/Q- and R-types bypharmacological and electrophysiological characteristics of the pore-forming a_1 subunit [1-3]. The P/Q-type Ca^{2+} channel has theα_{1A} subunit ($Ca_v2.1$),which is produced by alternating the splicing of an a_1 subunit gene family [4], andexpresses prominentlythroughout the brain[5].

Rolling mouse Nagoya carries a recessive mutant allele of the *tottering* locus (tg^{rol}) on chromosome 8 [6], whichencodes a $Ca_v2.1$ gene [7], and is known as a model for human Ca^{2+} channelopathies such as episodic ataxia type 2 and familial hemiplegic migraine [8]. This mutant mouse is characterized by a severe ataxic gait and abnormal hindlimb extension, but does not exhibit epilepsy [9;10] as seen in allelic mutants, tottering, leaner [11] and rockermice [12]. Those phenotypes areinvolved in a selective reduction of Ca^{2+}currents through the P/Q-type channel[7], which is highly expressed in cerebellar Purkinje cells not only in normal mice [5] but also in $Ca_v2.1$ mutants [12;13], including rolling mice [14]. On the other hand, the$Ca_v2.1$is known to be expressed in the cerebral cortex[5;15], andmediates Ca^{2+} inflow in pyramidal cells of layersII/IIIof the cerebral cortex [15;17].Considering the pivotal roles of the $Ca_v2.1$ channel, the presence of the mutated $Ca_v2.1$ channel in pyramidal neurons in the superficial pyramidal layer predicts dysfunction of corticocortical neuronal networks involved in abnormal synaptic signaling.The present study aimed to clarify whether fundamental laminar cytoarchitecture of the cerebral cortex was altered in rolling mice. The thicknessof cortical lamina was measured in threedifferentcortical areas, i.e., primary motor (M1), somatosensory (S1) and primary visual areas (V1)inrolling mice.

Materials and Methods

Animals

All experimental procedures were conducted in accordance with the guidelines of the NationalInstitutes 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 approvedthe 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^{rol}/tg^{rol}), raised by intercrossing heterozygous pairs, were readily identifiable by their ataxiclocomotion between postnatal days 10 and14. Wild-type (+/+) mice were used as controls.

Tissue preparation

A total of 5 male rolling and 5 male wild-type mice at 2 months of age were used. Animals were perfused with

0.9% NaCl, followed by 4% paraformaldehyde and 0.1% picric acid in 0.1 M phosphate buffer, pH. 7.4, under deep anesthesia with sodium pentobarbital (25mg/10g body weight).Brains were immersed in the same fixative. The brains were weighed just after separating them from the spinal cord at the C1 level, and photographs of their dorsal surfaceswere taken (Fig. 1). Then, the cerebella were separated by cutting the cerebellar peduncles, and weighed. Brains were cytoprotected in 30% sucrosein 10 mM phosphate-buffered saline overnight. The specimens werethen frozen in optimal cutting temperature (OCT) embedding compound, and sectioned serially in the coronal plane at 40 μm by aRetratome (REM-700; Yamato Koki Industrial, Osaka, Japan) with a refrigeration unit (Electro Freeze MC-802A, Yamato Koki Industrial Co., Ltd.).Sections were stained with Klüver–Barrera according to the protocols of Sheehan and Hrapchak (1980) [18].

Quantitative analysis

Three distinct cortical areas, i.e.,the M1,S1 and V1, weredefined on the KlüverBarrera'sstainedsectionswith references to the atlas of Paxinos and Franklin (2004) [19].The thickness oflayers I, II/III, IV, Vand IV of each cortical area as shown in Figure 2 was respectively measured using Image J software (National Institutes of Health, Washington, USA) oncaptured images.

Statistical analyses

Brain weights were statistically analyzed by Student's *t*-test.In order to evaluate mutant- and cortical region-related changes in the cortical thickness, two-way ANOVA testwas carried out using genotypes (rolling and wild-type), and cortical areas (the M1, S1 and V1) as factors. Furthermore, mutant- and cortical region-related changes in the thickness of each cortical layer were statistically analyzed by three-way ANOVA using genotypes, cortical areas and cortical layers (layers I, II/III, IV, V and VI) as factors.

