OPEN ACCESS Freely available online

PLoS one

# Deletion of RAGE Causes Hyperactivity and Increased Sensitivity to Auditory Stimuli in Mice

**Seiichi Sakatani[1☯], Kazuyuki Yamada[2☯], Chihiro Homma[2], Seiichi Munesue[3], Yasuhiko Yamamoto[3], Hiroshi Yamamoto[3], Hajime Hirase[1,4*]**

**1** Hirase Research Unit, RIKEN Brain Science Institute, Wako, Saitama, Japan, **2** Research Resource Center, RIKEN Brain Science Institute, Wako, Saitama, Japan, **3** Department of Biochemistry and Molecular Vascular Biology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan, **4** Saitama University Brain Science Institute, Saitama, Japan

## Abstract

The receptor for advanced glycation end-products (RAGE) is a multi-ligand receptor that belongs to the immunoglobulin superfamily of cell surface receptors. In diabetes and Alzheimer's disease, pathological progression is accelerated by activation of RAGE. However, how RAGE influences gross behavioral activity patterns in basal condition has not been addressed to date. In search for a functional role of RAGE in normal mice, a series of standard behavioral tests were performed on adult RAGE knockout (KO) mice. We observed a solid increase of home cage activity in RAGE KO. In addition, auditory startle response assessment resulted in a higher sensitivity to auditory signal and increased prepulse inhibition in KO mice. There were no significant differences between KO and wild types in behavioral tests for spatial memory and anxiety, as tested by Morris water maze, classical fear conditioning, and elevated plus maze. Our results raise a possibility that systemic therapeutic treatments to occlude RAGE activation may have adverse effects on general activity levels or sensitivity to auditory stimuli.

**Citation:** Sakatani S, Yamada K, Homma C, Munesue S, Yamamoto Y, et al. (2009) Deletion of RAGE Causes Hyperactivity and Increased Sensitivity to Auditory Stimuli in Mice. PLoS ONE 4(12): e8309. doi:10.1371/journal.pone.0008309

**Editor:** Mark A. Smith, Case Western Reserve University, United States of America

**Received** September 30, 2009; **Accepted** November 23, 2009; **Published** December 15, 2009



**Funding:** Funding was received from the Ministry of Education, Science, Sports and Culture grant-in-aids for Young Scientists (B) (21700368, SS) and Scientific Research (B) (19390085, HY) and RIKEN intramural fundings (HH). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: hirase@brain.riken.jp

☯ These authors contributed equally to this work.

## Introduction

The receptor for advanced glycation end-products (RAGE) is a multi-ligand receptor that belongs to the immunoglobulin superfamily of cell surface receptors [1,2]. A full-length RAGE has one transmembrane domain and the extracellular region contains one V-type and two C-type immunoglobulin (ligand binding) domains [1]. In situ hybridization and RT-PCR studies suggest a widespread existence of RAGE in body organs with the highest expression level in the lung [3]. In addition to the full length RAGE, various splice variants have been identified including the endogenous secretory form of RAGE (esRAGE) which may act as a decoy receptor in extracellular space [4,5].

Ligands of RAGE include high mobility group box 1 (HMGB1, also known as amphoterin) [6], amyloid β-peptide (Aβ) [7], and S100B [8]. These ligands are known to be upregulated in neuropathological conditions. For instance, accumulation of Aβ occurs from an onset of Alzheimer's disease [9]. HMGB1 and S100B levels are increased in neuroinflammatry conditions such as in epilepsy and ischemia [10,11]. Interestingly, S100B knockout mice have been reported to enhance spatial memory and context dependent fear memory [12]. Recently, S100B-RAGE interaction has been implicated in the brain *in vivo* in a condition that mimics epileptic seizures by kainic acid administration [13].

RAGE KO mice have been generated by a multiple number of laboratories [14,15]. Although RAGE KO mice have been utilized in biochemical and physiological experiments to address the roles of RAGE in progression of various pathological conditions, consequences of lacking RAGE in normal condition have hardly been addressed. In this study, we performed a series of standard behavioral tests to identify the phenotype of RAGE KO mice.

## Results

Prior to the behavioral experiments, genotyping was performed by PCR (Fig. 1 A) and the body weight was measured for each mouse. Two sets of behavioral experiments with different sets of mice were performed to assure the results. The first set of animals (Set 1) consisted of a WT population ($n = 10$) that weighed $27.42 \pm 3.66$ g and a RAGE KO population ($n = 10$) that weighed $26.88 \pm 2.73$ g. The second set of mice (Set 2) consisted of a WT population ($n = 10$) that weighed $23.61 \pm 1.16$ g and a RAGE KO population ($n = 10$) that weighed $22.27 \pm 1.33$ g. There was no significant difference in the mean body weight between WT and KO (t-test, $p = 0.713$) in Set 1, however, the mean body weight was significantly different in Set 2, (t-test, $p < 0.05$), although the difference was small. There were no mice with obvious abnormal appearance. In some mice, genotypes were reconfirmed at the protein level by Western blotting (Fig. 1B).

The mice were assessed for home cage activity. As the room illumination is controlled at 12/12 hour light/dark cycle, the animals' activity was modulated accordingly with more activity

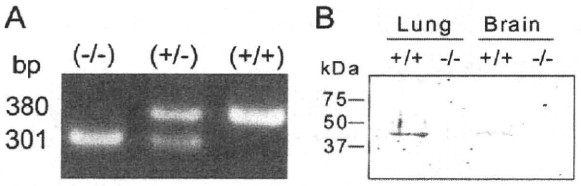


**Figure 1. RAGE deletion in RAGE KO mice was confirmed by both DNA and protein levels.** (A) PCR for ear samples shows RAGE(−/−) mice have a single band at 301 bp, RAGE(+/+) mice have a single band at 380 bp, and RAGE(+/−) mice have both bands, as described in Myint et al. [14]. (B) Western blotting analysis shows that RAGE is present in both the lung and brain in a RAGE(+/+) mouse.
doi:10.1371/journal.pone.0008309.g001

during the dark phase (Fig. 2A) During the seven days of continuous monitoring, KO displayed more activity than WT (two-way ANOVA with repeated measurements for genotype, $F(1,18) = 6.426$, $p<0.05$ for Set 1; $F(1,17) = 6.581$, $p<0.05$ for Set 2). Overall, KO showed more activity in the dark phase (Fig. 2B and C). Both WT and KO showed gradual decrease in activity in the dark phase during the course of the seven days, whereas activity in the light phase remained low.

In the open field test, both genotypes had similar exploration distance in fifteen minutes (WT vs. KO: 5421.1±833.1 cm vs. KO 5211.4±320.5 cm, $p = 0.619$ for Set 1; 6283.5±1401.9 cm vs. 5563.6±1189.3 cm, $p = 0.232$ for Set 2). The mean distance traveled in one minute could not be distinguished by genotype throughout the fifteen minutes of the experiment. The total time spent in the center of the arena was similar between WT and KO in Set 1 (224.7±117.5 s vs. 242.0±70.8 s, $p = 0.695$, t-test), however KO tended to stay longer in the center position in Set 2 (163.8±41.7 s vs. 281.6±73.5 s, $p<0.01$, t-test).

In the light-dark box test, the results varied between Set 1 and Set 2 (as summarized in Table S1). Therefore, we decided that the test does not delineate behavioral phenotypes of RAGE KO mice.

Both WT and KO displayed comparable behavioral patterns in the elevated plus maze test under 70 lx condition (Set 1) and 40 lx condition (Set 2). The proportion of the time spent in the open arm (WT vs. KO: 14.5±19.6% vs. 24.5±28.1%, $p = 0.597$ for Set 1; 23.1±15.4% vs. 13.3±14.0%, $p = 0.09$ for Set 2; Mann-Whitney's U-test,) and the relative frequency of open arm entry (31.0±16.9% vs. 32.1±24.3%, $p = 0.971$ for Set 1; 31.6±11.0% vs. 24.0±14.0% $p = 0.307$ for Set 2, Mann-Whitney's U-test) were not significantly different.

Auditory startle response assessment resulted in a higher sensitivity to auditory signal in KO. In both Set 1 and 2, WT were virtually irresponsive to auditory signals up to 90 dB, whereas KO showed response from 85 dB (Fig. 3A). The WT displayed startle response at 95 dB or larger. Prepulse inhibition showed a clear difference between WT and KO (Fig. 3B). For all the tested prepulse tones (i.e. 70 dB, 75 dB and 80 dB), KO startle response was more inhibited by the prepulse sound (t-test, $p<0.05$ for all of the cases for Set 1, $p<0.01$ for all cases for Set 2). Similar results were obtained with a startle stimulus of 110 dB tested in Set 2, in that KO showed significantly more prepulse inhibition for all the tested prepulse tones ($p<0.05$ for 70 dB, $p<0.01$ for 75 and 80 dB).

Morris water maze test was done to test animals' spatial learning ability. There was no significant difference in the total distance traveled to find the target during four days of training between the genotypes (two-way ANOVA for genotype, $F(1,54)$, $p = 0.962$ for Set 1; $F(1,54)$, $p = 0.06$ for Set 2). Similarly, the probe test did not yield any performance differences in the target ratio measured by stay time (WT vs. KO: 34.3±12.1% vs. 32.4±9.6, $p = 0.734$ for Set 1; 26.8±19.9% vs. 36.3±13.0%, $p = 0.273$ for Set 2; Mann-Whitney's U-test) or in the target ratio measured by number of crosses (44.0±15.2% vs. 34.8±16.4%, $p = 0.167$ for Set 1; 35.3±33.3% vs. 34.9±21.9%, $p = 1.00$ for Set 2; Mann-Whitney's U-test).

The experimental animals were tested for fear conditioning. During conditioning trials, both WT and KO showed similar freezing response after electric foot shocks (final bin freezing behavior percentage WT vs. KO: 40.7±21.6% vs. 61.2±23.5%, $p = 0.070$ for Set 1; 29.3±23.1% vs. 34.5±19.2%, $p = 0.956$ for Set 2, Mann-Whitney's U-test, Fig. 3C). In the assessment of context dependence of the fear, both genotypes appeared to elicit similar degree of freezing behavior (25.5±11.8% vs. 28.7±15.9%, $p = 0.705$ for Set 1; 38.3±24.8% vs. 51.9±10.8%, $p = 0.131$ for Set 2, Mann-Whitney's test). There was no difference in the freezing response tested against the conditioned sound cue Set 1, (38.4±22.3% vs. 47.1±20.7, $p = 0.406$, Mann-Whitney's U-test), however KO displayed significantly higher freezing response in Set 2 (23.0±15.8% vs. 57.5±16.9% $p<0.01$). Combined population statistics show the difference overall is significant ($p<0.01$). Interestingly, there is a statistical difference in the freezing response in the cue test cage without the conditional stimuli (10.7±7.7% vs. 19.6±10.9%, $p<0.05$ for Set 1; 4.5±7.1% vs. 28.0±18.0%, $p<0.01$ for Set 2, Mann-Whitney's U-test).

## Discussion

Among the series of behavioral tests, the most striking behavioral difference was observed in the home cage activity. RAGE KO mice displayed ~30% higher activity in darkness on day 1 and persistently higher activity during the seven days of observation. In addition, auditory startle response assessment resulted in a higher sensitivity to auditory signal in KO mice. The higher sensitivity to auditory signal provides an explanation for the increased prepulse inhibition ratio in KO animals and auditory cue-dependent classical fear conditioning. The animals' curiosity or anxiety should be excluded from the subject of the difference, as the open field test and the elevated plus maze test yielded no significantly different scores.

Our results indicate that deletion of RAGE has minimal effects on the animals' spatial learning ability (as tested with Morris water maze and context-dependent classical fear conditioning). Therefore, it appears that RAGE does not have a critical importance in synaptic plasticity of the hippocampus and the associated areas. In fact, long-term potentiation in the entorhinal cortex has been reported to be not affected in RAGE KO mice [16]. By contrast, genetic manipulations of S100B, a ligand for RAGE, result in more visible effects on learning and memory. S100B KO mice improve performance in spatial learning and become more sensitive to context-dependent fear conditioning [12], whereas S100B overexpressing transgenic mice have inferior performance in spatial learning [17]. The behavioral differences between RAGE KO and S100B KO imply that RAGE may not be a crucial receptor of S100B for learning and memory. It is, however, noted that attenuation of kainate-induced gamma oscillations in S100B KO [18] has recently been demonstrated to be dependent on activation of RAGE [13], suggesting a role of RAGE in hyperactive brain states. One potential caveat is that the mice used in the current study have been backcrossed eight times to C57BL6, so that the expected percentage of genetic material from the original strain is below 0.4%.

