Evaluation of Pax6 Mutant Rat as a Model for Autism

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Abstract

Autism is a highly variable brain developmental disorder and has a strong genetic basis. Pax6 is a pivotal player in brain development and maintenance. It is expressed in embryonic and adult neural stem cells, in astrocytes in the entire central nervous system, and in neurons in the olfactory bulb, amygdala, thalamus, and cerebellum, functioning in highly context-dependent manners. We have recently reported that Pax6 heterozygous mutant (rSey²/+ ) rats with a spontaneous mutation in the Pax6 gene, show impaired prepulse inhibition (PPI). In the present study, we further examined behaviors of rSey²/+ rats and revealed that they exhibited abnormality in social interaction (more aggression and withdrawal) in addition to impairment in rearing activity and in fear-conditioned memory. Ultrasonic vocalization (USV) in rSey²/+ rat pups was normal in male but abnormal in female. Moreover, treatment with clozapine successfully recovered the defects in sensorimotor gating function, but not in fear-conditioned memory. Taken together with our prior human genetic data and results in other literatures, rSey²/+ rats likely have some phenotypic components of autism.

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Introduction

Autism is a highly variable brain developmental disorder defined by three core symptoms with onset prior to 3 years of age: atypical social behavior; disrupted verbal and non-verbal communication; and unusual patterns of highly restricted interests and repetitive behaviors [1–4]. In addition to the major symptoms, there are several associated emotional manifestations in autism, such as depression and increased anxiety and fear [5–7]. Furthermore, the concept of autism itself has been broadened and now includes the group of syndromes referred to as pervasive developmental disorders including Asperger’s disorder, pervasive developmental disorder not otherwise specified, childhood disintegrative disorder, and Rett’s disorder in the Diagnostic and Statistical Manual of Mental Disorders IV [4]. Collectively, these disorders are known as the autism spectrum disorders (ASD). The existence of multiple symptom domains and the spectrum of related disorders demonstrate the complexity of the autism phenotype.

Several twin studies indicate that concordance rates for monozygotic twins (70–90%) are several-fold higher than those for dizygotic twins (−10%) [2,8–10]. These indicate that genetic factors play important roles in its etiology. However, the identification of susceptibility genes has been hindered by the heterogeneity of the syndrome, insufficient numbers of analyzed samples and small effect size of each risk gene, compared to other physical complex disorders [11]. Under these circumstances, to search for rare risk variants with substantial effects may be a fruitful approach [12].

Autism is psychiatric illnesses in which complicated information processing might be disturbed at different levels of brain development, introducing substantial heterogeneity [13]. Fundamental differences in the underlying neurodevelopmental disruptions probably lead to the heterogeneity in both symptoms and developmental course that are characteristic of autism. Various genes operate to form the brain through neurogenesis, gliogenesis, area formation and neuronal circuit formation. Pax6 gene encodes a transcription factor that is essential for neurodevelopment, and is expressed in restricted regions of the forebrain, hindbrain, and spinal cord appearing as early as at embryonic day 8.5 (E8.5) and throughout life in certain brain regions such as the amygdala, olfactory bulb, pyriform cortex, and dentate gyrus and also in astrocytes [14–17]. Human Pax6 gene is originally identified in chromosomal region 11p13 as one related with WAGR (Wilm’s tumor, Aniridia, Genitourinary malformations and mental Retardation) syndrome [18,19], which is a rare genetic disorder caused by chromosomal deletion of the 11p12-p14 region. The majority of WAGR patients have mental retardation and behavioral problems, and importantly, more than 20% of the patients also have features of autism [20,21]. Recent studies have identified Pax6 mutations in individuals who manifest mental retardation, aniridia and autism.
[22–24]. Furthermore, chromosome 11p13, on which PAX6 is located, is implicated as a possible locus for autism susceptibility by a linkage study [25]. These accumulating lines of evidence suggest that PAX6 mutations display concomitant phenotypes of autism. Recently, our group has carried out resequencing analysis of the gene in autistic patients with aniridia, with the aim of searching for additional mutations of PAX6, which might be associated with the disease. As a result we have detected a novel missense mutation of PAX6 in an autistic patient, not found in 2,120 non-autistic subjects [26]. These suggest that a part of autistic patients carry rare PAX6 mutations and that Pax6 dysfunction during neurodevelopment might be responsible for autistic behaviors.

