

Decreased Level of Anxiety-like Behaviors and Decreased Expression of Brain Tryptophan Hydroxylase in Aralar-deficient Mice: A Preliminary Study

Yasuhide Matsumoto¹⁾, Kyoko Shimotsu¹⁾, Mikio Iijima²⁾, Takeyori Saheki³⁾, Shiro Nakagawa¹⁾

¹⁾ Laboratory for Neuroanatomy, Graduate School of Medical and Dental Sciences, Kagoshima University,

²⁾ Department of Biochemistry and Genetics, Graduate School of Medical and Dental Sciences, Kagoshima University,

³⁾ Institute for Health Sciences, Tokushima Bunri University

(Accepted 31 October 2011)

Abstract

Aralar, an isoform of the calcium-binding aspartate-glutamate mitochondrial carrier, is distributed specifically in the brain and skeletal muscles. The aim of the present study was to investigate behaviors induced by the decreased expression of aralar in the brain and to assess whether expression of brain tryptophan hydroxylase, a key enzyme in serotonin synthesis, is correlative with behavioral changes in aralar-deficient mice. In the elevated plus-maze test, heterozygous mice (Aralar^{+/-}) spent more time in the open arms and showed a significant increase in the number of entries to the open arms compared to wild-type littermates (Aralar^{+/+}). In addition, Aralar^{+/-} mice spent more time in the light compartment in a light-dark exploration test compared to Aralar^{+/+} mice. Western blot analysis showed a decreased expression of brain tryptophan hydroxylase in Aralar^{+/-} mice compared to Aralar^{+/+} mice. These results suggested that Aralar^{+/-} mice exhibit a lower level of anxiety-like behaviors compared to Aralar^{+/+} littermates. This might be correlative with the reduced serotonin synthesis.

Key words: aralar, serotonin, tryptophan hydroxylase, elevated plus-maze test, light-dark exploration test

Introduction

Aralar, an isoform of the calcium-binding aspartate-glutamate mitochondrial carrier, is highly expressed in the brain and skeletal muscles, and functions in the transport of aspartate from mitochondria to the cytosol in the exchange for glutamate¹⁾. It also plays a role in the transport of nicotinamide adenine dinucleotide (NAD), reducing equivalents from the cytosol to mitochondria as a member of the malate-aspartate shuttle²⁾. Aralar is a member of the mitochondrial solute carrier family of nuclear encoded, membrane-embedded proteins that promote solute transport across the inner mitochondrial membrane (SLC 25)³⁾. It is encoded by SLC25A12⁴⁾.

Recent research has focused on the role of amino acid transporters because malfunctions in these proteins are known to contribute to inherited and acquired diseases [for review, see ref. 5]. For example, aralar is thought to

play an important role in myelin formation by supplying aspartate for the synthesis of N-acetylaspartate in neurons in the central nervous system, and aralar-knockout mice show hypomyelination in the brain⁶⁻⁸⁾. We have reported a total loss of aralar protein in the brains of aralar-knockout mice (Aralar^{-/-}) and approximately half of wild-type levels in heterozygous mice (Aralar^{+/-}) by Western blot⁶⁾.

The elevated plus-maze test is one of the most widely used animal models of anxiety. It was developed based on the idea that rodents avoid open, elevated alleys, presumably because of fear⁹⁾. When exposed to the elevated plus-maze test, a naïve mouse will show signs of conflict, exploring the novel area and escaping the open arm. This “pullback” behavior is decreased by classic anxiolytic drugs and increased by anxiogenic agents^{10,11)}. A previous report suggested that a combination of three widely used tests of anxiety, namely the elevated plus-maze test, the light-dark exploration test, and the open-

field test, provides comprehensive, reliable, and rapid assessment of the emotional profile of rodents¹²⁾.

Results of many studies have indicated interesting correlations between brain serotonin content and anxiety-like behaviors¹³⁻¹⁵⁾. The biosynthesis of serotonin is regulated by two rate-limiting enzymes, tryptophan hydroxylase-1 and -2¹⁶⁾. Tryptophan hydroxylase-2 is specifically expressed in the brain and is referred to as the central isoform, whereas tryptophan hydroxylase-1 is mainly present in the pineal gland, thymus, spleen, and gut, and is referred to as the peripheral isoform¹⁷⁾.

