Integrative Molecular Medicine

Research Article ISSN: 2056-6360

Age-related difference in nociceptive behavior of Cav2.1α_1 mutant mice, *rolling Nagoya*

Ying Zhou[1], Kimie Niimi[2], Weidong Li[1] and Eiki Takahashi[1,2*]

[1]Bio-X Institutes, Key Laboratory for the Genetics of Developmental and Neuropsychiatric Disorders (Ministry of Education), Shanghai Jiao Tong University, Shanghai, 200240, People's Republic of China

[2]Research Resources Center, RIKEN Brain Science Institute, Saitama, 351-0198, Japan

Abstract

Although *rolling Nagoya* mice carrying a mutation in the α_1 subunit of the Cav2.1 channel demonstrate severe ataxia, heterozygous *rolling Nagoya* (*rol*/+) mice seem no apparent behavioral abnormalities. However, in our previous study, aged rol/+ mice show deficits with regard to spatial short-term memory in the object location test. These results indicate that onset of phenotypes in *rol*/+ mice is age dependence. However, it is not yet clear whether nociceptive transmission deficits accompany accelerated aging in *rol*/+ mice. To assess whether *rol*/+ mice exhibit an age-related abnormality of nociceptive transmission, we examined a battery of tests using the von Frey test for mechanically induced response, the hot plate test for thermally induced response, and the formalin paw test for chemically induced response. In the von Frey test and the hot plate test, although 2-month-old *rol*/+ and +/+ mice had similar responses, 22-month-old *rol*/+ mice showed nociceptive transmission deficits. In the formalin paw test, 2-month-old *rol*/+ and +/+ mice had similar responses, while 22-month-old *rol*/+ mice exhibited attenuated Phase 2 response, but normal Phase 1 response. These findings indicate that the *rol*/+ mice could be useful to delineate the involvement of age-related nociceptive mechanisms.

Introduction

Neuronal voltage-dependent Ca^{2+} channels (VDCCs) including Cav2.1 (P/Q-type), Cav2.2 (N-type), and Cav2.3 (R-type) channels mediate a number of neuronal functions including neuronal excitation, neurite outgrowth, synaptogenesis, neurotransmitter release, neuronal survival, differentiation, plasticity, and regulation of gene expression [1-3]. The VDCC is a molecular complex comprised of several subunits: α1, α2-δ, β, and γ [1]. The α1 subunit is essential for channel functioning and determines fundamental channel properties [1]. The α1 subunit of the Cav2.1 channel (Cav2.1α1) broadly expresses in the presynaptic terminals [1]. Thus, alterations in the Ca^{2+} current through Cav2.1α1 would induce neuronal and circuit dysfunction.

Rolling Nagoya (*rol/rol*) mice carrying a mutation in the Cav2.1α1 demonstrate severe ataxia [4,5]. It has been reported that Ca^{2+} influx through Cav2.1 decreases with aging [6]. In a previous study, we examined age-related cognitive alterations [7]. Although we found no difference between 2-month-old heterozygous *rolling Nagoya* (*rol*/+) and wild-type (+/+) mice, 22-month-old *rol*/+ mice showed decreased spatial learning compared with age-matched +/+ mice.