To address this issue using experimental animals in the current study, we performed the detailed analyses of rSey2+/+ rats that have spontaneous nonsense mutations in the Pax6 gene [27,28], in terms of behavioral tests, biochemical analysis and pharmacological examination.

Results

Abnormal exploratory behavior toward a novel environment in rSey2+/+ rats

rSey2+/+ rats were fertile and showed no change in sexual behavior and litter size compared with the wild type rats (WT). Apparently, rSey2+/+ rats exhibited no ataxia or seizure, and moved normally as judged from their footprint patterns (data not shown). To assess whether rSey2+/+ rats display autistic phenotypes, we executed several behavioral tests using only male rats to avoid potential influence of menstrual cycles except for USV test.

Firstly we conducted open-field tests using a box equipped with photobeam sensors given a 15 minutes session [29,30]. In this test, no significant differences were observed in times spent in locomotion, distances of locomotion, speed of movement and times spent in the central and peripheral zone of the open field (Fig. 1A–C, data not shown). However, rearing activity was significantly lower in rSey2+/+ rats than in WT (two-way analysis of variance (ANOVA) with repeated measures (genotype × time); main effect of factor ‘genotype’: F(1) = 6.54, P<0.02; Fig. 1D). The result suggests that rSey2+/+ rats may be less exploratory or more anxious to their novel environment [31,32].

To assess the level of anxiety in rSey2+/+ rats, we next carried out a light-dark (LD) choice test [33]. rSey2+/+ rats spent more time in the light side of the test box than that WT did (two-way ANOVA with repeated measures (genotype × time); main effect of factor ‘genotype’: F(1) = 7.06, P<0.02; Fig. 2A). In addition, the number of entries into the dark box was increased in rSey2+/+ rats compared with that of WT (two-way ANOVA with repeated measures (genotype × time); main effect of factor ‘genotype’: F(1) = 6.02, P<0.02; Fig. 2B). Because rodents prefer the dark environment, rSey2+/+ rats might be less anxious. However, we have to bear in mind that rSey2+/+ rats have eye defects such as decreased eye size, and various levels of cataract and iris hyperplasia, though these phenotypic variation seems to be less than those in Sey mice [34]. rSey2+/+ rats could discriminate a difference between lightness and darkness since the time percentages spent in the light and dark sides were not equal. Anyway, the results of LD test should be interpreted with caution.

To further examine emotional behavior in rSey2+/+ rats, we performed forced swim test [35]. There were no differences between WT and rSey2+/+ rats in immobility times at 1st and 2nd trials of the tests (Fig. 2C, D). Taken altogether, it is reasonable to assume that impaired rearing activity in rSey2+/+ rats may not be related to increased anxiety or depressed state but rather to a less exploratory phenotype.

Abnormality in social interactions in rSey2+/+ rats

A critical component in a model animal of autism is a quantitative measure of appropriate social interaction. To address this issue, we examined social interactions of WT and rSey2+/+ rats by measuring time spent in locomotion, aggression, following, passive body contact, allo-grooming, mounting, sniffing, and isolation (WT, 14 pairs; rSey2+/+ rats, 16 pairs).

At 14–17 week-old, rSey2+/+ rats clearly showed more aggressive behavior than WT (t-test; P<0.01; Fig. 3A), while the former showed less following behavior than the latter (t-test; P<0.01; Fig. 3A). There were no differences in the other behaviors (locomotion, passive body contact, allo-grooming, mounting, sniffing and isolation). Interestingly, these features became much more obvious at full adult stages (36–40 weeks). rSey2+/+ rats of these ages still showed aggressive behavior, although aged WT rats never did (t-test; P<0.001; Fig. 3B). Moreover, rSey2+/+ rats exhibited significantly less following (t-test; P<0.05; Fig. 3B) and passive body contact behaviors (t-test; P<0.01; Fig. 3B). Thus rSey2+/+ rats clearly exhibited abnormalities in social interaction; they were more aggressive at fighting and less interested in a strange partner.