In the present preliminary study, we used the elevated plus-maze test and the light-dark exploration test to assess anxiety-like behaviors in aralar-deficient mice, and used Western blot to quantify brain tryptophan hydroxylase levels.

Materials and methods

Generation of mice and genotyping

Mice with targeted disruption of the aralar gene were obtained by gene trapping at Lexicon Genetics (The Woodlands, TX) in SVJ129 ES cells with the use of an insertion vector and gene trap technology of Lexicon, as described previously⁶⁾. Aralar- knockout congenic mice with a C57BL/6J genetic background were obtained by backcrossing for at least nine generations. Genotyping was determined by polymerase chain reaction and genomic DNA, as reported previously⁶⁾.

Animals

Mice were housed under a 12-hour light/ dark cycle at 22°C±2°C with *ad libitum* access to food and water. All experimental procedures were carried out in accordance with the Guide for Animal Experimentation of Kagoshima University and were approved by the Committees of the Kagoshima University for Animal and Genetic Recombination Experimentations.

Behavioral experiments

Six 9-month-old male littermates, three Aralar^{+/-} and three Aralar^{+/+}, were used. Behavioral experiments were performed at 22°C±2°C, between at 4:00 PM and 7:00 PM, by a single researcher. For each group of three mice (Aralar^{+/-} and Aralar^{+/+}), one experimental test per day was performed for 3 days.

Mice were tested for anxiety-like behaviors in the

elevated plus-maze test, according to a previously reported method^{18,19)}. Briefly, the apparatus consisted of a central platform (5 cm × 5 cm) with two open arms (25-cm long, 5-cm wide, with 1-cm high walls) and two closed arms (same dimensions as the open arms but with 15-cm high walls) elevated 50 cm above the floor. The mice were placed individually on the platform facing the open arms and allowed to explore freely for 5 min. The total number of entries into the open and closed arms (an entry was defined as all four legs in an arm), as well as the time spent in the open and closed arms, were measured with the use of an overhead video camera.

The light-dark exploration test was carried out according to a previously reported method^{18,19)}. The light-dark exploration box (30-cm long, 15-cm wide and 15-cm high) had one aperture (5 cm×10 cm) at floor level between the light and dark compartments. Light intensity of the light compartment maintained at 2,300 lux by a desk lamp 30 cm above the apparatus. Mice were placed individually in the light compartment and allowed to cross freely from one compartment to the other. The time spent in the light compartment and the number of crosses from the dark compartment to the light one was measured for 10 min with the use of a videotape recorder. Entrance into a compartment was defined as all four paws crossing the threshold into the compartment¹⁸⁾.

Western blot analysis

Male Aralar^{+/+} and Aralar^{+/-} mice (four in each group) were killed by decapitation at 9 to 12 months of age. Samples of cerebral hemisphere and brainstem were rapidly removed, frozen at -80°C, and stored at -70°C until use. Homogenate supernatants were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically on Clear Blot Membrane-p (Atto Corp., Tokyo, Japan). After blocking with Tris-HCl buffered saline solution containing 5% nonfat milk and 0.1% Tween 20 for 1 h at room temperature, the membranes were incubated with specific antibodies raised against tryptophan hydroxylase (1: 2,000; Protos Biotech Corp., New York, NY) or β -actin (1: 20,000; Sigma-Aldrich, St. Louis, MO) overnight at room temperature. This was followed by incubation with a secondary horseradish peroxidase-labeled goat antibody (1: 2,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 90 min at room temperature. To visualize the peroxidase

Table 1. Results of the elevated plus-maze test (mean \pm S.E.M., n=9).

	Time in open arm (s)	Time in closed arm (s)	Time in center area (s)	Number of entries	
				Open arm	Closed arm
Aralar ^{+/+}	8.7 \pm 2.5	231.7 \pm 8.0	59.7 \pm 7.6	1.8 \pm 0.3	11.0 \pm 0.7
Aralar ^{+/-}	30.4 \pm 4.6*	197.8 \pm 7.2*	71.8 \pm 5.8	4.4 \pm 0.3*	11.0 \pm 1.8

* $p < 0.01$ compared with Aralar^{+/+}Table 2. Results of the light-dark exploration test (mean \pm S.E.M., n=9).