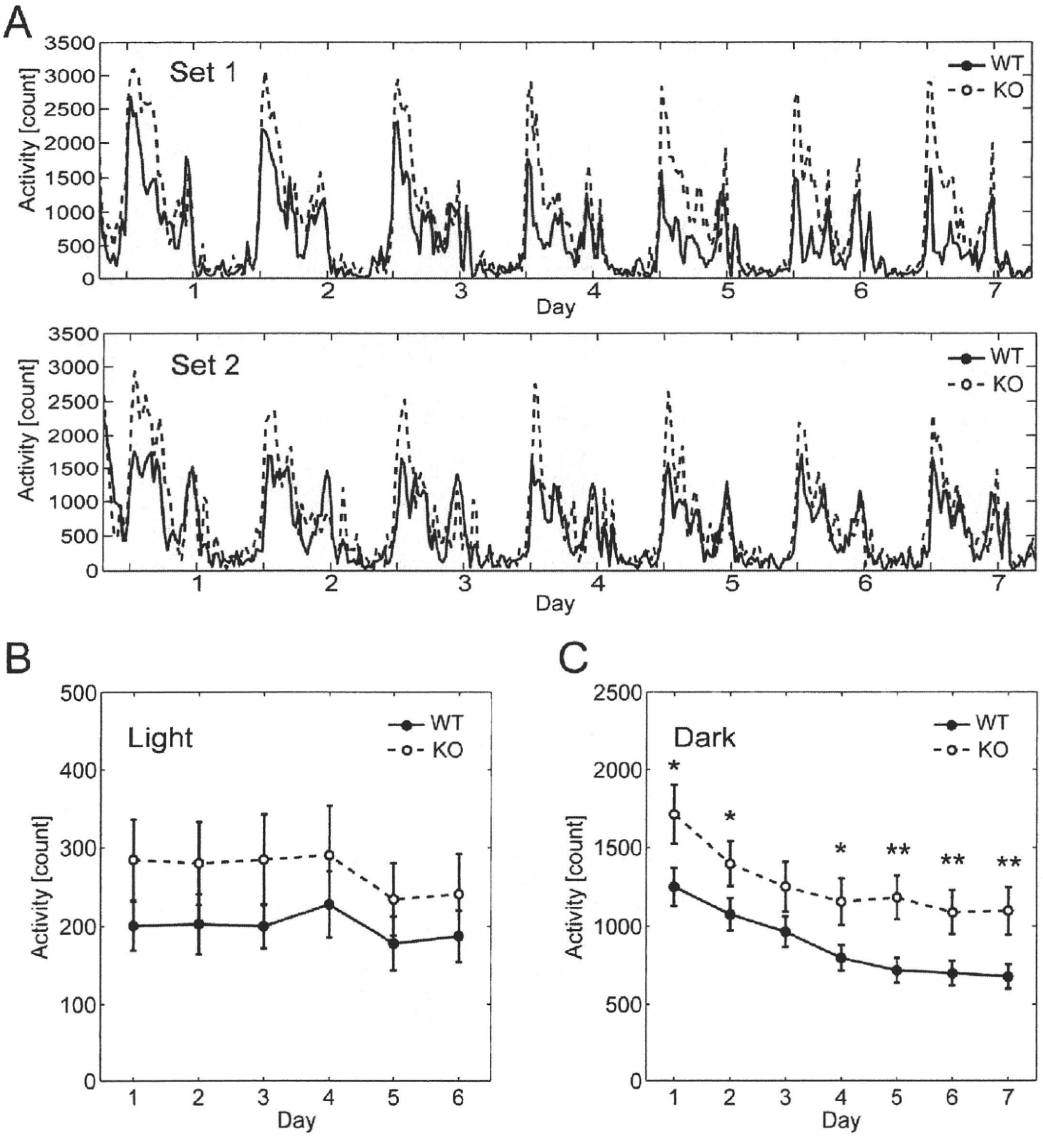


**Figure 2. Enhanced home cage activity in RAGE KO mice.** (A) Average home cage activity records of WT (solid line) and RAGE KO (dashed line) mice are shown for two independent sets of experiments (see main text for more details). The light and dark phases are indicated by white and grey backgrounds, respectively. (B) Group comparison of home cage activity in the light phase. The activity in the light phase is similar and remained constantly low. (C) Group comparison of home cage activity in the dark phase. The activity of KO mice in dark phase is higher than that of WT mice. Note that both WT and KO showed a gradual decrease in activity in the dark during the course of the seven days. For B and C, Set 1 and Set 2 are combined. Data are mean±S.E.M. * $p<0.05$, ** $p<0.01$.
doi:10.1371/journal.pone.0008309.g002

As the KO phenotypes were dependent on presentation of sensory stimulus, RAGE may play an active role in sensory organs or the brain. Immunohistochemical localization of RAGE in the brain has remained controversial to date [19,20,21,22]. Furthermore, esRAGE, a soluble and secretory form of RAGE, could play an important role. Interestingly, reduced immunoreactivity against esRAGE in CA3 hippocampal neurons were found in Alzheimer's patients [23]. Future investigations on localization of membrane bound and soluble forms of RAGE, as well as RAGE induced biochemical pathways shall further identify the role of RAGE in the central nervous system.

As activation of RAGE accelerates pathological progression of diabetes or Alzheimer's disease, therapeutic treatments to attenuate activation of RAGE have been suggested [24] and experimented in animal disease or inflammation models [8,25,26,27]. Our results raise a possibility that systemic therapeutic treatments to occlude RAGE activation may have adverse effects as demonstrated by the home cage activity and prepulse inhibition behavioral tests. Further investigations using mice of different background strains and identification of biochemical pathways that elucidates the behavioral phenotypes are needed for better understanding of RAGE in basal states.

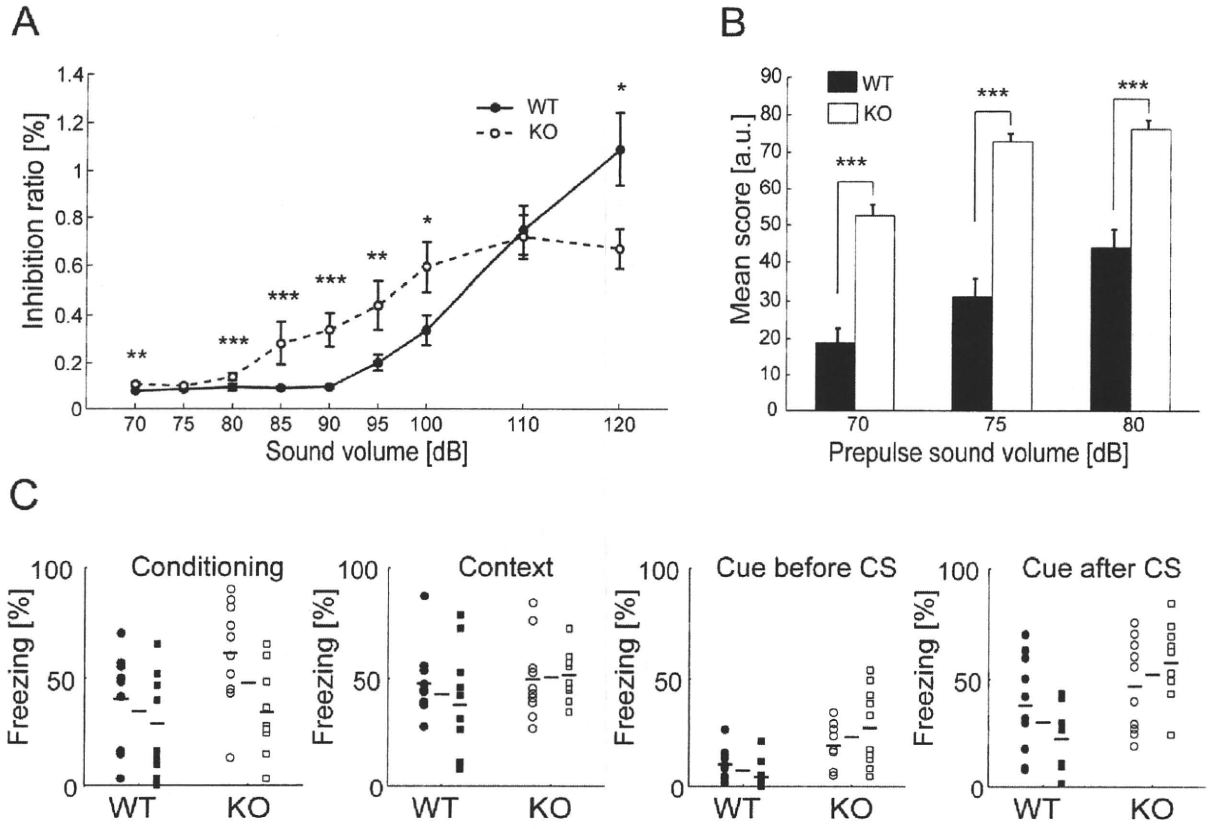


**Figure 3. Auditory startle response assessment resulted in a higher sensitivity to auditory signal and cue-dependent fear memory was affected in RAGE KO.** (A) KO mice are more sensitive to auditory stimulation (Set 1 & 2 combined). (B) Prepulse inhibition showed the response is more inhibited in KO mice. Abscissa values indicate the volume of prepulse tones. Data are mean±S.E.M. for A and B. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (C) Four stages of freezing response in the classical fear conditioning test are plotted. The freezing responses at final bin (30 s period, 1 min after the final (second) shock) of the conditioning phase (Conditioning) were not significantly different between WT and KO mice. Both WT and KO show similar freezing responses in the context test (Context). In the cue test, there is a significant difference in the freezing response in the cue test cage *without* the conditional stimuli (Cue before CS). Overall, KO mice show a higher sensitivity to the conditional auditory stimuli in the cue-dependent test (Cue after CS). Set 1 and Set 2 data are represented by circles and squares, respectively. Horizontal bars correspond to the median values for Set 1, Set 1 & 2, and Set 2. See the main text for detailed statistics.
doi:10.1371/journal.pone.0008309.g003

## Materials and Methods

### Subjects

RAGE (−/−) (KO) mice were generated similar to as described in Myint et al. [14]. Briefly, the RAGE mutant mice were originally created using E14.1 ES cells (129 background). After the chimeric mice were made, they were crossbred with Cre-transgenic mice (CD-1 background) that transiently express Cre recombinase in eggs [28]. The resultant RAGE KO mice were then backcrossed to C57BL/6J (Charles River Japan) for eight generations. Two independent populations of ten mature male RAGE KO mice and ten mature male wild type (WT) RAGE (+/+) mice were used. Littermates and non-littermates were mixed. The first group consisted of mixed yet age-matched population ranging from postnatal eight to eighteen weeks. In the second group, the age was more tightly matched so that the ages of the mice were all eight weeks. Mice were genotyped prior to the behavioral experiments, but the identities of the mice were not exposed to the experimenter during the behavioral experiments. Mice were housed individually before transferring to the behavioral laboratory. The light condition was 12/12 hour light-dark cycle with light phase starting at 8:00 a.m. The temperature and humidity of the laboratory were maintained at 22–23°C and 50–60%, respectively. Food and water were freely available for entire period of the home cage activity measurement and when the mice were housed in their home cage. Large blunt tongs wrapped with silicon rubber were used to handle mice to avoid individual variability in the handling procedure. All of the experiments were conducted in the light phase.

### PCR Genotyping

Tissue samples from the ear were dissolved in a buffer containing (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2 mM MgCl2, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween-20, 0.5 mg/ml proteinase K) at 55°C for overnight. The lysate, dNTP mixture, TaKaRa Ex Taq, Taq buffer and the following three primer were mixed; 5′-CCAGAGTGACAACAGAGCAGAC-3′ (primer 1), 5′-GGTCAGAACATCACAGCCCGGA-3′ (primer 2), and 5′-CCTCGCCTGTTAGTTGCCCGAC-3′ (primer 3) (nucleotides 73915-73936, 74523-74544, and 74881-74902 in GenBank accession no. AF030001, respectively). The thermocycle for the PCR reaction consisted of the following sequences: 94°C

(1 min) followed by 35 cycles of 95°C (30 s), 62°C (30 s), 72°C (30 s), followed by 74°C (10 min) incubation. The mixtures were separated in 1% agarose gel and the band images were captured by a CCD camera system (Dolphin-View, Wealtec).

## Column Chromatography and Western Blotting

A polyclonal anti-RAGE antibody (H-300, Santa Cruz Biotech. Inc.) was coupled to HiTrap NHS-activated HP Columns (GE Healthcare) according to the manufacturer's instructions. Tissue homogenates (1 ml) from lung (0.18 g or 0.2 g) and brain (0.5 g or 0.5 g) of RAGE KO or WT mice, respectively, in tissue lysis buffer of 50 mM Tris-HCl (pH 7.5), 1% TritonX-100, 150 mM NaCl, and proteinase inhibitors (10 KIU/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM benzaminidin, and 1 mM EDTA) were applied to the HiTrap-anti-RAGE antibody column previously equilibrated with the lysis buffer. After washing with a 5 bed volume of the equilibration buffer, bound proteins were eluted with 0.1 M glycine–HCl (pH 2.5). The eluate was precipitated with 10% trichloroacetic acid (TCA) at 4°C for 15 min. The pellet was re-suspended in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue) and boiled at 95°C for 5 min. Proteins in the lysates were resolved by SDS-PAGE (5–20%) and transferred onto a polyvinylidene fluoride membrane (Millipore Corp.). The membranes were incubated with a polyclonal anti-RAGE antibody [14] and an IRDye 680 donkey-anti-rabbit antibody (LI-COR Biosciences, NE) was used as a second antibody. The signal was monitored using a LI-COR Odyssey IR imaging system (Lincoln, NE).

## Behavioral Tests

The experimental animals were subject to a series of behavioral tests performed according to the schedule described in Table 1. The procedure for each behavioral test is described below (further details of the procedures are described in Kato et al. [29]). Dimensions of experimental apparatuses are represented as (width × length × height). After each trial (except the auditory startle response test and the water maze test), the apparatuses were wiped and cleaned with 80% alcohol and damp towel. In the auditory startle response test, holding chambers were washed by tap water, wiped by paper towel, and dried after each trial. All experimental protocols were approved by the RIKEN Institutional Animal Care and Use Committee.

