Behavioral/Systems/Cognitive

X11-Like Protein Deficiency Is Associated with Impaired Conflict Resolution in Mice

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Understanding how emotion is generated, how conflicting emotions are regulated, and how emotional states relate to sophisticated behaviors is a crucial challenge in brain research. Model animals showing selective emotion-related phenotypes are highly useful for examining these issues. Here, we describe a novel mouse model that withdraws in approach-avoidance conflicts. X11-like (X11L)/Mint2 is a neuronal adapter protein with multiple protein-protein interaction domains that interacts with several proteins involved in modulating neuronal activity. X11L-knock-out (KO) mice were subordinate under competitive feeding conditions. X11L-KO mice lost significantly more weight than cohoused wild-type mice without signs of decreased motivation to eat or physical weakness. In a resident-intruder test, X11L-KO mice showed decreased intruder exploration behavior. Moreover, X11L-KO mice displayed decreased marble-burying, digging and burrowing behaviors, indicating aberrant ethological responses to attractive stimuli. In contrast, X11L-KO mice were indistinguishable from wild-type mice in the open field, elevated plus maze, and light/dark transition tests, which are often used to assess anxiety-like behavior. Neurochemical analysis revealed a monoamine imbalance in several forebrain regions. The defective ethological responses and social behaviors in X11L-KO mice were rescued by the expression of X11L under a *Camk2a* promoter using the Tet-OFF system during development. These findings suggest that X11L is involved in the development of neuronal circuits that contribute to conflict resolution.

Introduction

Behavior arises from the integration of multiple emotional processes, which may themselves conflict. Approach-avoidance conflict in response to various emotional stimuli (e.g., seeking friendship, overcoming shyness or desiring success, avoiding competition) is a common everyday experience. Studies of human genetics and genetically engineered mice have gradually begun to unravel the molecular and neuronal mechanisms underlying emotion-related behavior, and cumulative evidence supports a crucial role of monoaminergic systems (Lesch et al., 1996; Zhuang et al., 1999; Gross et al., 2002; Finn et al., 2003; Holmes et al., 2003; Reif and Lesch, 2003). Behavioral paradigms designed to assess anxiety-related behaviors are relatively well established. In many of these paradigms, anxiety-like behavior is inferred by the avoidance of unpleasant stimuli under conflicting situations. However, more sophisticated paradigms and model animals that allow us to distinguish various emotional states are required to clarify how conflicting emotions are regulated in the brain and how emotional states underlie behavior.

The neuronal adaptor protein X11L (also called X11β and Munc18–1 interacting protein 2; Mint2) interacts with amyloid precursor protein (APP) and regulates APP metabolism (Tomita et al., 1999; Araki et al., 2003; Lee et al., 2004; Sano et al., 2006a; Saito et al., 2008). X11L suppresses amyloidogenic processing of APP via intracellular sorting machinery, and thus the lack of X11L in mice is associated with an increase in amyloid β (Aβ) 40 and 42 in the hippocampus (Sano et al., 2006a). X11L constitutes the X11 protein family with X11/X11α/Mint1 and X11L2/X11γ/Mint3, which comprises a poorly conserved N-terminal region, a conserved central phosphotyrosine interaction domain, and two C-terminal PDZ [postsynaptic density-95/*Drosophila* disks-large/zona occludens-1] domains (Tomita et al., 1999). X11L and X11 are brain-specific proteins that interact with many molecules, such as Munc18–1, calcium channels, β-neurexin, and hyperpolarization-activated cyclic nucleotide-gated potassium channel 2, involved in the regulation of neuronal/synaptic activity (Okamoto and Südhof, 1997; Maximov et al., 1999; Tomita et al., 1999; Biederer and Südhof, 2000; Kimura et al., 2004; Rogelj et al., 2006). Approximately 80% of Mint1/2 (X11/X11L) double-knock-out (KO) mice, but not single-gene KO mice, die at birth, and presynaptic release probability is decreased in the hippocampal excitatory neurons of double-KO mice, but not Mint1 (X11) single-KO mice (Ho et al., 2003, 2006), indicating substantial redundancies between X11 and X11L functions. The physiological roles of X11L, however, are not well understood.

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Here, we performed studies to identify the neuronal mechanisms regulated by X11L and to determine whether X11L-KO mice could model Alzheimer's disease (AD). Thus, we extensively analyzed the behavioral phenotypes, and neurochemical and neurophysiological characteristics of X11L-KO mice. Although no deficits in learning, memory, or synaptic properties were detected in X11L-KO mice, we observed characteristic deficits in motivated approach behaviors associated with emotional conflict. Restoration of expression of X11L during development restored normal performance in the resident-intruder and ethological tests designed to observe species-typical behaviors to attractive stimuli, such as unfamiliar-objects/thick-bedding and a clogged-tunnel. We suggest that X11L-KO mice provide a unique opportunity to address the neuronal mechanisms that underlie emotional conflict.