	Time spent in light compartment (s)	Number of entries into light compartment
Aralar ^{+/+}	95.1 \pm 8.5	13.3 \pm 1.6
Aralar ^{+/-}	136.4 \pm 9.2*	13.9 \pm 1.1

* $p < 0.01$ compared with Aralar^{+/+}

reaction product, membranes were treated with 0.2% diaminobenzidine in 0.0005 % hydrogen peroxidase for 10 to 30 min at room temperature. Semi-quantitative analysis of band density was performed with the use of NIH Image-J Gel Analyzer software.

Statistical analysis

Statistical evaluation was performed with the use of the unpaired Student *t* test. A probability value of 0.05 was set as the level of significance. All data are expressed as mean \pm standard error of the mean (S.E.M.).

Results

Results of the behavioral experiments are summarized in Tables 1 and 2. For the elevated plus-maze test (Table 1), Aralar^{+/-} mice made significantly more entries into the open arms ($t=6.10$, $p < 0.01$) and spent significantly more time in the open arms ($t=4.12$, $p < 0.01$) than their wild-type littermates. In contrast, the Aralar^{+/-} mice spent significantly less time in the closed arms ($t=3.16$, $p < 0.01$) than Aralar^{+/+} mice. However, there was no effect of genotype on the number of entries to the closed arms ($t=0.00$, $p=1.00$) or on the time spent in the center area ($t=1.26$, $p=0.226$).

For the light-dark exploration test (Table 2), Aralar^{+/-} mice spent significantly more time in the light compartment ($t=3.31$, $p < 0.01$) compared to their wild-type littermates. However, there was no effect of genotype on the number of transitions between the light and dark

compartments ($t=0.28$, $p=0.78$), suggesting similar general exploratory behaviors between Aralar^{+/-} and Aralar^{+/+} mice.

Results of semi-quantitative Western blot analysis are shown in Figs. 1 and 2. The levels of tryptophan hydroxylase in the cerebral hemisphere and brainstem of Aralar^{+/-} mice were slightly but significantly lower than those of Aralar^{+/+} mice ($t=3.04$, $p < 0.05$; $t=3.69$, $p < 0.05$, respectively).

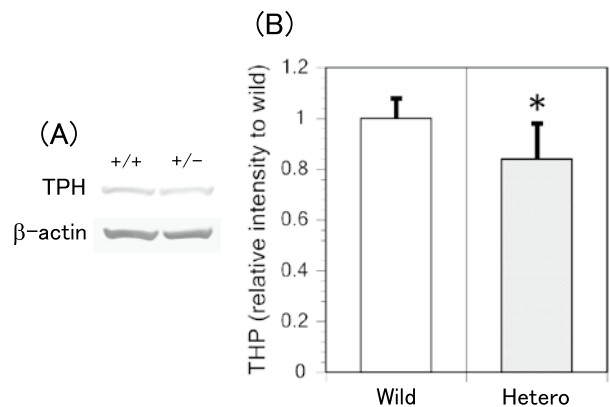


Fig.1. Effect of aralar-deficiency on the expression of tryptophan hydroxylase (TPH) in the cerebral hemispheres of Aralar^{+/-} mice (Hetero). (A) Levels of TPH expression in the cerebral hemispheres were determined by Western blot with an anti-TPH antibody. In all blots, staining for β -actin was used as a loading control. (B) The level of TPH expression obtained in the Aralar^{+/-} mice was calculated as an intensity relative to that of Aralar^{+/+} mice (Wild). Data are expressed as mean \pm S.E.M. (n = 4). * $p < 0.05$ vs. Wild.