Abnormality in fear-conditioned memory in rSey2+/+ rats

Next we examined performance in memory of rSey2+/+ rats. Because behavioral tasks requiring visual function may be influenced by potential visual impairment in rSey2+/+ rats due to eye abnormalities, we chose a tone fear-conditioning test using acoustic stimuli in a context-independent way [36]. For conditioning, an electrical shock (0.3 mA) was given just after 20 s tone (Fig. 4A). Although freezing response immediately after the foot shock (Fig. 4A) was not different between WT and rSey2+/+ rats, the latter exhibited significantly reduced freezing response 48 h (two-way ANOVA with repeated measures (genotype × time); main effect of factor ‘genotype’: F(1) = 9.20, P<0.01; Fig. 4B), and 96 h (two-way ANOVA with repeated measures (genotype × time); main effect of factor ‘genotype’: F(1) = 11.40, P<0.005; Fig. 4C) after the initial training session. Since auditory ability and electric foot shock sensitivity may affect freezing responses, we performed auditory threshold test (Fig. 4D) and shock sensitivity test (Fig. 4E). There were no differences in auditory threshold for orienting sound source and shock sensitivity. Therefore, rSey2+/+ rats had low performance in the context-independent tone-fear conditioned memory.

Abnormality in ultrasonic vocalization test

Next we tested communicative behavior of rSey2+/+ rats by recording isolation-induced USV [37] of male postnatal day 7 (P7) rat pups that were separated from their dams. Number of ultrasonic calls (Fig. 5A), and mean duration of calls (Fig. 5B), latency to start calling (Fig. 5C), and peak frequency (Fig. 5D) were not different between WT and rSey2+/+ male rat pups, suggesting that communicative behavior of rSey2+/+ male rats was normal. We also conducted USV test on rSey2+/+ female rat pups since they were not yet influenced by sexual cycles. It is of note that rSey2+/+ female rat pups emitted fewer calls than WT (two-way ANOVA measures (genotype × gender); interaction: F(1) = 4.20, P<0.05; main effect of factor ‘gender’: F(1) = 10.53, P<0.002; not significant main effect of factor ‘gender’; Fig. 5B). These results imply that rSey2+/+ rat female pups exhibit less property toward their mothers as similarly observed in autistic infants. This finding is quite interesting since we reported an autistic girl who has a mutation in the PAX6 gene inherited from her father who is not diagnosed as autism [26].
Decreased serotonin levels in plasma and brain samples of rSey2/+ rats

Although the relationship between autism and abnormal serotonin (5-HT) levels is still unclear, an imbalance of 5-HT concentrations in brain and/or in blood is believed to cause many of the characteristic symptoms of autism [38–40]. We measured platelet-poor-plasma (PPP) 5-HT levels in adult male rats (18–20 weeks) using high-performance liquid chromatography (HPLC) equipped with an electrochemical detection system. As shown in Fig. 6A, PPP 5-HT levels in rSey2/+ male rats were lower than in WT (t-test: P < 0.05). We also measured the 5-HT level in the hippocampus. Intriguingly, 5-HT level in the rSey2/+ hippocampus was marginally reduced compared to that in WT (t-test: P = 0.083; Fig. 6B).

Anti-psychotic drug recovers PPI

One goal to establish a rodent model for mental diseases is to use it for discovery of new drugs for therapy. As described above, rSey2/+ rats showed several phenotypes related to autism together with impairment of PPI [41]. In addition, 5-HT levels were abnormal in the brain and serum of rSey2/+ rats. Therefore, we tested effects of clozapine, an anti-psychotic drug that targets both 5-HT receptor and dopamine receptor D4 [42]. WT and rSey2/+ rats at 12–16 weeks were intraperitoneally treated either with saline (control) or with clozapine (1.5 mg/kg body weight) 30 min before behavior tests. Interestingly, rSey2/+ rats treated with clozapine improved scores of PPI (t-test: P < 0.05; Fig. 7A). In marked contrast, clozapine-treated rSey2/+ rats showed no recovery on rearing behavior and on tone-fear conditioned memory at 48 h and 96 h (data not shown). Moreover, clozapine had no effects on foot shock sensitivity and acoustic startle response (data not shown). These results suggest that clozapine is effective to improve sensorimotor deficits in rSey2/+ rats but does not alter other behavioral phenotypes.