Materials and Methods

Animals

All experimental protocols were approved by the Animal Care and Use Committees of the RIKEN Brain Science Institute. X11L-KO mice were previously generated as a C57BL/6 coisogenic strain (Sano et al., 2006a). A tTA transgenic mouse line, B6;CBA-Tg(Camk2a-tTA)1Mmay/J, under control of the *Camk2a* promoter (Mayford et al., 1996), was obtained from The Jackson Laboratory. The constructs of *FLAG-humanX11L (hX11L)* (Taru and Suzuki, 2004) and *NLS-LacZ* (Sassa et al., 2004) have been described previously. These coding sequences together with splicing signals derived from the pMSG vector (GE Healthcare) were inserted downstream from the bidirectional tet-response element (TRE)-dependent promoters of the pBl Tet vector (Clontech). The resulting *FLAG-X11L-TRE-NLS-LacZ* construct was microinjected into fertilized C57BL/6 eggs. The generated mouse lines were crossed with *Camk2a-tTA* mice. We selected a representative line that bidirectionally expressed FLAG-X11L and NLS-LacZ at the highest level depending on the presence or absence of doxycycline (DOX) (Sigma-Aldrich).

Experimental groups of wild-type (WT) and X11L-KO mice were generated by intercrossing X11L heterozygous mutant mice. To obtain *FLAG-X11L-TRE-NLS-LacZ/Camk2a-tTA* double-transgenic (DTg) mice under the X11L-KO background (DTg:X11L-KO mice), we first generated each single-transgenic mouse under the X11L-KO background and intercrossed them. X11L-KO littermates from the crosses were used for the behavioral studies. These mice theoretically carry 99% or more of the C57BL/6-derived genome. Genetic background-matched and age-matched WT mice were obtained independently from crosses between X11L-mutation heterozygotes of the same generation.

Adult male mice >3 months old were used. Mice were maintained under a 12 h light and dark cycle (lights on, 8:00 A.M.–8:00 P.M.), and *ad libitum* feeding and drinking conditions. Mice were group-housed by combining equal numbers of each genotype, unless otherwise stated. An investigator ignorant to the genotype of the animals performed all behavioral, pharmacological, and electrophysiological experiments.

Antibodies

The mouse monoclonal anti-X11L C-terminal domain (347–5) was described previously (Sumioka et al., 2003). Anti-Mint2/X11L (611033) was purchased from BD Biosciences; horseradish peroxidase (HRP)-conjugated anti-β-tubulin (s.c.-9104) was from Santa Cruz Biotechnology; anti-FLAG M2 (F1804) was from Sigma-Aldrich; anti-galactosidase (55976) was obtained from Cappel Laboratories; and HRP-conjugated anti-mouse IgG (NA931) and HRP-conjugated anti-rabbit IgG (NA934) were purchased from GE Healthcare.

Western blotting analysis

Western blotting was performed as described previously (Sano et al., 2006a,b). Details are described in the supplemental materials, available at www.jneurosci.org.

Behavioral analysis

Competitive food restriction. Groups of mice were cohoused in the same cage for at least a week before food restriction. In mixed genotype housing cages, equal numbers of WT and X11L-KO mice [(2 + 2) or (3 + 3) per cage] were cohoused. In uniform genotype housing cages, genotype-matched mice (4–6 mice/cage) were cohoused. During the food restriction period, mice were provided with 2 or 3 food pellets, equivalent to 4% of their free-feeding body weight, per day. During all test periods, mice were allowed water *ad libitum*. Weight was measured daily before feeding.

Food intake. To examine food intake of the mice under noncompetitive conditions, mice that were food-restricted as described above in mixed genotype housing cages were housed individually and then fed *ad libitum* for 90 min. The amount of food consumed and weight-gain after feeding was measured. Calculated food intake and weight-gain were normalized to the body weight measured before feeding.

Grip strength. A spring balance attached to the distal end of a wire-mesh end was used to test grip strength (O'Hara & Co.). Mice were allowed to grip the wire-mesh with their forelimbs, and were then pulled gently in a horizontal direction by their tails, away from the spring balance. The force applied to the spring balance at the moment the mice released the wire-mesh was recorded. Measurements were repeated twice with a 30 min intertrial interval. The mean of the two trials was calculated. In this test, mice were normally housed without food restriction.

Resident-intruder test. The resident-intruder test was performed according to the procedure described previously (Wada et al., 1997). Resident mice were maintained in individual housing at least for 5 weeks before experimentation. Corn cob (GreenTru; Green Products Company) was used for the bedding material to allow for easy observation of the mouse behavior. Intruder mice (male, DBA/2J strain; JCL Inc.; 7 weeks) were housed in groups of 4–5 mice per cage with standard bedding material (Tek-Fresh; Harlan) for 2 weeks before the experiment.