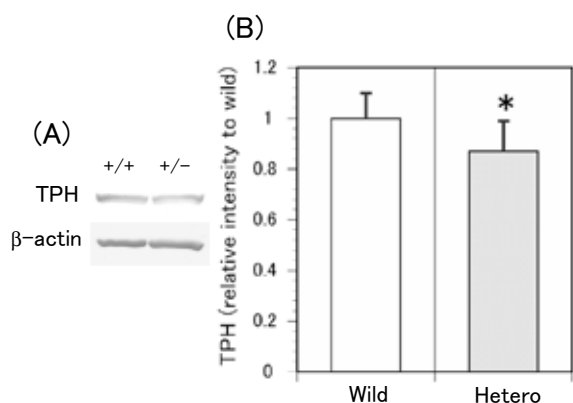


Fig.2. Effect of aralar-deficiency on the expression of tryptophan hydroxylase (TPH) in the brainstems of Aralar^{+/-} mice (Hetero). (A) Levels of TPH expression in the brainstems were determined by Western blot with an anti-TPH antibody. In all blots, staining for β-actin was used as a loading control. (B) The level of TPH expression in Aralar^{+/-} mice was calculated as an intensity relative to that of Aralar^{+/+} mice (Wild). Data are expressed as mean ± S.E.M. (n = 4). **p* < 0.05 vs. Wild.

Discussion

Aralar deficiency led to specific behavioral effects in mice in the present study. The major findings of the elevated plus-maze test were that Aralar^{+/-} mice showed a significantly greater number of open-arms entries compared to their wild-type littermates. In addition, Aralar^{+/-} mice spent more time in the open arms than Aralar^{+/+} mice. These behavioral differences of Aralar^{+/-} mice were similar to those of the mice treated with standard anxiolytic drugs (e.g., diazepam), suggesting decreased anxiety-like behavior^{18,20}. Similar results were obtained with the dark-light exploration test, in which Aralar^{+/-} mice spent more time in the more aversive light compartment than their wild-type littermates. However, the number of the transitions between the two compartments was unchanged between the two genotypes, suggesting similar effects of anxiolytic drug treatment²¹. Bourin and Hascoët²¹ reported that preliminary screening of locomotor activity is necessary to avoid false-positive results in the dark-light exploration test. In fact, general locomotor activity in a novel environment and explorative drive were not profoundly affected by the decreased expression of aralar protein; the number of spontaneous movements and activities during the open-field test did not differ significantly between Aralar^{+/+} and Aralar^{+/-} animals (our unpublished data).

Interestingly, Savelieva et al.²² reported that genetic deletion of serotonin synthesis did not affect locomotion or exploratory behavior, given that no differences were found in the open-field test between deficient and wild-type animals.

Our present results also showed a decrease in tryptophan hydroxylase expression in the cerebral hemisphere and brainstem of Aralar^{+/-} mice compared to Aralar^{+/+} mice, suggesting a decrease in serotonin content in the brain. Interestingly, it has been reported that the expression of tryptophan hydroxylase in the brain is markedly decreased in response to abnormal circumstances such as a hypoxic environment²³, oxidative stress²⁴, dioxin exposure^{25,26}, and ethanol intake²⁷. Therefore, it is reasonable to suggest that aralar deficiency may alter the synthesis of tryptophan hydroxylase via a decrease in the transfer of NADH-reducing equivalents from the cytosol to mitochondria.

Our results from the behavioral tests as well as Western blot analysis suggest that decreased anxiety-like behaviors in Aralar^{+/-} mice may be related to a decrease in brain tryptophan hydroxylase level. There is accumulating evidence that serotonergic signaling is a major modulator of emotional behavior, including fear and anxiety, as well as aggression, and that it integrates complex brain functions such as cognition, sensory processing, and motor activity²⁸. A cumulative decrease in serotonergic signaling to septohippocampal and other limbic and cortical areas involved in the control of anxiety is believed to underlie the anxiolytic effects of ligands with selective affinity for the 5-HT_{1A} receptor in some animal models of anxiety-related behavior²⁸.

Further studies are needed to elucidate the mechanism whereby decreased aralar protein expression, perhaps accompanied by a increased level of cytosolic NADH, results in decreased expression of tryptophan hydroxylase in Aralar^{+/-} mice.

Acknowledgements

We are grateful to Mr. T. Shinyama and Ms. M. Hatoya, Laboratory for Neuroanatomy, Graduate School of Medical and Dental Sciences, Kagoshima University, for their helpful assistances.