Results

Photographs of the dorsal view of rolling and wild-type micebrains were shown in Figure 1.No obvious differences in the gross appearance of the brainswerenoted between rolling and control mice. The weights of the brainsincluding the olfactory bulb, cerebrum, diencephalone, brainstemand cerebellum were not significantly different between the two genotypes examined.

Klüver-Barrera's stained coronal sections of three functional cortical areas, i.e., the M1, S1, and V1,are shown in Figure 2.Six layers of the cortex were defined in all three cortical areas examined: layer I consisted of neuropil with a few scattered neurons adjacent to the pial surface; layer II/III contained small- and medium-sized pyramidal neurons, and small granular neurons; layer IV contained small granular neurons; layer V contained large-sized pyramidal neurons; and layer VI contained the polymorphic neurons.Such cortical layers were distinguishable in each cortical area, except for layer IV in the M1, which was too thin to define.While cortical thickness varied among each cortical area,no obvious difference in the cortical organization was obtained between rolling and wild-type mice. The cortical thickness of each cortical area was quantified, and the results are shown in Figure 3. Two-way ANOVA revealed significant effects on cortical areas ($F_{2,23}$ = 101.83, P<0.001), but not on genotypes (rolling and control mice) and found an interaction between these two factors.

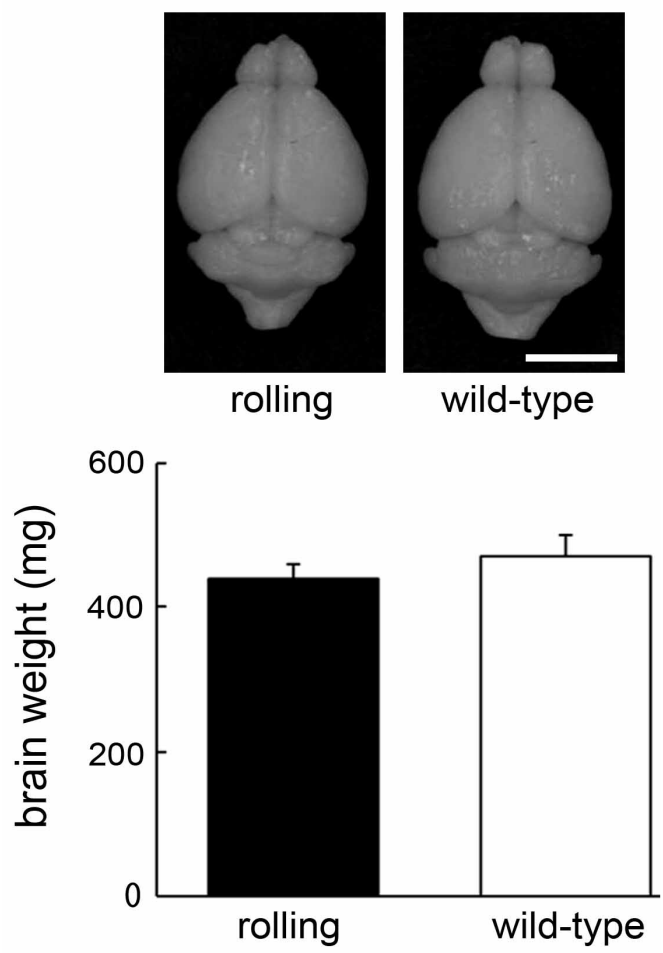

Fig. 1

Figure 1. Gross appearances (upper photographs) and brain weight(lower histogram) of rolling mouse Nagoyaand wild-type mice.*Results of brain weight are presented as the mean ±SD. Scale bar = 5mm.*

The thickness of cortical layers was examined in each functional cortical area, and the results are shown in Figure 4. Three-way ANOVA revealed significant effects on cortical areas ($F_{2,116}$= 221.52, P<0.001),cortical layers ($F_{4,116}$ = 683.71, P <0.001), and interactions between