In the resident-intruder test, residents' cages were placed in an area with low lighting (120 lux). The intruder was transferred to the resident home cage, then social behaviors of the resident mouse toward the intruder mouse were observed for 5 min. The test was conducted twice (24 h intertrial interval) using different intruder mice for each trial. The following behavioral indices were measured. Approaching: exploratory behavior toward the intruder, sometimes involving contact with the intruder and sniffing behavior; Following: exploratory following and chasing of the intruder; and Aggression: aggressive movements against the intruder, such as fighting, biting, and other aggressive responses (aggressive-sniffing, -grooming, -following). As some indices may or may not involve aggression, behaviors were counted as aggressive only when the intruder responded with a submissive posture (Grant and Mackintosh, 1963). Behaviors of the resident mice were analyzed and counted using commercial software (VideoStudio, Corel) after the experiment.

Marble burying test and digging test. The marble burying test (MBT) was performed according to the procedure described previously (Yamada et al., 2002) with some modifications. Twenty clear blue glass marbles (17 mm diameter), white paper bedding material (Paperclean; Japan SLC), and transparent cages (24 × 45 × 21 [H] cm) were used for each trial. The cage was filled with bedding (5 cm deep). Mice were individually habituated to the cages for 30 min (habituation session) and then returned to their home cages. The 20 glass marbles were placed on the bedding surface in the habituated cage, evenly spaced at 5 cm intervals. The mice were then placed into the same cages to which they were habituated (MBT session) and allowed to remain for 20 min. After 20 min, the mice were removed from the cage and the number of marbles buried more than two-thirds in the bedding was counted. In both the habituation and MBT sessions, horizontal and vertical activity of the mice was measured using a two-level infrared beam apparatus (Scanet, Melquest). In the digging test, the mice were placed on the bedding (5 cm deep) without any marbles present. Mouse behavior was recorded from above for 30 min using a video camera, and the time spent digging was measured manually.

Burrowing test. The burrowing test was performed according to previously described procedures (Deacon et al., 2002) with some modifications. Both WT and X11L-KO mice were maintained in individual housing. Two gray vinyl chloride tubes (inside tube diameter, 56 mm; length, 150 mm; open end of the tube raised 30 mm; lower end closed with a cap) were placed in their home cage at ~3 h before the start of dark period. One tube was filled with 200 g of the usual food pellets, each weighing ~2 g, while other tube was empty to provide a shelter. The weight of displaced food pellets from the tube was weighed after a 15 h period.

Methods for the other behavioral tasks are described in the supplemental materials, available at www.jneurosci.org.

β-Galactosidase (LacZ) staining

LacZ staining was performed according to previously described procedure (Iwasato et al., 2000; Tanaka et al., 2008) with some modifications. Mice were deeply anesthetized with 2,2,2-tribromoethanol (Avertin, Sigma-Aldrich) and transcardially perfused with physiological saline and then 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4, at 4°C for 20 min. The brains were excised, postfixed with the same fixative at 4°C for 2 h, and equilibrated in 30% (w/v) sucrose in PB as a cryoprotectant. The brains were embedded in OCT compound (Sakura Finetech), and frozen sections (20 μm) were prepared. Sections were then washed in phosphate buffered saline (PBS) on ice for 5 min and stained in 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 20 mM Tris/HCl pH 7.5, and 2 mM $MgCl_2$ in PB at 37°C overnight. LacZ-stained sections were washed in PBS and then counter-stained with eosin.

Monoamine measurement

Circular tissue punches (1 mm diameter) were taken from 150 μm thick frozen coronal brain sections obtained from 4- to 5-month-old mice ($n = 8$ per group) and stored at −80°C until assayed. Monoamines and metabolites were extracted by sonication in 0.1 M perchloric acid containing a mixture of internal standards (200 nM isoproterenol, 200 μM L-norleucine, and 5 μM ethylhomocholine). Protein was then measured using a DC Protein Assay kit (Bio-Rad). The remainder of the sample was centrifuged for 15 min at 15,000 rpm and the supernatant filtered through polyvinylidene fluoride 0.22 μm micropore filters (Millipore Corp.) by centrifugation (14,000 rpm for 5 min). The filtrate was analyzed by high pressure liquid chromatography coupled to electrochemical detection (Eicom). Briefly, an Eicompak SC-5ODS 3.0 × 150 mm column was used for separations, perfused with a mobile phase consisting of citrate (41.4 mM), sodium acetate (39.2 mM), methanol (17%), sodium 1-octanesulfonate (190 mg/L), and EDTA (5 mg/L), adjusted to pH 3.7 using glacial acetic acid and pumped at a rate of 0.5 ml/min. The working electrode (WE-3G) potential was set at +0.75 V. Column temperature was maintained at 25°C. Data were collected and analyzed by Ezchrom Elite software (Scientific Software Inc.). All analyte information, including the retention times, peak heights, concentrations, and recovery rate of internal standards, was calculated by comparison to standard curves generated for known concentrations of external standards that were run daily. Turnover rates were calculated as follows: noradrenaline (NA) turnover rate = [3-methoxy-4-hydroxyphenylglycol (MHPG)]/[NE]; dopamine (DA) turnover rate = [3, 4-dihydroxyphenylacetic acid (DOPAC)] + [homovanillic acid (HVA)]/[DA]; serotonin (5-HT) turnover rate = [5-hydroxyindoleacetic acid (5-HIAA)]/[5-HT]. Correlation maps were constructed using iVici software (Michnick Laboratory, http://michnick.bcm.umontreal.ca/ivici/) using correlation coefficients calculated by pairwise comparisons (Tarassov and Michnick, 2005).