The aging brain shows age-associated abnormalities in peptidergic, cholinergic, monoaminergic, and amino acid neurotransmitter release [8-10], suggesting that changes in these domains might contribute to deficits in neuronal functions. The nervous systems express conspicuous signs of aging in the form of memory dementia, which commonly affect the elderly human population. Elderly people, especially those over eighty years of age, are the fastest growing segment of the population throughout the world, and this trend has important implications for the study of pain. It is important to understand the relationship between aging and pain, since it has been reported that the pain threshold is significantly increased in aged human subjects [11]. Several studies have examined age differences in nociceptive behaviors in the rat, and the results showed that older animals are less responsive than younger animals [12,13]. There are two different types of nociception: phasic and tonic nociception. Phasic nociception refers to a brief transient pain elicited by a brief noxious stimulus, and standard tests include the von Frey and hind paw pressure tests for mechanically induced response and the tail flick and hot plate tests for thermally induced response. Tonic nociception refers to continuous pain elicited by inflammation, and the standard test is the formalin paw test for chemically induced response. It was reported that 24-month-old rats were less sensitive than 3-month-old rats in terms of pain responsiveness in the hind paw pressure test, tail flick test, hot plate test [12], and formalin paw test [13]. The expression level of Cav2.1α1 in synapses decreases with aging [14]. Given that the precise regulation of Ca^{2+} signaling is important for functions of neurons and circuits, alterations in Ca^{2+} current through Cav2.1 in aged *rol*/+ mice would affect nociceptive behaviors. However, it is not yet clear whether nociceptive transmission deficits accompany accelerated aging in *rol*/+ mice.

In this study, we examined age-related nociceptive responses of *rol*/+ mice by using the von Frey test for mechanically induced response, the hot plate test for thermally induced response, and the formalin paw test for chemically induced response.

Correspondence to: Eiki Takahashi, DVM, PhD, Research Resources Center, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama, 351-0198, Japan, Tel: +81-48-467-5871; Fax: +81-48-467-9692; **E-mail:** etakahashi@brain.riken.jp

Key words: *aging, Cav2.1, nociceptive behavior, Rolling Nagoya mice*

Received: April 20, 2015; **Accepted:** May 14, 2015; **Published:** May 17, 2015

Materials and methods

Mice

All animal procedures were approved by the Animal Experiments Committee of Shanghai Jiao Tong University and RIKEN. The *Rolling Nagoya* strain, with a mixed C57BL/6J and SIII genetic background, was provided by the RIKEN BRC with the support of the National BioResource Project of the Ministry of Education, Culture, Sports, Science, and Technology in Japan. *Rolling Nagoya* was backcrossed with C57BL/6J mice for 12 generations. Male *rol*/+ and +/+ F1 progeny were derived from a cross between *rol*/+ mice and genotyped by PCR using tail DNA [7]. The mice were given free access to water and food pellets (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and were kept under a 12/12-h light/dark cycle (lights on from 08:00 to 20:00) at 23 ± 1°C and 55 ± 5% humidity. We used 2-month-old (young) and 22-month-old (aged) *rol*/+ and +/+ mice and all tests were performed during the light phase on male mice only.

Histologic examination

Sections of the dorsal root ganglion (DRG), lumbar level of the spinal cord, and thalamus from 2-month-old (*rol*/+, +/+: *n*=6, 5) and 22-month-old (*rol*/+, +/+: *n*=5, 5) were fixed in 4% buffered formalin and embedded in paraffin. Paraffin sections 4 μm thick were stained with hematoxylin and eosin (HE).

Assessment of nociceptive behavior

Experiments including the von Frey test, the hot plate test, and the formalin paw test were performed according to our previous report [15]. We used separate groups of mice for each of three behavioral tests. The von Frey test and the hot plate test were conducted between 10:00 AM and 4:00 PM by a well-trained experimenter who was blinded as to the mouse strains. The formalin paw test was conducted between 10:00 AM and 12:00 PM by a well-trained experimenter who was blinded as to the mouse strains, because it has been reported that there is a marked decrease in tonic pain response in the afternoon [16].

In the von Frey test, 2-month-old (*rol*/+, +/+: *n*=12, 12) and 22-month-old ((*rol*/+, +/+: *n*=8, 8) were placed individually in plastic cages (11 x 18 x 15 cm) with a wire mesh bottom. After an acclimation period of at least 60 min, von Frey hairs (Touch Test Sensory Evaluator, North Coast Medical, San Jose, CA, USA) of different bending forces (0.02, 0.04, 0.07, 0.16, 0.40, 0.60, 1.00, 1.40, 2.00, 4.00 g) were pressed perpendicularly against the plantar skin until they slightly buckled. Enough pressure to just bend the hair was used and maintained for 6 sec on the left hind paw. If the mouse withdrew its foot, the next lowest force hair was tested. If the mouse did not withdrew its foot, the next highest force hair was tested. Once the mouse responded, a total of six stimulations of the same intensity was applied to the left hind paw at intervals of 6 sec. The minimum force (*g*) at which mice exposed six times to the same stimulus showed at least four instances of paw-lifting was recorded.