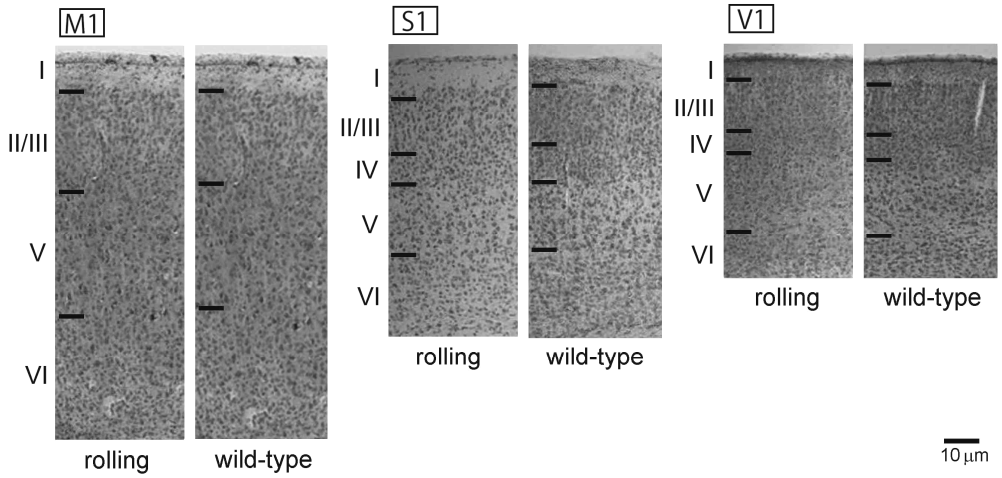

Fig. 2

Figure 2. Klüver Barrera's stainedcoronal sections of primary motor (M1), primary sensory (S1) and primary visual (V1) areas of cerebral cortex of rolling and wild-type mice. *The layer IV in the M1 was too thin to be distinguishable in either rolling or wild-type mice. Scale bar = 10μm.*

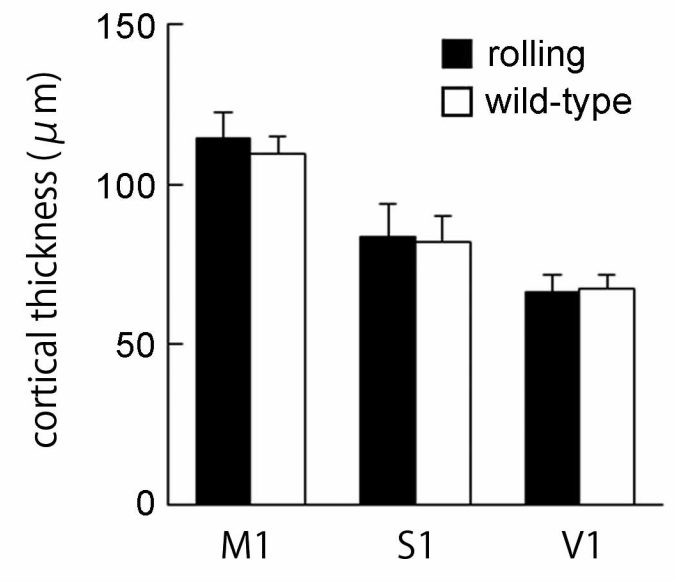

Fig. 3

Figure 3. Histogram showing cortical thickness of primary motor (M1), somatosensory (S1) and primary visual (V1) areas of cerebrum in rolling and wild-type mice. *Results are presented as the mean ± SD. No significant effect on the cortical thickness of three cortical areas between rolling and wild-type mice was noted by three-way ANOVA.*