**Home cage activity measurement.** Spontaneous activity of mice in their home cage was measured using a 24 channel activity monitoring system (O'Hara, Tokyo, Japan). Cages were individually set into the compartments made of stainless steel in the negative breeding rack (JCL, Tokyo, Japan). A piezoelectric sensor was equipped on the ceiling of each compartment to detect the mouse movements. Activity counts represent the number of active time bin (approximately 0.20–0.25 s each) in which spontaneous activity including locomotor activity, rearing and other voluntary stereotypic movements were detected. Home cage activity was measured for seven consecutive days during which bedding materials were not changed.

**Open field test.** Open field test apparatus was placed in a small sound-proof room (185×185×225 cm). The apparatus consisted of four white plastic boxes (50×50×40 cm), two electric fans for ventilation and background noise (35 dB), white LED light source (70 lx at the center of the field) which served as the sole source of illumination during the experiment. For each box, a CCD camera is attached on the ceiling for monitoring mice. Mice were individually introduced at the center of the arena and were allowed to move freely for 15 min. Distance traveled (cm) and % duration of staying at the center area of the field (30% of the field) were adopted as the indices, and they were collected every 1 min.

**Light-dark (L-D) box test.** A light-dark box system was equipped in the same sound-proof room as the open field test. The light box was made of white plastic (20×20×20 cm) and illuminated by LEDs (250 lx at the center of the box) and a CCD camera was equipped on the ceiling, and the dark box was made of black plastic (20×20×20 cm) and an infrared camera was

**Table 1.** Behavioral battery test schedule.

| Set 1 | | |
|---|---|---|
| Day | Time | Behavioral paradigm |
| 1 | AM | Introduction to behavioral experiment room |
| | PM | Home cage activity test started (at 15:00) |
| 8 | PM | Home cage activity test finished |
| 14 | PM | Open field test (15 min, 70 lx) |
| 15 | PM | Light-Dark box test (10 min) |
| 19 | PM | Elevated plus maze test (5 min, 70 lx) |
| 21 | PM | Startle response & PPI test (120 dB) |
| 22 | PM | Startle response & PPI test (120 dB) |
| 25 | AM/PM | Water maze test: training day 1 |
| 26 | AM/PM | Water maze test: training day 2 |
| 27 | AM/PM | Water maze test: training day 3 |
| 28 | AM/PM | Water maze test: training day 4 |
| 29 | PM | Water maze test: probe test |
| 33 | PM | Fear conditioning test (conditioning trial) |
| 34 | PM | Fear conditioning test (context trial) |
| 35 | PM | Fear conditioning test (cued trial) |
| Set 2 | | |
| Day | Time | Behavioral paradigm |
| 1 | AM | Introduction to behavioral experiment room |
| | PM | Home cage activity test started (at 15:00) |
| 8 | PM | Home cage activity test finished |
| 14 | PM | Open field test (15 min, 70 lx) |
| 20 | PM | Open field test (15 min, 250 lx) |
| 26 | PM | Light-Dark box test (10 min) |
| 32 | PM | Elevated plus maze test (5 min, 40 lx) |
| 39 | PM | Startle response & PPI test (110 dB) |
| 40 | PM | Startle response & PPI test (110 dB) |
| 46 | PM | Startle response & PPI test (120 dB) |
| 47 | PM | Startle response & PPI test (120 dB) |
| 53 | AM/PM | Water maze test: training day 1 |
| 54 | AM/PM | Water maze test: training day 2 |
| 55 | AM/PM | Water maze test: training day 3 |
| 56 | AM/PM | Water maze test: training day 4 |
| 57 | PM | Water maze test: probe test |
| 60 | PM | Fear conditioning test (conditioning trial) |
| 61 | PM | Fear conditioning test (context trial) |
| 62 | PM | Fear conditioning test (cued trial) |

doi:10.1371/journal.pone.0008309.t001

equipped on the ceiling. The light box and dark box was connected by a gate for transition on the center panel between the light box and dark box (5×0.5×3 cm) with a slide door. Mice were individually introduced into the light box, and the door of the tunnel automatically opened after two seconds. Then mice were allowed to move freely for ten min. Total distance traveled, % distance traveled in the light box, % duration staying in the light box, number of the transitions between the light and dark boxes and the latency to first enter the dark box were measured.

**Elevated plus maze test.** An elevated plus maze consisted of a pair of closed arms (25×5×15 cm) and a pair of open arms 25×5×0.3 cm) was placed in the same sound-proof room as the open field test. The floor of each arm was made of white plastic and the walls of the closed arms and ridges of the open arms were made of clear plastic. The closed arms and open arms were arranged orthogonally. The apparatus was elevated 60 cm above the floor and illuminated at 70 lx at the center platform of the maze (5×5 cm). Mice were individually put on the center platform facing to an open arm, and then mice were allowed to move freely in the maze for 5 min. Total distance traveled, % time stayed in the open arms, % number of the open arm entry were measured.

**Auditory startle response.** Each mouse was put into a small cage for startle response (30 or 35 mm diameter, 12 cm long) and set on the sensor block in a sound-proof chamber (60×50×67 cm) with dim illumination (10 lx at the center of the sensor block). White noise (65 dB) was presented as background noise. Experimental session began after the mouse was acclimatized to the environment for five min. In the first session, only startle stimuli (SS, 120 dB, 40 ms) were presented for ten times in random inter-trial intervals (ITI, 10–20 s). In the second session, startle response to stimuli at various intensities were assessed. Five rounds of 70 to 120 dB white noise stimuli (in 5 or 10 dB increments, 40 ms) were presented in quasi-random order and random ITI. In the prepulse inhibition (PPI) session, mice experienced five types of trials; no stimulus, SS only, and prepulse (20 ms, lead time 100 ms)-SS pairings with three different prepulse volumes (70 dB, 75 dB, and 80 dB). Each trial repeated ten times in quasi-random order and random ITI. In the final session, only SS were again presented for ten times in random ITI.

**Morris water maze test.** A standard Morris' water maze test was performed [30]. Briefly, a circular maze made of white plastic (1 m diameter, 30 cm depth) was filled with white-colored water to about 20 cm in depth (22 to 23°C). There were some extra-maze landmark cues (i.e., calendar, figure, plastic box) that were visible from the mice in the maze. Mice underwent six trials per day for four consecutive days. Each acquisition trial was initiated by placing an individual mouse into the water facing the outer edge of the maze at one of the four designated starting points in quasi-random order. The submerged platform remained constant for each mouse throughout testing. A trial was terminated when the mouse reached the platform, and the latency and distance swam were measured. Mice that did not reach the platform within 60 s were placed on the platform for extra 30 s before being returned to their home cage. The inter-trial interval was about 6 min. After the four day training, a probe test was conducted. In the probe test, the platform was taken away and each mouse started from the point opposite from the target platform to swim for 60 s. The distance swam, the number of crossings the position of the target platform and other three platforms, time staying in the quadrants of the four platforms were measured.

**Classical fear-conditioning.** Classical fear conditioning test consisted of three parts; a conditioning trial, a context test trial, and a cued test trial. Fear conditioning was carried out in a clear plastic chamber equipped with a stainless steel grid floor (34×26×30 cm) connected to an electric shock generator. A CCD camera was equipped on the ceiling of the chamber. White noise (65 dB) was supplied as an auditory cue (CS). The conditioning trial consisted of a 2 min exploration period followed by two CS-US pairings separated by 1 min each. A US (foot-shock: 0.5 mA, 2 s) was administered at the end of the 30 s CS period. A context test was performed in the same conditioning chamber for three min in the absence of CS. The cued test was performed in an alternative context with different chamber (triangular shape, white color walls, 0–1 lx brightness, solid floor with thin bedding materials). The cued test consisted of a 2 min exploration period to evaluate the nonspecific contextual fear, followed by 2 min CS period (no US) to evaluate the acquired cued fear. Rate of freezing response (immobility excluding respiration and heartbeat) of mice was measured as an index of fear memory.

### Data Analysis

Behavioral experiments with mouse tracking information were analyzed with custom-modified ImageJ software (O'Hara, Tokyo, Japan). ImageJ is public domain software available from NIH (http://rsb.info.nih.gov/ij). The measured analyzed values are represented in terms of mean±standard deviation throughout the manuscript, unless otherwise noted.

## Supporting Information

**Table S1** Behavioral scores for the light-dark box test.
Found at: doi:10.1371/journal.pone.0008309.s001 (0.03 MB DOC)

## Acknowledgments

We are grateful to Dr. Ryusuke Nakagawa for his support and advice. We thank Ms. Kazuko Yahagi for technical assistance.

## Author Contributions

Conceived and designed the experiments: SS KY. Performed the experiments: SS KY CH SM YY. Analyzed the data: SS KY HH. Contributed reagents/materials/analysis tools: YY HY. Wrote the paper: SS KY HH.

## References

1. Neeper M, Schmidt AM, Brett J, Yan SD, Wang F, et al. (1992) Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. J Biol Chem 267: 14998–15004.
2. Schmidt AM, Vianna M, Gerlach M, Brett J, Ryan J, et al. (1992) Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface. J Biol Chem 267: 14987–14997.
3. Brett J, Schmidt AM, Yan SD, Zou YS, Weidman E, et al. (1993) Survey of the distribution of a newly characterized receptor for advanced glycation end products in tissues. Am J Pathol 143: 1699–1712.
4. Park IH, Yeon SI, Youn JH, Choi JE, Sasaki N, et al. (2004) Expression of a novel secreted splice variant of the receptor for advanced glycation end products (RAGE) in human brain astrocytes and peripheral blood mononuclear cells. Mol Immunol 40: 1203–1211.
5. Yonekura H, Yamamoto Y, Sakurai S, Watanabe T, Yamamoto H (2005) Roles of the receptor for advanced glycation endproducts in diabetes-induced vascular injury. J Pharmacol Sci 97: 305–311.
6. Hori O, Brett J, Slattery T, Cao R, Zhang J, et al. (1995) The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and

amphoterin in the developing nervous system. J Biol Chem 270: 25752–25761.
7. Yan SD, Chen X, Fu J, Chen M, Zhu H, et al. (1996) RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. Nature 382: 685–691.
8. Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, et al. (1999) RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. Cell 97: 889–901.
9. Glenner GG, Wong CW (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem Biophys Res Commun 120: 885–890.
10. Kim JB, Sig Choi J, Yu YM, Nam K, Piao CS, et al. (2006) HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. J Neurosci 26: 6413–6421.
11. Griffin WS, Yeralan O, Sheng JG, Boop FA, Mrak RE, et al. (1995) Overexpression of the neurotrophic cytokine S100 beta in human temporal lobe epilepsy. J Neurochem 65: 228–233.
12. Nishiyama H, Knopfel T, Endo S, Itohara S (2002) Glial protein S100B modulates long-term neuronal synaptic plasticity. Proc Natl Acad Sci U S A 99: 4037–4042.
13. Sakatani S, Seto-Ohshima A, Shinohara Y, Yamamoto Y, Yamamoto H, et al. (2008) Neural-activity-dependent release of S100B from astrocytes enhances kainate-induced gamma oscillations in vivo. J Neurosci 28: 10928–10936.
14. Myint KM, Yamamoto Y, Doi T, Kato I, Harashima A, et al. (2006) RAGE control of diabetic nephropathy in a mouse model: effects of RAGE gene disruption and administration of low-molecular weight heparin. Diabetes 55: 2510–2522.
15. Constien R, Forde A, Liliensiek B, Grone HJ, Nawroth P, et al. (2001) Characterization of a novel EGFP reporter mouse to monitor Cre recombination as demonstrated by a Tie2 Cre mouse line. Genesis 30: 36–44.
16. Origlia N, Righi M, Capsoni S, Cattaneo A, Fang F, et al. (2008) Receptor for advanced glycation end product-dependent activation of p38 mitogen-activated protein kinase contributes to amyloid-beta-mediated cortical synaptic dysfunction. J Neurosci 28: 3521–3530.
17. Gerlai R, Wojtowicz JM, Marks A, Roder J (1995) Overexpression of a calcium-binding protein, S100 beta, in astrocytes alters synaptic plasticity and impairs spatial learning in transgenic mice. Learn Mem 2: 26–39.
18. Sakatani S, Seto-Ohshima A, Itohara S, Hirase H (2007) Impact of S100B on local field potential patterns in anesthetized and kainic acid-induced seizure conditions in vivo. Eur J Neurosci 25: 1144–1154.
19. Muhammad S, Barakat W, Stoyanov S, Murikinati S, Yang H, et al. (2008) The HMGB1 receptor RAGE mediates ischemic brain damage. J Neurosci 28: 12023–12031.
20. Lue LF, Walker DG, Brachova L, Beach TG, Rogers J, et al. (2001) Involvement of microglial receptor for advanced glycation endproducts (RAGE) in Alzheimer's disease: identification of a cellular activation mechanism. Exp Neurol 171: 29–45.
21. Ma L, Carter RJ, Morton AJ, Nicholson LF (2003) RAGE is expressed in pyramidal cells of the hippocampus following moderate hypoxic-ischemic brain injury in rats. Brain Res 966: 167–174.
22. Chou DK, Zhang J, Smith FI, McCaffery P, Jungalwala FB (2004) Developmental expression of receptor for advanced glycation end products (RAGE), amphoterin and sulfoglucuronyl (HNK-1) carbohydrate in mouse cerebellum and their role in neurite outgrowth and cell migration. J Neurochem 90: 1389–1401.
23. Nozaki I, Watanabe T, Kawaguchi M, Akatsu H, Tsuneyama K, et al. (2007) Reduced expression of endogenous secretory receptor for advanced glycation endproducts in hippocampal neurons of Alzheimer's disease brains. Arch Histol Cytol 70: 279–290.
24. Schmidt AM, Yan SD, Yan SF, Stern DM (2001) The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. J Clin Invest 108: 949–955.
25. Lutterloh EC, Opal SM, Pittman DD, Keith JC Jr, Tan XY, et al. (2007) Inhibition of the RAGE products increases survival in experimental models of severe sepsis and systemic infection. Crit Care 11: R122.
26. Hofmann MA, Drury S, Hudson BI, Gleason MR, Qu W, et al. (2002) RAGE and arthritis: the G82S polymorphism amplifies the inflammatory response. Genes Immun 3: 123–135.
27. Lalla E, Lamster IB, Feit M, Huang L, Spessot A, et al. (2000) Blockade of RAGE suppresses periodontitis-associated bone loss in diabetic mice. J Clin Invest 105: 1117–1124.
28. Kato I, Yamamoto Y, Fujimura M, Noguchi N, Takasawa S, et al. (1999) CD38 disruption impairs glucose-induced increases in cyclic ADP-ribose, [Ca2+]i, and insulin secretion. J Biol Chem 274: 1869–1872.
29. Kato T, Ishiwata M, Yamada K, Kasahara T, Kakiuchi C, et al. (2008) Behavioral and gene expression analyses of Wfs1 knockout mice as a possible animal model of mood disorder. Neurosci Res 61: 143–158.
30. Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11: 47–60.

