nucleus that exhibits morphological sex differences. This subnucleus, which is known as the principal nucleus of the BNST (BNSTp) or the medial part of the posteromedial subdivision of the BNST, is larger and has more neurons in males than in females in adult rats and adult mice [3–5]. The BNSTp of rat is involved in the regulation of masculine sexual behavior, because lesions of the BNSTp induce deficits in male behavioral expression [6, 7]. Accordingly, morphological sex differences in the BNSTp are responsible for the sex-specific functions of this nucleus. The vasopressinergic system of the BNST also exhibits sexual dimorphism; males have more vasopressinergic neurons [8], and this sexual dimorphism is involved in sex difference in regulation of aggressive behavior [9]. In humans, a component of the BNST, which is called the darkly staining posteromedial component of the BNST or the central part of the BNST (hereafter the BNSTc), also exhibits morphological sex differences: it is larger in men than in women [10, 11]. Furthermore, the volume and neuron number of the BNSTc in male-to-female transsexuals, but not in homosexual men, are comparable to those in heterosexual women [11, 12], suggesting that the BNSTc in humans is related to gender identity.

Neonatal castration in male rodents results in a decrease in the volume and neuron number of the BNSTp, while testosterone propionate injection in newborn females results in an increase in both [13, 14], indicating that the morphological sex differences in the BNSTp can be eliminated and reversed by manipulation with androgen during the neonatal period. In addition, BNSTp volume on the left side, but not on the right side, in testicular feminization mutation male rats, in which androgen receptors are less functional, is as small as that in normal female rats [15], suggesting that androgen binding to androgen receptors is implicated in masculinization of the BNSTp. Moreover, in rodents, it is generally accepted that perinatal testicular androgen exerts significant effects on organization of male-typical structures on the brain after it is converted to estrogen in the brain by the cytochrome P450 enzyme aromatase [1, 2], although sexual differentiation of the brain is proposed to be partially sex chromosome-dependent [16, 17]. Hisasue et al. [18] recently reported that treatment with estradiol benzoate (EB) or a selective agonist of estrogen receptor (ER)-α or -β into newborn female mice increases the number of neuron in the BNSTp and enlarges BNSTp volume in adulthood, although activation of either ERα or ERβ is not sufficient for masculinization of the BNSTp. Thus, estrogen actions via both ERα and ERβ may be required for masculinization of the BNSTp in mice. However, Hisasue et al. [18] also showed that neonatal EB treatment was ineffective in increasing BNSTp volume in female mice. Therefore, it remains unclear whether the estrogen that is synthesized from testicular androgen by aromatase has all the functions necessary for masculinization of the BNSTp in male mice.

To assess the role of aromatase in masculinization of the BNSTp, we examined the morphology of the BNSTp in both sexes of aromatase knockout (ArKO) mice. Similarly, we examined the morphology of the BNSTp in ERα knockout (αERKO) and ERβ knockout (βERKO) mice to assess the involvement of these ERs in BNSTp masculinization. We next examined the BNSTp of ArKO male mice treated with EB in the early postnatal period to determine whether this treatment could rescue the effects of an aromatase gene deletion on the BNSTp in males.

Animals and Methods

Animals

ArKO [19, 20], αERKO [21], and βERKO mice [22] and their respective wild-type littermates were obtained from litters resulting from matings between heterozygous mice derived from the respective breeding colonies, which were maintained at the University of Tsukuba. Mice from the breeding colonies were all completely backcrossed to C57BL/6J mice. All animals were housed in a room maintained at 22°C with a 12-hour light/12-hour dark lighting cycle (light on at 08:00 h) with free access to a standard diet and tap water. This study was performed in accordance with the National Institutes of Health guidelines and approved by the University of Tsukuba Animal Care and Use Committee.

Experimental Design

Experiment 1

Coronal brain sections obtained from male and female adult ArKO, αERKO, or βERKO mice and their respective wild-type littermates were stained with cresyl fast violet (ArKO male: n = 4 (17–20 weeks old); wild-type male littermate of ArKO mice: n = 4 (15–30 weeks old); ArKO female: n = 4 (17–21 weeks old); wild-type female littermate of ArKO mice: n = 4 (15–30 weeks old); αERKO male: n = 5 (15–27 weeks old); wild-type male littermate of αERKO mice: n = 6 (14–27 weeks old); αERKO female: n = 5 (13–32 weeks old); wild-type female littermate of αERKO mice: n = 4 (13–32 weeks old); βERKO male: n = 5 (13–25 weeks old); wild-type male littermate of βERKO mice: n = 4 (13–25 weeks old); βERKO female: n = 5 (13–20 weeks old); wild-type female littermate of βERKO mice: n = 5 (14–20 weeks old)). Using a stereological method (see below section 'Stereological Analysis of the BNSTp' for details), BNSTp volume and neuronal and glial cell numbers in the BNSTp were measured to determine the effects of aromatase, ERα, or ERβ gene deletions on masculinization of the BNSTp. Neuronal cell density in the BNSTp was calculated by dividing BNSTp volume with neuronal cell number; glial cell density was also calculated using this method. These cell densities were also assessed to determine the effects of aromatase, ERα, or ERβ gene deletions. Additionally, we measured testosterone concentrations in plasma, which were taken from animals used for histological analyses, by enzyme-linked immunosorbent assay to examine whether there was a causal relationship between the morphology of the BNSTp and testosterone levels in adulthood.

Experiment 2

We next examined whether the effects of the aromatase gene deletion on masculinization of the BNSTp can be rescued in ArKO male mice by postnatal treatment with estrogen. ArKO male mice (n = 6) and wild-type male mice (n = 3) were subcutaneously injected with 1.5 μg of EB dissolved in 5 μl of sesame oil on postnatal days 1, 2, and 3 (postnatal day 1 = day of birth). As a negative control, sesame oil (5 μl/day) was injected into ArKO male mice (n = 4) and wild-type male mice (n = 5) on the same days as the EB injection. Postnatal treatment with 7.5- or 15-μg of 17β-estradiol restores reproductive ability and aggressive behavior in ArKO male mice [20, 23], and the binding affinity of EB to murine ERα is approximately 6–10 times higher than that of 17β-estradiol [24]. We referred to these reports to determine the dose of EB used in this experiment.

After sexual maturation of these animals (17–21 weeks), coronal brain sections that included the BNSTp were stained with cresyl fast violet, and the volume of the BNSTp and the neuronal and glial cell numbers in the BNSTp were measured using a stereological method (see below section 'Stereological Analysis of the BNSTp' for details). The densities of neuronal and glial cells in the BNSTp were measured described above. The densities were also assessed to determine the effects of postnatal EB treatment on the BNSTp of ArKO males.

General Procedures

Specimen Preparation

Animals deeply anesthetized with sodium pentobarbital (60 mg/kg body weight) were perfused intracardially with 0.1 M phosphate-buffered saline (PBS; pH 7.5), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.5). Brains were postfixed at 4°C overnight with 4% paraformaldehyde in 0.1 M PB and then immersed in 30% sucrose in 0.1 M PB for 2 days at 4°C. Coronal brain sections (30 μm thickness) that included the BNSTp were prepared with a cryostat and collected from rostral to caudal levels of the BNSTp at 60-μm intervals.

Brain sections were mounted on gelatin-coated slides, placed in a graded series of ethanol solutions for delipidation, and then stained with 0.1% cresyl fast violet solution for 5–10 min. The sections were placed in distilled water, sequentially incubated in a graded series of ethanol solutions appropriate for bleaching of the dye, dehydrated with absolute ethanol, cleared with xylene, and covered with embedding medium and a coverslip. The stained sections were used for stereological analysis.

Stereological Analysis of the BNSTp

We observed the BNSTp of cresyl-fast-violet-stained sections (thickness 30 μm/section, collecting interval 60 μm) on a PC monitor with the aid of a CCD camera connected to a light microscope (DM5000B; Leica Microsystems, Wetzlar, Germany) and measured BNSTp volume and the numbers of neuronal and glial cells in the BNSTp with the aid of a computer and Stereo Investigator software (MBF Bioscience, Inc., Williston, Vt., USA). To avoid bias, identification numbers of animals were coded, and the person who performed the stereological analysis was blinded to the source of the materials. In this study, we analyzed the BNSTp on the left side because the BNSTp on the left side is known to be larger than that on the right and because the sex difference in BNSTp volume is prominent on the left side [15]. The rostro-caudal levels of brain sections used in analysis were from the septo-preoptic region to the anterior hypothalamic region, and the region of interest corresponded to the posteromedial part of the medial division of the BNST (BSTMPM) described in the mouse brain atlas by Paxinos and Franklin [25]. The average numbers of sections collected per animal were as follows: 5.5 for males and 6 for females among ArKO mice and their wild-type littermates, 6.7 for males and 6 for females among αERKO and their wild-type mice, and 4.9 for males and 4 for females among βERKO and their wild-type mice in experiment 1, and 4.9 per animal in experiment 2.

To measure BNSTp volume and neuronal and glial cell numbers in the BNSTp on the left side, we used the optical fractionator method in accordance with the system workflow of the Stereo Investigator (MBF Bioscience, Inc.). Briefly, we traced outlines of the BNSTp on the images of the sections to calculate the estimated volume of the BNSTp and select the region for cell counts. After setting the counting frame size to 20 × 20 μm, the grid size for arrangement of the counting frame at 200 × 200 μm, the highest optical dissector setting at 10–12 μm, and the highest top guard zone setting at 2–4 μm, neuronal and glial cells within the counting frames were then counted manually to estimate the total numbers of neuronal and glial cells in the BNSTp. As the criteria for counting neurons, cells containing blue- or purple-colored rough endoplasmic reticulum (Nissl bodies) in the cytoplasm and having an oval or spherical nucleus with a blue- or purple-colored nucleolus were defined as neuronal cells. For measuring the number of glial cells, we counted cells that had blue- or purple-colored nucleus without colored cytoplasm, excepting cells contacting capillary wall.

Enzyme-Linked Immunosorbent Assay for Testosterone

Blood was taken from the right atrium before animals were perfused with PBS for histological processing (see *Specimen Preparation*). The lipophilic fraction of the plasma was extracted with diethyl ether according to a procedure described previously [26]. Testosterone in extracted samples was assayed using a Testosterone EIA Kit (Cayman Chemical, Ann Arbor, Mich., USA) in accordance with the manufacturer's protocol. The intra- and inter-assay coefficients of variation were 5.9% (testosterone concentration, 143.4 pg/ml) and 10.7% (testosterone concentration, 68.5 pg/ml), respectively.

Statistical Analyses

Two-way analysis of variance (ANOVA) was used to determine the main effects of genotype and sex, and the effects of interaction between genotype and sex on BNSTp volume, neuronal and glial cell numbers, neuronal and glial cell densities, and plasma testosterone concentration in experiment 1. Two-way ANOVA was also preformed to determine main effects of hormonal treatment and sex and effects of interaction between hormonal treatment and sex on BNSTp volume, neuronal and glial cell numbers, and neuronal and glial cell densities. When significant effects of an interaction between main factors were detected by two-way ANOVA, Fisher's PLSD test was performed for post-hoc analysis. In the post-hoc analysis, a probability value of $p < 0.05$ was considered significant.

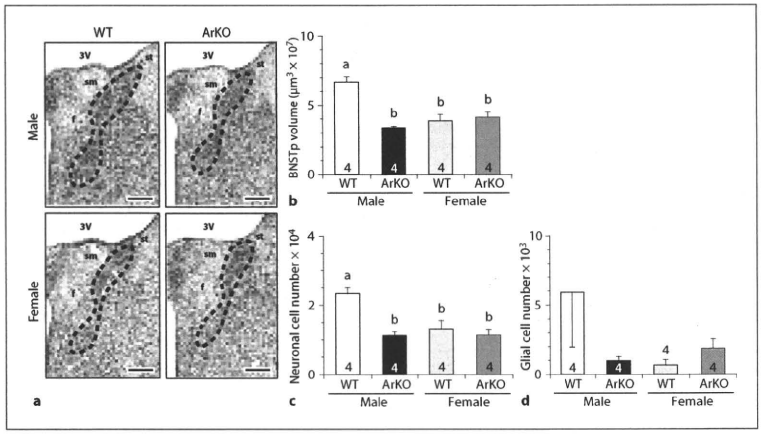


Fig. 1. Effects of the aromatase gene deletion on the BNSTp of mice. Photomicrographs of the BNSTp in cresyl-fast-violet-stained coronal brain sections of ArKO and wild-type (WT) mice (**a**). Dashed line in each panel indicates the outline of the BNSTp. Scale bars indicate 300 μm. f, fornix; sm, stria medullaris of thalamus; st, stria terminalis; 3V, third ventricle. BNSTp volume (**b**) numbers of neuronal cells (**c**) and glial cells (**d**) in the BNSTp of ArKO mice and their wild-type littermates. The number in each column indicates the number of animals used. Values (mean ± SEM) with different letters differ significantly ($p < 0.05$) from each other.

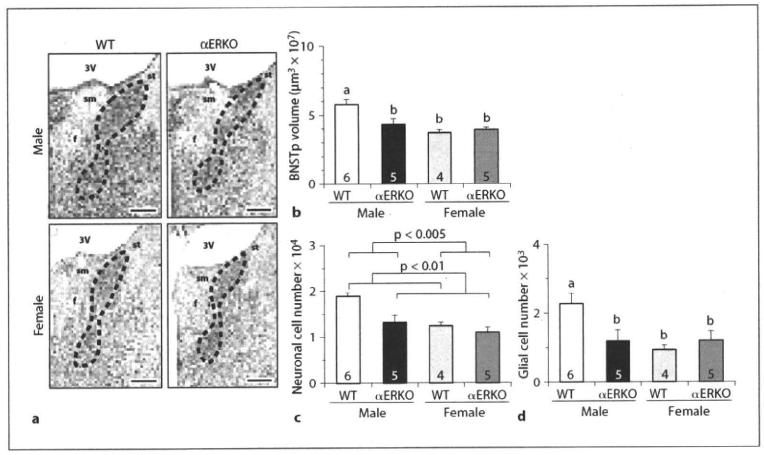


Fig. 2. Effects of the ERα gene deletion on the BNSTp of mice. Photomicrographs of the BNSTp in cresyl-fast-violet-stained coronal brain sections of αERKO and WT mice (**a**). BNSTp volume (**b**) numbers of neuronal cells (**c**) and glial cells (**d**) in the BNSTp of αERKO mice and their wild-type littermates. The number in each column indicates the number of animals used. Values (mean ± SEM) with different letters differ significantly ($p < 0.05$) from each other. Scale bars indicate 300 μm. See figure 1 for abbreviations.

Results

Experiment 1

Effects of Deletion of the Aromatase Gene on BNSTp

The BNSTp, which was occupied with cresyl-fast-violet-stained neuronal cell bodies in specimen preparations, was larger in wild-type males than in wild-type females (fig. 1a). In contrast, the size of the BNSTp in ArKO male mice was comparable to that in ArKO female mice and was smaller than that in male wild-type littermates. Stereological analysis revealed that the aromatase gene deletion decreased the BNSTp volume in male but not in female mice (fig. 1b). Two-way ANOVA indicated that there were significant effects of genotype ($F_{1, 12} = 17.8$, $p < 0.005$) and of sex ($F_{1, 12} = 7.92$, $p < 0.05$) on BNSTp volume among ArKO mice and their wild-type littermates. The effect of an interaction between genotype and sex was significant ($F_{1, 12} = 24.0$, $p < 0.0005$). Based on post-hoc analysis, BNSTp volume in wild-type males was significantly larger than that in wild-type females. In contrast, there was no significant difference in BNSTp volume between the sexes in ArKO mice, and this apparent similarity was due to a significant reduction in BNSTp volume of ArKO males.

The aromatase gene deletion decreased not only BNSTp volume but also neuronal cell number in the BNSTp of males (fig. 1c). There were significant effects of genotype ($F_{1, 12} = 15.1$, $p < 0.005$), of sex ($F_{1, 12} = 7.87$, $p < 0.05$), and of an interaction between genotype and sex ($F_{1, 12} = 8.34$, $p < 0.05$) on the neuron number among ArKO mice and their wild-type littermates. Based on the post-hoc analysis, the number of neurons in the BNSTp of ArKO males was significantly smaller than that in wild-type male littermates, while there was no significant difference in the neuron number between ArKO females and their wild-type female littermates. There was no significant difference in the neuron number between male and female ArKO mice, and this apparent similarity was due to a significant reduction of the number of neurons of ArKO males.

The number of glial cells in the BNSTp did not significantly differ by sex or genotype among ArKO mice and their wild-type littermates (fig. 1d). There was no effect of an interaction between sex and genotype on the number of glial cells in the BNSTp, although the glial cell number had tendency to be larger in the wild-type male littermates. There was no significant effect of genotype, of sex, or of an interaction between genotype and sex on neuronal and glial cell densities in the BNSTp among ArKO mice and their wild-type littermates (data not shown).

Effects of Deletion of the ERα Gene on BNSTp

Based on images from the light microscope, the BNSTp was thinner along the mediolateral axis in αERKO males than in wild-type males, although no apparent difference in the morphology of BNSTp was found between αERKO females and wild-type females (fig. 2a). Based on measurement of BNSTp volume, we found significant effects of genotype ($F_{1, 16} = 5.49$, $p < 0.05$), of sex ($F_{1, 16} = 20.2$, $p < 0.0005$), and of an interaction between genotype and sex ($F_{1, 16} = 9.55$, $p < 0.01$) among αERKO mice and their wild-type littermates. Based on post-hoc analysis, BNSTp volume was significantly larger in wild-type males than in wild-type females (fig. 2b). Moreover, BNSTp volume in αERKO male mice was significantly smaller than that in wild-type males and did not significantly differ from that in female mice.