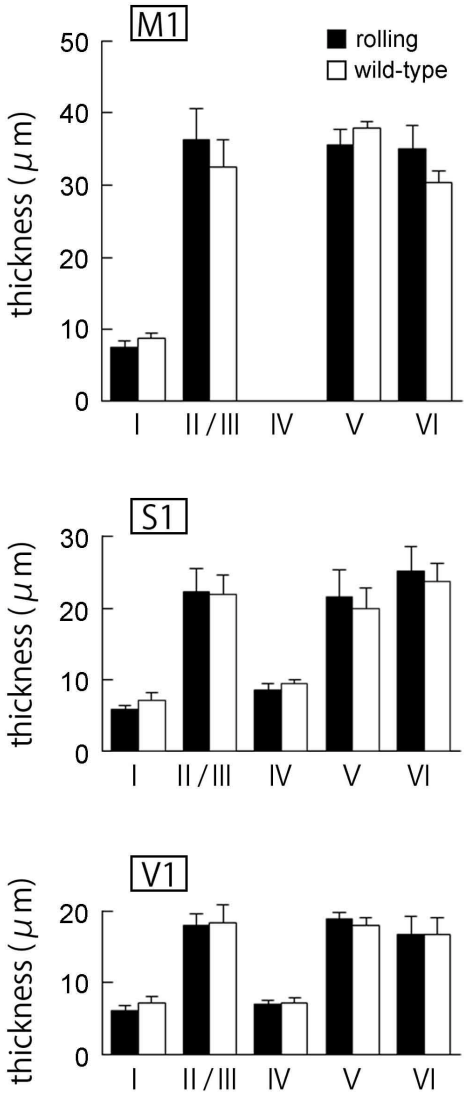

Fig. 4

Figure 4. Histogram showing thickness of cortical layers I-VI in primary motor (M1), somatosensory (S1) and primary visual (V1) areas of cerebrum of rolling and wild-type mice. The layer IV in the M1 was too thin to be distinguishable in either rolling or wild-type mice. *Results are presented as the mean ± SD. No significant effect on the thickness of each layer between rolling and wild-type mice was noted in three cortical areas by three-way ANOVA.*

these two factors($F_{8,116}$ = 83.35, P<0.001). However, there were no significant effects on genotypes(rolling and wild-type mice)or on theinteractions of genotypes with the other two factors. Thus, the thickness of cortical layers varied among the cortical areas with no differences between rolling and wild-type mice.

Discussion

The $Ca_v2.1$ channel is known to be expressed in various regions of the central nervous system (CNS),the most prominent expression of which is observed in the cerebellum [5]. Since homozygous rolling mice exhibit motor deficits characterized by a severe ataxic gait and abnormal hindlimb extension [9;10], a number of studies have been reported in terms oftheir cerebellar abnormalities [15;20;21]. Furthermore, heterozygous rolling mice (tg^{rol}/+) are involved in functions of the central and peripheral nervous systems: NMDA-mediated signaling in the hippocampus and nucleus accumbens in short term learning [22] and age-related emotional changes by alterations in the serotonergic system [25]. Thus, the presence of the mutated $Ca_v2.1$ channel is involved in functions of various regions of the CNS. In the present study, laminar cytoarchitecture of the cerebral cortex revealed by thickness of cortical layers was not altered throughout the M1, S1 and V1 in homozygous rolling mice. On the other hand, the cerebellum is known as the CNS region with the most prominent expression of $Ca_v2.1$ [5], althoughno cytoachitectural abnormalities weredetectedthere[15;18;21]. That evidence suggests that the $Ca_v2.1$ gene mutation does not alter the fundamental laminar organization of the cerebrum as well as the cerebellum, but that it is involved in the functions of those CNS regions.

In the present study, the brain weights were not different between homozygous rolling and wild-type mice. A number of studies have been reported concerning the weights of various CNS regions of homozygous rolling mice, and the reduced weights were marked in specific regions, i.e., the cerebellum and brainstem [28] The reduced cerebellar weight of homozygous rolling mice is thought to be linked with apoptosis of a significant number of cerebellar granule cells [29]. Furthermore, susceptibility to the $Ca_v2.1$ gene mutation was heterogeneous among different Purkinje cell populations in another $Ca_v2.1$ mutant mice, tottering, which exhibited selective loss ofanonphosphorylated form of neurofilament heavy chain (SMI-32) immunopositive Purkinje cells [30;31]. In the present study, the cortical thickness and the thickness of the cortical layers were not altered throughout the M1, S1 and V1 in homozygous rolling mice. The results suggest that expression of the mutated $Ca_v2.1$ channel is not involved in the laminar organization and/or neuronal degeneration in the cerebral cortex.

In our previous studies and those by others, the abnormality in Purkinje cell morphology characterized by axonal torpedoes (or swellings) have been obtained in homozygous rolling mice [15;18;21;32;33] and other $Ca_v2.1$ mutant mice [34]. Such torpedoes are known as neuropathological signs characterized by local accumulations of malalignedneurofilaments and mitochondria [35-37]. Therefore, expression of the mutated $Ca_v2.1$ channel may alter the morphology of Purkinje cell axons, which is related to $Ca_v2.1$ channel-related neuronal functions, i.e., neuronal transmitter release [38]. The $Ca_v2.1$ channel is known to be expressed in the cerebral cortex [6;11], and mediates Ca^{2+} inflow in pyramidal cells of layers II/III of the cerebral cortex [12;13]. Therefore, the mutated $Ca_v2.1$ channel may be involved in neuronal functions of pyramidal neurons by impairing axonal transport in rolling mice.

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