The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.34, Special Issue II, SP279-SP286, 2009



# Intrauterine environment-genome interaction and Children's development (2): Brain structure impairment and behavioral disturbance induced in male mice offspring by a single intraperitoneal administration of domoic acid (DA) to their dams

**Kentaro Tanemura, Katsuhide Igarashi, Toshiko-R Matsugami, Ken-ichi Aisaki, Satoshi Kitajima and Jun Kanno**

*Division of Cellular & Molecular Toxicology, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*

(Received February 17, 2009)

**ABSTRACT** — To demonstrate induction of delayed central nervous toxicity by disturbing neuronal activities in the developing brain, we administered a single intraperitoneal dose of domoic acid (DA; 1 mg/kg), a potent glutamate receptor agonist, to pregnant female mice at the gestational day of 11.5, 14.5 or 17.5. The dams had recovered from acute symptoms within 24 hr, followed by normal delivery, feeding and weaning. All male offspring mice after weaning were apparently normal in response to handlers during cage maintenance, body weight measurement and to mate mice in group housing conditions. At the age of 11 weeks, our neurobehavior testing battery revealed severe impairment of learning and memory with serious deviances of anxiety-related behaviors. The developed brain of prenatally exposed mice showed myelination failure and the overgrowth of neuronal processes of the limbic cortex neurons. This study indicates that the temporal disturbance of neurotransmission of the developing brain induces irreversible structural and functional damage to offspring which becomes monitorable in their adulthood by a proper battery of neurobehavioral tests.

**Key words**: Domoic acid, Prenatal exposure, Brain structure, Behavior

## INTRODUCTION

Adequate neural activities are necessary for the maturation of neural networks during brain development (Rice and Barone, 2000). Historically, the presence of such plasticity-driven mechanisms has been demonstrated by a series of studies of eyelid suture in kittens or monkeys and corresponding findings reported in young human cataracta patients (Wiesel, 1982; Gu *et al.*, 1989; Fonta *et al.*, 2000). These processes require proper stimuli to the brain that trigger the release of neurotransmitters from the neurons and subsequent receptor-mediated signal transduction (Ooi and Wood, 2008; Greer and Greenberg, 2008; Cohen-Cory, 2002). Therefore, it is highly conceivable that disturbance of neural activities by neuroactive xenobiotics leads to malformation of the fine structure of the brain. Even when the exposure was transient, it would result in anomaly of higher brain functions in adulthood without overt signs of brain damage during maturation.

Glutamate receptors begin to express in the late embryonic stages, and their expression increases with the advance of brain development (Luján *et al.*, 2005; Manent *et al.*, 2005). Prenatal exposure of xenobiotic chemicals that interfere with the glutamate receptor function could induce malformation of the fine structure of the brain which should lead to anomaly of higher brain function that is different from acute neurotoxicity known for such chemicals to induce in adults (Bondy and Campbell, 2005). A marine biotoxin domoic acid (DA) which is structurally related to glutamate, and activates ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kinate subtypes of glutamate receptors (Pulido, 2008) is known to cause acute symptoms of diarrhea, seizures and memory loss in adult human by eating contaminated shellfish (Tryphonas and Iverson, 1990), and DA induced acute neurotoxicity in animal

Correspondence: Jun Kanno (E-mail: kanno@nihs.go.jp)

model (Chandrasekaran *et al.*, 2004). Additionally, DA is also known to cross the placenta, and enters prenatal brain tissue in rats (Maucher and Ramsdell, 2007). Therefore, prenatal exposure of DA may disrupt the neural activities by excessive stimulation of glutamate receptors, and should induce fine structural and functional disorganization in the developing brain. Here, we report that a transient transplacental DA exposure *in utero* induced alteration of the neurobehavioral parameters and corresponding fine brain structure of the male C57BL/6 mice in their adulthood.

## MATERIALS AND METHODS

### Animal treatment

All experiments were carried out under approval of Experimental Animal Use Committee of National Institute of Health Sciences, Japan. Pregnant C57LB/6 female mice obtained from Japan SLC, Inc., were individually housed in plastic breeding cages with free access to water and pellet diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) in a 12 hr light-dark cycle conventional condition. Four groups with five pregnant mice each were prepared. All groups received three intraperitoneal injections on gestational day 11.5 (E11.5) as a late embryonic period, 14.5 (E14.5) and 17.5 (E17.5) as early and late fetal period respectively. Group A (Control) received three i.p. shots of saline on E11.5, E14.5 and E17.5. Group B (DA@ E11.5) received one shot of DA (Calbiochem, San Diego, CA, USA) at a dosage of 1 mg/kg on E 11.5 and two shots of saline on E14.5 and E17.5. Group C (DA@14.5) received a shot of saline on E11.5, a shot of DA on E14.5 and another saline on E17.5. Group D (DA@E17.5) received two shots of saline on E11.5 and E14.5 , and a shot of DA on E17.5. The pups were weaned at 4 weeks of age, and four male mice per liter were randomly selected and housed in one cage with free access to water and CRF-1 pellet until 11 weeks of age.

### Immunohistochemical analysis

Brains (n = 4 male mice per group) were fixed with methacarn fixative (methanol: chloroform:acetic acid, 60:30:10 v/v) and paraffin-embedded sections were prepared. Mouse monoclonal anti-microtubule associated protein 2 (MAP2, sc-32791; Santa Cruz, CA, USA), mouse monoclonal anti-neurofilament-m (NF-M, sc-20013; Santa Cruz, CA, USA), rabbit polyclonal anti-myelin associated glycoprotein (MAG, sc-15324; Santa Cruz, CA, USA), and rabbit polyclonal anti MAP2 (sc-20172; Santa Cruz, CA, USA) were used. Deparaffinized sections were pretreated with HistoVT-One (Nacalai Tesque, Kyoto, Japan.) as previously described (Tanemura *et al.*, 2005) and incubated with primary antibodies. Secondary antibodies were Alexa 568-conjugated anti-mouse IgG and Alexa 488-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR, USA). Fluorescent images were obtained with an FV-300 confocal laser scanning microscope (Olympus, Tokyo, Japan). For semi-quantitative analysis of images, we calculated the ratio of fluorescence intensity compared to control mice (group A), by using the IMAGE J program (http://rsb.info.nih.gov/ij/index.html. National Institute of Health, Bethesda), after adjusting background noise (n = 4 images per mouse).

### Neurobehavioral tests

A battery of neurobehavioral tests were conducted on open field test (OF), light/dark transition test (LD), elevated plus maze test (EP) and contextual/cued fear conditioning test (FZ). Experimental apparatuses and image analyzing softwares were obtained from O'Hara & Co., Ltd., Japan. Image analyzing softwares (Image OF4, Image LD2, Image EP2 and Image FZ2) were developed from the public domain IMAGE J program. All experiments were done with 8 mice per group (32 mice total), and were conducted between 13:30 and 16:30. The level of background noise during behavioural testing was about 50 dBA. After each trial, the apparatuses were wiped and cleaned.

### Open field test

The locomotor activity was measured for 10 min using an open field apparatus made of white plastic (50 x 50 x 40 (H) cm).

An LED light system was positioned 50 cm above the centre of the field (50 lux at the centre of field). Total distance travelled (cm), time spent in the central area (30% of the field) (sec), and the frequencies of movement were measured (Tanemura *et al.*, 2002).

### Light/dark transition test

The apparatus used for the light/dark transition test consisted of a cage (21 x 42 x 25(H) cm) divided into two chambers by a partition with an opening. One chamber is brightly illuminated (250 lux), whereas the other chamber is dark (2 lux). A mouse is placed into the dark area and allowed to move freely between the two chambers through the opening for 5 min. The latency for the first move to the light area, the total number of transitions and the time spent on each side were measured.

### Elevated plus maze test

The plus-shaped apparatus consisted of four arms (25

x 5 cm) connected to a central square area (5 x 5 cm). Opposite two arms are enclosed with 20 cm-high transparent walls and other two are left open. The floor of the maze is made of white plastic plate and is elevated 60 cm above the room floor (200 lux at the centre of the apparatus). A mouse is placed to the central square area of the maze, facing one of the open arms, and the behavior was recorded for 10 min: total distance traveled (cm), total time on open arms and central square area (sec) and the total number of entry to any of the arms (Tanemura *et al.*, 2002).

### Contextual/cued fear conditioning test

The apparatus consists of a conditioning chamber (or a test chamber) (17 x 10 x 10 (H) cm) made of clear plastic with ceiling and placed in a sound proof box. The chamber floor has stainless steel rods (2-mm diameter) spaced 5 mm apart for giving electric foot shock (0.1 mA, 3 sec duration) to the mouse. The soundproof box consists of white-coloured wood, and is equipped with an audio speaker and light source (35 lux at the centre of the floor). A CCD camera is positioned 20 cm above the ceiling of the chamber. During the conditioning trial (Day 1), mice are placed individually into the conditioning chamber in the sound proof box and, after 90 sec, they are given three tone-shock pairings (30 sec of tone, 75 dB, 10 KHz followed by 3 sec of electric shock at the end of tone, 0.1 mA) separated by 90 sec. Then they are returned to their home cage. Next day (Day 2), as a "contextual fear test", they are returned to the conditioning chamber without tone and shock for a 6-min. On the third day (Day 3), they are brought to a novel chamber of different make without stainless steel rods place in the sound proof box and, after a period of 3 min, only the conditioning tone is presented for 3 min (no shock was presented, 35 lux at the centre of the floor). The freezing response of mice was defined as a consecutive 2 sec period of immobility. Freezing rate (%) was calculated as [time freezing/session time] x 100 (Tatebayashi *et al.*, 2002).

### Statistical analysis

Data were indicated as means ± S.D. Statistical analysis was conducted with student's t-test by using StatView (SAS Institute, Cary, NC, USA). A p-value of $< 0.05$ compared to the results of control male mice (group A) was considered statistically significant.

## RESULTS

### Effects on morphology of brain by prenatal exposure to DA

Offspring mice of all groups after weaning up to the age of 11 weeks were apparently normal in response to handlers during cage maintenance, body weight measurement and to mate mice in group housing conditions. Routine histological observation of the brain at 11 weeks old by hematoxylin-eosin staining could not reveal difference among the groups (data not shown). By immunohistochemical study on the same brain sections, reduced immuno-reactivity against the MAG, the marker for myelin, was detected in the cortices of group B (DA@11.5) and C (DA@14.5) compared to control (Figs. 1A-D and I). In contrast, increased immuno-reactivity against MAP2, the marker for neuronal dendrite, was indicated in the same area of group B (DA@11.5), C (DA@14.5) and D (DA@17.5) compared to control (Figs. 1E-H and J). Increased immuno-reactivity against MAP2 was also found in lateral area of CA3 hippocampus of group B (DA@11.5), C (DA@14.5) and D (DA@17.5) compared to control, whereas immuno-reactivity for MAP2 showed no significant difference in medial area of CA3 hippocampus among the groups (Figs. 2A-D and I). Immuno-reactivity against NF-M; the marker for neuronal axon, also showed no significant difference in the same area among the groups (Figs. 2E-H and J).

### Effects on behavior by prenatal exposure of DA

In the OF test, the distance traveled was not different among the groups (Fig. 3A), the time spent in center area was significantly prolonged in group D (DA@17.5) mice (Fig. 3B). In the LD test, group C (DA@14.5) mice stayed in light area for longer time (Fig. 4A), and latency for the first move to light area was significantly shorter in group C (DA@14.5) and D (DA@17.5) (Fig. 4B). In the EP test, significantly increased distance traveled and time spent in the open area were detected for group B (DA@11.5), C (DA@14.5) and D (DA@17.5) (Figs. 5A and B). In the FZ test, both Day 1 and Day 2 freezing responses of group C (DA@14.5) and D (DA@17.5) were significantly reduced compared to control (Figs. 6A and B).