In the hot plate test, 2-month-old (*rol*/+, +/+: *n*=12, 10) and 22-month-old (*rol*/+, +/+: *n*=8, 8) were placed on a hot plate (Hot plate Analgesia Meter, OTS40, Medizintechnik, Burgdorf, Germany) heated to 50 °C. The cut-off time was 30 sec. The latency until mice showed the first sign of discomfort (forepaw or hind paw licking/biting) was recorded.

In the formalin paw test, 20 μl of 3% formalin solution was injected subcutaneously under the dorsal surface of the left hind paw of 2-month-old (*rol*/+, +/+: *n*=10, 10) and 22-month-old (*rol*/+, +/+: *n*=8, 8). The animals' responses, such as licking or biting the left hind paw, were recorded between 0 and 15 min (phase 1) and 15 and 60 min (phase 2) after the injection.

Statistical analyses

Data are presented as the means ± SEM. Statistical analysis was conducted by using Excel Statistics 2006 (SSRI, Tokyo, Japan). Data were analyzed using Dunnett's test or by analysis of variance (ANOVA) followed by Bonferroni's correction for multiple comparisons between groups, if appropriate The results were considered significant if the probability of error was 5% or less.

Results

There was no apparent morphological abnormality of the DRG, lumbar spinal dorsal horn and laternal ventroposterior nucleus of 2- and 22-month-old *rol*/+ mice on HE staining (data not shown).

The nociceptive responses to mechanical stimuli were measured using the von Frey test. No significant difference was observed between 2-month-old *rol*/+ (0.7 ± 0.08 g) and +/+ (0.6 ± 0.09 g) mice ($F(1,22)=0.025$, $P>0.05$). On the other hand, there was significant difference between 22-month-old *rol*/+ (1.6 ± 0.11 g) and +/+ (1.0 ± 0.17 g) mice ($F(1,14)=5.232$, $P<0.05$).

Thresholds for noxious heat stimuli were measured using the hot plate test. Although no significant difference was observed between 2-month-old *rol*/+ (14.3 ± 0.94 s) and +/+ (13.9 ± 0.67 s) mice ($F(1,22)=0.108$, $P>0.05$) at 50°C, thermal nociceptive threshold was significant difference between 22-month-old *rol*/+ (29.7 ± 1.81 s) mice and +/+ (22.1 ± 1.73 s) mice of the same age ($F(1,14)=5.561$, $P<0.05$).

Nociceptive responses to chemical stimuli were measured using the formalin paw test in the 2-month-old (Figure 1A) and 22-month-old (Figure 1B) mice. Saline injection in the formalin test did not induce a pain response in any of the mice (data not shown). Subcutaneous injection of formalin produced a characteristic biphasic licking/biting response consisting of an initial, rapidly decaying acute phase (phase 1, 0–15 min after injection) and a long-lived tonic phase (phase 2, 15–60 min after injection). In phase 1, injected formalin directly stimulated the peripheral nociceptors and elicited pain responses, while in phase 2, inflammation induced by formalin injection elicited the response. There was no significant difference in licking/biting time in phase 1 between 2-month-old *rol*/+ and +/+ mice ($F(1,18)=0.017$, $P>0.05$) or between 22-month-old *rol*/+ and +/+ mice ($F(1,18)=0.016$, $P>0.05$). On the other hand, although the total licking/biting times of phase 2 responses in 2-month-old *rol*/+ and +/+ mice were similar ($F(1,18)=0.014$, $P>0.05$), the total licking/biting time of phase 2 responses in 22-month-old *rol*/+ mice was significantly decreased compared with that of age-matched +/+ mice ($F(1,14)=8.657$, $P<0.01$).