Discussion

In this study, we examined whether and/or how much Pax6 heterozygous rats model autism. Pax6 homozygous mutant mice/rats, in which Pax6 functions are completely lost, die at birth with severe defects in the formation of the eyes, nose, forebrain, and spinal cord [see review by [43]]. There is a case report describing that a fetus with compound homozygous mutations in PAX6 gene exhibits similar congenital defects [44]. On the other hand, Pax6 heterozygous mice/rats are viable and fertile, and show slight defects in formation of the eyes, olfactory bulb and cerebrum [34,45–47]. In human, haploinsufficiency of PAX6 causes the absence or hypoplasia of the anterior commissure, decreased volumes of the corpus callosum and smaller brain size, in addition to aniridia and various eye abnormalities [48–52]. In addition, 11p12-13 locus covering PAX6 gene is suggested as one of the autism linkage loci [25], and we have previously reported a mutation in PAX6 found in an autistic patient [26]. These data suggest that PAX6 haploinsufficiency may give rise to subtle abnormality in brain structures, which may lead to developmental

Figure 1. General motor activity of WT and rSey2/+ rats in an open-field test. Time spent in locomotion (A), distance of locomotion (B), speed of movement (C), rearing counts (D) were measured in WT (blue, n = 22) and rSey2/+ rats (magenta, n = 28). rSey2/+ rats exhibited decrease in the number of rearing compared to WT (genotype: F(1) = 6.54, P < 0.02; time: F(5) = 17.13, P < 0.001; genotype × time: F(5) = 3.74, P < 0.005; D), although time spent in locomotion (A), distance of locomotion (B), and speed of movement (C) were not significantly changed. Data are expressed by mean ± SEM. n.s., not significant ***P < 0.001, compared to WT as determined by Bonferroni post hoc test.

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Figure 2. Anxious and depressive-like behavior in WT and rSey2+/+ rats. Anxious-like behavior was evaluated using a LD test. The mean percentage of time spent in the light compartment (A) and the number of transitions between the two compartments (B) were measured in WT (blue, n = 23) and rSey2+/+ rats (magenta, n = 28). rSey2+/+ rats spent longer time in the light compartment (genotype: F(1) = 7.06, P < 0.02; time: F(3.86) = 6.91, P < 0.001; genotype × time: F(3.86) = 0.56, not significant; A) and exhibited more number of transitions between the two compartments (genotype: F(1) = 6.02, P < 0.02; time: F(3.41) = 5.56, P < 0.001; genotype × time: F(3.41) = 0.49, not significant; B), compared to WT. Depressive-like behavior was evaluated using a forced swim test. Immobility time was calculated in first trial of test (C) and in second trial performed 24 h after the first trial (D). Immobility times were not changed between WT (blue, n = 11) and rSey2+/+ rats (magenta, n = 16). Data are expressed by mean ± SEM. n.s., not significant ***P < 0.001, compared to WT as determined by Bonferroni post hoc test.
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Figure 3. Social interaction test in WT and rSey2+/+ rats. Time spent for social behaviors, including locomotion, aggression, following, passive body contact, allo-grooming, mounting, sniffing and isolation were measured in WT and rSey2+/+ rats using an open-field box (A, B). A pair of WT (blue, 14 pairs) or a pair of rSey2+/+ rats (magenta, 16 pairs) at 14–17 weeks old was placed in an open-field box. rSey2+/+ rats exhibited more aggressive behavior and less following compared with WT (A). A pair of WT (blue, 12 pairs) or a pair of rSey2+/+ rats (magenta, 13 pairs) at 36–40 weeks old also exhibited more aggressive behavior but less following. In addition, decreased passive body contact was observed (B). Data are expressed by mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, compared to WT as determined by t-test.
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disorders such as autism. Our present animal study revealed that rSey2/+ rats indeed displayed some behavioral abnormalities and 5-HT system deficits related to autism.

Comparison of phenotypes between rSey2/+ rats and conditional Pax6 KO mice

In a recent study, conditional Pax6 knockout mice (Pax6fl/fl; Emx1-Cre mutants; Pax6 cKO mice) in which Pax6 expression was completely and specifically abolished in the cortex and hippocampus were generated [53]. They exhibit drastically severe cortical deficits such as loss of upper layers and prefrontal cortex markers, which are not seen in human autism. These Pax6 cKO mice demonstrated a decreased locomotor activity and ataxia due to defects in motor performance and prefrontal deficits, whereas locomotor activity of rSey2/+ rats was normal. Intriguingly, phenotypes of recent memory and extinction of cued fear that are related to amygdala functions, are different between Pax6 cKO mice and rSey2/+ rats. It is considered that Pax6 function may be normal in the Pax6 cKO amygdala because promoter activity of Emx1 is not
working in this brain area [54], whereas expression levels of Pax6 in the amygdala are deemed to be decreased in rSey2/+ rats. As expected, this amygdala-dependent cued-fear conditioned recent memory is normal in Pax6 cKO mice but impaired in rSey2/+ rats.