Among αERKO mice and their wild-type littermates, neuron number in the BNSTp in αERKO males was lower than that of the wild-type males and similar to that of females (fig. 2c). Two-way ANOVA indicated that there were significant effects of genotype ($F_{1, 16} = 10.3$, $p < 0.01$) and of sex ($F_{1, 16} = 15.1$, $p < 0.005$) on the number of neurons in the BNSTp, though the effect of the interaction between genotype and sex was not significant. There was no significant effect of genotype, of sex, or of the interaction between genotype and sex on the density of neurons in the BNSTp among αERKO mice and their wild-type littermates (data not shown).

The ERα gene deletion decreased not only BNSTp volume and neuron number but also glial cell number in the BNSTp of mutant males (fig. 2d). There were significant effects of sex ($F_{1, 16} = 5.23$, $p < 0.05$) and of an interaction between genotype and sex ($F_{1, 16} = 5.47$, $p < 0.05$) on the glial cell number among αERKO mice and their wild-type littermates, although the overall effect of genotype was not significant. Based on the post-hoc analysis,

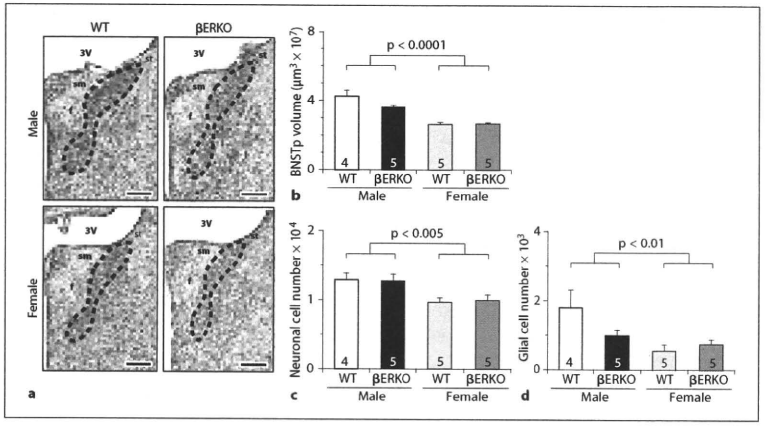


Fig. 3. Effects of the ERβ gene deletion on the BNSTp of mice. Photomicrographs of the BNSTp in cresyl-fast-violet-stained coronal brain sections of βERKO and WT mice (**a**). BNSTp volume (**b**) numbers of neuronal cells (**c**) and glial cells (**d**) in the BNSTp of βERKO mice and wild-type littermates. The number in each column indicates the number of animals used. Values are the mean ± SEM. Scale bars indicate 300 μm. See figure 1 for abbreviations.

αERKO males had a significantly smaller number of glial cells in the BNSTp than did the wild-type males, and the number in αERKO males was similar to and not significantly different from that in wild-type and αERKO females. However, the density of glial cells in the BNSTp was not affected by sex, genotype, or an interaction between sex and genotype (data not shown).

Effects of Deletion of the ERβ Gene on BNSTp

The BNSTp was larger and had greater neuronal and glial cell numbers in the βERKO males and their wild-type male littermates than in the βERKO females and their wild-type female littermates (fig. 3). We observed a significant effect of sex on BNSTp volume ($F_{1, 15} = 55.4$, $p < 0.0001$), neuronal cell number in the BNSTp ($F_{1, 15} = 12.8$, $p < 0.005$), and glial cell number in the BNSTp ($F_{1, 15} = 8.78$, $p < 0.01$) among βERKO and wild-type mice. However, the values of these endpoints were not significantly changed by genotype or by an interaction between sex and genotype. There was no significant effect of genotype, of sex, or of an interaction between genotype and sex on neuronal and glial cell densities in the BNSTp among βERKO mice and their wild-type littermates (data not shown).

Plasma Testosterone Levels in ArKO, αERKO, and βERKO Mice

Plasma testosterone concentrations were higher in males than in females, and the levels of plasma testosterone were higher in ArKO animals (fig. 4a). Two-way ANOVA revealed that there were significant effects of sex ($F_{1, 12} = 13.8$, $p < 0.005$) and genotype ($F_{1, 12} = 38.2$, $p < 0.0001$) on plasma testosterone concentrations among ArKO mice and their wild-type littermates. However, plasma testosterone level was not affected by an interaction between genotype and sex.

We found a significant effect of genotype on plasma testosterone concentrations among αERKO mice and wild-type littermates ($F_{1, 16} = 26.7$, $p < 0.0001$). Deletion of the ERα gene increased testosterone levels in both sexes, and the testosterone levels in αERKO females was comparable to those in αERKO males (fig. 4b).

βERKO males and their wild-type male littermates had significantly higher concentrations of plasma testosterone than did their βERKO and wild-type female littermates ($F_{1, 15} = 16.6$, $p < 0.005$) (fig. 4c). On the other hand, deletion of the ERβ gene did not significantly change plasma testosterone levels in either sex. Two-way ANOVA indicated that neither genotype nor an interaction between sex and genotype significantly affected plasma testosterone levels.

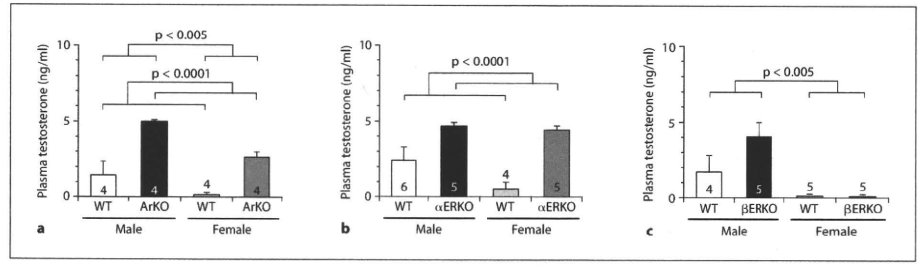


Fig. 4. Effects of the aromatase, ERα, or ERβ gene deletions on plasma testosterone concentration in mice. Plasma testosterone concentrations of ArKO mice (**a**), αERKO mice (**b**), and βERKO mice (**c**). Values are the mean ± SEM. The number in each column indicates the number of animals used.

Experiment 2

We examined the effects of postnatal EB treatment on the BNSTp in ArKO and wild-type adult male mice and found that there was no significant effect of postnatal EB treatment or of an interaction between hormonal treatment and genotype. However, two-way ANOVA revealed significant differences in BNSTp volume between ArKO and wild-type males ($F_{1, 14} = 27.9$, $p < 0.0005$). BNSTp volume was smaller in ArKO males than in wild-type males (fig. 5a).

Neuronal cell number in the BNSTp was significantly changed by an interaction between genotype and hormonal treatment ($F_{1, 14} = 5.56$, $p < 0.05$), although no significant effect of either main factor alone was found. Based on post-hoc analysis, oil-treated wild-type males had a significantly greater number of neurons in the BNSTp than did oil-treated ArKO males (fig. 5b). Postnatal EB treatment significantly increased neuron number in the BNSTp of ArKO males, but not in the wild-type males. There was a significant effect of genotype on neuronal cell density in the BNSTp among ArKO and wild-type males with or without postnatal EB treatment ($F_{1, 14} = 6.22$, $p < 0.05$), and the density of ArKO males was higher than that of the wild-type males (fig. 5c).

The number of glial cells in the BNSTp did not significantly differ among ArKO and wild-type male mice with or without EB treatment (fig. 5d). The density of glial cells in the BNSTp was not significantly different among ArKO and wild-type males with or without EB treatment (fig. 5e).

Discussion

The BNSTp is a sexually dimorphic nucleus that is larger and contains more neurons in male rodents than in female rodents [3–5]. We confirmed this sexual dimorphism in wild-type littermates of ArKO, αERKO, and βERKO mice. Our study further showed that the sexual dimorphism in the volume and neuron number of the BNSTp was completely eliminated in ArKO mice. This elimination of sexual dimorphism was entirely due to a significant reduction in the volume and neuron number of the BNSTp in ArKO males, and not to changes in ArKO females. These findings indicated that aromatase played an essential role in the formation of the male-typical structure of the BNSTp. The BNST contains more arginine-vasopressin neurons in males than in females [8]. The number of arginine-vasopressin neurons in the BNST of ArKO male mice is smaller than that of the wild-type males [27]. Reduction of neuron number in the BNSTp of ArKO males may be caused, in part, by a decrease in arginine-vasopressin neurons. Aromatase is an enzyme that converts androgen to estrogen. Therefore, it is likely that the aromatase deficiency results in a loss of estrogen function. As we expected, elimination of the sex difference in the volume and neuron number of the BNSTp occurred not only in ArKO mice but also in αERKO mice, which was due to a reduction of the volume and neuron number in knockout male mice rather than to changes in female mice. αERKO mice used in this study are known to have a small number of ERα-containing cells in the medial preoptic area, but not in other regions of the brain, including the BNST [28], indicating

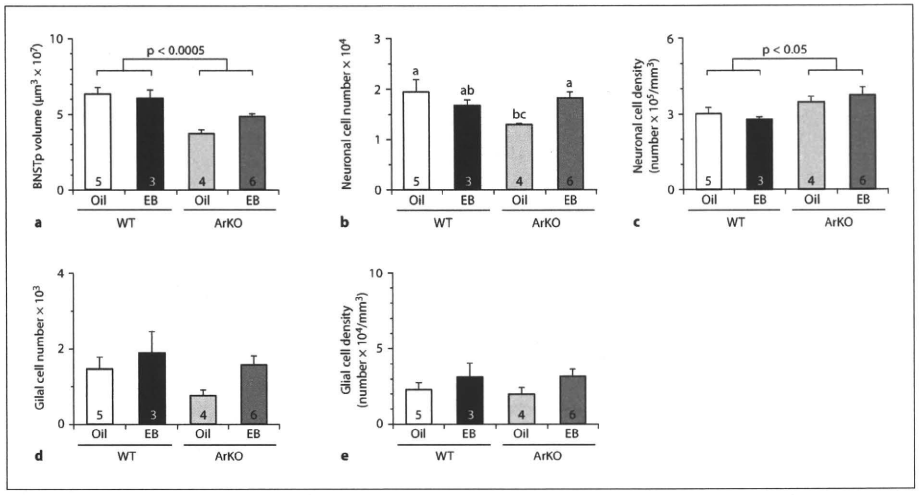


Fig. 5. Effects of postnatal EB treatment on BNSTp volume (**a**), the number and density of neuronal cells (**b**, **c**) and the number and density of glial cells (**d**, **e**) in the BNSTp of ArKO male mice. The number of animals used in each group is indicated in each column. Values (mean ± SEM) with different letters differ significantly ($p < 0.05$) from each other.

that the αERKO mice do not completely lack ERα expression in the brain. Morphological alteration in the BNSTp of αERKO males may be mainly due to the lack of ERα within the BNSTp. In contrast, the volume and neuron number of the BNSTp did not change with deletion of the ERβ gene, and βERKO mice exhibited morphological sex differences in the BNSTp as did the wild-type littermates. Taken together, the findings of our current study suggested that the actions of estrogen synthesized from androgen by aromatase were essential for organization of male-typical structures in the BNSTp of mice and that the actions of this estrogen are mostly mediated through its binding to ERα rather than to ERβ.

In the current study, we also found a sex difference in glial cell number in the BNSTp of mice. Morphometrical analysis demonstrated that the number of glial cells was significantly greater in the male BNSTp than in the female BNSTp in the wild-type littermates of αERKO and βERKO mice. Furthermore, this sex difference was eliminated by deletion of the ERα gene, but not the ERβ gene. Based on these findings, we believe that sex difference in glial cell number contributed to the sexual dimorphism of the BNSTp, at least in part, and that estrogen actions via ERα were involved in producing sex difference in glial cell number. However, among ArKO mice and the wild-type littermates, we did not observe a significant effect of sex or genotype on glial cell number in the BNSTp, although the number of glial cells tended to be higher in wild-type males than in wild-type females and ArKO males. In addition, no significant difference in the number of GFAP-immunoreactive cells was reported in the BNSTp of mice [29]. Further study is needed to determine whether and how sex differences in glial cell number arise in the BNSTp.

In rodent models of sexual dimorphism, masculinization and defeminization of the brain involve the actions of estrogen, which is synthesized locally in the brain from testicular androgen by aromatase during the perinatal period [1, 2]. In this context and as shown in this study, it is likely that the morphological sex differences in the BNSTp of mice are eliminated by a deletion of the aromatase gene. In this study, we also examined whether postnatal EB treatment rescued the effects of the aromatase gene deletion on the BNSTp of ArKO male mice. We found that postnatal EB treatment significantly increased the number of neurons in the BNSTp of ArKO males and that the neuron number in EB-treated ArKO males was similar to that of the wild-type males. This finding suggested that estrogen functions during the early postnatal period to maintain neuron number of the BNSTp in male mice. Also, in female mice, postnatal EB treatment has been shown to increase the number of BNSTp neurons [18]. Neurons expressing aromatase are located in the BNSTp of rats during the late prenatal period to the early postnatal period [30]. Cellular colocalization of ERα and aromatase has been reported in the BNST of mice in the postnatal period [31]. These results suggest that maintenance of the number of BNSTp neurons in male mice may involve the effects of estrogen, which is locally produced by aromatase and binds to ERα in BNSTp in the postnatal period.

EB treatment in the early postnatal period significantly increased the number of BNSTp neurons in ArKO males to a level comparable to that in wild-type males. However, EB-treated ArKO males had a significantly smaller BNSTp volume than did wild-type males. This last observation indicated that the hormonal treatment used in this study was not sufficient to reverse the effects of an aromatase gene deletion. It is unclear why the EB treatment did not result in a complete recovery of BNSTp volume. Also, a study using female mice did not clearly determine whether postnatal EB treatment can masculinize the volume of female BNSTp because it showed that BNSTp volume in adulthood was increased by subcutaneous injection of EB into newborn females, but it also showed no effect of this same treatment [18]. However, this previous report clearly demonstrated that EB treatment could increase the number of BNSTp neurons in female mice [18]. There are several possible hypotheses that could explain why postnatal EB treatment did not restore BNSTp volume in male ArKO mice. First, differences in the effects of EB between BNSTp volume and neuron number would be explained if neuronal cell size were smaller in EB-treated ArKO males even though neuron number had increased. Sex difference in BNSTp volume is due to sex differences in cell number and cell size because cells are larger in the BNSTp of male mice than that of female mice [3]. Alternatively or additionally, the timing of EB treatment may be appropriate for recovery of neuron number but not for recovery of BNSTp volume. In ferrets, pharmacological blockade of estrogenic actions during the prenatal period suppresses masculine sexual behavior [32] and disrupts the male-typical formation of a sexually dimorphic nucleus of the preoptic/anterior hypothalamic area [33]. Such evidence suggests that prenatal, rather than postnatal, EB treatment may be able to recover BNSTp volume in ArKO males; however, our study did not test this possibility. It is also possible that estrogen exerting its actions during the perinatal period was not sufficient for complete masculinization of the BNSTp, for example, a sensitive period important for organizational actions of sex steroids may begin during the perinatal period and end in late adolescence [34].

Reportedly, ArKO mice have higher circulating levels of testosterone than do wild-type mice of both sexes [20, 35]. αERKO mice also have higher blood testosterone concentrations than wild-type mice [36–39]. We confirmed that the circulating levels of testosterone are elevated in both ArKO and αERKO mice. When considering the effects of deletion of the aromatase or ERα gene on the BNSTp and on circulating testosterone levels, it is reasonable to think that the morphometrics of the BNSTp, such as volume and neuronal and glial cell numbers, are not altered by circulating testosterone levels in adulthood. However, it was reported that androgen affects the medial amygdala of adult rats to increase the volume, neuronal soma size, and number of glial cells [40].

Apoptosis during the postnatal period may contribute to sex differences in neuron numbers in the BNSTp because the number of apoptotic cells in the BNSTp between postnatal days 3 and 6 is greater in female than in male mice [41]. The sex difference in the number of apoptotic cells is inversely related to that in the number of living neurons in adulthood. Reduced estrogen levels caused by an aromatase gene deletion induce apoptotic cell death in the BNSTp of postnatal male mice [42]. We further showed that postnatal EB treatment increased the number of neurons in the BNSTp of ArKO male mice. Taking this evidence and our findings together, estrogen synthesized by aromatase during the postnatal period may protect BNSTp neurons from apoptosis. The mechanisms by which estrogen protects BNSTp neurons from apoptosis are largely unknown. However, the modulatory effects of estrogen on the expression of the Bcl-2 family, which is involved in the mitochondrial signaling pathway that regulates apoptosis, may play a role in creating the sex difference in number of neurons in BNSTp. Bax is a pro-apoptotic member of the Bcl-2 family, and in Bax-deficient mice, there is no significant sex difference in number of neurons in BNSTp in adult mice [3] or in number of apoptotic cells in BNSTp in postnatal mice [41]. Post-

natal EB treatment in female rats can reduce the degree of sex difference in the size of SDN-POA by decreasing the number of apoptotic cells [43], and this decrease is due to the up-regulation of anti-apoptotic Bcl-2 expression and the down-regulation of pro-apoptotic Bax expression in the SDN-POA of these females [44]. Therefore, estrogen may modulate the expression of the Bcl-2 family members in the BNSTp of postnatal mice. Further studies are needed to determine the exact mechanisms responsible for the effects of aromatized androgen on masculinization of the BNSTp in mice.