Discussion

In this study, nociceptive responses were examined using the von Frey test for mechanically induced response, the hot plate test for thermally induced response, and the formalin paw test for chemically induced response in *rol*/+ mice of different ages.

The pain signals produced by the von Frey test enter DRG where the primary afferents are located, leading to excitation of the lumbar levels of the spinal cord [17]. The pain signals produced by the hot plate test and formalin paw test stimulate the DRG and then this information is conveyed to the lumbar levels of the spinal cord and supraspinal

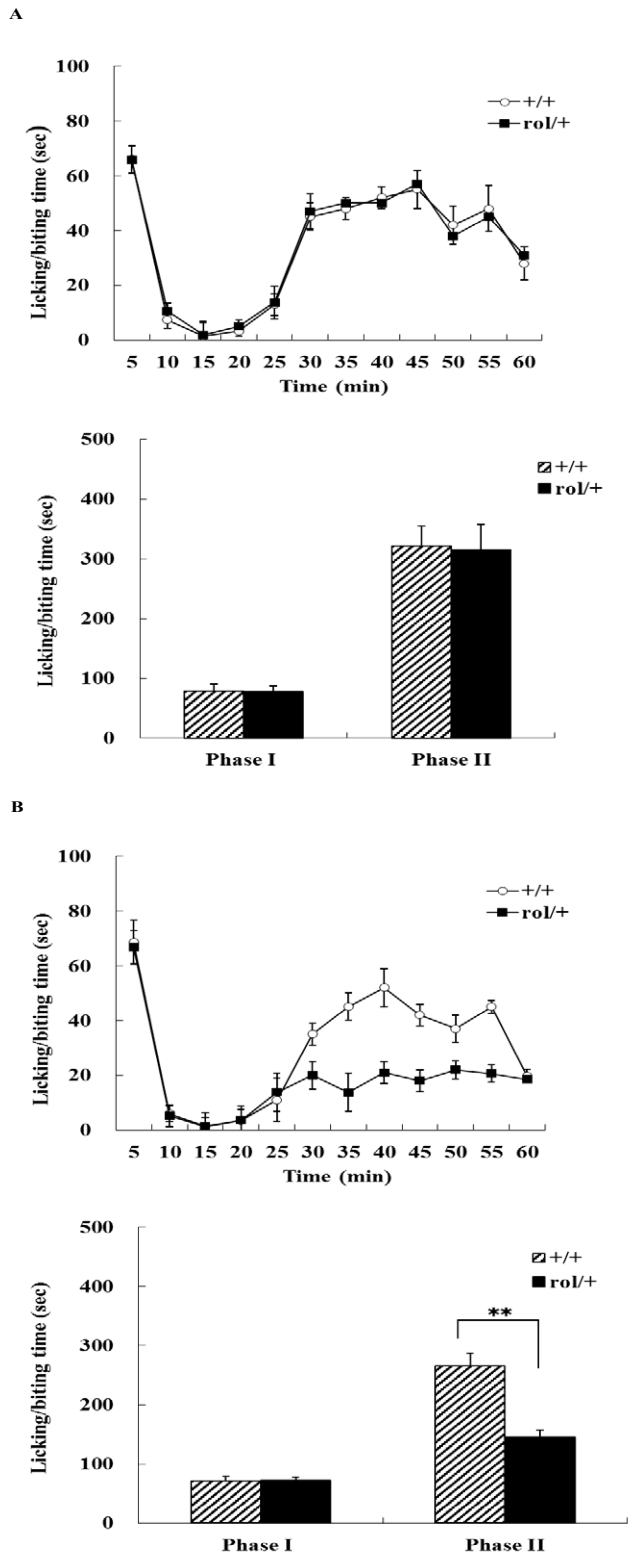

Figure 1. Chemically evoked nociceptive responses. Time–effect curve of nociceptive responses (licking/biting) of *rol*/+ and +/+ mice at the age of 2 month (A, upper) and 22 months (B, upper) in the formalin test. Licking/biting time in every 5 min period was plotted. Histograms show the licking/biting time during phase 1 and phase 2 of *rol*/+ and +/+ mice at the age of 2 month (A, lower) and 22 months (B, lower). Asterisks indicate a significant difference from age-matched +/+ mice (**, P<0.01).

levels (e.g., thalamus) [17]. Therefore, we examined the structure of the DRG, lumbar spinal dorsal horn and laternal ventroposterior nucleus of 2- and 22-month-old *rol*/+ mice. There was no apparent morphological abnormality on HE staining. It is therefore unlikely that morphological changes during development led to impairment of nociceptive response.

In the von Frey test, significant difference was observed between 22-month-old *rol*/+ and +/+ mice. In the hot plate test, significant difference was also observed between 22-month-old *rol*/+ and +/+ mice. Nociceptive information from mechanical stimuli is modulated by a simple spinal reflex and by descending pathways [17,18]. Thermally induced pain behavior is known to be associated with both spinal and supraspinal involvement in nociception [17, 19]. The *rol*/+ mice showed age-related nociceptive transmission deficits in the von Frey test and the hot plate test. These results indicate that alterations in Ca^{2+} current through Cav2.1 in aged *rol*/+ mice would affect inputs via primary afferents from mechanical and thermal stimulus, or spinal and supraspinal reflexes.