## DISCUSSION

The expression levels of glutamate receptors starts to elevate at the fetal period, i.e. approximately from E14 (Luján *et al.*, 2005; Manent *et al.*, 2005). Exogenous glutamatergic stimuli at this period would affect the for-

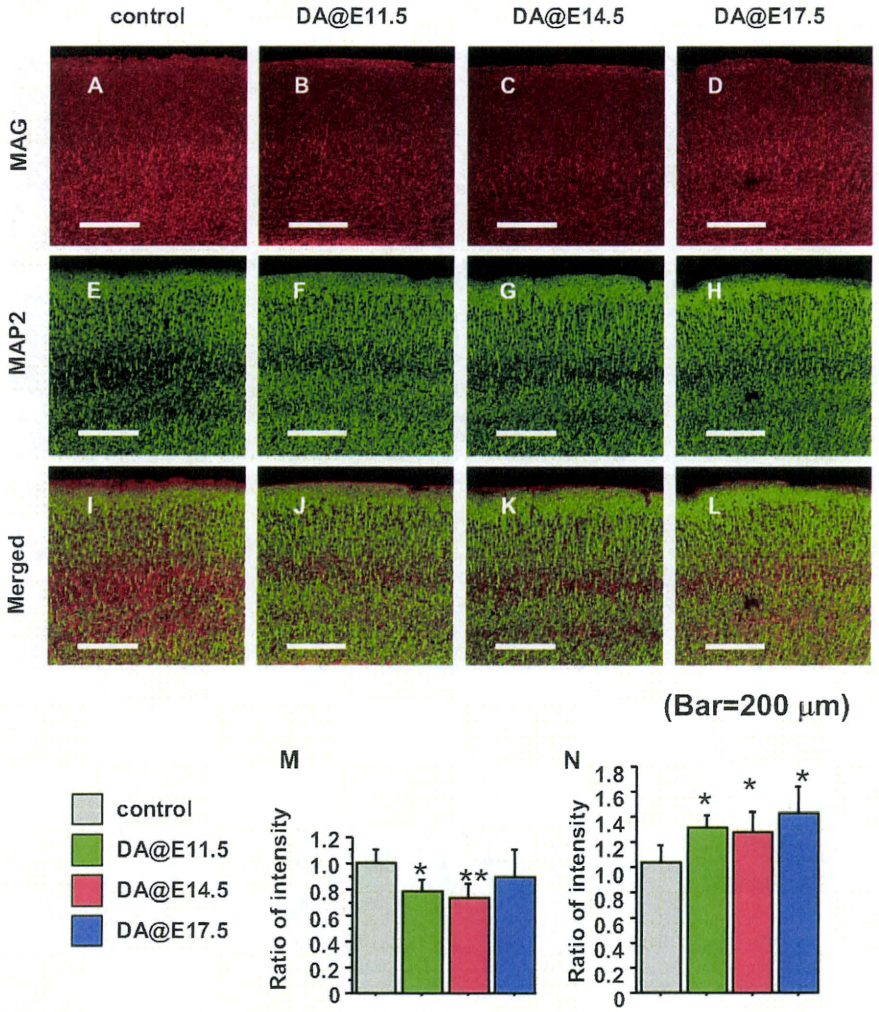


**Fig. 1.** Delayed effects on cerebral cortex induced by prenatal exposure of DA.
A-D, Immunohistochemical staining against MAG; E-H, immunohistochamical staining against MAP2; I-L, merged images of the cerebral cortex. A, E, I, group A (control), B, F, J, group B (DA@11.5), C, G, K, group C (DA@14.5) and D, H, L, group D (DA@17.5). Scale bar = 200 mm. M, Quantitative analysis in intensity ratio to control of MAG expression, and J, MAP2 expression among the groups (mean ± S.E.M.). Asterisk (**) and (*) indicate significant difference compared to control ($P < 0.01$) and ($P < 0.05$).

mation of the neural circuits. An extreme example to support this hypothesis would be the phenotype of the double knockout mouse of glutamate transportors GLT1 and GLAST (Matsugami *et al.*, 2006). Lack of these transportors is considered to result in abnormally high concentration of glutamate in the brain. In fact, morphological anomaly became apparent in synchronization with the expression of glutamine receptors. In our study, corresponding to the hypothesis, the neurobehavioral symptoms as a whole was severer for those exposed at fetal periods, i.e. E14.5 and E17.5, compared to those at embryonic period, i.e. E11.5 (Fig. 7).

We demonstrated that a prenatal exposure of a relatively low dose of DA induced a spectrum of neurobehavioral anomalies which became monitorable at the adult stage accompanied by alteration in fine brain structures detectable by immunohistochemistry. It is emphasized that this amount of DA did not induce abnormal responses dur-

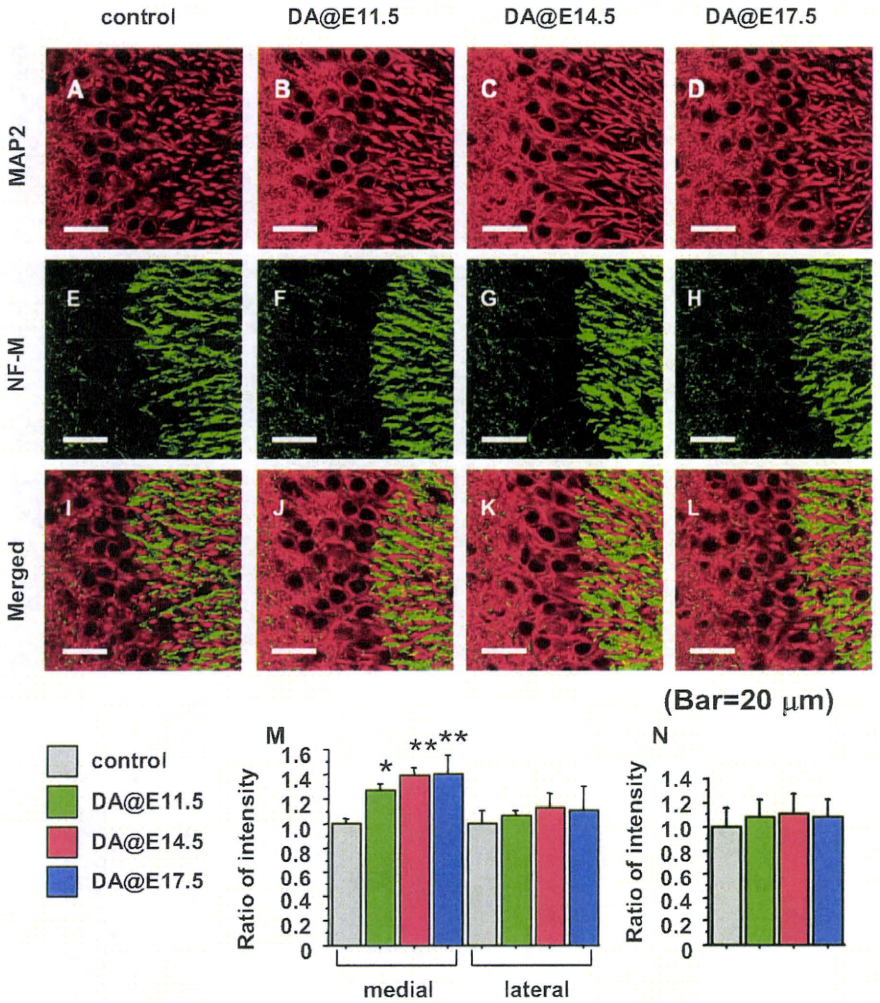


**Fig. 2.** Delayed effects on hippocampus induced by prenatal exposure of DA.
A-D, Immunohistochemical staining against MAP2; E-H, immunohistochamical staining against NF-M; I-L, merged images, of CA3 hippocampus. A, E, I, group A (control), B, F, J, group B (DA@11.5), C, G, K, group C (DA@14.5) and D, H, L, group D (DA@17.5). Scale bar = 200 mm. M, Quantitative analysis of MAP2 expression, and N, NF-M expression among the groups (mean ± S.E.M.). Asterisk (**) and (*) indicated significant difference compared to control ($P < 0.01$) and ($P < 0.05$).

ing maturation, such as hyperreactivity to handling and to cage mates, and did not present overt malformation of the brain detectable by the routine H&E histology at the age of 2 weeks (data not shown). It is also noted that the spectrum of the neurobehavioral symptoms induced in mice exposed to DA at adulthood was different from those monitored in this study (data not shown).

Although progressive hippocampal neuronal damages were reported to be induced by prenatal administration of DA (0.6 mg/kg intravenous injection to the dam) (Dakshinamurti *et al.*, 1993), we did not find notable neuronal loss or neuronal cell death as the delayed effects in adult mouse brain by prenatal exposure. On the other hand, we found myelination failure (Miller and Mi, 2007) in cortex of group B (DA@11.5) and C (DA@14.5) mice. And we also detected a finding compatible with the overgrowth of neuronal processes in cortex and hippocampus of group B (DA@11.5), C (DA@14.5) and D (DA@14.5)

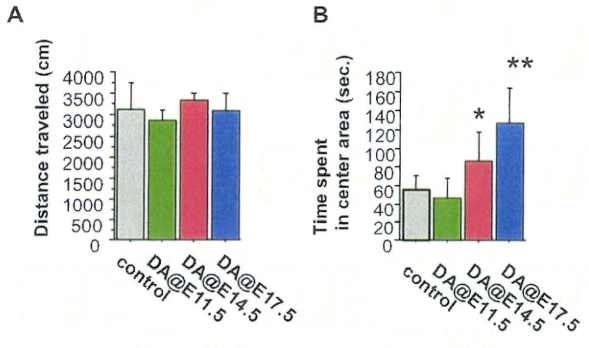


**Fig. 3.** Delayed effects on locomotor activity (OF test) induced by prenatal exposure of DA.
A, Mean distance travelled (total distances divided by total duration of trial, 10 min) and B, mean time spent in center area (30% of the field) in the open field apparatus (mean ± S.E.M.). Asterisk (**) and (*) indicated significant difference compared to control ($P < 0.01$) and ($P < 0.05$).

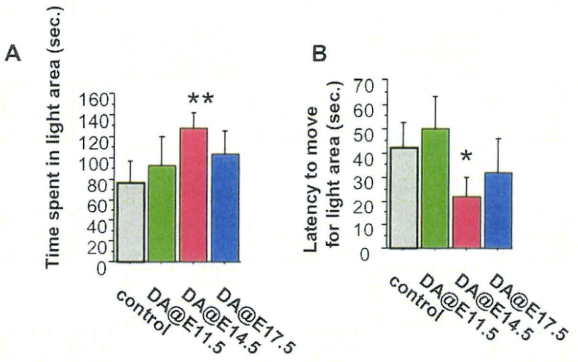


**Fig. 4.** Delayed effects on anxiety-related behavior (LD test) induced by prenatal exposure of DA.
A, Total time spent in light area, and B, latency time to move to light area in the LD apparatus (mean ± S.E.M.). Asterisk (**) and (*) indicated significant difference compared to control ($P < 0.01$) and ($P < 0.05$).

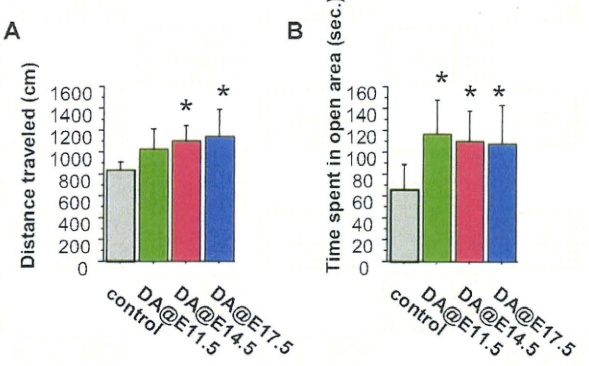


**Fig. 5.** Delayed effects on anxiety-related behavior (EP test) induced by prenatal exposure of DA.
A, Total distance travelled, and B, total time spent in open area in the elevated plus maze apparatus (mean ± S.E.M.). Asterisk (*) indicated significant difference compared to control ($P < 0.05$).

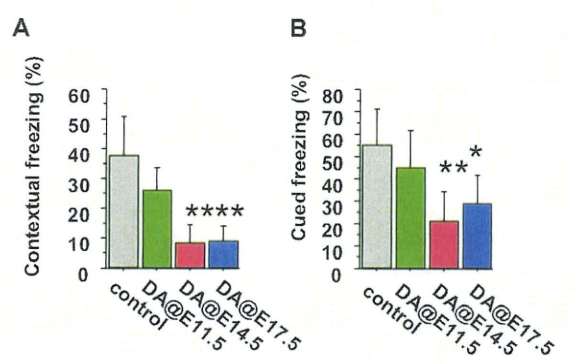


**Fig. 6.** Delayed effects on learning and memory (FZ test) induced by prenatal exposure of DA.
A, Contextual fear test and B, cued fear test. Memory performance is expressed as a mean percent duration of freezing responses (mean ± S.E.M.). Asterisk (**) and (*) indicated significant difference compared to control ($P < 0.01$) and ($P < 0.05$).

mice by using cytoskeletal marker. These findings indicated that the disorganization of brain was induced by the prenatal exposure of DA, and remained irreversibly up until the maturation period.