PPI that reflects the sensorimotor gating system is reported to be abnormal across neuropsychiatric disorders including autism [55] and Asperger’s disorder [56]. PPI scores were not changed in both Pax6 cKO mice and rSey2/+ rats at juvenile. Interestingly, rSey2/+ rats exhibited decreased PPI at 12 weeks and afterwards [41]. These results may mean that impaired Pax6 functions could elicited altered inhibitory control of sensory input which is not obvious in younger animals.

Sex differences in impairment of USV in rSey2/+ rats

Abnormal reciprocal social interactions and communication deficits are the two of the three diagnostic symptoms of autism.

Figure 5. Ultrasonic vocalization in WT and rSey2/+ rat pups. The USV of individual rat pups (WT male: n = 18; WT female: n = 9; rSey2/+ male: n = 11; rSey2/+ female: n = 13) were measured during the isolation condition on P7. Spectrograms (frequency, kHz × time, s) of USV produced by WT (Upper) and rSey2/+ male pups (Lower) (A). Column graphs comparing among WT male (closed blue line), rSey2/+ male (closed magenta line), WT female (hatched blue line) and rSey2/+ female (hatched magenta line) pups in the number of calls per trial (B), mean duration of one call (s) (C), latency of the first vocalization (s) (D), and the peak frequency (kHz) (E). rSey2/+ female rats exhibited decrease in the number of calls compared to WT female rats (genotype: F(1) = 10.53, P < 0.002; gender: F(1) = 1.87, not significant; genotype x gender: F(1) = 4.20, P < 0.05; B). Data are expressed by mean ± SEM. **P < 0.01, compared to WT female rats as determined by Bonferroni post hoc test.

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These results might suggest that female \( rSey2^{+} \) work is needed to reveal detailed pathogenic mechanisms of sex-dependent differences in the hippocampal 5-HT system [69,70]. Elevated HTR2A activation by 5-HT could potently attenuate PPI [72]. Interestingly, we showed recovery of impaired PPI in \( rSey2^{+} \) rats using acute administration of clozapine, an anti-psychotic drug that shows strong binding to HTR2A [73] and decreases HTR2A mRNA [74]. We consider that HTR2A expression might increase in compensation to decreased 5-HT levels in synaptic clefts, which might induce impaired PPI in \( rSey2^{+} \) rats.

In summary, \( rSey2^{+} \) rats may potentially be used for discovery of effective drugs for autism, because these animals mimic at least some phenotypes of the disease.

### Methods

#### Animals

Large colonies of \( rSey2^{+} \) rats and wild type Sprague-Dawley (SD) rats (littermates of \( rSey2^{+} \) rats) were maintained in Tohoku University Graduate School of Medicine and in Yamanouchi Pharmaceutical Company. All rats were maintained with a 12/12 light cycle (lights on at 8:00 am) under temperature (22–24°C) and humidity (50–60%) controlled conditions. Food and water were available ad libitum. All the behavioral tests were conducted between 13:00 and 18:00 h. One week before the beginning of behavioral tests, the rats were housed one per cage and were handled once a day for 5 days. Genotype of \( rSey2^{+} \) rats was externally distinguishable because they have eye defects [27].

#### Altereds 5-HT levels and PPI in \( rSey2^{+} \) rats

Several studies have demonstrated high levels of 5-HT in whole blood or platelets in approximately 25–30% of autistic patients [see review by [67]]. Given that 99% of circulating 5-HT are accumulated in platelets, the measurement of platelet-poor plasma (PPP) 5-HT is essential for the evaluation of ‘free’ 5-HT, since this may reflect amount of brain synaptic 5-HT. In the present study, we found decreased 5-HT levels both in PPP and in the hippocampus of \( rSey2^{+} \) rats, representing the first rodent model that shows impaired 5-HT conditions. Although there are few reports on PPP 5-HT levels in autistic adults, one paper has reported that lower PPP 5-HT levels might relate to the pathophysiology and symptomatology of autism [68].