8-Hydroxy-2-dipropylaminotetralin-induced hypothermia

(±)-8-Hydroxy-2-dipropylaminotetralin (8-OH-DPAT) hydrobromide was purchased from Tocris Bioscience. The 8-OH-DPAT induced hypothermia experiment was performed according to the procedure described previously (Li et al., 1999) with minor modifications. Mice were handled every day for 1 week before the experiment. Mouse body temperature was measured using a digital thermometer with a temperature probe (Physitemp BAT-12R and RET-3) inserted 2–2.5 cm into the rectum, with the mouse slightly restrained by the tail. WT and X11L-KO mice were subcutaneously injected with saline or 8-OH-DPAT at doses of 0.1 or 0.5 mg/kg. Body temperature was monitored every 10 min for 60 min before the injection and every 15 min for 90 min after the injection. Mean temperature before injection was used as the basal temperature.

Statistics

Statistical analyses were performed using SPSS 14.0J or Excel statistics 2008 (Social Survey Research Information Co.) software. For comparison of two groups in most experiments, we applied an unpaired two-tailed *t* test. A repeated measures ANOVA was used to analyze learning data through training from the water maze and radial maze tests, and the 2 d of data from the resident-intruder test. We used a single-factor ANOVA followed by Scheffe's *post hoc* analysis to test for differences among three or more independent groups. Nonparametric tests were used to analyze data from the marble buying, digging and burrowing tests (Mann–Whitney *U* test for comparison of two groups or Steel test for rescue experiments). Differences were considered to be statistically significant when the probability value was <0.05.

Results

X11L-KO mice do not exhibit an AD-model like phenotype in terms of learning and memory

Amyloid β40 and 42 levels are increased in X11L-KO mice (Sano et al., 2006a). Patients with AD as well as AD mouse models that exhibit Aβ accumulation are cognitively impaired (Chen et al., 2000; Janus et al., 2000; Morgan et al., 2000). To determine whether X11L-KO mice have an AD-like phenotype, we analyzed their learning and memory ability. The Morris water maze task is a hippocampus-dependent task that is most frequently used to detect dysfunction of learning and memory performance in mouse models of AD. Despite the increase in Aβ in X11L-KO hippocampi, the learning and memory performance of X11L-KO and WT mice was comparable (supplemental Fig. 1*A–C*, available at www.jneurosci.org as supplemental material). Working memory in the 8-arm radial maze test and associative memory in context-dependent and cue-dependent fear conditioning tests were not impaired in X11L-KO mice compared with WT mice (supplemental Fig. 1*D*, *E*, available at www.jneurosci.org as supplemental material). These data suggest that learning and memory functions in X11L-KO mice are largely intact.

An accumulation of Aβ may depress neuronal transmission (Chapman et al., 1999; Kamenetz et al., 2003). Additionally, X11L interacts with the synaptic machinery that regulates neurotransmitter release such as Munc 18–1 and β-neurexin (Okamoto and Südhof, 1997; Biederer and Südhof, 2000; Ho et al., 2003, 2006). Expression of X11L is most abundant in the CA3 pyramidal neurons (Nakajima et al., 2001). Thus, we next examined the synaptic properties of the CA3-CA1 synapse in hippocampal slices from WT and X11L-KO mice. Basal synaptic transmission, paired-pulse facilitation (a form of short-term plasticity), and long-term potentiation in slices from X11L-KO mice were similar to those of WT littermates (supplemental Fig. 2*A–C*, available at www.jneurosci.org as supplemental material). In the hippocampus, no differences were detected between X11L-KO and WT mice in the amounts of X11/X11L binding, or of the levels of presynaptic and postsynaptic molecules examined (supplemental Table 1, available at www.jneurosci.org as supplemental material).

Therefore, the behavioral and physiological data do not support the tenet that X11L-KO mice are AD-like animal model associated with dementia.

X11L-KO mice are subordinated by their WT peers under competitive conditions

We noticed that X11L-KO mice showed a unique behavioral phenotype during a set of repeated radial maze tests. When food restriction was implemented by limiting the amount of time during which food was available (90 min free feeding/d), group housed X11L-KO and WT mice had a similar rate of body weight loss. When the food restriction regimen was transiently shifted to