Among multiple endpoints of the behavioral test battery we used, serious deviances in anxiety-related behaviors of group C (DA@14.5) and D (DA@17.5) mice were observed. Mice in those groups showed low performances in adaptations for novel circumstances, i.e., strange and broad area in OF test, beamish place in LD test, high and narrow space in EP test. Additionally, we also found severe impairment of learning and memory. Although the low performances of memory task have been reported in rats with prenatal DA exposure (Levin *et al.*, 2005),

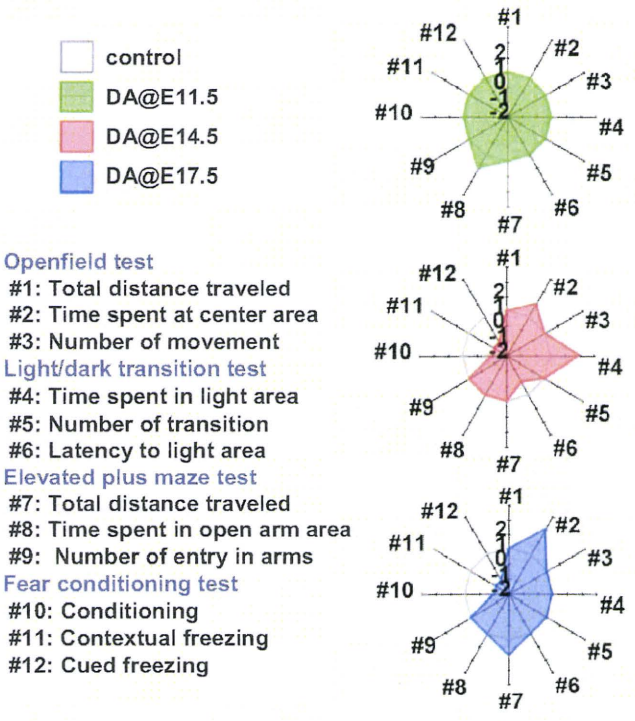


**Fig. 7.** Summary radar chart of the neurobehavioral battery test results. Radial axis indicates the direction (increase or decrease) of the deviation, and the p value of the endpoints compared to the control (+1 and −1, $0.01 <= p < 0.05$, +2 and −2, $p < 0.01$). Regular dodecagon of radius 0 indicates no deviation from control.

we showed serious deviances about affective (emotional) behaviors additional to severe memory deficit.

In conclusion, we clearly indicated that the disturbance against the adequate neural activity during developmental period when glutamate receptors became active have induced delayed memory defect and unnatural adoptive behaviors that became monitorable at the maturation period in mice. The responsible foci deduced from these behavioral disturbances are the limbic cortex and hippocampus. Our morphological findings are consistent with the interpretation. A combination of neurobehavioral and pathomorphological analysis was shown to be an effective method to assess delayed neurotoxic effects which dose not induce immediate organic brain damage and related symptoms after exposure. Having adopted the hypothesis that exogenous stimuli to neural signaling systems during the development of the brain can be a cause of delayed anomaly of higher brain function, stimuli toward systems other than glutamate receptors should also induce such anomaly of different targets and symptoms in concert with the distribution of the corresponding receptor(s) in the developing brain. Such data on other system would be reported elsewhere.

## ACKNOWLEDGMENTS

The authors thank Mr. Yusuke Furukawa and Ms. Maki Otsuka for technical support. This study was supported by Health Sciences Research Grants H17- -Kagaku- 001 from the Ministry of Health, Labour and Welfare, Japan.

This peer-reviewed article is based upon a lecture presented at the 35th Annual Meeting of Japanese Society of Toxicology, June 2008 in Tokyo under the theme of "Children's Toxicology", June 2008 in Tokyo.

## REFERENCES

Bondy, S.C. and Campbell, A. (2005): Developmental neurotoxicology. J. Neurosci. Res., **81**, 605-612.

Chandrasekaran, A., Ponnambalam, G. and Kaur, C. (2004): Domoic acid-induced neurotoxicity in the hippocampus of adult rats. Neurotox. Res., **6**, 105-117.

Cohen-Cory, S. (2002): The developing synapse: construction and

modulation of synaptic structures and circuits. Science, **298**, 770-776.

Dakshinamurti, K., Sharma, S.K., Sundaram, M. and Watanabe, T. (1993): Hippocampal changes in developing postnatal mice following intrauterine exposure to domoic acid. J. Neurosci., **13**, 4486-4495.

Fonta, C., Chappert, C. and Imbert, M. (2000): Effect of monocular deprivation on NMDAR1 immunostaining in ocular dominance columns of the marmoset Callithrix jacchus. Vis. Neurosci., **17**, 345-352.

Greer, P.L. and Greenberg, M.E. (2008): From synapse to nucleus: calcium-dependent gene transcription in the control of synapse development and function. Neuron, **59**, 846-860.

Gu, Q.A., Bear, M.F. and Singer, W. (1989): Blockade of NMDA-receptors prevents ocularity changes in kitten visual cortex after reversed monocular deprivation. Brain Res. Dev. Brain Res., **47**, 281-288.

Levin, E.D., Pizarro, K., Pang, W.G., Harrison, J. and Ramsdell, J.S. (2005): Persisting behavioral consequences of prenatal domoic acid exposure in rats. Neurotoxicol. Teratol., **27**, 719-725.

Luján, R., Shigemoto, R. and López-Bendito, G. (2005): Glutamate and GABA receptor signalling in the developing brain. Neuroscience, **130**, 567-580.

Manent, J.B., Demarque, M., Jorquera, I., Pellegrino, C., Ben-Ari, Y., Aniksztejn, L. and Represa, A. (2005): A noncanonical release of GABA and glutamate modulates neuronal migration. J Neurosci., **25**, 4755-4765.

Matsugami, T.R., Tanemura, K., Mieda, M., Nakatomi, R., Yamada, K., Kondo, T., Ogawa, M., Obata, K., Watanabe, M., Hashikawa, T. and Tanaka, K. (2006): Indispensability of the glutamate transporters GLAST and GLT1 to brain development. Proc. Natl. Acad. Sci. USA, **103**, 12161-12166.

Maucher, J.M. and Ramsdell, J.S.(2007): Maternal-fetal transfer of domoic acid in rats at two gestational time points. Environ Health Perspect., **115**, 1743-1746.

Miller, R.H. and Mi, S. (2007): Dissecting demyelination. Nat. Neurosci., **10**, 1351-1354.

Ooi, L. and Wood, I.C. (2008): Regulation of gene expression in the nervous system. Biochem. J., **414**, 327-341.

Pulido, O.M. (2008): Domoic acid toxicologic pathology: a review. Mar. Drugs, **6**, 180-219.

Rice, D. and Barone, S.Jr. (2000): Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ. Health Perspect., **108**, 511-533.

Tanemura, K., Murayama, M., Akagi, T., Hashikawa, T., Tominaga, T., Ichikawa, M., Yamaguchi, H. and Takashima, A. (2002): Neurodegeneration with tau accumulation in a transgenic mouse expressing V337M human tau. J Neurosci., **22**, 133-141.

Tanemura, K., Ogura, A., Cheong, C., Gotoh, H., Matsumoto, K., Sato, E., Hayashi, Y., Lee, H.W. and Kondo, T. (2005): Dynamic rearrangement of telomeres during spermatogenesis in mice. Dev. Biol., **281**, 196-207.

Tatebayashi, Y., Miyasaka, T., Chui, D.H., Akagi, T., Mishima, K., Iwasaki, K., Fujiwara, M., Tanemura, K., Murayama, M., Ishiguro, K., Planel, E., Sato, S., Hashikawa, T. and Takashima, A. (2002): Tau filament formation and associative memory deficit in aged mice expressing mutant (R406W) human tau. Proc. Natl. Acad. Sci. USA, **99**, 13896-13901.

Tryphonas, L. and Iverson, F. (1990): Neuropathology of excitatory neurotoxins: the domoic acid model. Toxicol Pathol., **18**, 165-169.

Wiesel, T.N. (1982): Postnatal development of the visual cortex and the influence of environment. Nature, **299**, 583-591.

Biochemical and Biophysical Research Communications 385 (2009) 319–323

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Abnormalities in aggression and anxiety in transgenic mice overexpressing activin E

Kazunari Sekiyama [a], Osamu Hashimoto [a,*], Yuuki Ushiro [a], Chika Adachi [a], Takefumi Kikusui [b], Kentaro Tanemura [c], Yoshihisa Hasegawa [a]

[a] Laboratory of Experimental Animal Science, Faculty of Veterinary Medicine, Kitasato University, School of Veterinary Medicine, 35-1 Higashi 23 Bancho, Towada-shi, Aomori 034-8628, Japan
[b] Companion Animal Research, Azabu University, 1-17-1 Fuchinobe, Sagamihara-shi, Kanagawa 229-8501, Japan
[c] National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

ARTICLE INFO

*Article history:*
Received 21 April 2009
Available online 20 May 2009

*Keywords:*
Activin E
Aggression
Anxiety
Transgenic mouse

ABSTRACT

To study the function of activin E, a TGF-β superfamily member, in the regulation of affective behavior, we investigated the behavior of transgenic mice overexpressing activin E (TgActβE mice). Male TgActβE mice showed aggressive behavior in resident–intruder tests. In elevated plus-maze tests, the percentage of open arm entries was significantly increased in female TgActβE mice compared with that in wild-type mice. Furthermore, female TgActβE mice stayed in the central area for a significantly longer time than wild-type mice in open field tests. These results indicated that TgActβE mice had less anxiety-like behavior. The number of restraint-stress-evoked c-Fos-positive cells in the hypothalamic paraventricular nucleus in TgActβE mice was significantly decreased compared with that in wild-type mice. This suggests that synthesis of corticotrophin-releasing hormone induced by stress was decreased in TgActβE mice. Taking these results together, activin E may act as a regulator of the hypothalamic–pituitary–adrenal axis.


## Introduction

The TGF-β superfamily has more than 30 members, including TGF-βs, growth and differentiation factors (GDFs), bone morphogenetic proteins (BMPs) and activins [1]. They carry out multifunctional regulation in a wide variety of cell and tissue types in an autocrine or paracrine manner.

Activins are disulfide-linked homo- or heterodimers of the β subunits of inhibin/activin A and B. Activin A (βAβA), activin AB (βAβB) and activin B (βBβB) were initially identified as stimulators of FSH secretion from pituitary cells [2]. However, it is now known that activins have multifunctional effects including promotion of erythroid differentiation, nerve cell survival, induction of mesoderm in *Xenopus laevis* embryos, promotion of bone growth, augmentation of somatostatin expression, and induction of cell cycle arrest and apoptosis [2].

Additional inhibin/activin β subunit genes (βC and βE) have been identified in mammals [3]. Analysis of the amino acid sequence of the mature region of known activin β subunits has indicated that the subunits have approximately 50% overall homology. The activin βC and βE subunits have the same general structure as the βA and βB subunits, with seven cysteine residues generally conserved among TGF-β superfamily members, as well as an additional two cysteines at the N-terminal region of mature proteins that are typically conserved in activins. Similar to activin βA and βB subunits, βC and βE subunits can form disulfide-linked homo- and heterodimers [3]. It has been reported that activin E is associated with cell growth and apoptosis in some cell types [3]. However, gene targeting has revealed that activin E null mice have no abnormalities so far [4]. Therefore, further study is needed to understand the function of activin E.

In the central nervous system (CNS), activins have roles in neuroprotection and antidepressant activity [5,6]. Intracerebroventricular infusion of activin A rescues neuronal damage from excitotoxic and hypoxic–ischemic brain injury. Furthermore, forebrain-specific expression of activin A leads to abnormal anxiety-related behavior [7]. These findings suggest that the regulation of activin and its signaling in the CNS are important physiologically for many aspects of behavior. Recently, aberration of mRNA expression for activins, especially activin E, has been observed in the rat brain infected with Borna disease virus (BDV), which induced abnormal behavior [8].

We have reported previously the generation and characterization of transgenic mice that overexpress human activin E [9]. It has been found that activin E plays a role in pancreatic exocrine cell growth. However, the function of activin E in the CNS is unclear. To study the role of activin E in the regulation of emotional

* Corresponding author. Fax: 81 176 23 8703.
*E-mail address:* ohashim@vmas.kitasato-u.ac.jp (O. Hashimoto).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.05.054

behavior, we examined transgenic mice that overexpressed activin E (TgActβE mice). We demonstrated that the mice exhibited abnormal aggressive behavior and anxiety, which suggests that activin E has a function in the brain. Furthermore, the transgenic mice may be a useful animal model for the study of mood disorders.

## Materials and methods

*Animals.* C57BL/6 and A/J mice were obtained from SLC Japan (Hamamatsu, Japan). The generation of TgActβE mice has been described previously [9]. Two lines, 16 and 19 (TgActβE16 and TgActβE19) were backcrossed to C57BL/6 mice and analyzed together with littermate controls after 9–17 generations of backcrossing. Male mice were housed individually from the age of 8 weeks. Female mice were housed in groups of three from the age of 6 weeks. Mice were maintained in a 12-h light–dark cycle at 24 ± 2 °C and given standard chow and water *ad libitum*. Experimental procedures and the care of animals were performed in accordance with the requirements of the Institutional Animal Care Committee at Kitasato University, in compliance with National Institutes of Health guidelines.