In summary, we showed that ArKO and αERKO male mice had smaller BNSTp volume and neuron number in the BNSTp than did their wild-type male littermates. The number of glial cells in the BNSTp of αERKO males was also lower than that of wild-type males. However, the BNSTp did not change with deletion of the ERβ gene. We further showed that postnatal EB treatment could rescue some effects of deletion of the aromatase gene on the number of BNSTp neurons. These findings suggest that masculinization of the BNSTp in mice involves the postnatal actions of estrogen that is synthesized from androgen by aromatase and that this estrogen mostly acts via ERα.

Acknowledgments

We thank K. Watai for his technical assistance. This work was supported in part by the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (No. 19681006 to S.T., No. 17330151, and No. 17052001 to S.O.), by the Project Research Grant from the Research Management Bureau of Saitama University to S.T. (A10-20), and by the University of Tsukuba Research Project, Special Research Grant to S.O.

References

1 MacLusky NJ, Naftolin F: Sexual differentiation of the central nervous system. Science 1981;211:1294–1302.
2 Morris JA, Jordan CL, Breedlove SM: Sexual differentiation of the vertebrate nervous system. Nat Neurosci 2004;7:1034–1039.
3 Forger NG, Rosen GJ, Waters EM, Jacob D, Simerly RB, de Vries GJ: Deletion of bax eliminates sex differences in the mouse forebrain. Proc Natl Acad Sci USA 2004;101:13666–13671.
4 del Abril A, Segovia S, Guillamon A: The bed nucleus of the stria terminalis in the rat: regional sex differences controlled by gonadal steroids early after birth. Brain Res 1987;429:295–300.
5 Hines M, Allen LS, Gorski RA: Sex differences in subregions of the medial nucleus of the amygdala and the bed nucleus of the stria terminalis of the rat. Brain Res 1992;579:321–326.
6 Liu YC, Salamone JD, Sachs BD: Lesions in medial preoptic area and bed nucleus of stria terminalis: Differential effects on copulatory behavior and noncontact erection in male rats. J Neurosci 1997;17:5245–5253.
7 Claro F, Segovia S, Guilamon A, Del Abril A: Lesions in the medial posterior region of the bst impair sexual behavior in sexually experienced and inexperienced male rats. Brain Res Bull 1995;36:1–10.
8 van Leeuwen FW, Caffe AR, De Vries GJ: Vasopressin cells in the bed nucleus of the stria terminalis of the rat: sex differences and the influence of androgens. Brain Res 1985;325:391–394.
9 De Vries GJ, Miller MA: Anatomy and function of extrahypothalamic vasopressin systems in the brain. Prog Brain Res 1998;119:3–20.
10 Allen LS, Gorski RA: Sex difference in the bed nucleus of the stria terminalis of the human brain. J Comp Neurol 1990;302:697–706.
11 Zhou JN, Hofman MA, Gooren LJ, Swaab DF: A sex difference in the human brain and its relation to transsexuality. Nature 1995;378:68–70.
12 Kruijver FP, Zhou JN, Pool CW, Hofman MA, Gooren LJ, Swaab DF: Male-to-female transsexuals have female neuron numbers in a limbic nucleus. J Clin Endocrinol Metab 2000;85:2034–2041.
13 Guillamon A, Segovia S, del Abril A: Early effects of gonadal steroids on the neuron number in the medial posterior region and the lateral division of the bed nucleus of the stria terminalis in the rat. Dev Brain Res 1988;44:281–290.
14 Chung WC, Swaab DF, De Vries GJ: Apoptosis during sexual differentiation of the bed nucleus of the stria terminalis in the rat brain. J Neurobiol 2000;43:234–243.
15 Durazzo A, Morris JA, Breedlove SM, Jordan CL: Effects of the testicular feminization mutation of the androgen receptor gene on bstmpm volume and morphology in rats. Neurosci Lett 2007;419:168–171.
16 Carruth LL, Reisert I, Arnold AP: Sex chromosome genes directly affect brain sexual differentiation. Nat Neurosci 2002;5:933–934.
17 De Vries GJ, Rissman EF, Simerly RB, Yang LY, Scordalakes EM, Auger CJ, Swain A, Lovell-Badge R, Burgoyne PS, Arnold AP: A model system for study of sex chromosome effects on sexually dimorphic neural and behavioral traits. J Neurosci 2002;22:9005–9014.
18 Hisasue S, Seney ML, Immerman E, Forger NG: Control of cell number in the bed nucleus of the stria terminalis of mice: Role of testosterone metabolites and estrogen receptor subtypes. J Sex Med 2010;7:1401–1409.
19 Toda K, Takeda K, Okada T, Akira S, Saibara T, Kaname T, Yamamura K, Onishi S, Shizuta Y: Targeted disruption of the aromatase p450 gene (cyp19) in mice and their ovarian and uterine responses to 17beta-oestradiol. J Endocrinol 2001;170:99–111.
20 Toda K, Okada T, Takeda K, Akira S, Saibara T, Shiraishi M, Onishi S, Shizuta Y: Oestrogen at the neonatal stage is critical for the reproductive ability of male mice as revealed by supplementation with 17beta-oestradiol to aromatase gene (cyp19) knockout mice. J Endocrinol 2001;168:455–463.
21 Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O: Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci USA 1993;90:11162–11166.
22 Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O: Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc Natl Acad Sci USA 1998;95:15677–15682.
23 Toda K, Saibara T, Okada T, Onishi S, Shizuta Y: A loss of aggressive behaviour and its reinstatement by oestrogen in mice lacking the aromatase gene (cyp19). J Endocrinol 2001;168:217–220.
24 Matthews J, Celius T, Halgren R, Zacharewski T: Differential estrogen receptor binding of estrogenic substances: A species comparison. J Steroid Biochem Mol Biol 2000;74:223–234.
25 Paxinos G, Franklin KJ: The mouse brain in stereotaxic coordinates. San Diego, Academic Press, 2001.
26 Tsukahara S, Nakajima D, Kuroda Y, Hojo R, Kageyama S, Fujimaki H: Effects of maternal toluene exposure on testosterone levels in fetal rats. Toxicol Lett 2009;185:79–84.
27 Plumari L, Viglietti-Panzica C, Allieri F, Honda S, Harada N, Absil P, Balthazart J, Panzica GC: Changes in the arginine-vasopressin immunoreactive systems in male mice lacking a functional aromatase gene. J Neuroendocrinol 2002;14:971–978.
28 Ogawa S, Lubahn DB, Korach KS, Pfaff DW: Behavioral effects of estrogen receptor gene disruption in male mice. Proc Natl Acad Sci USA 1997;94:1476–1481.
29 Holmes MM, McCutcheon J, Forger NG: Sex differences in NeuN- and androgen receptor-positive cells in the bed nucleus of the stria terminalis are due to Bax-dependent cell death. Neuroscience 2009;158:1251–1256.
30 Shinoda K, Nagano M, Osawa Y: Neuronal aromatase expression in preoptic, strial, and amygdaloid regions during late prenatal and early postnatal development in the rat. J Comp Neurol 1994;343:113–129.
31 Tsuruo Y, Ishimura K, Osawa Y: Presence of estrogen receptors in aromatase-immunoreactive neurons in the mouse brain. Neurosci Lett 1995;195:49–52.
32 Tobet SA, Baum MJ: Role for prenatal estrogen in the development of masculine sexual behavior in the male ferret. Horm Behav 1987;21:419–429.
33 Tobet SA, Zahniser DJ, Baum MJ: Differentiation in male ferrets of a sexually dimorphic nucleus of the preoptic/anterior hypothalamic area requires prenatal estrogen. Neuroendocrinology 1986;44:299–308.
34 Schulz KM, Molenda-Figueira HA, Sisk CL: Back to the future: The organizational-activational hypothesis adapted to puberty and adolescence. Horm Behav 2009;55:597–604.
35 Fisher CR, Graves KH, Parlow AF, Simpson ER: Characterization of mice deficient in aromatase (arko) because of targeted disruption of the cyp19 gene. Proc Natl Acad Sci USA 1998;95:6965–6970.
36 Lindzey J, Wetsel WC, Couse JF, Stoker T, Cooper R, Korach KS: Effects of castration and chronic steroid treatments on hypothalamic gonadotropin-releasing hormone content and pituitary gonadotropins in male wild-type and estrogen receptor-alpha knockout mice. Endocrinology 1998;139:4092–4101.
37 Akingbemi BT, Ge R, Rosenfeld CS, Newton LG, Hardy DO, Catterall JF, Lubahn DB, Korach KS, Hardy MP: Estrogen receptor-alpha gene deficiency enhances androgen biosynthesis in the mouse leydig cell. Endocrinology 2003;144:84–93.
38 Couse JF, Yates MM, Rodriguez KF, Johnson JA, Poirier D, Korach KS: The intraovarian actions of estrogen receptor-alpha are necessary to repress the formation of morphological and functional leydig-like cells in the female gonad. Endocrinology 2006;147:3666–3678.
39 Couse JF, Yates MM, Walker VR, Korach KS: Characterization of the hypothalamic-pituitary-gonadal axis in estrogen receptor (ER) null mice reveals hypergonadism and endocrine sex reversal in females lacking eralpha but not erbeta. Mol Endocrinol 2003;17:1039–1053.
40 Morris JA, Jordan CL, Breedlove SM: Sexual dimorphism in neuronal number of the posterodorsal medial amygdala is independent of circulating androgens and regional volume in adult rats. J Comp Neurol 2008;506:851–859.
41 Gotsiridze T, Kang N, Jacob D, Forger NG: Development of sex differences in the principal nucleus of the bed nucleus of the stria terminalis of mice: role of bax-dependent cell death. Dev Neurobiol 2007;67:355–362.
42 Wu MV, Manoli DS, Fraser EJ, Coats JK, Tollkuhn J, Honda S, Harada N, Shah NM: Estrogen masculinizes neural pathways and sex-specific behaviors. Cell 2009;139:61–72.
43 Arai Y, Sekine Y, Murakami S: Estrogen and apoptosis in the developing sexually dimorphic preoptic area in female rats. Neurosci Res 1996;25:403–407.
44 Tsukahara S, Hojo R, Kuroda Y, Fujimaki H: Estrogen modulates bcl-2 family protein expression in the sexually dimorphic nucleus of the preoptic area of postnatal rats. Neurosci Lett 2008;432:58–63.

Early life stress disrupts peripubertal development of aggression in male mice

Mumeko C. Tsuda, Naoko Yamaguchi and Sonoko Ogawa

To investigate the effects of early life stress on the development of social behaviors in male mice, we examined behavioral responses toward same sex stimulus mice in the social investigation test and aggressive behaviors in peripubertal male mice exposed to maternal separation (MS) during the first 2 weeks of life. MS suppressed aggressive behaviors from 5–9 weeks of age, but had no effect on social investigative behaviors in the social investigation test. Investigation of neuroendocrine bases of behavioral effects of MS showed that MS reduced plasma testosterone levels and decreased arginine vasopressin and increased oxytocin immunoreactivity in the paraventricular nucleus of peripubertal males. These results collectively suggest that early life stress disrupts the development of male aggressive behaviors and associated neuroendocrine systems. *NeuroReport* **22:259–263 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

NeuroReport 2011, **22**:259–263

Keywords: adolescence, maternal separation, oxytocin, paraventricular nucleus, social behavior, testosterone, vasopressin

Laboratory of Behavioral Neuroendocrinology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan

Correspondence to Sonoko Ogawa, PhD, Laboratory of Behavioral Neuroendocrinology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Research Building D-409, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8577, Japan
Tel/fax: +81 29 853 2966;
e-mail: ogawa@kansei.tsukuba.ac.jp

Received 23 November 2010 **accepted** 3 January 2011

Introduction

Adolescence is a developmental period between childhood and adulthood that involves cognitive, emotional, sexual, and social maturation [1]. Growing evidence indicates that early adverse experiences, such as childhood maltreatment, is associated with an increased risk of developing social adjustment disorders and onset of these disorders can be seen as early as the adolescent age [2,3]. Maternal separation (MS) is an animal model established to mimic the stress of early adverse experiences. In a recent study, daily MS stress during the first 2 weeks of life has been reported to modify aggressive behaviors in juvenile male rats, as indicated by increased levels of offensive play-fighting behaviors compared with control males [4]. Moreover, MS has been reported to have species-dependent consequences on male aggression in adulthood. Specifically, aggression levels were enhanced by MS in male rats [5], but reduced in male mice [6]. Thus far, the effects of MS stress on the development of social behaviors during the adolescent period have not been studied in mice. Therefore, this study examined the effects of MS on the development of aggressive behavior and behavioral responses to a same sex opponent in the social investigation test (SIT) in peripubertal male mice.

The adolescent period is generally associated with major physiological and behavioral changes triggered by testicular maturation and the production of adult levels of testosterone [7,8]. In rodents, male aggression is known to be positively correlated with circulating levels of testosterone [9] and recent studies reported that pubertal maturation of testosterone levels have organizational effects on male aggressive behaviors in adulthood [10]. To date, the effects of early life stress on pubertal development of testosterone levels remains to be determined. In mice, puberty onset has been found to occur around postnatal day (PND) 40 [11]. Therefore, we examined the effect of MS on the maturation of plasma testosterone levels from 4 weeks (before puberty) to 6 weeks (after puberty) of age in male mice.

Arginine vasopressin (AVP) and oxytocin (OT) are neuropeptides mainly synthesized in the paraventricular nucleus (PVN) and supraoptic nuclei of the hypothalamus and released peripherally through the posterior pituitary and centrally to various brain regions. Central release of both neuropeptides is reported to play a role in stress responses [12] and also in the regulation of social behaviors, including aggression [13,14]. Both AVP and OT systems are known to undergo developmental changes from the perinatal period through adulthood. Recently, MS was shown to disrupt age-dependent changes in both AVP and OT systems in the brain [4,15], which possibly contributed to enhanced levels of aggressive behaviors in juvenile and adult male rats [4,5]. However, it is still not known whether MS modifies AVP and OT systems during the peripubertal development in mice. Therefore, we investigated MS effects on AVP and OT immunoreactivity in the PVN around the peripubertal period (4–6 weeks of age).

Materials and methods

Animals and treatments

Adult C57BL/6J female and male mice (CLEA, Tokyo, Japan) were mated. Pregnant female mice were individually housed in plastic cages (29 × 19 × 12 cm) with cotton nesting material during their third week of gestation. On the day after parturition, designated as PND 1, each litter was culled to six pups (female:male ratio was as close to 1:1 as possible). MS pups were separated daily from their dam for 3 h between 15:00 h and 18:00 h from PND 1 to 14. First, MS dams were placed in a separate cage and transferred to a separate room during the separation period. Then, MS pups were placed together in a separate small container with clean bedding and kept warm at a temperature of 35°C. At 18:00 h, all pups were returned to their home-cage, immediately followed by the dam. For nonseparated (control) litters, dams and pups were handled in the same manner as MS groups, except for the separation procedure. On PND 21, all pups were weaned and group-housed with littermates of the same sex. All mice were maintained on a 12 : 12-light/dark cycle (lights off at 12:00 h) and at a constant temperature (23 ± 2°C). Food and water were provided *ad libitum*. All procedures were carried out in accordance with the National Institutes of Health guidelines and the University of Tsukuba Animal Care and Use Committee. Only male offspring were used in this study.

Behavioral testing

At 4 weeks of age, male mice from control and MS treatment groups were single-housed and assigned to one of two behavioral testing groups: (i) control ($n = 5$) and MS ($n = 7$) male mice were tested in SIT at 5 and 6 weeks of age for measurement of behavioral responses toward an unfamiliar same sex stimulus mouse and (ii) control ($n = 13$) and MS ($n = 19$) male mice were tested for aggressive behaviors from 5 to 9 weeks of age. All behavioral tests were conducted during the dark phase, 3–6 h after lights off, under red light illumination.

Social investigation test

Experimental male mice were tested in their home-cage against an unfamiliar gonadally intact C57BL/6J male mouse for 15 min. Stimulus mice were placed in clear Plexiglas cylinders (7 cm in diameter at the bottom, 5 cm in diameter at the top, 11.5 cm in height) consisting of 28 holes (6 mm in diameter) near the bottom 3 cm of the cylinder (Mouse Cylinder SIOT2, O'Hara & Co., Ltd, Tokyo, Japan) and introduced into the center of the males' home-cage. Behaviors of mice during SIT were video-recorded and cumulative duration of sniffing toward perforated parts of the cylinder (social investigation) was scored off-line using a digital event recorder program (Recordia 1.0b, O'Hara & Co., Ltd).

Aggressive behavior test

Male aggression was assessed in the resident-intruder paradigm for 3 consecutive days every week from 5 to 9 weeks of age, for a total of 15 tests. Each male was tested in his home-cage (as a resident) against a weight-matched, gonadally intact, olfactory bulbectomized C57BL/6J male intruder mouse for 15 min. Tests were video-recorded and cumulative duration of aggressive bouts in each test was scored with a digital event recorder program. Data were averaged for each week (three tests per week) for each experimental male. An aggressive bout was defined as a series of behavioral interactions consisting of at least one of the following: chasing, boxing, tail rattling, wrestling, biting, and offensive lateral attack. If the interval between two aggressive bouts exceeded 3 s, the two bouts were scored as two separate bouts.