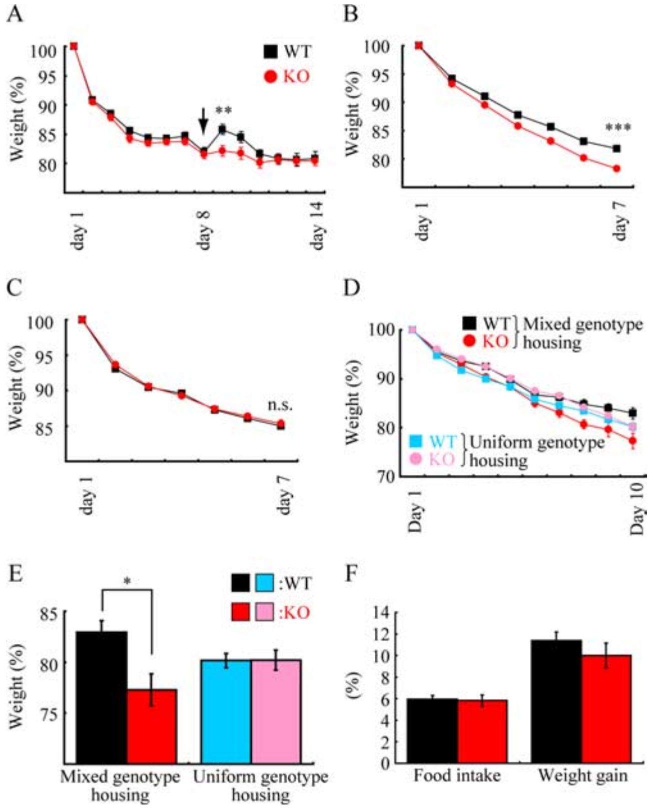

Figure 1. X11L-KO mice are subordinated in competitive feeding conditions. ***A–C***, The percentages of weight reduction by food deprivation for the mixed genotype housing condition (WT mice, black square; X11L-KO mice, red circle). Free-feeding body weight was defined as 100%. ***A***, From day 1 to day 7, mice could freely feed for 90 min per day. At day 8 (arrow), only an amount of food equivalent to 8% of the sum of the mouse body weight per cage was provided. ***B***, Food pellets (2 pellets/4 mice; amount of food = 4% of the sum of the mouse body weights per cage under free-feeding conditions) were placed into the cages from day 1 to 7. ***C***, Food was restricted by a time-limited manner of free feeding for 90 min/d. ***D***, The percentages of weight reduction by food deprivation are shown for 10 d under different housing conditions (WT mice in mixed genotype housing, black square; X11L-KO mice in mixed genotype housing, red circle; WT mice in uniform genotype housing, blue square: X11L-KO mice in uniform genotype housing, pink circle). Body weights in the *ad libitum* feeding condition were defined as 100%. ***E***, Percentage body weight at day 10 is shown. In only the mixed genotype housing, X11L-KO mice lost significantly more weight than did WT mice. ***F***, The percentage of food intake and weight gain during the 90 min free feeding after 11 d of competitive food restriction was the same between genotypes. Data represent mean ± SE. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (WT mice, black and blue columns; X11L-KO mice, red, and pink columns).

restricting the amount of food available on day 8 (amount of food = 8% of the summed body weight per cage), WT mice had a transiently larger weight gain than cohoused X11L-KO mice (7–8 months old, $n = 12$ per group; at day 9, $p < 0.01$) (Fig. 1*A*). This differential weight loss in X11L-KO mice was reproduced in another experiment. X11L-KO mice lost significantly more weight than cohoused WT mice under the amount-limited (competitive) feeding regimen (amount food = 4% of the summed body weight per cage per day; 3–4 months old, $n = 12$ per group; at day 7, $p < 0.001$) (Fig. 1*B*). Under the time-limited (no or very low-competitive) condition (90 min free feeding per day), there were no differences in body weight between genotypes (8–9 months old, $n = 12$ per group, $p = 0.68$) (Fig. 1*C*).

Based on these observations, we hypothesized that X11L-KO mice were subordinated under competitive feeding conditions. To test this hypothesis, we restricted feeding using two types of group housing conditions: equal numbers of WT and X11L-KO mice per cage (referred to as "mixed genotype housing") or groups of a single genotype mice per cage (referred to as "uniform genotype housing"). To avoid the influence of body weight at the start, the WT and X11L-KO mice in the mixed genotype housing were weight matched. During 10 d of feeding restriction using the limited-amount regimen, the X11L-KO mice in the mixed genotype housing cages lost significantly more weight than the WT mice (5–6 months old, $n = 6$ per group; weight ratio at day 10; WT, 82.9 ± 1.14%; X11L-KO, 77.3 ± 1.56%). No differences between genotypes were induced by the same treatment in uniform genotype housing (weight ratio at day 10; WT, 80.2 ± 0.68%, $n = 5$; X11L-KO, 80.2 ± 0.97%, $n = 6$). ANOVA and *Post hoc* Scheffe's test revealed significant differences between genotypes only in the mixed genotype housing groups ($F_{(3,19)} = 4.05$, $p < 0.05$; Scheffe's test, WT vs X11L-KO in mixed genotype housing, $p < 0.05$; in uniform genotype housing, $p = 1.00$) (Fig. 1*D*,*E*).