References

- 1) del Arco A, Satrústegui J. Molecular cloning of Aralar, a new member of the mitochondrial carrier superfamily that binds calcium and is present in human muscle and brain. *Journal of Biological Chemistry* 1998; 273: 23327-23334.
- 2) Palmieri L, Pardo B, Lasoras FM, del Arco A, Kobayashi K, Iijima M, et al. Citrin and aralar 1 are Ca²⁺-stimulated aspartate/glutamate transporters in mitochondria. *EMBO Journal* 2001; 20: 5060-5069.
- 3) Casimir M, Lasorsa FM, Rubi B, Caille D, Palmieri F, Meda P, et al. Mitochondrial glutamate carrier GC1 as a newly identified player in the control of glucose-stimulated insulin secretion. *Journal of Biological Chemistry* 2009; 284: 25004-25014.
- 4) Sanz R, del Arco A, Ayuso C, Ramos C, Satrústegui J. Assignment of the calcium-binding mitochondrial carrier Aralar 1 gene (SLC25A12) to human chromosome band 2q31 by in situ hybridization. *Cytogenetics and Cell Genetics* 2000; 89: 143-144.
- 5) Bröer S, Palacín M. The role of amino acid transporters in inherited and acquired diseases. *Biochemical Journal* 2011; 436: 193-211.
- 6) Jalil Md A, Begum L, Contreras L, Pardo B, Iijima M, Li MX, et al. Reduced N-acetylaspartate levels in mice lacking aralar, a brain- and muscle-type mitochondrial aspartate-glutamate carrier. *Journal of Biological Chemistry* 2005; 280: 31333-31339.
- 7) Satrústegui J, Contreras L, Ramos M, Marmol P, del Arco A, Saheki T, et al. Role of aralar, the mitochondrial transporter of aspartate-glutamate, in brain N-acetylaspartate formation and Ca²⁺ signaling in neuronal mitochondria. *Journal of Neuroscience Research* 2007; 85: 3359-3366.
- 8) Sakurai T, Ramoz N, Barreto M, Gazdciu M, Takahashi N, Gertner M, et al. Slc25a12 disruption alters myelination and neurofilaments: a model for a hypomyelination syndrome and childhood neurodevelopmental disorders. *Biological Psychiatry* 2010; 67: 887-894.
- 9) Montgomery KC. The relation between fear induced by novel stimulation and exploratory behavior. *Journal of Comparative and Physiological Psychology* 1955; 48: 254-260.
- 10) Rodgers RJ, Cole JC. The elevated plus-maze : pharmacology, methodology and ethology. In: Cooper SJ, Hendrie CA, editors, *Ethology and Psychopharmacology*, New York: John Willey and Sons, 1994: 9-44.
- 11) Handley SL, McBlane JW. 5-HT drugs in animal models of anxiety. *Psychopharmacology* 1993; 112: 13-20.
- 12) Ramos A, Pereira E, Martins GC, Wehrmeister TD, Izídio GS. Integrating the open field, elevated plus maze and light/dark box to assess different types of emotional behaviors in one single trial. *Behavioural Brain Research* 2008; 193: 277-288.
- 13) Argyropoulos SV, Hood SD, Adrover M, Bell CJ, Rich AS, Nash JR, et al. Tryptophan depletion reverses the therapeutic effect of selective serotonin reuptake inhibitors in social anxiety disorder. *Biological Psychiatry* 2004; 56: 503-509.
- 14) Bechtholt AJ, Hill TE, Lucki I. Anxiolytic effect of serotonin depletion in the novelty-induced hypophagia test. *Psychopharmacology* 2007; 190: 531-540.
- 15) Hood SD, Hince DA, Davies SJC, Argyropoulos S, Robinson H, Potokar J. et al. Effects of acute tryptophan depletion in serotonin reuptake inhibitor-remitted patients with generalized anxiety disorder. *Psychopharmacology* 2010; 208: 223-232.
- 16) Walther DJ, Peter JU, Bashammakh S, Hörtnagl H, Voits M, Fink H, et al. Synthesis of serotonin by a second tryptophan hydroxylase isoform. *Science* 2003; 299: 76.
- 17) Walther DJ, Bader M. A unique central tryptophan hydroxylase isoform. *Biochemical Pharmacology* 2003; 66: 1673-1680.
- 18) Crawley J, Goodwin FK. Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. *Pharmacology Biochemistry and Behavior* 1980; 13: 167-170.
- 19) Pellow S, Chopin P, File SE, Briley M. Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *Journal of Neuroscience Methods* 1985; 14: 149-167.
- 20) Treit D, Robinson A, Rotzinger S, Pesold C. Anxiolytic effects of serotonergic interventions in the shock-probe burying test and the elevated plus-maze test. *Behavioural Brain Research* 1993; 54: 23-34.
- 21) Bourin M, Hascoët M. The mouse light/dark box test. *European Journal of Pharmacology* 2003; 463: 55-65.