*Behavioral tests.* All the behavioral experiments performed under 640-lx light conditions and were carried out between 1330 and 1630 h. Elevated plus-maze behavior was measured when mice were 9–11-weeks-old. The elevated plus-maze apparatus consisted of four arms elevated 45 cm above the floor, with each arm positioned at 90° relative to the adjacent arms. Two of the arms were enclosed with high walls (280 × 50 × 150 mm), and the other arms were open (280 × 50 mm). Open arms and closed arms were connected via a central area (50 × 50 mm) to form a plus sign. The maze was made of polypropylene and glued on cardboard. The behavior of the mice in the maze was recorded by a video camera. The camera was suspended above the maze and the experimenter was not in close proximity to the experimental setup for the duration of the study. Mice at the start of each trial were placed in the central platform of the maze, facing an open arm. A standard 5-min test was employed for each mouse. The maze was thoroughly cleaned with damp and dry towels between experimental periods. The number of entries into, and percentage of time spent in the open and closed arms were counted. Frequency and percentage of total time of other activities (self grooming, stretching, peering and rearing) were also measured in the test.

For the resident–intruder test, male TgActβE mice (14–16-weeks-old) were housed in individual cages for more than 3 weeks before testing. A male A/J mouse (8–9-weeks-old) was introduced into the cage as an intruder and we observed the aggressive confrontation. The observation was continued for 5 min after the first bite was confirmed, but when no attack took place, observation was discontinued at 10 min after introduction of the intruder. The tests were repeated twice, at 3-day intervals, for each mouse. All the experimental sessions were recorded on videotape, and the behavior parameters at the second session were scored. The durations of the following behaviors were measured: offensive biting, sideways threat, tail rattling, and jumping.

*Acute stress test.* TgActβE mice were stressed by restraint in a 50-ml plastic centrifuge tube for 30 min and then returned to their home cages. After 15 min, mice were anesthetized with pentobarbital (50 mg/kg, i.p. injection), venous blood samples were taken from the postcaval vein and perfused transcardially with PBS followed by Bouin's fixative. Serum corticosterone was measured using Corticosterone EIA Kits (No. 500651, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The brains were subjected to histological analysis.

*Histological analysis.* Mice were anesthetized with pentobarbital (50 mg/kg, i.p. injection) and perfused transcardially with PBS followed by Bouin's fixative. The brain was isolated, re-fixed in Bouin's solution for 16 h and embedded in paraffin.

For detection of c-Fos, 6-μm sections of the brain were deparaffinized and heated by microwaves at an upper temperature limit of 95 °C for 30 min in 10 mM sodium citrate buffer (pH 6.0). After cooling, anti c-Fos antibody (SC-52; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:500 dilution in TBS-BSA was incubated with the sections, and the immunoreactivity was visualized as described above. To quantify the c-Fos expression in the paraventricular nucleus (PVN), c-Fos-positive cells were counted in randomly selected fields (0.5 $mm^2$ per PVN) as described elsewhere [10].

*Statistical analysis.* Results were expressed as mean ± SE. Differences between groups were analyzed for statistical significance by using the $\chi^2$ test (StatView 5.0 software; Abacus Concepts, Berkeley, CA, USA) for the resident–intruder test, multivariate analysis of variance (MANOVA) with the Hotelling–Lawley trace was conducted for the genotype and sex comparisons, followed by Tukey's post hoc test for elevated plus-maze test and open field test. Also, differences between groups were analyzed for statistical significance by using the unpaired $t$ test. $P < 0.05$ was considered to be statistically significant.

## Results

### *Abnormal behavior of transgenic mice*

To determine aggressive behavior of male TgActβE mice, we performed the resident–intruder test. TgActβE mice showed anogenital contact and rearing at first, similar to that in wild-type-mice. However, some TgActβE mice displayed aggressive behavior, i.e., offensive biting, sideways threat and tail rattling to the intruder mice (Table 1). These results suggested that TgActβE mice exhibited more response to the intruders than wild-type mice did. Since TgActβE16 mice showed similar phenotypes to TgActβE19 mice in the test, we hereinafter describe the behavioral abnormalities in TgActβE19 mice.

To further understand the abnormal emotion of TgActβE mice, we next performed the elevated plus-maze and open field tests. MANOVA revealed genotype differences in 13 dependent variables: total arm entries, percentage of total time and entries in the open and closed arms, and the frequency and total time of self grooming, rearing, stretching and peering [genotype: $F(13, 42) = 2.05$, $P < 0.05$]. TgActβE mice showed a significantly increased percentage of open arm entries [$F(1, 54) = 9.7$, $P < 0.01$] compared with that of wild-type mice, using bivariate analysis. TgActβE mice showed a significantly deceased percentage of closed arm entries [$F(1, 54) = 9.7$, $P < 0.01$] and total time spent in closed arms [$F(1, 54) = 7.33$, $P < 0.01$]. In addition, the total time and frequency of stress-evoked self grooming [time: $F(1, 54) = 4.08$, $P < 0.05$, frequency: $F(1, 54) = 7.76$, $P < 0.01$] were significantly increased in TgActβE mice. Univariate analysis by $t$ test revealed that arm entries of male TgActβE mice were comparable with those of wild-type mice in the elevated plus-maze (Table 2). Furthermore, there was no significant difference in other parameters such as

**Table 1**
Resident–intruder test in male transgenic mice.

| | Wild-type mice (%) | TgActβE mice (%) | Significance |
|---|---|---|---|
| Offensive biting | 0.0 | 27.3 | $P = 0.0624$ |
| Sideways threat | 0.0 | 54.5 | $P < 0.01$ |
| Tail rattling | 0.0 | 9.1 | $P = 0.3061$ |
| Jumping | 0.0 | 54.5 | $P < 0.01$ |

Percentage of behavior elements is shown; $n = 11$.
$\chi^2$ test was performed to determine differences between the groups.

**Table 2**
Behavioral analysis of transgenic mice on the elevated plus maze.

| Genotype | Total arm entries | % Open arm entries | % Closed arm entries | % Time in open arms | % Time in closed arms | Stretching | | Peering | | Self grooming | | Rearing | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Time (%) | Frequency | Time (%) | Frequency | Time (%) | Frequency | Time (%) | Frequency |
| Male | | | | | | | | | | | | | |
| Wild-type | 8.7 ± 1.4 | 9.3 ± 3.5 | 90.6 ± 3.5 | 8.1 ± 4.2 | 82.3 ± 4.8 | 2.1 ± 0.4 | 7.3 ± 1.1 | 1.0 ± 0.2 | 5.0 ± 0.9 | 4.5 ± 1.0 | 4.0 ± 0.7 | 4.1 ± 0.7 | 14.4 ± 2.2 |
| TgActβE | 9.6 ± 1.5 | 19.3 ± 4.9 | 80.7 ± 4.9 | 7.7 ± 2.4 | 72.5 ± 2.8 | 2.7 ± 0.5 | 9.5 ± 1.0 | 1.4 ± 0.4 | 7.4 ± 1.6 | 2.4 ± 0.4* | 2.1 ± 0.3* | 4.2 ± 0.7 | 13.0 ± 2.1 |
| Female | | | | | | | | | | | | | |
| Wild-type | 7.5 ± 1.2 | 20.5 ± 0.4 | 17.0 ± 5.0 | 17.0 ± 5.0 | 74.2 ± 5.0 | 3.2 ± 0.4 | 7.3 ± 0.7 | 2.1 ± 0.4 | 8.5 ± 1.4 | 5.6 ± 1.0 | 3.7 ± 0.5 | 5.4 ± 0.8 | 13.3 ± 1.7 |
| TgActβE | 6.6 ± 0.9 | 38.5 ± 5.9* | 33.7 ± 8.5 | 33.7 ± 8.5 | 52.8 ± 8.1* | 3.6 ± 1.0 | 7.4 ± 1.2 | 2.4 ± 0.4 | 10.8 ± 1.9 | 4.2 ± 0.8 | 2.6 ± 0.5 | 3.5 ± 0.7 | 8.8 ± 1.7 |

Values are mean ± SE. $n = 11–16$. *$P < 0.05$, compared with the wild-type mice of same sex.

stretching, peering from closed arms, and rearing. However, the total time and frequency of stress-evoked self grooming in closed arms were significantly decreased in male TgActβE mice (Table 2). On the other hand, in female TgActβE mice, the percentage of the open arm entries was significantly increased, and the percentage of closed arm entries and time spent in the closed arms were significantly decreased compared with those in wild-type mice (Table 2). The results indicated that female TgActβE mice had less anxiety than wild-type mice.

Furthermore, open field tests indicated that TgActβE mice had more exploratory behavior and less anxiety-like behavior than wild-type mice did (Supplementary data).

*Decrease of c-Fos-positive cells in PVN and serum corticosterone level in TgActβE mice*

Male and female TgActβE mice showed aggressive and anti-anxiety behavior, respectively. Therefore, we hypothesized that TgActβE mice have increased stress tolerance. To elucidate this hypothesis, the mice were exposed to restraint stress. Acute stress is known to evoke a discrete expression pattern of c-Fos, an inducer of neuronal activity, in the brain [11]. Corticotropin-releasing factor (CRF) is a central regulator of the hormonal stress response. It is thought that CRF-producing cells are abundant in the PVN [12,13]. c-Fos-expressing cells in transgenic hypothalamic PVN was detected by immunohistochemistry (Fig. 1A–D). c-Fos-positive cells were detected in the PVN of wild-type mice. However, the number of c-Fos-positive cells in the PVN of TgActβE mice was significantly decreased compared with that in wild-type mice (Fig. 1E). Consistently, serum corticosterone level after acute stress in TgActβE mice was lower than that in wild-type mice (Fig. 2). These results suggested that synthesis of CRF in response to stress was decreased in TgActβE mice.

## Discussion

In the present study, we demonstrated that TgActβE mice exhibited abnormal behavior. Ectopic expression of activin E was detected in the transgenic choroid plexus of the brain (Supplementary data). It has been proposed that activin E is a negative regulator of cell growth. This concept has been supported by experiments in which overexpression of activin E in some types of cells has led to inhibition of cell growth in vivo and in vitro [3]. However, no prominent abnormalities, including apoptosis, were observed histologically in the transgenic brain. Furthermore, the relative weight of the brain to total body weight in the transgenic mice was comparable with that in wild-type mice (Supplementary data). These results indicated that ectopic expression of activin E in the brain caused the behavioral abnormalities. How does misexpressed activin E regulate the emotional behavior of TgActβE mice?

Ligands of the TGF-β superfamily exert their biological effects via a hetero-oligomeric complex of two types (type I and II) of transmembrane serine/threonine kinase receptors and Smads, which are intracellular receptor-associated proteins [14,15]. There are numerous combinations of ligand–receptor complexes that act as multiple TGF-β superfamily ligands. For example, activin receptors can bind activins, BMPs, Vg1, nodal, Lefty2 and GDF11. Based on similarities in the protein structure of activins, it is possible that activin E shares the receptors with other ligands.

Activin type I and II receptors are highly expressed in the forebrain [16,17]. It has been reported that activin A promotes neuron survival [18] and protects them from excitotoxic and ischemic damage [5]. A recent study by Agata et al. has shown that the transgenic mice with forebrain-specific expression of activin A (ACM4 mice) exhibit reduced anxiety in behavioral analysis [7]. The behavioral profiles of ACM4 mice resemble those of TgActβE mice. In female TgActβE mice, reduction of anxiety was also seen in elevated plus-maze tests and open field tests (Supplementary data). Therefore, activin E may mimic the activin A signal via the same receptor system and induce the behavior disorder in TgActβE mice. However, since ACM4 mice have enhanced locomotor activity that was not seen in TgActβE mice, the signal transduction of activin E may be slightly different from that of activin A. Further studies are needed to clarify the target cells and the signal transduction of activin E in the CNS.

*Drosophila schnurri* (Shn) and its vertebrate orthologs (shn-1, 2 and 3) are large zinc-finger-containing proteins [19,20]. Shn can interact with Smad proteins and regulate the signaling of TGF-β superfamily members [21]. Shn-2-deficient mice (Shn-$2^{-/-}$ mice) show hypersensitivity to stress, accompanied by anxiety-like behavior [10]. The sensitivity to anxiety of TgActβE mice was opposite to the phenotype of Shn-$2^{-/-}$ mice, therefore, Shn-2 may transduce the activin E signal that regulates the CNS.