Pharmacological studies suggest differential roles of 5-HT receptor subtypes in the modulation of PPI [69,70]. 5-HT2A receptor (HTR2A) is widely expressed in the central nervous system [71]. Elevated HTR2A activation by 5-HT could potentially attenuate PPI [72]. Interestingly, we showed recovery of impaired PPI in \( rSey2^{+} \) rats using acute administration of clozapine, an anti-psychotic drug that shows strong binding to HTR2A [73] and decreases HTR2A mRNA [74]. We consider that HTR2A expression might increase in compensation to decreased 5-HT levels in synaptic clefts, which might induce impaired PPI in \( rSey2^{+} \) rats.

In summary, \( rSey2^{+} \) rats may potentially be used for discovery of effective drugs for autism, because these animals mimic at least some phenotypes of the disease.
matched male rats of littersmates at various postnatal stages were used in this study. All the animal experiments were carried out in accordance with the National Institute of Health guidance for the care and use of laboratory animals and were approved by The Committees for Animal Experiments in Tohoku University Graduate School of Medicine (21-252), Mitsubishi Kagaku Institute of Life Sciences (MITILS-00-003) and RIKEN (H19-2B109).

General activity test

General activity was measured in an open-field box (80×80×40 cm) made of gray vinyl chloride plates as described previously [30]. The apparatus was placed in a sound-attenuating room where external noise was greatly reduced (~45 dB at 500 Hz). Two pairs of 7×7 array infrared photosensors were attached to the outer wall equally spaced in lower and upper rows at intervals of 2 cm and 4.5 cm above the floor. The lower row of photocells was used to measure locomotor activity and the upper row to detect rearing behavior. A computer recorded the number of horizontal photobeam interruptions caused by animal movement. Each rat at 12–15 weeks old was placed into the apparatus and remained for 30 min.

Social behavior recording

A pair of WT or a pair of rSey2/± rats were placed in an open-field box (80×80×40 cm) and behavior of the two rats were video recorded for 15 min. Recorded behavior was analyzed by measuring periods for various types of behavior. The aggressive behaviors (aggression) were kicking, wrestling, and defeating. Locomotion and isolation indicate that both animals behaved independently with movement and rest, respectively. Passive body contact indicates that both animals rested in contact with each other. The other behaviors were following, allo-grooming, mounting, and sniffing the partner. The scoring was carried out by an observer blind to the genotype. A blind observer manually measured time spent in each categorized behavior.

Light–dark choice test

The apparatus consisted of two compartments made of gray vinyl chloride plates and placed in a darkened and sound-attenuating room. One compartment was a bright (250 lux) chamber (40×80×40 cm) illuminated by a white bulb (100 W) and the other was a dark (0.5 lux) chamber (40×80×40 cm). The two compartments were separated by a wall and connected by a small opening (8×20 cm) through which the photobeams of the sensors passed. A rat at 13–16 weeks old was placed in the center of the light chamber, and its behavior was recorded for 30 min. The rat was considered to have entered a new area when all four sensors passed. A rat at 15–16 weeks old was removed from the chamber 2 min after the foot shock and returned to their home cages. Forty-eight h or 96 h after the training, the rats were placed into a different square chamber in a different room, and 2 min later the same tone was sounded for 5 min without a foot shock. The amount of fear conditioned to the tone was assessed by scoring freezing behavior.

Auditory threshold test

Rats at 17–21 weeks old were placed in the conditioning chamber for 1 min and were then given 3 s tones (1,000 Hz and 5,000 Hz) of increasing sound intensity. The interval between tones was 10 s. We determined the threshold of sound level required to elicit the orienting reflex to the sound source.

Electric shock sensitivity test

For the last of the behavioral tests, we measured the sensitivity of rats at 17–21 weeks old to footshock. In this test, each rat was placed in the conditioning chamber and received 1 s shocks of increasing intensity. The sequence of shocks was 29 s. The sequence of the current used was as follows: 0.05 mA, 0.08 mA, 0.1 mA, 0.2 mA, 0.3 mA, 0.4 mA, 0.5 mA, 0.6 mA and 0.8 mA. We determined the minimal level of current required to elicit the following stereotypical responses: flinching, running, vocalization, and jumping. These experiments were performed blindly.

Measurement of ultrasonic vocalizations

A total of 51 rat pups (WT male; 18, WT female; 9, rSey2/± male; 11, rSey2/± female; 13) born to 4 dams were tested in USV during the isolation condition on P7. At first, we removed a dam from the home cage. To maintain the pups’ body temperature, the home cage was placed on a heat pad maintained at 35°C. A pup was transported in a plastic chamber (170×280×130 cm) with absorbent cotton which was placed in a soundproof box and vocalizations were recorded for 5 min.