- 22) Savelieva KV, Zhao S, Pogorelov VM, Rajan I, Yang Q, Cullinan E, et al. Genetic disruption of both tryptophan hydroxylase genes dramatically reduces serotonin and affects behavior in models sensitive to antidepressants. *PLoS ONE* 2008; 3: e3301.
- 23) Hedner T, Lundborg P. Regional changes in monoamine synthesis in the developing rat brain during hypoxia. *Acta Physiologica Scandinavica* 1979; 106: 139-143.
- 24) Kuhn DM, Sykes CE, Geddes TJ, Jaunarajs KLE, Bishop C. Tryptophan hydroxylase 2 aggregates through disulfide cross-linking upon oxidation: possible link to serotonin deficits and non-motor symptoms in Parkinson's disease. *Journal of Neurochemistry* 2011; 116: 426-437.
- 25) Kuchiiwa S, Cheng SB, Nagatomo I, Akasaki Y, Uchida M, Tominaga M, et al. In utero and lactational exposure to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin decreases serotonin-immunoreactive neurons in raphe nuclei of male mouse offspring. *Neuroscience Letters* 2002; 317: 73-76.
- 26) Ahmed RG. Perinatal TCDD exposure alters developmental neuroendocrine system. *Food and Chemical Toxicology* 2011; 49: 1276-1284.
- 27) Oreland S, Raudkivi K, Oreland L, Harro J, Arborelius L, Nylander I. Ethanol-induced effects on the dopamine and serotonin systems in adult Wistar rats are dependent on early-life experiences. *Brain Research* 2011; 1405: 57-68.
- 28) Lesch KP, Zeng Y, Reif A, Gutknecht L. Anxiety-related traits in mice with modified genes of the serotonergic pathway. *European Journal of Pharmacology* 2003; 480: 185-204.

Aralar欠損マウスにおける脳内トリプトファン水酸化酵素の発現の減少と不安様行動の減少についての予報

松元 泰英¹⁾, 下津 京子¹⁾, 飯島 幹雄²⁾, 佐伯 武頼³⁾, 中河 志朗¹⁾

¹⁾ 鹿児島大学大学院医歯学総合研究科神経解剖学, ²⁾ 鹿児島大学大学院医歯学総合研究科医化学,

³⁾ 徳島文理大学健康科学研究所

aralarは、ミトコンドリアに局在するカルシウム依存性のアスパラギン酸・グルタミン酸輸送体のアイソフォームの一つで、脳と骨格筋に特異的に発現することが報告されている。本研究の目的は、脳内aralarの減少に伴う行動様式の変化を調べるとともに、セロトニン合成で鍵酵素として働くトリプトファン水酸化酵素の発現を調べ、aralar欠損マウスにおける行動様式の変化とその関係を解明することにある。高架式十字迷路試験では、aralarヘテロマウスは、オープンアームに同腹の野生型マウスに比べて長く滞在し、オープンアームへの侵入回数も同腹の野生型マウスと比較し有意に増加した。さらに明暗試験において、aralarヘテロマウスは、野生型マウスに比べて、より長く明るい区画にとどまっていた。一方、ウエスタンブロット解析では、脳内トリプトファン水酸化酵素の発現が、野生型マウスと比較し、aralarヘテロマウスにおいて減少していることを示した。これらの結果は、同腹の野生型マウスと比べて、aralarヘテロマウスの不安様行動が減少していることを示唆し、この特異な行動様式が脳内セロトニンの減少と関連性を有する可能性を示している。