Immunohistochemistry

A separate set of 4, 5, and 6-weeks-old control ($n = 8$ per age) and MS ($n = 8$ per age) male mice were deeply anesthetized with an intraperitoneal injection of a 1 : 1 solution containing sodium pentobarbital (60 mg/kg) and heparin (1000 units/kg) and perfused transcardially with 0.1 M phosphate buffered saline, pH 7.2, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. Brains were removed, postfixed in 4% paraformaldehyde in phosphate buffer, immersed in 30% sucrose in 0.1 M phosphate buffer for 48 h at 4°C, and coronally sectioned at 30 μm on a freezing microtome. Free-floating sections were incubated in (i) either anti-AVP (0.025 mg/ml; AB1565; Chemicon International Inc., Temecula, California, USA) or anti-OT (0.05 mg/ml; AB911; Chemicon International) antiserum in 0.05 M tris-buffered saline (TBS), pH 7.2, containing 1% Triton X-100, 3% normal goat serum (Vector Laboratories, Burlingame, California, USA), and 3% bovine serum albumin (Sigma-Aldrich, St. Louis, Missouri, USA) overnight at 4°C; (ii) a 1 : 250 dilution of biotinylated goat antirabbit secondary antibody (Vector Laboratories) in TBS containing 1% Triton X-100, 3% normal goat serum, and 3% bovine serum albumin for 2 h at room temperature; and (iii) the avidin–biotin complex (Vectastain ABC Elite kit, Vector Laboratories) in TBS for 1 h at room temperature. Sections were visualized with 0.03% diaminobenzidine and 0.03% hydrogen peroxide in TBS. Three anatomically matched sections for the PVN (Bregma, 0.70–0.94 mm) were selected for each animal. Images of the PVN were obtained at × 10 magnification with a digital camera mounted on an Olympus microscope and total number of immunoreactive (-ir) cells was bilaterally counted for each animal by an experimenter who was unaware of the treatment groups of the tissues.

Enzyme immunoassay for serum testosterone

For testosterone assay, serum samples were obtained from 4, 5, and 6-weeks-old control and MS male mice used for immunohistochemistry. Testosterone concentrations of serum samples were determined using a testosterone enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, Michigan, USA) according to the manufacturer's protocol. The intra-assay standard variation was 8.7% and the interassay coefficient of variation was 10.3%.

0959-4965 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins

DOI: 10.1097/WNR.0b013e328344495a

Statistics

Data were analyzed by a two-way analysis of variance (ANOVA) for repeated measurements (SIT and aggression data) or independent measurements (immunohistochemical and hormonal data) for the main effects of treatment, age, and their interactions. Differences in the percentages of animals that exhibited aggression were analyzed with a χ^2 test and differences in the average number of weeks mice showed aggression out of 5 weeks of testing were analyzed by *t*-tests. All data are presented as mean ± standard error of the mean. When appropriate, ANOVAs were followed by Bonferroni post-hoc test. Significant differences were considered when P value was less than 0.05.

Results

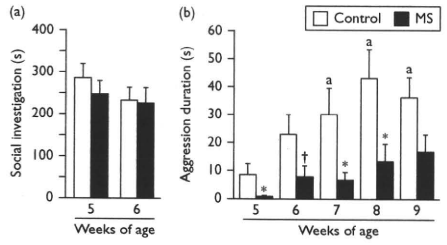
Social investigation

Social behavioral responses toward an unfamiliar same sex stimulus mouse were not altered by MS stress in 5 weeks and 6-weeks-old male mice. Control and MS male mice exhibited similar levels of social sniffing toward the stimulus mouse at both ages (Fig. 1a).

Aggressive behavior

MS greatly modified aggression levels of peripubertal male mice, as indicated by significant main effects of treatment and age on cumulative duration of aggression [(treatment: $F(1,30) = 9.45$, $P < 0.01$; age: $F(4,120) = 6.84$, $P < 0.0001$; treatment × age: not significant); Fig. 1b]. Post-hoc analysis showed that compared with 5 weeks, control male mice began to exhibit higher levels of aggression from 6 weeks of age and continued to be aggressive for the following weeks. In contrast, MS male mice displayed very little increase from 5 weeks of age and consistently showed significantly lower levels of aggression throughout all ages compared with control male mice. Moreover, number of male mice that displayed aggression increased with age in control male mice [5 vs. 8 weeks: $\chi^2(1) = 6.50$, $P < 0.05$; 5 vs. 9 weeks: $\chi^2(1) = 7.08$, $P < 0.05$], in which by 9 weeks of age, 92% of mice showed aggression; however, no such increase was seen in MS male mice (Table 1). Therefore, from 7 weeks of age, significantly less MS males exhibited aggression compared to control male mice [7 weeks: $\chi^2(1) = 4.39$, $P < 0.05$; 8 weeks: $\chi^2(1) = 11.57$, $P < 0.01$; 9 weeks: $\chi^2(1) = 6.91$, $P < 0.01$]. Furthermore, average number of weeks MS male mice displayed aggression out of 5 weeks of testing was significantly less compared with control mice [$(t(30) = 3.72$, $P < 0.001)$; Table 1].

Fig. 1



Effects of maternal separation (MS) on social investigation duration in (a) social investigation test and (b) cumulative duration of aggression in peripubertal male mice. All data are presented as mean ± standard error of the mean. †$P < 0.1$ and *$P < 0.05$ versus control of same age; [a]$P < 0.05$ versus 5 weeks of age in the same treatment group.

Serum testosterone levels

Out of 48 samples measured for serum testosterone levels, five samples did not yield detectable levels and were therefore discarded from further analysis. Two-way ANOVAs of the remaining samples showed that MS greatly reduced serum testosterone levels compared with control male mice $[F(1,37) = 9.09$, $P < 0.01$; Fig. 2a]. Furthermore, serum testosterone levels in control male mice significantly increased with age, whereas no such age-dependent changes were found in MS male mice [age: $F(2,37) = 5.82$, $P < 0.01$; age × treatment: $F(2,37) = 3.80$, $P < 0.05$].

Oxytocin immunoreactivity in the paraventricular nucleus

There was a significant main effect of MS on the number of OT immunopositive cells in the PVN $[(F(1,42) = 7.85$, $P < 0.01)$; Fig. 2b], but no effect of age or interaction. Further analysis showed that MS male mice had a higher number of OT positive cells at 4 weeks ($P < 0.05$) and 6 weeks ($P < 0.05$; Fig. 2d and e) of age compared with control male mice.

Arginine vasopressin immunoreactivity in the paraventricular nucleus

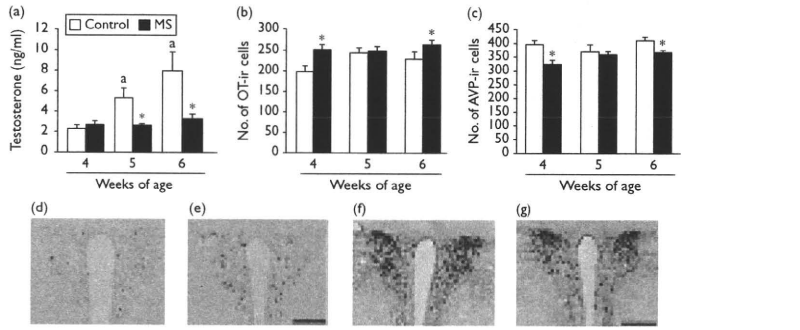
An MS effect on the number of AVP positive cells in the PVN was found $[(F(1,42) = 16.57$, $P < 0.01)$; Fig. 2c], but there was no effect of age or interaction.

Table 1 Number and percentage of mice that showed aggression in at least one test each week and average number of weeks male mice exhibited aggression

	Control (n=13)	MS (n=19)
Age (weeks)		
5	6/13 (46.2%)[a]	4/19 (21.1%)
6	7/13 (53.8%)	6/19 (31.6%)
7	10/13 (76.9%)	6/19 (31.6%)*
8	12/13 (92.3%)†	6/19 (31.6%)**
9	12/13 (92.3%)†	9/19 (47.4%)**
Average number of weeks showed aggression	3.7 ± 0.4[b]	1.6 ± 0.3*

[a]Number of mice that showed aggression/total number of mice tested, percentage of mice that showed aggression (in parentheses).
[b]Average number of weeks showed aggression out of 5 weeks of testing.
*$P < 0.05$ versus control.
**$P < 0.01$ versus control.
†$P < 0.05$ versus 5 weeks of age.

Fig. 2



(a) Maternal separation (MS) effects on plasma testosterone levels and (b) number of oxytocin (OT) and (c) arginine vasopressin (AVP) immunopositive cells in the paraventricular nucleus (PVN) of peripubertal male mice. Representative photomicrographs of OT immunopositive cells of (d) control and (e) MS male mice and AVP immunopositive cells of (f) control and (g) MS male mice in the PVN at 6 weeks of age. The scale bar represents 200 μm. All data are presented as mean ± standard error of the mean. *$P < 0.05$ versus control in the same age group; [a]$P < 0.05$ versus 4 weeks of age in the same treatment group. ir, immunoreactive.

Subsequent post-hoc analysis showed that MS reduced the number of AVP immunopositive cells in 4 weeks ($P < 0.05$) and 6-weeks-old male mice ($P < 0.05$; Fig. 2f and g).

Discussion

In this study, we provide new evidence that neonatal MS stress may specifically disrupt the pubertal development of male aggressive behaviors, as indicated by reduced levels of aggression, but no MS effects on social investigative behaviors toward a same sex opponent in SIT during the peripubertal period. Furthermore, our results showed that unlike control male mice, MS male mice failed to show a significant increase of aggression with age, suggesting the possibility of a delayed or inhibited pubertal onset of aggression. In rodents, adolescence is considered to last from approximately PND 21 to PND 60 [11] and onset of agonistic and aggressive behaviors during this period is essential for the further development of adequate adult social behaviors [16]. In an earlier study, MS was reported to decrease levels of aggressive behaviors in male mice subjected to MS stress during the first 2 weeks of life when tested in adulthood [6]. Taken together, these results suggest that MS-induced disruption of the development of pubertal aggressive behaviors may have led to reduced aggression levels found in adult MS male mice.

Onset of puberty in rodents is considered to occur around PND 40 [11] and it is known that there is a pubertal maturation of testosterone levels at this time [1,7,8]. Studies that investigated the effect of prenatal stress on the onset of puberty in male rats and mice reported no significant disruptions in the timing of puberty onset, as measured by luteinizing hormone secretion and balanopreputial gland separation [17,18]. In contrast, our results showed that plasma testosterone levels were significantly lower in peripubertal MS male mice and these male mice also failed to exhibit an increase in testosterone levels at 5 weeks of age as seen in control male mice. As we found that body weight of peripubertal MS male mice did not differ from control male mice (unpublished observation), low testosterone levels did not necessarily affect the physical development of MS male mice. However, it is still possible that neonatal MS stress may disrupt the timing of puberty onset through alterations in testosterone levels in male mice. Furthermore, plasma testosterone levels are known to be positively correlated with male aggression levels in most rodents [9]. Therefore, reduced levels of plasma testosterone in MS male mice might be partly involved in reduced aggressive behaviors found in this study.

In this study, we found that MS treatment decreased the number of AVP and increased the number of OT immunopositive cells in the PVN compared with control male mice. Central release of AVP and OT from PVN neurons to other hypothalamic and limbic brain regions play an important role in the regulation of social behaviors such as male aggression [19,20]. Furthermore, AVP and OT are known to often exert an opposite action in the regulation of male aggression, in which AVP can facilitate and OT can inhibit the levels of aggression [13]. In this respect, MS-induced changes in AVP and OT activity in opposing directions corroborates the suppressed levels of aggressive behaviors in peripubertal MS male mice. It should be noted that MS effects on the number of AVP

Behavioural Brain Research 221 (2011) 172–181

Contents lists available at ScienceDirect

Behavioural Brain Research

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

Research report

Automated test of behavioral flexibility in mice using a behavioral sequencing task in IntelliCage

Toshihiro Endo[a], Fumihiko Maekawa[c], Vootele Võikar[d], Asahi Haijima[a], Yukari Uemura[b], Yan Zhang[a], Wataru Miyazaki[a], Shigetomo Suyama[c], Kuniko Shimazaki[c], David P. Wolfer[e,f], Toshihiko Yada[c], Chiharu Tohyama[a], Hans-Peter Lipp[d], Masaki Kakeyama[a,*]

[a] Laboratory of Environmental Health Sciences, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, Japan
[b] Department of Biostatistics, School of Public Health, The University of Tokyo, Japan
[c] Department of Physiology, Division of Integrative Physiology, Jichi Medical University, School of Medicine, Japan
[d] Institute of Anatomy, University of Zurich, Switzerland
[e] Institute of Anatomy and Zurich Center for Integrative Human Physiology, University of Zurich, Switzerland
[f] Institute of Human Movement Sciences, ETH Zurich, Switzerland

ARTICLE INFO

Article history:
Received 6 September 2010
Received in revised form 23 February 2011
Accepted 28 February 2011

Keywords:
Mouse
Automated analysis
Serial reversal learning
Behavioral flexibility
IntelliCage

ABSTRACT

There has been a long-standing need to develop efficient and standardized behavioral test methods for evaluating higher-order brain functions in mice. Here, we developed and validated a behavioral flexibility test in mice using IntelliCage, a fully automated behavioral analysis system for mice in a group-housed environment. We first developed a "behavioral sequencing task" in the IntelliCage that enables us to assess the learning ability of place discrimination and behavioral sequence for reward acquisition. In the serial reversal learning using the task, the discriminated spatial patterns of the rewarded and never-rewarded places were serially reversed, and thus, mice were accordingly expected to realign the previously acquired behavioral sequence. In general, the tested mice showed rapid acquisition of the behavioral sequencing task and behavioral flexibility in the subsequent serial reversal stages both in intra- and inter-session analyses. It was found that essentially the same results were obtained among three different laboratories, which confirm the high stability of the present test protocol in different strains of mice (C57BL/6, DBA/2, and ICR). In particular, the most trained cohort of C57BL/6 mice achieved a markedly rapid adaptation to the reversal task in the final phase of the long-term serial reversal test, which possibly indicated that the mice adapted to the "reversal rule" itself. In conclusion, the newly developed behavioral test was shown to be a valid assay of behavioral flexibility in mice, and is expected to be utilized in tests of mouse models of cognitive deficits.

1. Introduction

The behavioral characterization of genetically modified mice as well as wild-type strains has become a powerful tool for investigating not only the molecular bases of normal brain functions but also the pathogenesis and treatment of neuropsychological disorders [1–9]. However, the levels of efficiency, standardization, and reproducibility of the testing methods for mouse behavioral assessment have been considered still inadequate [10–14]. More importantly, the limited number of established "higher-order" cognitive test paradigm for mice makes it difficult for researchers to determine the accurate neurobehavioral phenotypes of both wild-type and pathological mice [15].

To overcome this problem, a number of computer-assisted technologies for automatically capturing rodent behavior over long periods have become available [16–18]. Among them, IntelliCage (New Behavior AG; http://www.newbehavior.com/) used in the present study is a unique approach in the sense that this system is specially designed for the cognitive assessment of group-housed mice. The IntelliCage system can be a powerful tool for the behavioral characterization of mice by at least fivefold. First, its use makes it possible to achieve a sensitive and highly standardized experiment by minimizing the artifacts that arise from unavoidable differences among experimenters or other laboratory-specific conditions. Second, long term monitoring of mouse behavior can be performed in a familiar and thus less stressful environment. Third, high-throughput testing is possible by analyzing a maximum of 16 mice simultaneously. Fourth, experimenters can design and use their own original cognitive task depending on their research objective. Fifth, IntelliCage can be run in a fully automated manner,

* Corresponding author. Tel.: +81 3 5841 1415; fax: +81 3 5841 1434.
E-mail address: kake@m.u-tokyo.ac.jp (M. Kakeyama).

0166-4328/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbr.2011.02.037

utilizing sensors and four operant conditioning units placed in each corner of the cage.

Recent studies have already shown the advantages of using this IntelliCage system in the spontaneous and learning behavioral analysis of mice [19–27]. By contrast, there are still few established protocols for IntelliCage that focus on higher-order cognitive skills typified by executive brain functions. The executive brain function is a shorthand description of a set of cognitive processes that are responsible for appropriately organizing, performing, and maintaining goal-directed actions under ever-changing environmental contexts [28–32]. The quality of life based on intellectual and mental integrity is largely dependent on such brain function, and its dysfunction is widely seen in people with aging-associated cognitive decline and various neuropsychological disorders [29,33–37] as well as patients with frontal lobe damage caused by traumatic brain injury and cerebrovascular disease [38,39]. Although researchers have recently established executive function tests for mice such as attentional set-shifting task [40–43] and selective attention [44], few reports that assessed the executive functions of group-housed mice in a computerized, high-throughput manner are available.