To rule out the possibility that the larger weight loss in X11L-KO mice was caused by a loss of appetite, the amount of food ingested and of weight gain after eating were measured under noncompetitive conditions in which each food-deprived mice were fed individually. Food intake and weight gain in X11L-KO mice were indistinguishable from those of WT mice (3–4 months old, $n = 12$ per group; food intake, $p = 0.85$; weight gain, $p = 0.33$) (Fig. 1*F*). Lever-pressing behavior to obtain food pellets in an operant conditioning test, in which mice received a 20 mg pellet when they pressed the lever 1, 10, or 50 times, was not different between genotypes (data not shown). These data indicate that the weight loss in X11L-KO mice in the competitive condition was not likely caused by a decreased motivation to eat, or a reduction in the incentive value of the food.

We considered the possibility that X11L-KO mice were less able to access food when competing with WT mice due to a physical limitation, such as weaker muscle strength. We assessed muscle (grip) strength of the forelimbs of WT and X11L-KO mice, and observed no differences between genotypes (7–8 months old, $n = 24$ per group; WT, 0.71 ± 0.03 Newtons (N); X11L-KO, 0.80 ± 0.04 N; grip strength, $p = 0.08$). Moreover, neither X11L-KO nor WT mice had wounds consistent with aggressive behavior in the home cage. Together, these findings suggested that X11L-KO mice are subordinated only under highly competitive conditions, and that this response is not due to a physical factor.

X11L-KO mice exhibit reduced social interaction

Because the subordinate feeding behaviors of the X11L-KO mice were observed under competitive conditions, we hypothesized that X11L-KO might show aberrant social interaction. To assess social behavior in X11L-KO mice, we used the resident-intruder test. We measured nonaggressive approach and following, and aggressive behaviors against intruder mice (see experimental procedure for details). In our experiment, fighting behavior was rarely observed. Approaching and following behaviors were lower in resident X11L-KO mice compared with WT mice (5–6 months old, $n = 10$ per group; approach, $F_{(1,18)} = 4.27$, $p = 0.05$; following, $F_{(1,18)} = 4.43$, $p < 0.05$; aggression, $F_{(1,18)} = 1.59$, $p = 0.22$; Figure 2*A–C*). The total number of resident social interactions with intruder mice was significantly decreased in X11L-KO mice compared with WT mice ($F_{(1,18)} = 9.14$, $p < 0.01$) (Fig. 2*D*). These data indicate that social interaction is suppressed in X11L-KO mice.

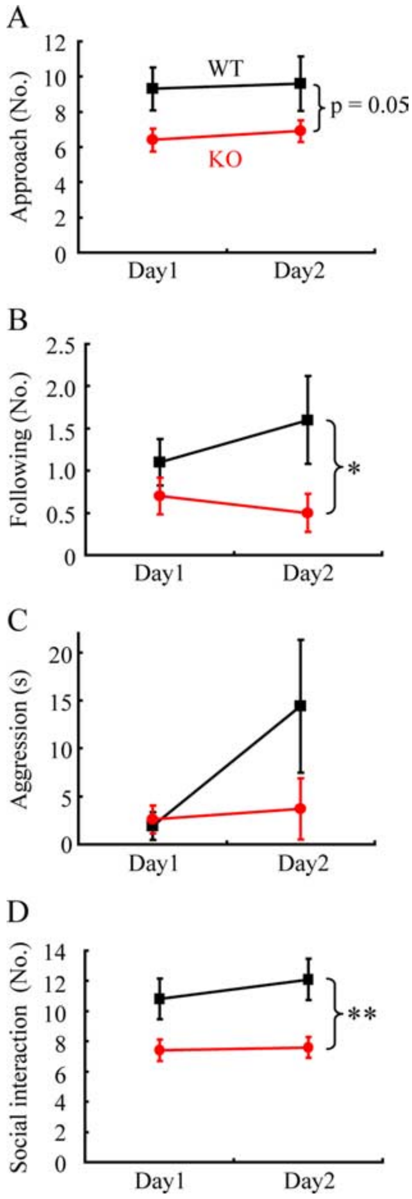

Figure 2. Social interaction against an intruder is significantly decreased in X11L-KO mice. ***A–C***, The resident-intruder test (WT mice, black square; X11L-KO mice, red circle) was performed for 2 consecutive days using different intruder mice for each trial. The numbers of approaches (***A***) and following behaviors (***B***) of resident mice against intruder mice were lower in X11L-KO mice. ***C***, Differences in the amount of time spent by resident mice exhibiting aggressive behavior in which the intruder mice showed a typical submissive pose was not statistically significant between genotypes. ***D***, The summed number of social interactions of resident mice toward intruder mice was significantly decreased in X11L-KO mice compared with WT mice. Data represent mean ± SE. $^{*}p < 0.05$; $^{**}p < 0.01$.