The stress response is thought to be mediated by CRF, which is known to be a regulator of the hypothalamic–pituitary–adrenal (HPA) axis [22]. Alterations in the HPA axis have been related to various behavioral responses, including aggression and anxiety. c-Fos expression is considered to be one of the markers of brain activity, and its induction is often used to evaluate stress-related responses because it can be triggered by various stressors [11]. The stress-induced c-Fos expression in PVN cells in TgActβE mice was lower than that in wild-type mice. Furthermore, TgActβE mice showed stimulated aggressive behavior in the resident–intruder test and less anxiety-like behavior in the elevated plus-maze and open field tests. Taken together, these results suggest that activin E may act as a regulator of the HPA axis. However, it is known that activation of the HPA axis is correlated positively with anxiety and aggressive behavior [13,23]. These observations are not consistent with the data from TgActβE mice. Although the concentration of serum corticosterone in female TgActβE mice after acute stress

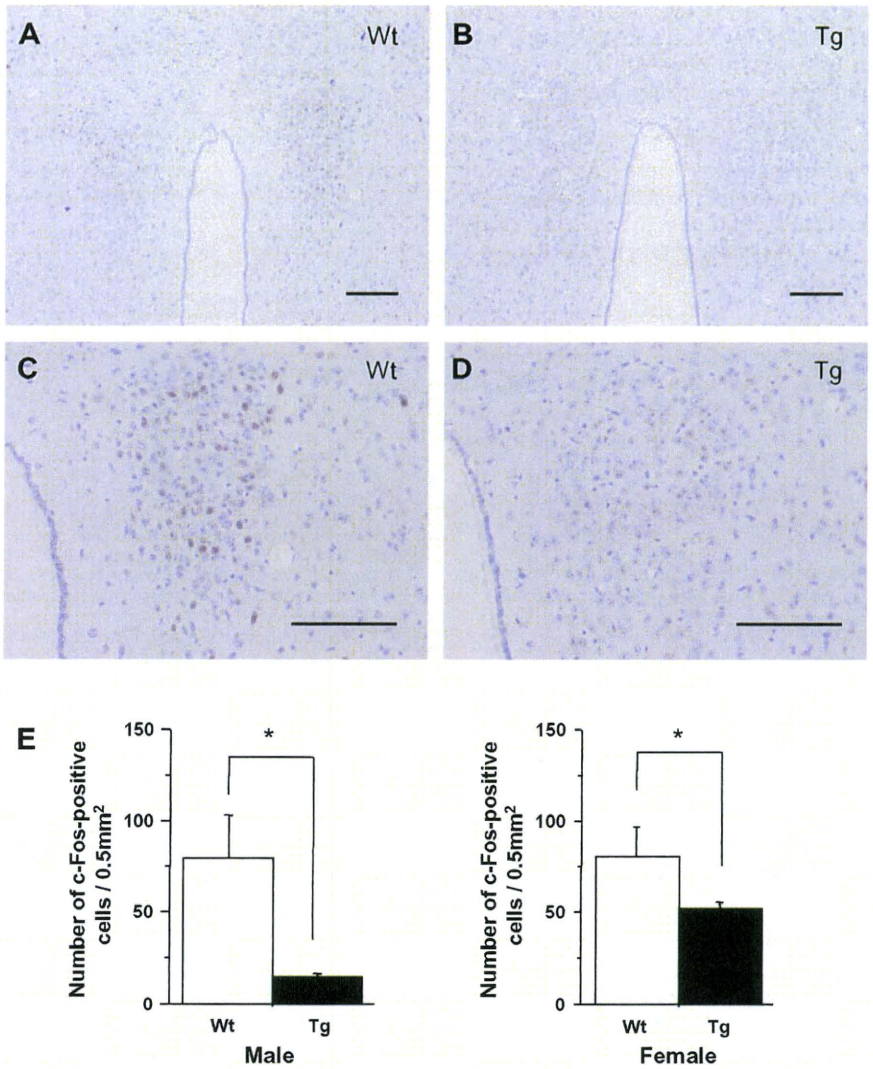


**Fig. 1.** c-Fos-positive cells in the PVN of TgActβE mice. Section from the mice subjected to restraint prior to sacrifice were immunoreacted for c-Fos. Photograph of male wild-type (A,C) and TgActβE (B,D) mice. Bar, 100 μm. Representative data are shown. The number of c-Fos-positive cells in the PVN was counted in one representative section (0.5 $mm^2$) from each mouse, and the mean for the male and female mice is shown (E). Values are means ± SE. $n = 4–5$. $^{*}P < 0.05$.

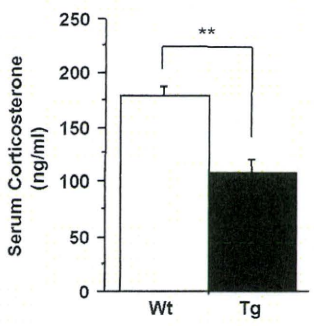


**Fig. 2.** Serum corticosterone level in TgActβE mice. Serum corticosterone concentration was determined after 30 min restraint stress in 14-week-old male TgActβE mice. Wt, wild-type mouse; Tg, TgActβE mice. Values are means ± SE. $n = 4$. $^{**}P < 0.01$.

was lower than that in wild-type mice, it was not statistically significant (Date not shown). Furthermore, the elevated plus-maze test revealed that the genotype differences in the behavior of TgActβE mice. Therefore, the abnormality in not only stress response but also other system might underlie the abnormal behaviors in the transgenic mice. Further analysis of the effect of activin E on the CNS is required to clarify the behavioral abnormalities in the transgenic mice.

The question arises as to what the behavioral abnormality induced by misexpression of activin E represents pathophysiologically. Recently, Nishino et al. have reported the involvement of the TGF-β superfamily, especially activin E, in neuronal disorders of rats infected with BDV [8]. Gene expression for the activin βE subunit in the rat brain is up-regulated by BDV infection. BDV is a neurotropic and single-stranded enveloped RNA virus that persistently infects the CNS of various animals and may cause behavioral abnormalities [24,25]. Experimental BDV infection in rodents also induces behavioral and neurological disturbances that resemble those in psychiatric patients, including those with autism, schizophrenia or mood disorders [26,27]. Interestingly, BDV genomic transcripts have been detected in neuropsychiatric patients [28]. Furthermore, viral RNA from BDV has been detected in patients

with schizophrenia or schizoaffective disorder, and in their biological relatives [29]. Taken together, these results suggest that activin βE subunit expressed in BDV-infected brain has a role in these mental disorders and is important for understanding the disease pathophysiologically.

In summary, we demonstrated that TgActβE mice have aggressive and low-anxiety behavior caused by reduced sensitivity to the stress. Ectopic expression of activin E in the choroid plexus might influence the CNS and behavior. TgActβE mice may be a useful animal model for further study of mental disorders in humans.

## Acknowledgments

We are grateful to Dr. Masayuki Funaba for helpful discussion. This work was supported by KAKENHI from the Japan Society for the Promotion of Science (to O.H.) and a Grant for Scientific Research from Kitasato University, School of Veterinary Medicine and Animal Sciences (to O.H.).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.05.054.

## References

[1] R. Derynck, K. Miyazono, TGF-β and the TGF-β family, in: R. Derynck, K. Miyazono (Eds.), The TGF-β Family, Cold Spring Harbor Laboratory Press, New York, 2008, pp. 29–43.
[2] E. Wiater, W. Vale, Activins and inhibins, in: R. Derynck, K. Miyazono (Eds.), The TGF-β Family, Cold Spring Harbor Laboratory Press, New York, 2008, pp. 79–120.
[3] C. Rodgarkia-Dara, S. Vejda, N. Erlach, A. Losert, W. Brusch, W. Berger, R. Schulte-Hermann, M. Grusch, The activin axis in liver biology and disease, Mutat. Res. 613 (2006) 123–137.
[4] A.L. Lau, T.R. Kumar, K. Nishimori, J. Bonadio, M.M. Matzuk, Activin βC and βE genes are not essential for mouse liver growth, differentiation, and regeneration, Mol. Cell. Biol. 20 (2000) 6127–6137.
[5] Y.P. Tretter, M. Hertel, B. Munz, G. ten Bruggencate, S. Werner, C. Alzheimer, Induction of activin A is essential for the neuroprotective action of bFGF in vivo, Nat. Med. 6 (2000) 812–815.
[6] A.L. Dow, D.S. Russell, R.S. Duman, Regulation of activin mRNA and Smad2 phosphorylation by antidepressant treatment in the rat brain: effects in behavioral models, J. Neurosci. 25 (2005) 4908–4916.
[7] H. Ageta, A. Murayama, R. Migishima, S. Kida, K. Tsuchida, M. Yokoyama, K. Inokuchi, Activin in the brain modulates anxiety-related behavior and adult neurogenesis, PLoS ONE 3 (2008) e1869.
[8] Y. Nishino, R. Ooishi, S. Kurokawa, K. Fujino, M. Murakami, H. Madarame, O. Hashimoto, K.M. Carbone, M. Funaba, Gene expression of the TGF-β family in rat brain infected with Borna disease virus, Microbes Infect., 2009, doi:10.1016/j.micinf.2009.04.014.
[9] O. Hashimoto, Y. Ushiro, K. Sekiyama, O. Yamaguchi, K. Yoshioka, K. Mutoh, Y. Hasegawa, Impaired growth of pancreatic exocrine cells in transgenic mice expressing human activin betaE subunit, Biochem. Biophys. Res. Commun. 341 (2006) 416–424.
[10] T. Takagi, W. Jin, K. Taya, G. Watanabe, K. Mori, S. Ishii, Schnurri-2 mutant mice are hypersensitive to stress and hyperactive, Brain Res. 1108 (2006) 88–97.
[11] E. Senba, K. Matsunaga, M. Tohyama, K. Noguchi, Stress-induced c-fos expression in the rat brain: activation mechanism of sympathetic pathway, Brain Res. Bull. 31 (1993) 329–344.
[12] E.R. De Kloet, E. Vreugdenhil, M. Oitzl, M. Joels, Brain corticosteroid receptor balance in health and disease, Endocrinol. Rev. 19 (1998) 269–301.
[13] L. Arborelius, M.J. Owens, P.M. Plotsky, C.B. Nemeroff, The role of corticotropin-releasing factor in depression and anxiety disorders, J. Endocrinol. 160 (1999) 1–12.
[14] J.L. Wrana, B. Ozdamar, LeC Roy, H. Benchabane, Signaling receptors of the TGF-β family, in: R. Derynck, K. Miyazono (Eds.), The TGF-β Family, Cold Spring Harbor Laboratory Press, New York, 2008, pp. 151–177.
[15] C-H. Heldin, TGF-β signaling from receptors to smads, in: R. Derynck, K. Miyazono (Eds.), The TGF-β Family, Cold Spring Harbor Laboratory Press, New York, 2008, pp. 259–285.
[16] V.A. Cameron, E. Nishimura, L.S. Mathews, K.A. Lewis, P.E. Sawchenko, W.W. Vale, Hybridization histochemical localization of activin receptor subtypes in rat brain, pituitary ovary and testis Endocrinol. 134 (1994) 799–808.
[17] M. Funaba, T. Murata, H. Fujimura, E. Murata, M. Abe, K. Torii, Immunolocalization of type I or type II activin receptors in the rat brain, J. Neuroendocrinol. 9 (1997) 105–111.
[18] D. Schubert, H. Kimura, M. LaCorbiere, J. Vaughan, D. Karr, W.H. Fischer, Activin is a nerve cell survival molecule, Nature 344 (1990) 868–870.
[19] K. Arora, H. Dai, S.G. Kazuko, J. Jamal, M.B. O'Connor, A. Letsou, R. Warrior, The *Drosophila schnurri* gene acts in the Dpp/TGFβ signaling pathway and encodes a transcription factor homologous to the human MBP family, Cell 81 (1995) 781–790.
[20] K. Staehling-Hampton, A.S. Laughon, F.M. Hoffmann, A *Drosophila* protein related to the human zinc finger transcription factor, PRDII/MBPI/HIV-EP1 is required for dpp signaling, Development 121 (1995) 3393–3403.
[21] L.C. Yao, I.L. Blitz, D.A. Peiffer, S. Phin, Y. Wang, S. Ogata, K.W. Cho, K. Arora, R. Warrior, Schnurri transcription factors from *Drosophila* and vertebrates can mediate Bmp signaling through a phylogenetically conserved mechanism, Development 133 (2006) 4025–4034.
[22] T.L. Bale, W.W. Vale, CRF and CRF receptors: role in stress responsivity and other behaviors, Annu. Rev. Pharmacol. Toxicol. 44 (2004) 525–557.
[23] M.R. Kruk, J. Halász, W. Meelis, J. Haller, Fast positive feedback between the adrenocortical stress response and a brain mechanism involved in aggressive behavior, Behav. Neurosci. 118 (2004) 1062–1070.
[24] R. Rott, H. Becht, Natural and experimental Borna disease in animals, Curr. Top. Microbiol. Immunol. 190 (1995) 17–30.
[25] J.C. De la Torre, Bornavirus and the brain, J. Infect. Dis. 186 (2002) S241–S247.
[26] S.A. Rubin, R.W.D. Waltrip, J.R. Bautista, K.M. Carbone, Borna disease virus in mice: host-specific differences in disease expression, J. Virol. 67 (1993) 548–552.
[27] W. Kamitani, E. Ono, S. Yoshino, T. Kobayashi, S. Taharaguchi, B.J. Lee, M. Yamashita, M. Okamoto, H. Taniyama, K. Tomonaga, K. Ikuta, Glial expression of Borna disease virus phosphoprotein induces behavioral and neurological abnormalities in transgenic mice, Proc. Natl. Acad. Sci. USA 100 (2003) 8969–8974.
[28] L. Bode, W. Zimmermann, R. Ferszt, F. Steinbach, H. Ludwig, Borna disease virus genome transcribed and expressed in psychiatric patients, Nat. Med. 1 (1995) 232–236.
[29] S.O. Nunes, E.N. Itano, M.K. Amarante, E.M. Reiche, H.C. Miranda, C.E. de Oliveira, T. Matsuo, H.O. Vargas, M.A. Watanabe, RNA from Borna disease virus in patients with schizophrenia, schizoaffective patients, and in their biological relatives, J. Clin. Lab. Anal. 22 (2008) 314–320.