Thus, the aim of this study is to establish a behavioral test protocol for mice that enables one to evaluate behavioral flexibility as one of the executive brain functions using IntelliCage. The test is composed of a newly developed "behavioral sequencing task" followed by its serial reversals. The test paradigm was originally developed on the basis of the idea of the Brixton Spatial Anticipation Task [45] which has been utilized as one of the clinical assessment methods of human executive functions using a visuospatial sequencing task [45–52]. By using the IntelliCage-based test in this study, it became possible to address not only acquisition of spatial and temporal pattern of rewarded places but also behavioral flexibility of mice in various time scales in a fully automated manner. The reproducibility of the protocol was verified by different experimenters at three different laboratories located in the University of Tokyo (UT), Tokyo Japan, Jichi Medical University (JMU), Tochigi, Japan and the University of Zurich (UZH), Zurich, Switzerland, using three strains of mice (C57BL/6, DBA/2 and ICR) and of different ages (from 2 to 8 months old).

2. Materials and methods

2.1. Animals and facilities

The experiments described in this study were conducted using identical type of IntelliCage systems at three different laboratories located at the University of Tokyo (UT), Tokyo, Japan, Jichi Medical University (JMU), Tochigi, Japan and the University of Zurich (UZH), Zurich, Switzerland. All the animal experiments were performed in a humane manner in accordance with the local guidelines of each institution.

At UT, male C57BL/6 (B6-UT, 6 months old, $n=8$) and DBA/2 (D2-UT, 8 months old, $n=8$) mice were used. All mice were purchased from CLEA Japan (Tokyo, Japan) and housed in 22 ± 1 °C, $50\pm10\%$ humidity, 12 h LD cycle (lights on 8:00–20:00). At JMU, male C57BL/6 (B6-JMU, 3 months old, $n=15$) and ICR mice (ICR-JMU, 2 months old, $n=11$) were used. All mice were purchased from CLEA Japan and Japan SLC, Inc. (Shizuoka, Japan) and housed in 22 ± 1 °C, $60\pm10\%$ humidity, 12 h LD cycle (lights on 7:00–19:00). At UZH, young (3 months old) and aged (12 months old) male mice of C57BL/6 and DBA/2 strains were used (Young B6-UZH, Aged B6-UZH, Young D2-UZH and Aged D2-UZH, $n=11$, 12, 12 and 11, respectively). All mice were purchased from Charles-River Laboratories (Sulzfeld, Germany) and housed in 21 ± 1 °C, $50\pm5\%$ humidity, 12 h LD cycle (lights on 20:00–8:00 as reversed cycle). In total, eight cohorts of male mice were analyzed using the IntelliCage system as described below.

All the animals were subcutaneously implanted with a glass-covered transponder with unique ID codes (Datamars SA) for radio-frequency identification (RFID)-based animal identification before the start of the experiments under light-anesthesia with diethyl ether or isoflurane.

2.2. IntelliCage apparatus

IntelliCage (NewBehavior AG; http://www.newbehavior.com) is a computer-based, fully automated testing apparatus used to analyze the spontaneous and learning behavior of RFID-tagged mice in a home cage (Fig. 1A). (For further descriptions, see [19–27]). In short, a large standard plastic cage ($55\times37.5\times20.5\,cm^3$) equipped with four triangular operant learning chambers (corners, hereafter) ($15\times15\times21\,cm^3$) that fit into each corner of the cage, RFID readers, and other types of sensor allows simultaneous monitoring of up to 16 transponder-tagged mice living in the same cage. Mice were allowed to enter the corner (corner visit, hereafter) through a short narrow tunnel that functions as an RFID antenna. In this unit, only one mouse can enter a corner at a time because of the limited size of the corner and tunnel. In the inner space of the corner, mice can find two nosepoke holes with an infrared beam-break response detector. The "correct" nosepoke triggers the opening of a motorized access gate to water-bottle nipples (gate, hereafter). In IntelliCage, the time and duration of each behavioral event (corner visit, nosepoke and lick), mouse ID and corner ID were automatically recorded through RFID readers, infrared sensors and lickometers.

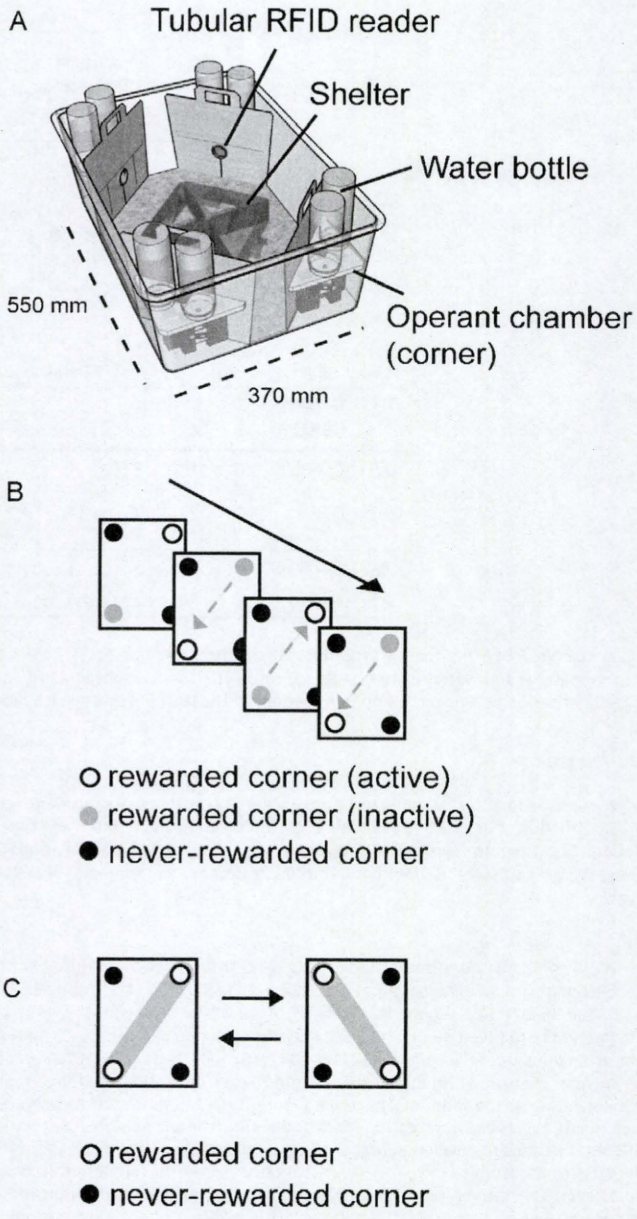
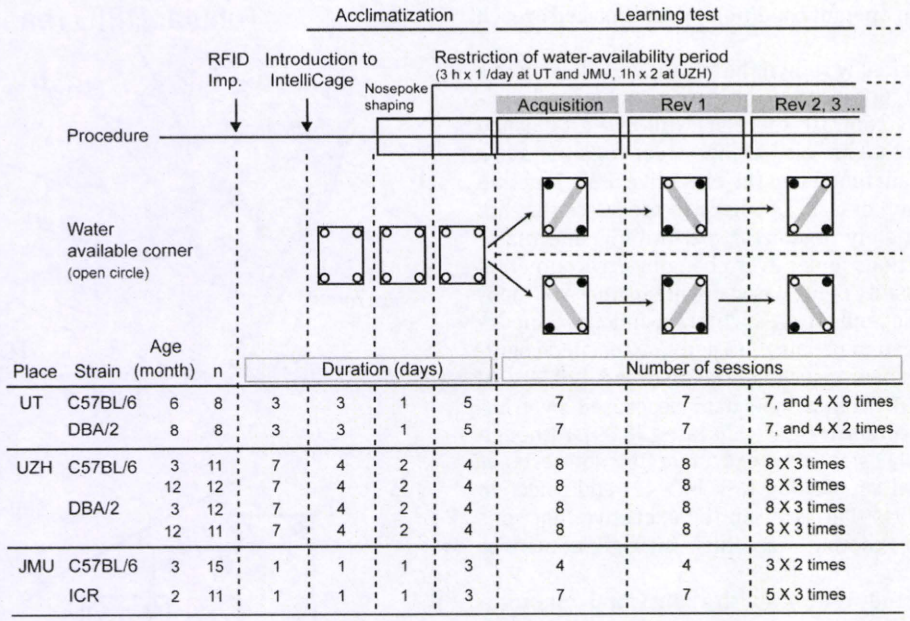


Fig. 1. Apparatus and task paradigm. (A) Overview of IntelliCage apparatus. Mice were group-housed and their behavioral responses (corner visits, nosepokes, and lickings) were monitored in a fully automated manner. The tubular RFID reader can record an implanted ID number when a mouse visits a corner where it can receive water as a reward. (B) Diagrams of behavioral sequencing task. Mice obtained a reward by alternately visiting the two distantly positioned rewarded corners (open circle). A visit to the never-rewarded corners (black circles) was counted as an "error" choice. (C) Serial reversal learning of the behavioral sequencing task. The patterns of corner conditions (rewarded or never-rewarded) were reversely switched every several sessions. The thick grey line in (C) indicates the expected shuttling path on which mice shuttle between the distantly positioned rewarded corners.



Place	Strain	Age (month)	n	Duration (days)				Number of sessions		
UT	C57BL/6	6	8	3	3	1	5	7	7	7, and 4 X 9 times
	DBA/2	8	8	3	3	1	5	7	7	7, and 4 X 2 times
UZH	C57BL/6	3	11	7	4	2	4	8	8	8 X 3 times
		12	12	7	4	2	4	8	8	8 X 3 times
	DBA/2	3	12	7	4	2	4	8	8	8 X 3 times
		12	11	7	4	2	4	8	8	8 X 3 times
JMU	C57BL/6	3	15	1	1	1	3	4	4	3 X 2 times
	ICR	2	11	1	1	1	3	7	7	5 X 3 times

Fig. 2. Overview of experimental procedures in the three laboratories (UT, JMU, and UZH). After RFID implantation and introduction to the IntelliCage, mice were habituated to the apparatus and water restriction period for 5–10 days and subsequently imposed with a learning test. Note that there are some modifications in terms of the duration of acclimatization period and number of sessions in the learning test in each laboratory.

2.3. Test protocols

We used an identical learning test protocol among the three laboratories except for small modifications such as schedule of acclimatization and number and time of sessions. The program was originally developed at UT, and provided to JMU and UZH where the program was used with minor modifications of experimental procedure (Fig. 2).

2.3.1. Acclimatization

We accommodated mice of the same age and strain in each IntelliCage at the three laboratories. In detail, groups named B6-UT (12), D2-UT (12), Young B6-UZH (11), Aged B6-UZH (12), Young D2-UZH (12), Aged D2-UZH (11), B6-JMU (15), and ICR-JMU (11) had the number of mice per cage as shown in parentheses. In order to adjust the number of mice per cage of B6-UT ($n = 8$) and D2-UT ($n = 8$) close enough to the other cohorts, additional mice were put to each cage and tested in the same manner. Although the additional mice were same in age and strain as those of mice in each cohort, their behavioral data were excluded from the analysis because they had different histories of developmental condition for the sake of another experiment. The gates through which mice can have access to water-bottle nipples in all corners were kept opened, and thus, the mice were allowed to have water *ad libitum* in each corner for the first three days (UT) of the acclimatization phase. Next, shaping of nosepoking behavior was performed for a total of six days (UT). In this phase, all the gates in front of the water bottles were closed initially so that mice had to nosepoke to open the gate and drink the water. The gate could be opened only by the first nosepoke in one corner visit, and was closed after 4 s. During the first day, mice obtained water by nosepokes at any time during the 24 h. Afterwards, nosepokes did not open the gates except during a 3 h period from 22:00 to 1:00 (UT) (i.e., all the mice were deprived of water outside these hours). During this 3 h period, a blue light-emitting diode (LED), which was attached to the outside of the cage, was dimly lighted to signal the water-availability period at UT. Likewise, during this period, red LEDs in the corner were lighted when mice made a visit. During acclimatization, all the mice visited all the four corners extensively. Their individual corner preferences varied between 20% and 30% (chance level 25%), indicating that all the mice became familiar with all the corners without any intensive preference for specific corner(s).

2.3.2. Behavioral sequencing task

After acclimatization, a behavioral sequencing task was imposed on the animals. This task, which was programmed using the graphic task designer software of the IntelliCage system, was imposed on mice and valid only during a 3 h period (UT) within a day (22:00–1:00). Thus, all the mice were deprived of water outside these hours in the same manner as in the last acclimatization phase. In the behavioral sequencing task (Fig. 1B), mice were imposed to discriminate the rewarded and never-rewarded corners, and to "shuttle" between the two distantly positioned rewarded corners. Each mouse could open the gate and drink water for 4 s only from the corners designated as "rewarded", while they could not do so in the "never-rewarded" corners. Additionally, each of the two distantly positioned rewarded corners had two distinct states, "active" or "inactive", in a mutually exclusive manner between them. That is, there is always one "active" rewarded corner, one "inactive" rewarded corner, and two never-rewarded corners at a time. The gates could be opened by a nosepoke action only in an "active" rewarded corner. When a mouse did a nosepoke in the "active" rewarded corner, and was presented a reward, the corner became "inactive". At the same time, the other rewarded corner, which was previously "inactive", became "active". This event-related alternation of the corner assignment was controlled for each mouse separately by IntelliCage software which ran throughout the experiment. Thus, the mice could not receive an additional reward by staying at one corner or by re-entering the same corner. Consequently, the mice had to shuttle between the two distantly positioned rewarded corners, avoiding temptations of entering the neighboring never-rewarded corners or just re-entering a specific corner. All the corner assignments were balanced within a group in the cage so that no specific corner would receive more traffic than others. As an "acquisition" stage, this behavioral sequencing task lasted for seven sessions in all the UT experiments, four sessions in a B6-JMU experiment, seven sessions in an ICR-JMU experiment, and eight sessions in all UZH experiments (Fig. 2). At UZH, the protocol had to be modified allowing 2 sessions per day (11:00–12:00 and 16:00–17:00), because the animal welfare guidelines at UZH did not allow water deprivation for more than 18 h.

2.3.3. Serial reversal learning

Subsequently, serial reversal task using the behavioral sequencing task paradigm were imposed on mice: Diagonal spatial patterns of rewarded and never-rewarded corners were reversely changed repetitively every 4–7 sessions. Thus, the mice had to learn to switch their shuttling behavior between the two diagonal spatial patterns (Fig. 1C). In total, 57 sessions, one session per day, were conducted in the UT experiment including the first seven sessions as an acquisition phase and further sessions with 11 serial reversals. At JMU, a total of 14 and 29 sessions, including 3 and 4 serial reversals, respectively, were carried out for B6-JMU and ICR-JMU, respectively, with one session per day. At UZH, a total of 40 sessions, including 4 serial reversals, were carried out with two sessions per day.

2.4. Behavioral scores

Corner visit of mice was defined as entering a corner chamber regardless of a nosepoke action. The records during each session were used to calculate the following four types of learning score: (1) Discrimination error rate = percentage of incorrectly visiting the two never-rewarded corners within the first 100 visits in each session; (2) Alternation score = percentage of alternate visits in specific diagonals (rewarded or never-rewarded) within total visits in a session or choice block (see Fig. 3, Fig. 7B and Supplemental Fig. S3); (3) Cumulative errors = a cumulative number of corner visits to never-rewarded corners; (4) Discrimination index = [10 − (number of errors within 10 choices) × 10]. This index was used to analyze the dynamics of within-session adaptation to the reversal learning.

2.5. Statistical analyses

All statistical analyses for possible difference in means in behavioral scores were carried out using SAS software (SAS Institute Inc.). In ANOVA, repeated

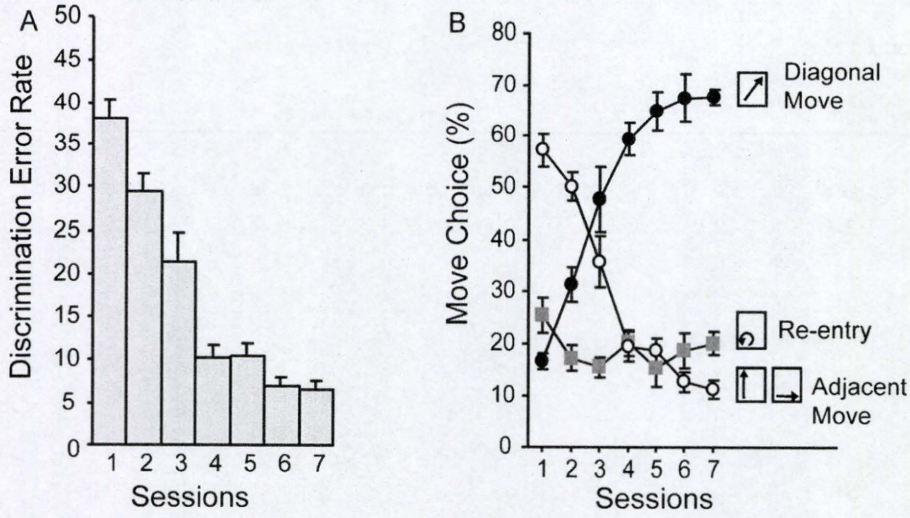


Fig. 3. Acquisition of behavioral sequencing task in B6-UT. (A) Reduction of visits to never-rewarded corners (errors) in the acquisition phase (sessions 1–7) in behavioral sequencing task. Discrimination error rate (mean ± S.E.M, $n = 8$) was defined as the number of visits to the two never-rewarded corners within the first 100 visits in each session, which makes the chance value of discrimination error rate 50%. Statistical analysis showed significant linear trends of error reduction ($p < 0.05$, one-way repeated measures ANOVA with session as a factor). See also Supplemental Fig. S2 for the other cohorts. (B) Acquisition of "shuttling" behavior in behavioral sequencing task. Three move choices in two consecutive corner visits were classified as follows: (1) diagonal move, (2) adjacent move (move to either of two adjacent corners), and (3) re-entry to the same corner, and thus chance values are calculated as 25%, 50%, and 25%, respectively. The rates of the three choices of move in the acquisition phase are shown (mean ± S.E.M, $n = 8$). The concurrent increase in diagonal move choice rate and the decrease in adjacent move choice rate indicated that the mice in this experiment acquired shuttling behavior.

analysis using a session or block of responses as a factor was adopted to assess a within-subject effect of training throughout sessions. Multiple comparisons were performed with Dunnett's test. For logarithmic curve fitting and R^2 value calculation, Excel (Microsoft) was used.