X11L-KO mice do not show altered anxiety-like behavior in conventional behavioral tests

Social interaction is the result of an inherent conflict in approach-avoidance factors, and is decreased under anxiogenic conditions (File, 1992; Crawley, 2007). Even if anxiety in the social interaction test qualitatively differs from that assessed on the elevated plus maze test, anxiolytic drugs are effective in both models (File, 1992; Nakamura and Kurasawa, 2001; Crawley, 2007). To gain insight into anxiety-related behaviors in X11L-KO mice, we subjected X11L-KO mice to open field, elevated plus maze, and light-dark transition tests. The behavior of the X11L-KO mice was comparable to that of WT mice in all of these tests (supplemental Table 2, available at www.jneurosci.org as supplemental material). Furthermore, X11L-KO mice did not show abnormalities in model behavioral tests for mental disorders such as the tail suspension test (a model of depression-like behavior) and prepulse inhibition test (a model for sensory motor gating activities associated with schizophrenia; supplemental Table 2, available at www.jneurosci.org as supplemental material). X11L-KO and WT mice showed comparable locomotor activity and circadian rhythms (supplemental Table 2, available at www.jneurosci.org as supplemental material). These results suggest that suppressed social interaction is not caused by enhanced anxiety-like, depressive behavior, or decreased locomotor activity.

X11L-KO mice show attenuated marble burying, digging, and burrowing behavior

The behavioral paradigms described above identify anxiety-related phenotypes based on avoidance behavior elicited by aversive stimuli. We examined whether behaviors that reflect approach motivation to some stimuli are selectively altered in X11L-KO mice. Behavior of X11L-KO mice and WT mice was therefore evaluated in the MBT. The MBT is proposed to be a model of defensive behavior, as mice actively bury glass marbles under a layer of bedding material (Broekkamp et al., 1986; Nicolas et al., 2006) (see also below). X11L-KO mice buried significantly fewer marbles than did WT mice (4–5 months old, $n = 12$ per group; WT, 11.2 ± 1.1; X11L-KO mice, 1.7 ± 0.76; number of buried marbles, $p < 0.001$) (Fig. 3*A*). Horizontal and vertical activity of X11L-KO mice was indistinguishable from that of WT mice in both the habituation and MBT sessions (locomotor activity in habituation session, $p = 0.75$; locomotor activity in MBT session, $p = 0.97$; rearing in habituating session, $p = 0.40$; rearing in MBT session, $p = 0.88$) (Fig. 3*B*,*C*). These data indicate that impaired marble burying behavior was not due to a reduction in general activity.

Marble burying behavior is argued to be the result of innate digging behavior (Njung'e and Handley, 1991a; Gyertyán, 1995; Deacon, 2006b). Thick bedding, which is used for the MBT, strongly motivates mice to dig the bedding material. This innate behavior is not habituated, is evoked in the absence of glass marbles, and continues even after all marbles are buried (Webster et al., 1981; Dudek et al., 1983; Gyertyán, 1995; Masuda et al., 2000; Deacon, 2006b). To assess whether impaired marble burying is associated with digging behavior, we measured the time spent digging during the habituation session. X11L-KO mice spent significantly less time digging (4–5 months old, $n = 12$ per group; WT, 288.9 ± 34.3 s; X11L-KO, 90.2 ± 15.3 s; $p < 0.001$) (Fig. 3*D*). Digging behavior was initiated within the first 10 min under conditions of a novel environment with thick bedding materials (supplemental Fig. 3*A*, available at www.jneurosci.org as supplemental material). In an independent experiment, strong and compulsive digging behavior was induced by bedding materials in the absence of marbles, depending on the bedding thickness (supplemental Fig. 3*B*, available at www.jneurosci.org as supplemental material). In this condition, X11L-KO mice again exhibited significantly reduced digging behavior. These data suggest that marble burying and digging behaviors are closely associated, and that the approach behavior motivated by incentives, such as marbles and bedding materials, is attenuated in X11L-KO mice.

Burying and digging behaviors are species-typical behaviors to some rodents. The burrowing test is conceptually related to the above ethological tests, as mice typically burrow