In the formalin paw test, *rol*/+ mice at the age of 22 months exhibited attenuated phase 2 response, but normal phase 1 response. It has been reported that Cav2.1 channel plays a role in the regulation of glutamate release [20,21]. Gene knockdown with intrathecal small interfering RNA of *N-methyl-D-aspartate* (NMDA) receptor NR2B subunit inhibits phase 2 of formalin-induced nociception [22]. These results indicate that aged *rol*/+ mice would affect glutamate release leading to nociceptive nociceptive transmission deficits.

The decrease in pain response could be related to motor deficits rather than pain response. However, because phase 1 of formalin paw test were normal in *rol*/+ mice at 22 months of age, this could be considered as evidence of intact motor response in the nociceptive behavioral tests. Although we would need to study age-related functional changes by means of electrophysiological analysis to examine the relationship between the Cav2.1 channel and neurotransmitter release in *rol*/+ mice, the present results indicate that age-related change in nociceptive response to mechanical, thermal, and chemical stimuli in *rol*/+ might arise from alterations of neurotransmitter mechanisms underlying the deterioration of depolarization-induced Ca^{2+} influx through the Cav2.1 channel.

Acknowledgments

This work was supported by China 973 project (2010CB529604) and National Scientific Foundation of China (81271511 and 30900432).

Authors' contributions

WL and ET designed and supervised the research, and wrote the manuscript. YZ and KN performed the surgeries and behavioral experiments. All authors read and approved the final version of the manuscript.

References

1. Catterall WA, Few AP (2008) Calcium channel regulation and presynaptic plasticity. *Neuron* 59: 882-901. [Crossref]

2. Evans RM, Zamponi GW (2006) Presynaptic Ca^{2+} channels--integration centers for neuronal signaling pathways. *Trends Neurosci* 29: 617-624. [Crossref]

3. Jarvis SE, Zamponi GW (2007) Trafficking and regulation of neuronal voltage-gated calcium channels. *Curr Opin Cell Biol* 19: 474-482. [Crossref]

4. Mori Y, Wakamori M, Oda S, Fletcher CF, Sekiguchi N, et al. (2000) Reduced voltage sensitivity of activation of P/Q-type Ca^{2+} channels is associated with the ataxic mouse mutation *rolling Nagoya* (tg(rol)). *J Neurosci* 20: 5654-5662. [Crossref]