3. Results

3.1. Acquisition of behavioral sequencing task

In the behavioral sequencing task, the mice were imposed to discriminate rewarded from never-rewarded corners with attainment of shuttling behavior between the two distantly positioned rewarded corners to obtain water continuously (Fig. 1B). The discrimination error rate, defined as the number of incorrectly visiting the two never-rewarded corners within the first 100 visits in each session, was significantly decreased in C57BL/6 mice examined at the UT (B6-UT), which is the representative cohort of the present study (Fig. 3A) ($F = 49.77$, df = 6/49, $p < 0.0001$, repeated measures ANOVA). A significant decrease in discrimination error rates occurred from sessions 2 to 4 (ANOVA, Dunnett, $p < 0.001$), followed by its drop to less than 10% during sessions 6 and 7. To analyze the attainment of shuttling behavior, three move choices in two consecutive corner visits were classified as follows: (1) diagonal move, (2) adjacent move (move to either of two adjacent corners), and (3) re-entry to the same corner, and thus chance values are calculated as 25%, 50%, and 25%, respectively. The rates of the three move choices in the acquisition phase of B6-UT are representatively shown in Fig. 3B. The concurrent increase in diagonal move choice rate and the decrease in adjacent move choice rate indicated that the mice in this experiment acquired the shuttling behavior between the diagonally positioned corners. The alternation score (percentage of alternate visits in each rewarded and never-rewarded diagonal) within a session are shown in Supplemental Fig. S3A (session 1) and S3B (session 7) and indicates that the mice showed shuttling specifically in a rewarded diagonal through the session 7. As the once acquired shuttling behavior had been kept at a high level through the subsequent sessions, we will focus on discrimination error rate as an index of behavioral flexibility in the following part in this article.

We found that all the other cohorts of mice, comprising different strains and ages used at the three independent laboratories, showed almost identical learning curves of discrimination error rates, with statistically significant linear trends of error reduction ($p < 0.05$, one-way repeated measures ANOVA with session as a factor) (see Supplemental Fig. S2). Although the reduction of errors varied according to strain, age and laboratory during the first three sessions, mice of all the cohorts showed a highly significant reduction in errors after session 4. It is notable that all the cohorts of mice robustly showed a "stepwise" acquisition of place discrimination in this task.

Throughout the experiments, we had the cages including the corners cleaned routinely once a week in the middle of the acquisition phase and the subsequent test phase, and did not find significant effects of the cleaning on the task performance. For information about a probe trial supplementarily conducted at UT, see Supplemental Fig.S1 and the legend.

3.2. Serial reversal learning

In serial reversal learning using the behavioral sequencing task paradigm, diagonal spatial patterns of rewarded and never-rewarded corners were reversely changed every 4 to 7 sessions (B6-UT). Thus, the mice were anticipated to switch their shuttling behavioral sequence between the two diagonals (Fig. 1C). We first analyzed the discrimination error rates for each session to clarify the inter-session dynamics of behavioral flexibility. The discrimination error rates of the three cohorts of C57BL/6 mice in the three laboratories are representatively shown in Fig. 4. In comparison with the last session of the acquisition phase and the first session of the first reversal stage (Rev 1), the discrimination error rate of all the three cohorts was found to be markedly increased to approximately 60% each, exceeding the chance level (=50%) in each cohort (see sessions 8, 9, and 5 of Fig. 4A–C, respectively). Subsequently, those cohorts displayed a significant reduction in the discrimination error rate during the following several sessions within Rev 1, showing inter-session enhancement of behavioral flexibility. This learning curve could be robustly found in all the subsequent reversal stages in each cohort (Rev 2 and later). It is also notable that

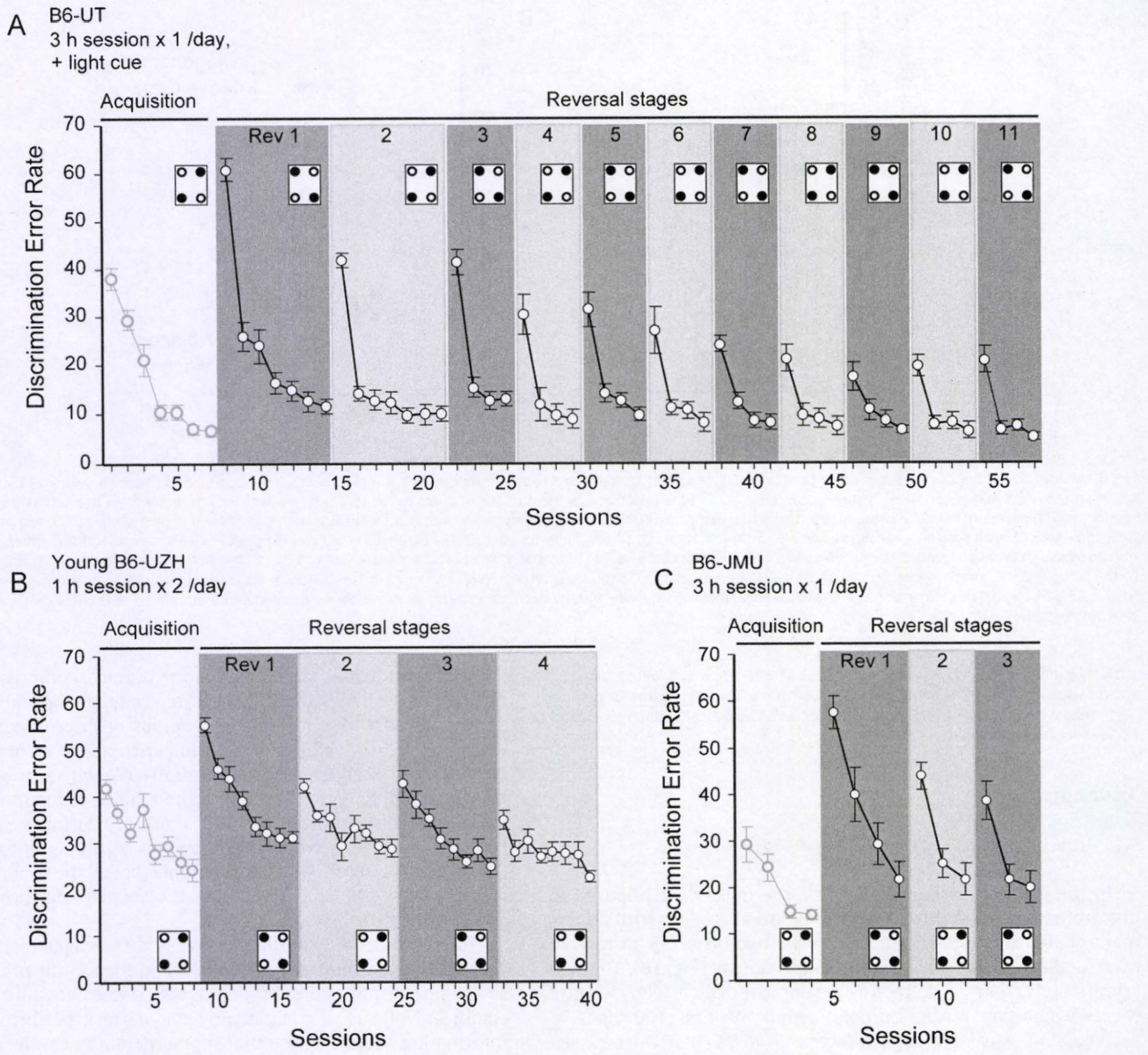


Fig. 4. Behavioral flexibility in serial reversal learning. Representative data on discrimination error rates (mean ± S.E.M) of C57BL/6 cohorts at (A) UT ($n = 8$), (B) UZH (young cohort, $n = 11$) and (C) JMU ($n = 15$) are shown. After the acquisition of place discrimination in the behavioral sequencing task, the patterns of corner conditions (rewarded or never-rewarded) were reversely changed repetitively every several sessions (reversal stages) as described in the figure. In the first session of each reversal stage (Rev), discrimination error rate transiently increased and gradually decreased within the following several sessions, indicating the dynamics of behavioral flexibility to the altered contingency.

a continued reduction of the discrimination error rate in the first sessions of each reversal stage (Rev) was observed in each cohort, which will be discussed in detail later as inter-reversal stage (Rev) improvement of behavioral flexibility.

We next analyzed the intra-session performance in detail. Fig. 5A representatively shows cumulative errors plotted for the first 100 visits in specific sessions in B6-UT mice. We found that the cumulative errors increase linearly in sessions 1 and 7 (the first and last sessions of the acquisition phase, respectively), and session 8 (the first session of Rev 1). In contrast, at session 15 (the first session of Rev 2), cumulative errors in the first half of the session exceeded the chance value (broken line), but then fell to below the chance value in the second half. This suggested that the mice realigned their behavior to the altered contingency rapidly within a session. The alternation scores (percentage of alternate visits) for both rewarded and never-rewarded diagonals also revealed a trinitarian process of the mice shifting the shuttling behavior in session 15 (Supplemental Fig. S3D), that is, the periods of (1) exceeded preference to the shuttling between the never-rewarded corners (from 0 to 15 choices), (2) similar preference between the correct and incorrect shuttling (from 20 to 45 choices), and (3) exceeded preference to the rewarded corners of mice. Since the x-axis of intra-session analyses is considered to reflect essentially a time, the time course of transition of mice responses, that is, an intra-session dynamics of behavioral flexibility can be observed. Subsequently, we plotted the discrimination index of the first sessions in different reversal stages (Revs 1, 3, 5, 7, and 9) to observe both short term and long term dynamics of behavioral flexibility (Fig. 5B). We found that logarithmic curves can be fitted well to each intra-session learning curve after Rev 3 ($R^2 = 0.835–0.940$), indicating that the reshaping of behavior mainly occurred in the early phase of each session. In addition, the step-by-step enhancement of the general adaptability was confirmed as mice proceeded to experience sequential reversal stages.

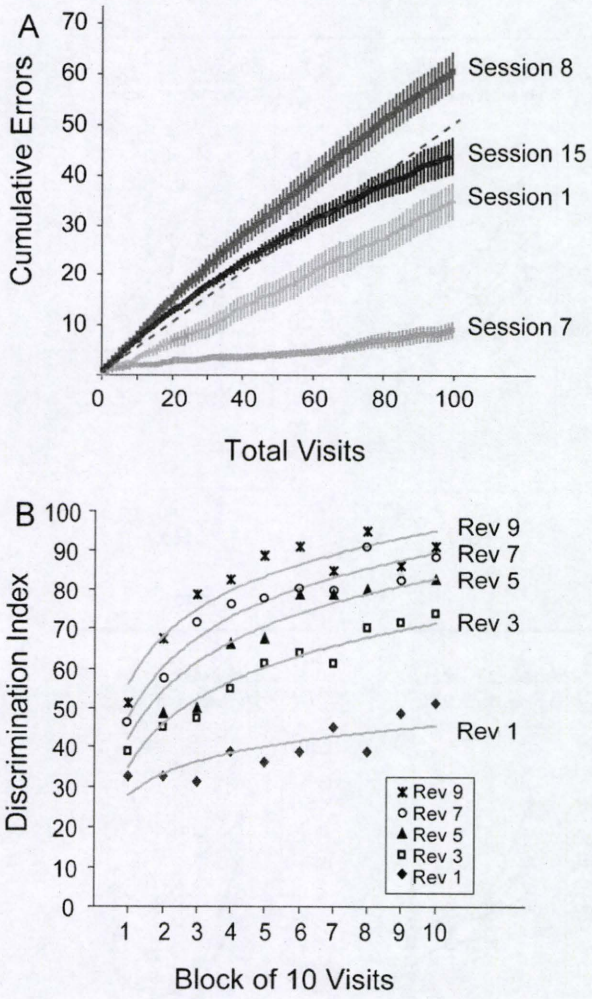


Fig. 5. Intra-session dynamics of behavioral flexibility in B6-UT mice. (A) Cumulative errors (mean ± S.E.M, $n = 8$) of the first 100 visits in sessions 1 and 7 (the first and last sessions of acquisition phase, respectively), and sessions 8 and 15 (the first sessions of Revs 1 and 2, respectively). The dotted line indicates a chance value (50 cumulative errors per 100 visits). (B) Means of discrimination index [10 − (number of errors within 10 visits) × 10] in the first sessions of Revs 1, 3, 5, 7, and 9 were plotted (mean ± S.E.M, $n = 8$). The logarithmic transformation was used for curve fitting to show the increased discrimination performance in the first sessions of Revs 1, 3, 5, 7, and 9 with reliable R^2 values (0.876, 0.939, 0.835, 0.940, and 0.651, respectively).

Lastly, we noted the fact that the mice progressively improved their behavioral flexibility performance in the serial reversals. So we directly compared the discrimination error rates in the first, second, third, and fourth sessions of the different reversal stages (Revs) (Fig. 6). In all the eight cohorts of mice, the discriminative performance consistently improved among the reversal stages (Revs), particularly in the first sessions with a significant linear trend of error reduction ($p < 0.05$, repeated measures ANOVA). In B6-UT mice, which were the most trained cohort in our study, this inter-reversal stage (Rev) improvement of behavioral flexibility finally resulted in a markedly rapid adaptation to the reversed contingency (Fig. 7A) and rapid reshaping of shuttling behavior (Fig. 7B and Supplemental Fig. S4).

4. Discussion

The significant outcome of the present study is that we established a fully automated behavioral test protocol for mice using IntelliCage to assess the acquisition of spatial and temporal patterns of rewarded place and behavioral flexibility as one of the modules of executive brain functions in mice. This test consists of a behavioral sequencing task followed by its serial reversals, which was originally developed based on the idea of Brixton Spatial Anticipation Task [45] which has been utilized as one of the clinical assessment methods of human executive functions using a visuospatial sequencing task [45–52]. The newly developed behavioral test protocol appears to be fairly robust, as indicated by similar patterns of learning curves observed in the results from the experiments conducted at the three different laboratories using three strains of mice (C57BL/6, DBA/2 and ICR) and of different ages (from 2 to 8 months old).

4.1. Behavioral indices for acquisition of behavioral sequencing task and behavioral flexibility

4.1.1. Acquisition of behavioral sequencing task

In the behavioral sequencing task, mice were found to rapidly learn place discrimination of rewarded and never-rewarded places with low individual differences. The alternating sequence rule of this task is of importance in accounting for such efficiency. That is, once a mouse received a reward in an "active" rewarded corner, the corner became "inactive", and at the same time, the other diagonally opposite rewarded corner conversely became "active". Thus, mice were obliged to shuttle between the two distantly positioned rewarded corners for additional rewards, avoiding the temptation of entering the neighboring never-rewarded corners. This rule enables to prevent mice from staying a specific corner to get additional rewards without active choice, and thus, to reduce the possibility of misinterpretation of pseudo-learning effect attributed to place preference. Additionally, the behavioral sequencing task concurrently demands cognitive processes for both reinforced alternation and place discrimination. Although this task can be considered to be relatively complex compared with each learning task of reinforced alternation and place discrimination for mice because of its mixed rule, they could acquire this behavioral sequence and show high discriminative performance within the first several sessions. When compared with other established spatial learning paradigms, the learning curves of the discriminative performance in the behavioral sequencing task paradigm were similar to those observed in the Morris water maze [3,53–56] and other maze tasks (e.g., [3,57,58]), despite the differences in experimental designs and indices. Considering the result of the probe trial (see Supplemental Fig. S1 and its legend), we concluded that the behavioral sequencing task paradigm using IntelliCage is an optimized method for the efficient cognitive assessment in mice.

4.1.2. Behavioral flexibility

To date, researchers have tried to establish the mouse as a useful animal model for assessing behavioral inflexibility that is found in human patients with frontal lobe damage, aging-associated cognitive decline, and neuropsychological disorders [29]. The reported methods are based on various approaches such as maze tasks [59,60] and operant conditioning procedures [42,61–65]. However, it is still unclear what types of behavioral measure are suitable for the integrated behavioral flexibility assessment in mice. Thus, the major aim of this study is to characterize a detailed process of behavioral adaptation to the altered action-outcome contingency in mice in a reproducible manner.