USV was recorded with a condenser microphone (CM16/ CMPA, Avisoft Bioacoustics, Berlin, Germany) connected to an amplifier/digitizer (Avisoft UltraSoundGate416H, Avisoft Bioacoustics, Berlin, Germany) at a sampling rate of 250 kHz with 125 kHz low-pass filter. The recorded files were transferred to a sound analysis software (SASLab Pro ver. 4.52, Avisoft Bioacoustics, Berlin, Germany) for fast Fourier transform (512 FFT-length, 100% frame size, Hamming window, 50% time window overlap). We analyzed the number of calls, mean
duration of one call, latency to start calling and peak frequency of each call.

Measurement of 5-HT concentration

Blood (2 ml) at 18–20 weeks old was taken from the abdominal vein and drawn into test tubes containing sodium fluoride and EDTA-2Na (Becton Dickinson, Tokyo, Japan), and immediately placed on ice. PPP was separated by centrifugation of the blood samples at 1,500 x g for 15 min at 4°C. The resulting PPP (800 µl) was collected and stored at −80°C till assayed. 5-HT analyses of the PPP samples were conducted within 1 week after the experiment. PPP samples (100 µl) were added to 100 µl of 0.5 M perchloric acid and 10 µl of 0.1 M isoproterenol as an internal standard. After vortex-mixing, the tubes were centrifuged at 2,000 x g for 4°C for 15 min, and 40 µl of the supernatant was then injected into the HPLC system, equipped with a reverse-phase chromatographic column (Eicom, SC-5ODS, 3 mm diameter x 150 mm, Eicom, Kyoto, Japan). The applied potential was +440 mV versus the Ag/AgCl electrode. The mobile phase consisted of a 0.1 M sodium phosphate buffer (pH 6.0)-methanol (8 : 2, v/v) containing 400 mg/L sodium 1-octanesulfonate and 50 mg/L EDTA-2Na. The flow rate was set at 0.5 ml/min and the column temperature was maintained at 25°C. Compounds were detected using an electrochemical detector (Eicom ECD-300, Eicom, Kyoto, Japan) and quantitated by comparison of peaks area to that of the internal standard [76].

Hippocampal samples at 12 weeks old were homogenized in 5 volumes of 0.2 M perchloric acid containing 0.1 M EDTA and the proper concentration of isoproterenol. After centrifugation of the homogenates, the pH of the supernatant was adjusted to 3.0 by adding 1 M sodium acetate. The mobile phase was 0.1 M sodium citrate, 0.1 M citric acid, 0.5 mM sodium octanesulfonate, 0.15 mM EDTA, and 12% methanol, pH 3.5 [77].

Measurement of auditory startle and prepulse inhibition of acoustic startle

Rats of 12–17 weeks old were tested in a startle chamber [SR-Lab Systems, San Diego Instruments, San Diego, CA] positioned within a soundproof cabinet in a sound-attenuating room according to the method previously described [78,79]. A constant background noise of 65 dB was presented throughout the test. To measure PPI scores rats were given 20-ms-long 68, 71, or 77 dB prepulse that preceded the 120 dB pulse (40 ms width) by 100 ms (pp68, pp71, pp77). Percent PPI of a startle response was calculated: 100 – [startle response on acoustic prepulse and startle stimulus trials/startle response alone trials] x100).

Treatment with clozapine

Rats of 12–17 weeks old were injected intraperitoneally with 0.5 mg/ml of clozapine (Sigma) to make the final dose at 1.5 mg/kg body weight or with the similar amount of saline (control) according to the body weight. Both rats were analyzed for the same behavior tests as described above.

Data analysis

Data were analyzed by Student’s t test, two-way ANOVA or two-way ANOVA with repeated measures followed by Bonferroni post hoc where appropriate, using SPSS version 16 software (SPSS Inc., Chicago, II, USA). A P value below 0.05 was considered to be significant. All values in the text and figure legends were expressed as means plus/minus standard error of the mean (SEM), and n is the number of rats tested except for social interaction test where n indicates the number of pairs of rats examined.

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Author Contributions

Conceived and designed the experiments: KI NO. Performed the experiments: TU TY KK KO KI NO. Performed experiments: Wrote the paper: TU TY KK KO NO. Performed experiments: MM.

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