5. Oda S (1973) The observation of rolling mouse Nagoya (rol), a new neurological mutant, and its maintenance (author's transl). *Jikken Dobutsu* 22: 281-288. [Crossref]

6. Tanaka Y, Ando S (2001) Age-related changes in the subtypes of voltage-dependent calcium channels in rat brain cortical synapses. *Neurosci Res* 39: 213-220. [Crossref]

7. Takahashi E, Niimi K (2009) Spatial learning deficit in aged heterozygous Cav2.1 channel mutant mice, rolling mouse Nagoya. *Exp Gerontol* 44: 274-279. [Crossref]

8. Dickstein DL, Kabaso D, Rocher AB, Luebke JI, Wearne SL, et al. (2007) Changes in the structural complexity of the aged brain. *Aging Cell* 6: 275-284. [Crossref]

9. Foster TC (2007) Calcium homeostasis and modulation of synaptic plasticity in the aged brain. Aging Cell 6: 319-325. [Crossref]

10. Mora F, Segovia G, del Arco A (2007) Aging, plasticity and environmental enrichment: structural changes and neurotransmitter dynamics in several areas of the brain. *Brain Res Rev* 55: 78-88. [Crossref]

11. Harkins SW, Price DD, Martelli M (1986) Effects of age on pain perception: thermonociception. *J Gerontol* 41: 58-63. [Crossref]

12. Akunne HC, Soliman KF (1994) Serotonin modulation of pain responsiveness in the aged rat. *Pharmacol Biochem Behav* 48: 411-416. [Crossref]

13. Gagliese L, Melzack R (1999) Age differences in the response to the formalin test in rats. *Neurobiol Aging* 20: 699-707. [Crossref]

14. Iwamoto M, Hagishita T, Shoji-Kasai Y, Ando S, Tanaka Y (2004) Age-related changes in the levels of voltage-dependent calcium channels and other synaptic proteins in rat brain cortices. *Neurosci Lett* 66: 277–281.

15. Takahashi E, Sagane K, Nagasu T, Kuromitsu J (2006) Altered nociceptive response in ADAM11-deficient mice. *Brain Res* 1097: 39-42. [Crossref]

16. Meunier CJ, Burton J, Cumps J, Verbeeck RK (1998) Evaluation of the formalin test to assess the analgesic activity of diflunisal in the rat. *Eur J Pharm Sci* 6: 311-316. [Crossref]

17. Chapman CR, Casey KL, Dubner R, Foley KM, Gracely RH, et al. (1985) Pain measurement: an overview. *Pain* 22: 1-31. [Crossref]

18. Garcia-Nicas E, Laird JM, Cervero F (2001) Vasodilatation in hyperalgesic rat skin evoked by stimulation of afferent A beta-fibers: further evidence for a role of dorsal root reflexes in allodynia. *Pain* 94: 283-291. [Crossref]

19. Wall PD, Melzack R (1999) Assessing transient and persistent pain in animals, In: R. Dubner and K. Ren (Eds.), Textbook of Pain, Churchill Livingstone, Edinburgh 360–362.

20. Kimura M, Katayama K, Nishizawa Y (1999) Role of glutamate receptors and voltage-dependent calcium channels in glutamate toxicity in energy-compromised cortical neurons. *Jpn J Pharmacol* 80: 351-358. [Crossref]

21. Lee CY, Chen CC. Liou HH (2009) Levetiracetam inhibits glutamate transmission through presynaptic P/Q-type calcium channels on the granule cells of the dentate gyrus. *Br J Pharmacol* 158: 1753–1762. [Crossref]

22. Tan PH, Yang LC, Shih HC, Lan KC, Cheng JT (2005) Gene knockdown with intrathecal siRNA of NMDA receptor NR2B subunit reduces formalin-induced nociception in the rat. *Gene Ther* 12: 59-66. [Crossref]