A unique feature of the behavioral flexibility assessment in this study is that it can be used to analyze behavioral data in various time scales that span from minutes to even months. In this study, mice were clearly found to develop behavioral flexibility to the altered action-outcome contingency in intra- and inter-session intervals. In the intra-session analysis, we found that mice shifted their preference to the newly assigned rewarded corners within the first sessions of each reversal stage (Rev) (see session 15 in Fig. 5A and B). These indices represent a dynamics of short term behavioral flexibility. In addition, mice showed day-to-day

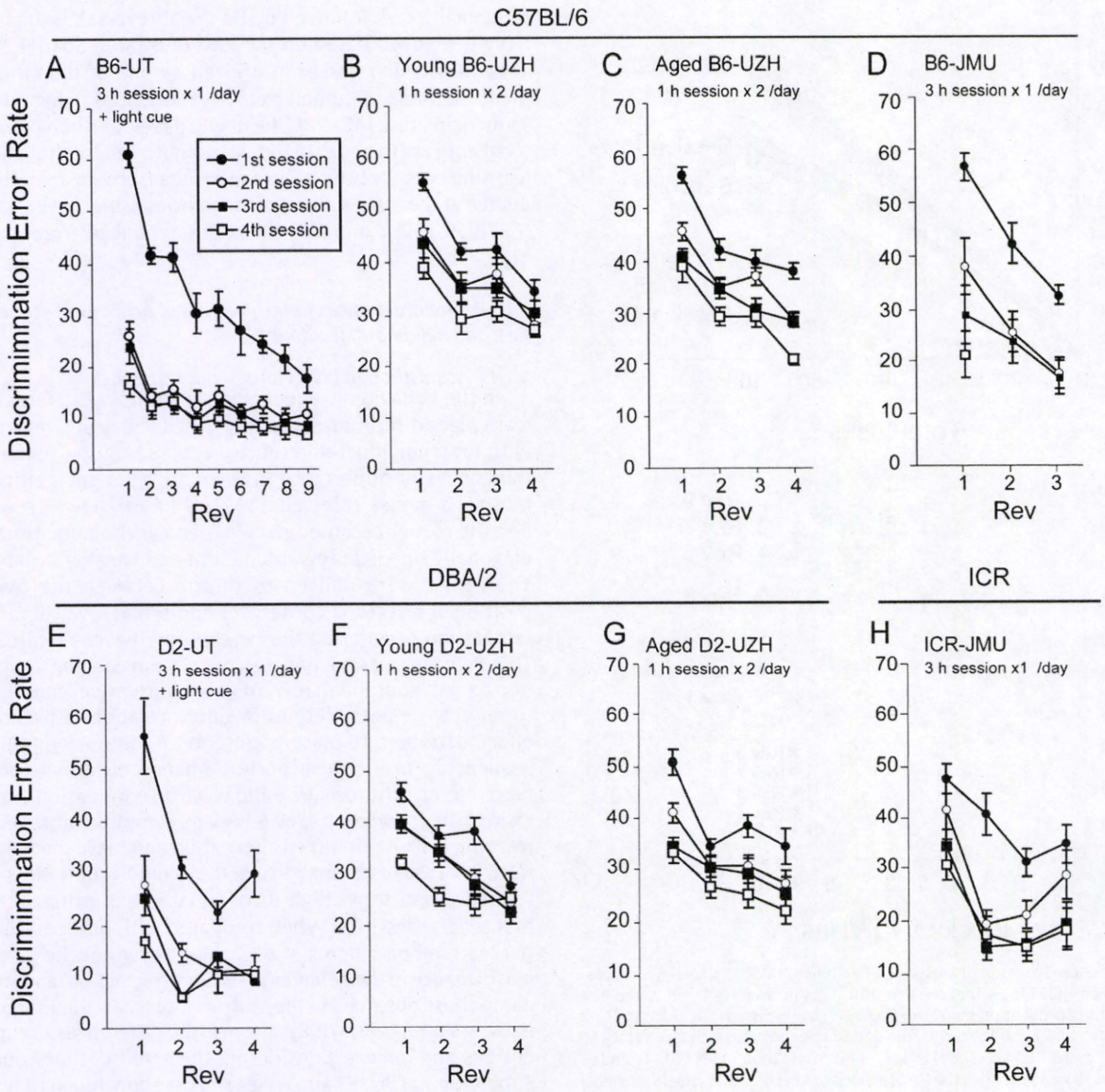


Fig. 6. Long term (inter-reversal stage) improvement of behavioral flexibility in the serial reversal task. (A) B6-UT, (B) Young B6-UZH, (C) Aged B6-UZH, (D) B6-JMU, (E) D2-UT, (F) Young D2-UZH, (G) Aged D2-UZH and (H) ICR-JMU. Panels (A), (B), and (D) were reconstructed using the data shown in Fig. 4. Discrimination error rates of the first, second, third, and fourth sessions from each reversal stage (Rev) were plotted to show the inter-reversal stage (Rev) dynamics of behavioral flexibility (mean ± S.E.M, $n = 8, 11, 12, 15, 8, 12, 11,$ and 11, respectively from panel (A) to (H)). The discriminative performance consistently improved among the reversal stages (Revs) in all the eight cohorts from Rev 2 to the subsequent stages, particularly in the first sessions with a significant linear trend of error reduction ($p < 0.05$, repeated measures ANOVA).

improvement of adaptive behavior. In other words, they were found to have inter-session dynamics of behavioral flexibility that forms typical learning curves (Fig. 4). The time course patterns of these learning curves on intra- and inter-sessions were repetitively observed, and thus, considered to be robust in a long term experiment. Therefore, we propose that the present method makes it possible to efficiently characterize the process of behavioral flexibility in mice.

Another feature of the present test is that the reversal learning of the behavioral sequencing task is distinct from other conventional tasks in terms of its behavioral requirements. That is, subjects were required not only to relearn the rewarded locations but also to develop shuttling behavior between the reversed diagonal corners. As in the case of the initial acquisition of the behavioral sequencing task, this paradigm prevents mice from staying at a specific corner to obtain additional rewards without an active choice in reversal learning. As a result, we found that this reversal learning yielded a very clear and consistent form of learning curve in mice. Thus, we concluded that the reversal learning using the behavioral sequencing task established in this study is useful for assessing behavioral flexibility in mice.

4.1.3. Adaptation to "reversal rule"

The serial reversal learning in this study exhibited another intriguing phenomenon, that is, a progressive enhancement in adaptation to reversal learning. In particular, the B6-UT mice, which were the most trained cohort in this study, swiftly shifted to the new spatial pattern in the final phase of the long term flexibility test (see Fig. 7A and Supplemental Fig. S4). The ability of the progressive shifting from trial-and-error to the immediate solving of new problems on the basis of previously learned experiences can be explained using the theory of "learning-set", which indicates that animals can learn to learn (a formation of learning-set). This theory was established by Harry F. Harlow following his pioneering experiments [66] using monkeys that showed errorless solving after a number of discrimination problems, indicating that they had grasped the rule characterizing the task. More specifically, a reversal learning-set is a phenomenon described as a progressive

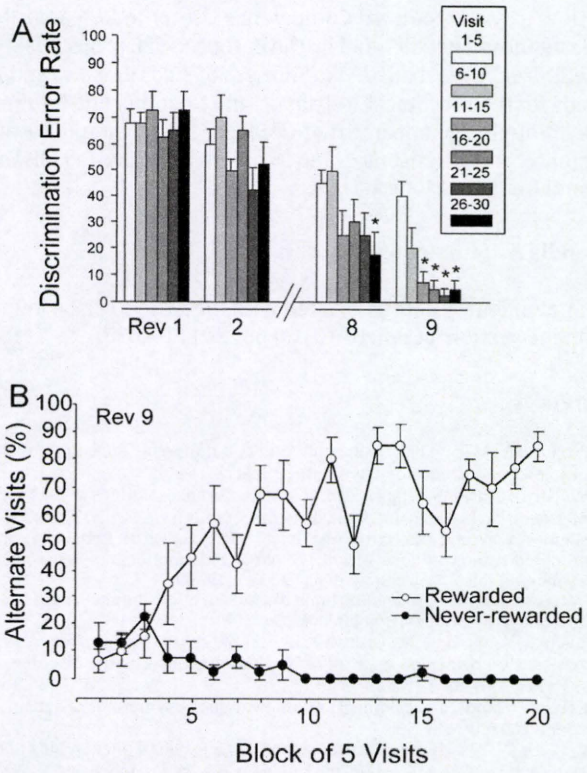


Fig. 7. Rapid adaptation to the reversed contingency in B6-UT mice. (A) Discrimination error rates (B6-UT) of the first 30 visits in the first sessions of Revs 1, 2, 8, and 9. Each bar represents the mean ± S.E.M ($n = 8$) of discrimination error rates compiled for every five-visit block. A significant linear trend of error reduction was observed in the first 30 visits in Revs 8 and 9 ($p < 0.05$, repeated measures ANOVA). The asterisks above the bars indicate a statistically significant difference in discrimination error rate (ANOVA and Dunnett's test, $^*p < 0.05$) compared with that in the first 5 visits in Rev 8 or 9. (B) Intra-session rapid progression of shuttling behavior between rewarded corners (B6-UT, mean ± S.E.M, $n = 8$) in the first session of Rev 9.

improvement over a series of reversal tasks, and has been observed in several animal species [67–70]. In this context, we speculate that the rapid adaptation to reversal learning shown after a series of reversals observed in this study reflects a long term learning effect which was attributed to an adaptation to "reversal rule" itself, or a reversal "learning-set".

4.2. Comparison among laboratories, ages and strains

Comparisons of the results from three independent laboratories showed that essentially the same results in the behavioral sequencing task and its serial reversals can be replicated in all the three strains (C57BL/6, DBA/2 and ICR) and in all of our laboratories (UT, JMU and UZH) (see Supplemental Fig.S2 and Figs. 4 and 6). However, despite the overall concordance in trends of the data, there were some differences among the laboratories particularly in the minimal discrimination error rates. Although the minimal error scores were relatively constant at each laboratory, best at UT (approx. 5–10%), somewhat higher at JMU, and highest at UZH (approx. 20–30%), the possible reasons for such differences as observed in the three laboratories are considered to be threefold. First, the highest minimal error scores in UZH might be attributable to the imposed shortening of the deprivation time, and with this, the motivation of the mice. The error visits of mice in UZH can be thought to reflect the exploratory patrolling of corners, which occurs spontaneously also under non-rewarded conditions. Thus, a sufficient deprivation time appears to be a critical factor in the protocol used. Second, the relatively small differences between UT and JMU may be attributable to the presence or absence of external LED light cues that indicated the task period (22:00–1:00), the light of which was not originally present in the IntelliCage system. This extra cue was installed at UT, but not at JMU or UZH, and it is considered to give mice a chance to reduce the minimal error scores at UT, but not at JMU or UZH. Third, a difference in the schedule of acclimatization, particularly the period after the time restriction was started (5, 4 and 3 days in UT, JMU and UZH, respectively) could be another factor that might produce the observed differences among the three laboratories. Although there were some uncontrolled conditions among the laboratories as mentioned above, it should be pointed out here that this study was not initially intended to carry out inter-laboratory comparisons. If such comparisons were planned, much more stringent experimental controls, such as raising all the mice in the same place, synchronized shipping and other requirements described in detail elsewhere [24,71], should have been made.

We compared the age and strain differences in learning performance using the UZH data. We could not detect any significant age-related difference in learning performances between the 3- and 12-month cohorts of C57BL/6 and between those of DBA/2. While some studies showed that mice in "middle-age" (8–18 months) had age-related cognitive or learning declines in the tasks of spatial learning, working memory, and contextual fear conditioning [72–77], other studies showed that middle-aged mice did not have a learning deficit, or even had a better learning performance than those of young mice [23,75,78]. Since most of the studies that showed the age-related cognitive and learning declines used mice over 20 months of age, further research using middle-aged mice to clarify its aging effect in details is necessary. Additionally, the possible effect of environmental enrichment by housing and testing mice in IntelliCage might contribute to preventing their age-related decline of learning performance. In the previous studies using mice, environmental enrichment (i.e., socially housed in a large cage with toys and/or running wheels) was found to have the capacity to cancel age-related learning impairments [73,74]. However, modifications of the task protocol may increase the sensitivity to detect more subtle signs of cognitive inflexibility in the middle-aged mice. This point should be given careful attentions in future studies. Second, strain difference analysis was also carried out on the data obtained at UZH (young B6-UZH vs. young D2-UZH, and aged B6-UZH vs. aged D2-UZH). As a result, significant main effects of strain for discrimination error rate were detected in Rev 1, 2, 3, and 4 between the young cohorts, and in Rev 1,2, and 3 between the aged cohorts, consistently showing higher discrimination error rates in C57BL/6 than in DBA/2 mice ($p < 0.05$, repeated measures ANOVA). These results were in line with a previous study by Krackow *et al.* [24] where a simple place learning task paradigm in IntelliCage was used, and better performance in reversal learning was shown in DBA/2 than in C57BL/6 mice. Although the strain difference in learning ability seems to be consistent, it may be difficult to simply speculate that the DBA/2 mice learned the task better than the C57BL/6 mice in this study. This is because a persistent significant difference in the average number of visits per session was also observed between those strains throughout the experiment (data not shown). The tested C57BL/6 mice showed consistently about 40% higher number of visits on average than the DBA/2 mice, and thus, the higher error rate shown in C57BL/6 than in DBA/2 might be attributed to differences in basic behavioral characteristics such as hyper locomotion or impulsivity. However, it is notable that all the cohorts of mice tested in this study showed similar and stable learning and relearning curves of discriminative performance in this behavioral flexibility test.

4.3. Applications of IntelliCage system

The use of IntelliCage has demonstrated distinct advantages over conventional manual or semi-automated testing methods for

individual mice. This fully automated system can minimize the negative effects of human handling, and thus mice can focus on events or tasks within their cage. IntelliCage also provides social context and environmentally enriched housing for mice.

On the other hand, some limitations for analyzing mice behavior are present in this task due to the more naturalistic approach that makes it difficult to exclude the possible effects of variability from social and individual behavioral contexts on the experimental results. In this regard, the variability in the social context and environmentally-enriched housing might induce some behavioral variability [71]. However, a recent report by Krackow *et al.* [24] showed that such behavioral variability did not seem to diminish the reproducibility of the main results in the specifically designed inter-laboratory comparisons using IntelliCage. The inter-laboratory comparison across three laboratories in this study also strongly indicates an excellent reproducibility of the main test results. By contrast, it is difficult to clearly evaluate whether and to what extent the behavioral context in the task, on individual animal basis, might affect the overall main result of the present study. For example, it is up to each mouse to decide when to start, stop, and resume their responses during the period of the task, which is difficult to be controlled strictly. However, we would speculate that the prolonged monitoring of individual animals over several weeks minimized the possibility of misreading the daily variability of task performance on individual animal basis, thus, allowed us to detect subtle differences in genetic or epigenetic individual characteristics [19–27].

The protocol we developed for the present study added significant assets to the IntelliCage system, in the following two aspects. First, the readout of learning effect in the behavioral sequencing task can be readily achieved because the observed learning curves of discrimination error rate were clear and consistent among cohorts. Second, the serial reversal learning paradigm for assessing behavioral flexibility in IntelliCage was established.

5. Conclusion

In conclusion, we established a test protocol for mice to assess behavioral flexibility as an advanced cognitive function in a group-housed environment and validated its reproducibility by inter-laboratory comparisons. It provides unique and detailed analyses of place discrimination in the behavioral sequencing task and behavioral flexibility in the subsequent serial reversals. Thus, the current protocol for IntelliCage not only lessens the shortcomings of the existing methods used to assess cognitive behavior of mice, namely, efficiency, reproducibility and standardization, but also offers a uniquely successful paradigm for unveiling the otherwise overlooked higher-order cognitive functions in this animal species.

Acknowledgements

The studies at UT were supported in part by a Health and Labour Sciences Research Grant from the Ministry of Health, Labour and Welfare, Japan (to MK), grants from the Environmental Technology Development Fund (to CT), a Grant-in-aid for Young Scientists (A) and (S) from the Japan Society for the Promotion of Science (JSPS) (to MK), and Global COE Program "Medical System Innovation on Multidisciplinary Integration" from MEXT, Japan (to TE). The studies at JMU were supported by Grant-in-Aid for Scientific Research on Priority Areas (15081101), for Scientific Research (B) (20390061), and for Challenging Exploratory Research (22659044) from Japan Society for the Promotion of Science (JSPS) to TY, and a grant from the 21st century Center of Excellence (COE) program (to TY) and the Japan Brain Foundation (to FM). The Swiss studies were supported by the Swiss National Science Foundation (to HPL and DPW), by the National Competence Center in Research "Neural Plasticity and Repair", and by the European FP6 project 0379065 ("IntelliMaze"). We thank Akiko Shimazaki, Yuki Hirasawa and Yuki Yoshida for their technical assistance, and Nozomi Endo for the artwork of IntelliCage apparatus at UT, Inger Drescher and Rosmarie Lang for technical assistance, and Drs. Sven Krackow and Elisabetta Vannoni for discussions at UZH.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbr.2011.02.037.

References

[1] Picciotto M, Wickman K. Using knockout and transgenic mice to study neurophysiology and behavior. Physiol Rev 1998;78:1131–63.
[2] Wolfer D, Crusio W, Lipp H. Knockout mice: simple solutions to the problems of genetic background and flanking genes. Trends Neurosci 2002;25:336–40.
[3] Holmes A, Wrenn CC, Harris AP, Thayer KE, Crawley JN. Behavioral profiles of inbred strains on novel olfactory, spatial and emotional tests for reference memory in mice. Genes Brain Behav 2002;1:55–69.
[4] Watase K, Zoghbi H. Modelling brain diseases in mice: the challenges of design and analysis. Nat Rev Genet 2003;4:296–307.
[5] Takao K, Yamasaki N, Miyakawa T. Impact of brain-behavior phenotypying of genetically-engineered mice on research of neuropsychiatric disorders. Neurosci Res 2007;58:124–32.
[6] Crawley J. Mouse behavioral assays relevant to the symptoms of autism. Brain Pathol 2007;17:448–59.
[7] Crawley JN. What's wrong with my mouse?: Behavioral phenotyping of transgenic and knockout mice. 2nd ed. Hoboken, N.J.: Wiley-Interscience; 2007.
[8] Moy S, Nadler J. Advances in behavioral genetics: mouse models of autism. Mol Psychiatry 2008;13:4–26.
[9] Wahlsten D, Metten P, Phillips T, Boehm Sn, Burkhart-Kasch S, Dorow J, et al. Different data from different labs: lessons from studies of gene-environment interaction. J Neurobiol 2003;54:283–311.
[10] Crabbe J, Wahlsten D, Dudek B. Genetics of mouse behavior: interactions with laboratory environment. Science 1999;284:1670–2.
[11] Brunner D, Nestler E, Leahy E. In need of high-throughput behavioral systems. Drug Discov Today 2002;7:S107–12.
[12] Wahlsten D, Rustay N, Metten P, Crabbe J. In search of a better mouse test. Trends Neurosci 2003;26:132–6.
[13] Tecott L, Nestler E. Neurobehavioral assessment in the information age. Nat Neurosci 2004;7:462–6.
[14] Fraser LM, Brown RE, Hussin A, Fontana M, Whittaker A, O'Leary TP, et al. Measuring anxiety- and locomotion-related behaviors in mice: a new way of using old tests. Psychopharmacology (Berl) 2010;211:99–112.
[15] Gerlai R. Behavioral tests of hippocampal function: simple paradigms complex problems. Behav Brain Res 2001;125:269–77.
[16] Gerlai R. Phenomics: fiction or the future? Trends Neurosci 2002;25:506–9.
[17] Spruijt B, de Visser L. Advanced behavioral screening; automated home cage ethology. Drug Discov Today 2006;3:231–7.
[18] Goulding E, Schenk A, Juneja P, MacKay A, Wade J, Tecott L. A robust automated system elucidates mouse home cage behavioral structure. Proc Natl Acad Sci USA 2008;105:20575–82.
[19] Galsworthy M, Amrein I, Kuptsov P, Poletaeva I, Zinn P, Rau A, et al. A comparison of wild-caught wood mice and bank voles in the Intellicage: assessing exploration, daily activity patterns and place learning paradigms. Behav Brain Res 2005;157:211–7.
[20] Knapska E, Walasek G, Nikolaev E, Neuhäusser-Wespy F, Lipp H, Kaczmarek L, et al. Differential involvement of the central amygdala in appetitive versus aversive learning. Learn Mem 2006;13:192–200.
[21] Onishchenko N, Tamm C, Vahter M, Hökfelt T, Johnson J, Johnson D, et al. Developmental exposure to methylmercury alters learning and induces depression-like behavior in male mice. Toxicol Sci 2007;97:428–37.
[22] Rudenko O, Tkach V, Berezin V, Bock E. Detection of early behavioral markers of Huntington's disease in R6/2 mice employing an automated social home cage. Behav Brain Res 2009;203:188–99.
[23] Mechan A, Wyss A, Rieger H, Mohajeri M. A comparison of learning and memory characteristics of young and middle-aged wild-type mice in the IntelliCage. J Neurosci Methods 2009;180:43–51.
[24] Krackow S, Vannoni E, Codita A, Mohammed A, Cirulli F, Branchi I, et al. Consistent behavioral phenotype differences between inbred mouse strains in the IntelliCage. Genes Brain Behav 2010.
[25] Barlind A, Karlsson N, Björk-Eriksson T, Isgaard J, Blomgren K. Decreased cytogenesis in the granule cell layer of the hippocampus and impaired place learning after irradiation of the young mouse brain evaluated using the IntelliCage platform. Exp Brain Res 2010;201:781–7.
[26] Codita A, Gumucio A, Lannfelt L, Gellerfors P, Winblad B, Mohammed A, et al. Impaired behavior of female tg-ArcSwe APP mice in the IntelliCage: a longitudinal study. Behav Brain Res 2010.

[27] Voikar V, Colacicco G, Gruber O, Vannoni E, Lipp H, Wolfer D. Conditioned response suppression in the IntelliCage: assessment of mouse strain differences and effects of hippocampal and striatal lesions on acquisition and retention of memory. Behav Brain Res 2010;213:304–12.
[28] Miller E. The prefrontal cortex and cognitive control. Nat Rev Neurosci 2000;1:59–65.
[29] Royall D, Lauterbach E, Cummings J, Reeve A, Rummans T, Kaufer D, et al. Executive control function: a review of its promise and challenges for clinical research. A report from the Committee on Research of the American Neuropsychiatric Association. J Neuropsychiatry Clin Neurosci 2002;14:377–405.
[30] Koechlin E, Summerfield C. An information theoretical approach to prefrontal executive function. Trends Cogn Sci 2007;11:229–35.
[31] Chan R, Shum D, Toulopoulou T, Chen E. Assessment of executive functions: review of instruments and identification of critical issues. Arch Clin Neuropsychol 2008;23:201–16.
[32] Robbins T, Arnsten A. The neuropsychopharmacology of fronto-executive function: monoaminergic modulation. Annu Rev Neurosci 2009;32:267–87.
[33] Marazziti D, Consoli G, Picchetti M, Carlini M, Faravelli L. Cognitive impairment in major depression. Eur J Pharmacol 2010;626:83–6.
[34] Kerns J, Nuechterlein K, Braver T, Barch D. Executive functioning component mechanisms and schizophrenia. Biol Psychiatry 2008;64:26–33.
[35] Hill E. Executive dysfunction in autism. Trends Cogn Sci 2004;8:26–32.
[36] Hill E, Bird C. Executive processes in Asperger syndrome: patterns of performance in a multiple case series. Neuropsychologia 2006;44:2822–35.
[37] Kipp K. A developmental perspective on the measurement of cognitive deficits in attention-deficit/hyperactivity disorder. Biol Psychiatry 2005;57:1256–60.
[38] Macmillan M. Restoring Phineas Gage: a 150th retrospective. J Hist Neurosci 2000;9:46–66.
[39] Barker-Collo S, Feigin V. The impact of neuropsychological deficits on functional stroke outcomes. Neuropsychol Rev 2006;16:53–64.
[40] Colacicco G, Welzl H, Lipp H, Würbel H. Attentional set-shifting in mice: modification of a rat paradigm, and evidence for strain-dependent variation. Behav Brain Res 2002;132:95–102.
[41] Bissonette GB, Martins GJ, Franz TM, Harper ES, Schoenbaum G, Powell EM. Double dissociation of the effects of medial and orbital prefrontal cortical lesions on attentional and affective shifts in mice. J Neurosci 2008;28:11124–30.
[42] Brigman JL, Mathur P, Harvey-White J, Izquierdo A, Saksida LM, Bussey TJ, et al. Pharmacological or genetic inactivation of the serotonin transporter improves reversal learning in mice. Cereb Cortex 2010;20:1955–63.
[43] Young JW, Powell SB, Geyer MA, Jeste DV, Risbrough VB. The mouse attentional-set-shifting task: a method for assaying successful cognitive aging? Cogn Affect Behav Neurosci 2010;10:243–51.
[44] Kolata S, Light K, Grossman HC, Hale G, Matzel LD. Selective attention is a primary determinant of the relationship between working memory and general learning ability in outbred mice. Learn Mem 2007;14:22–8.
[45] Burgess P, Shallice T. The Hayling Brixton tests. Thurston, U.K: Thames Valley Test Company; 1997.
[46] Shallice T, Burgess P. The domain of supervisory processes and temporal organization of behavior. Philos Trans R Soc Lond B Biol Sci 1996;351:1405–11, discussion 11-2.
[47] Burgess PW, Shallice T. Bizarre responses, rule detection and frontal lobe lesions. Cortex 1996;32:241–59.
[48] Andres P, Van der Linden M. Age-related differences in supervisory attentional system functions. J Gerontol B Psychol Sci Soc Sci 2000;55:373–80.
[49] Gioia GA, Isquith PK. Ecological assessment of executive function in traumatic brain injury. Dev Neuropsychol 2004;25:135–58.
[50] Wood RL, Liossi C. The ecological validity of executive tests in a severely brain injured sample. Arch Clin Neuropsychol 2006;21:429–37.
[51] Wood RL, Liossi C. The relationship between general intellectual ability and performance on ecologically valid executive tests in a severe brain injury sample. J Int Neuropsychol Soc 2007;13:90–8.
[52] Bielak AA, Mansueti L, Strauss E, Dixon RA. Performance on the Hayling and Brixton tests in older adults: norms and correlates. Arch Clin Neuropsychol 2006;21:141–9.
[53] Morris RG, Garrud P, Rawlins JN, O'Keefe J. Place navigation impaired in rats with hippocampal lesions. Nature 1982;297:681–3.
[54] Morris RG, Anderson E, Lynch GS, Baudry M. Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist AP5. Nature 1986;319:774–6.
[55] Gass P, Wolfer D, Balschun D, Rudolph D, Frey U, Lipp H, et al. Deficits in memory tasks of mice with CREB mutations depend on gene dosage. Learn Mem 1998;5:274–88.
[56] Chen G, Chen K, Kobayashi D, Barbour R, Motter R, Games D, et al. Active beta-amyloid immunization restores spatial learning in PDAPP mice displaying very low levels of beta-amyloid. J Neurosci 2007;27:2654–62.
[57] Lewejohann L, Skryabin B, Sachser N, Prehn C, Heiduschka P, Thanos S, et al. Role of a neuronal small non-messenger RNA: behavioral alterations in BC1 RNA-deleted mice. Behav Brain Res 2004;154:273–89.
[58] Dillon G, Qu X, Marcus J, Dodart J. Excitotoxic lesions restricted to the dorsal CA1 field of the hippocampus impair spatial memory and extinction learning in C57BL/6 mice. Neurobiol Learn Mem 2008;90:426–33.
[59] Voikar V, Vasar E, Rauvala H. Behavioral alterations induced by repeated testing in C57BL/6J and 129S2/Sv mice: implications for phenotyping screens. Genes Brain Behav 2004;3:27–38.
[60] Tanimura Y, Yang M, Lewis M. Procedural learning and cognitive flexibility in a mouse model of restricted, repetitive behavior. Behav Brain Res 2008;189:250–6.
[61] Izquierdo A, Wiedholz LM, Millstein RA, Yang RJ, Bussey TJ, Saksida LM, et al. Genetic and dopaminergic modulation of reversal learning in a touch screen-based operant procedure for mice. Behav Brain Res 2006;171:181–8.
[62] Delcasso S, Jeantet Y, Cho Y. A new test for long-term spatial memory using an operant chamber in mice. Behav Brain Res 2007;178:200–7.
[63] Brigman J, Feyder M, Saksida L, Bussey T, Mishina M, Holmes A. Impaired discrimination learning in mice lacking the NMDA receptor NR2A subunit. Learn Mem 2008;15:50–4.
[64] Brigman J, Ihne J, Saksida L, Bussey T, Holmes A. Effects of subchronic phencyclidine (PCP) treatment on social behaviors, and operant discrimination and reversal learning in C57BL/6J mice. Front Behav Neurosci 2009;3:2.
[65] Karlsson RM, Tanaka K, Saksida LM, Bussey TJ, Heilig M, Holmes A. Assessment of glutamate transporter GLAST (EAAT1)-deficient mice for phenotypes relevant to the negative and executive/cognitive symptoms of schizophrenia. Neuropsychopharmacology 2009;34:1578–89.
[66] Harlow H. The formation of learning sets. Psychol Rev 1949;56:51–65.
[67] Schusterman R. Successive discrimination-reversal training and multiple discrimination training in one-trial learning by chimpanzees. J Comp Physiol Psychol 1964;58:153–6.
[68] Schusterman R. Serial discrimination-reversal learning with and without errors by the California sea lion. J Exp Anal Behav 1966;9:593–600.
[69] Bond A, Kamil A, Balda R. Serial reversal learning and the evolution of behavioral flexibility in three species of North American corvids (Gymnorhinus cyanocephalus, Nucifraga columbiana, Aphelocoma californica). J Comp Psychol 2007;121:372–9.
[70] van der Plasse G, Feenstra M. Serial reversal learning and acute tryptophan depletion. Behav Brain Res 2008;186:23–31.
[71] Wolfer D, Litvin O, Morf S, Nitsch R, Lipp H, Würbel H. Laboratory animal welfare: cage enrichment and mouse behavior. Nature 2004;432:821–2.
[72] Frick KM, Burlingame LA, Arters JA, Berger-Sweeney J. Reference memory, anxiety and estrous cyclicity in C57BL/6NIA mice are affected by age and sex. Neuroscience 2000;95:293–307.
[73] Frick KM, Stearns NA, Pan JY, Berger-Sweeney J. Effects of environmental enrichment on spatial memory and neurochemistry in middle-aged mice. Learn Mem 2003;10:187–98.
[74] Frick KM, Fernandez SM. Enrichment enhances spatial memory and increases synaptophysin levels in aged female mice. Neurobiol Aging 2003;24:615–26.
[75] Magnusson KR, Scruggs B, Aniya J, Wright KC, Ontl T, Xing Y, et al. Age-related deficits in mice performing working memory tasks in a water maze. Behav Neurosci 2003;117:485–95.
[76] Verbitsky M, Yonan AL, Malleret G, Kandel ER, Gilliam TC, Pavlidis P. Altered hippocampal transcript profile accompanies an age-related spatial memory deficit in mice. Learn Mem 2004;11:253–60.
[77] Kaczorowski CC, Disterhoft JF. Memory deficits are associated with impaired ability to modulate neuronal excitability in middle-aged mice. Learn Mem 2009;16:362–6.
[78] Wong AA, Brown RE. Age-related changes in visual acuity, learning and memory in C57BL/6J and DBA/2J mice. Neurobiol Aging 2007;28:1577–93.

Legends to Supplemental Figures

Supplemental Figure S1. Probe trial for the validation of behavioral sequencing task as a place learning paradigm. For this additional test, another cohort of C57BL/6 mice (n=14) was used at UT. The mice were acclimatized to the IntelliCage apparatus and trained for the behavioral sequencing task with the same schedule used for other cohorts studied at UT (B6-UT and D2-UT) until session 7. On the following day, the cage and all the corner chambers were fully replaced with a different set of IntelliCage apparatus. Then, the mice were subjected to a probe trial. In the probe trial, the LED light cue was turned on at 22:00 to signal the start of the water-availability period in the past sessions, but they could not receive water even when visiting the previously learned rewarded corners. The probe trial was carried out based on the following hypothesis: if the mice are utilizing non-spatial cues such as odors in the cage to discriminate the corners, then changing a set of IntelliCage apparatuses will diminish their preference to visiting the correct corners. In addition, in the probe trial the mice were not given a reward even when they visited the correct corner. The reason was to observe whether the mice would show their preference to visit the correct corner by utilizing the memory that they acquired before the probe trial, but not the memory newly acquired from the probe trial.

As a result, the mice showed a significantly strong preference to the correct corner in the probe trial without a reward. This preference indicates that the mice certainly had "learned" the corner discrimination based more on spatial strategy instead of the local cues before the probe trial. Each bar represents the mean ± S.E.M of number of visits to correct (learned as rewarded) and incorrect (learned as never-rewarded) corners in every ten minutes after the beginning of the probe trial. Asterisks above bars indicate significantly smaller number of visits to incorrect corners compared to that of correct corners in each bin of 10 minutes (t-test, $*p<0.05$).

Supplemental Figure S2. Reduction of visits to never-rewarded corners (errors) in the acquisition phase in (A) young B6-UZH, (B) aged B6-UZH, (C) B6-JMU, (D) D2-UT, (E) young D2-UZH, (F) aged D2-UZH and (G) ICR-JMU. Discrimination error rate (mean ± S.E.M, n=11, 12, 15, 8, 12, 11, and 11, respectively from panel (A) to (G)) was defined as the number of visits to the two never-rewarded corners within the first 100 visits in each session, which makes the chance value of discrimination error rates 50%. Statistical analysis showed significant linear trends of error reduction ($p<0.05$, one-way repeated measures ANOVA with session as a factor) in all the cohorts.

Supplemental Figure S3. Shuttling behavior measures in sessions 1, 7, 8 (the first session of Rev 1), and 15 (the first session of Rev 2) of B6-UT mice. Means ± S.E.M (n=8) of ratio of alternate visits were plotted. Ratio of alternative choices = (number of alternate visits between the rewarded or never-rewarded corners)/block of five visits x 100.

Supplemental Figure S4. Profiling of corner visit of an individual mouse of B6-UT in sessions 1 and 7 (the first and last session in the acquisition phase, respectively), and the first sessions of Revs 1, 2, and 9. Blue (navy and sky) and red (red and pink) boxes indicate a visit to rewarded and never-rewarded corners, respectively. The patterns of moving from the previously visited corner to the indicated corner are further coded in the box. Navy blue and red boxes with a diagonal line indicate that the mouse visited the rewarded/never-rewarded corner from the diagonally opposite rewarded/never-rewarded corner. Navy blue and red boxes without a mark indicate that the mouse visited the corner from the adjacent corners. Sky blue and pink boxes without a mark indicate that the mouse continuously revisited the same corner.