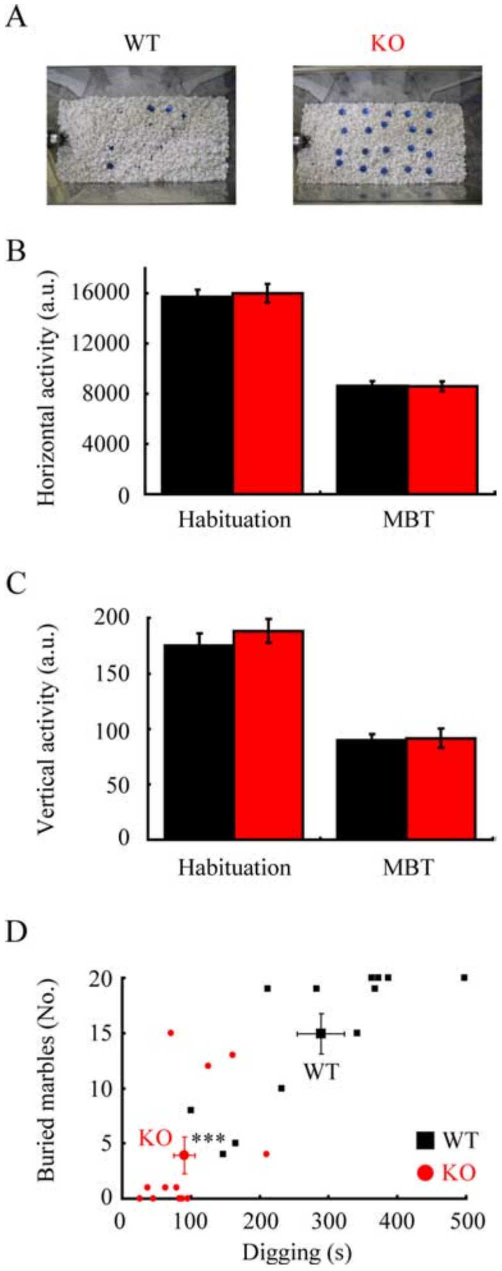

Figure 3. Marble burying and digging behaviors are impaired in X11L-KO mice. ***A***, Typical images of the cage after the marble burying test are shown. ***B***, ***C***, Horizontal (***B***) and vertical (***C***) activity in the habituation and MBT sessions was not significantly different in X11L-KO mice compared with WT mice. ***D***, The number of marbles buried and time spent digging during the habituation session (30 min) are plotted for each mouse. Data represent mean ± SE. $^{***}p < 0.001$ (WT mice, black column; X11L-KO mice, red column).

into tunnel-like containers filled with small-solid objects such as soils, small clay balls and food pellets (Deacon, 2006a). X11L-KO mice displaced significantly less food pellets from a tube (4–5 months old, $n = 14$ per group; WT, 80.6 ± 15.4 g; X11L-KO mice, 10.1 ± 1.9 g; weight of displaced pellets, $p < 0.001$). These data indicate that X11L plays a crucial role in these species-typical behaviors, which likely reflect defensive behaviors, seeking safety, exploration and hoarding food seen in the wild (Dudek et al., 1983; Deacon, 2006a,b), and further suggest X11L-KO mice are less attracted to what are usually attractive stimuli.

Impaired species-typical behaviors and reduced social interaction in X11L-KO mice are rescued by the expression of X11L during development

To confirm the causal link between X11L and a specific behavior impaired in X11L-KO mice, we performed a rescue experiment using the Tet-regulated system, which is used for conditional expression in mice (Mayford et al., 1996). In normal WT mice, X11L is highly expressed in the forebrain (Nakajima et al., 2001). Therefore, *Camk2a-tTA* mice (Mayford et al., 1996; Gross et al., 2002) were used to express X11L selectively in the forebrain after birth. In the *TRE-hX11L:Camk2tTA* DTg mice (Fig. 4*A*), FLAG-hX11L and β-galactosidase are expected to be coexpressed in the same cells. Western blot analyses using antibodies against the C-terminal epitope of X11L and FLAG successfully detected the expression of FLAG-hX11L in adult DTg brains (Fig. 4*B*). LacZ staining revealed that β-galactosidase was strongly expressed in the forebrain region, as well as in some other regions, such as the substantia nigra, ventral tegmental area, and raphe (Fig. 4*C*). Western blot analysis revealed that X11L protein expression was completely depleted by administering 2 mg/ml DOX in the drinking water for 3 weeks, and then recovered to ~80% of the initial expression level within 1 week after the withdrawal of DOX (Fig. 4*D*).

To perform a rescue experiment using this system, we generated DTg mice under the X11L-KO background (DTg:X11L-KO mice). We first focused on the MBT. DTg:X11L-KO mice buried significantly more marbles than X11L-KO mice (4–5 months old, WT, 8.1 ± 1.2, $n = 21$; X11L-KO, 2.7 ± 0.7, $n = 27$; DTg:X11L-KO, 5.6 ± 1.0, $n = 29$; X11L-KO vs DTg:X11L-KO, $p < 0.05$) (Fig. 4*G*). The increased burying behavior in DTg:X11L-KO mice was not likely due to an increase in general activity as the horizontal activity of DTg:X11L-KO mice was slightly but significantly decreased compared with X11L-KO mice in the habituation session and indistinguishable from X11L-KO mice in the MBT session (habituation session, X11L-KO vs DTg:X11L-KO, $p < 0.05$; MBT session, X11L-KO vs DTg:X11L-KO; $p = 0.53$) (Fig. 4*J*).

To test whether X11L protein regulates marble burying behavior during information processing, the expression of FLAG-hX11L was acutely inhibited in DTg:X11L-KO mice in the adult stage by administering DOX (DTg:X11L-KO/DOXad). FLAG-hX11L proteins were not detected in DTg:X11L-KO/DOXad mice administered DOX in their drinking water (2 mg/ml) for 3 weeks (Fig. 4*E*). The DTg:X11L-KO/DOXad mice, however, still buried significantly more marbles compared with X11L-KO mice (4–5 months old, WT/DOXad, 9.4 ± 1.4, $n = 21$, X11L-KO/DOXad, 3.0 ± 0.7, $n = 27$; DTg:X11L-KO/DOXad, 6.8 ± 1.0, $n = 30$; X11L-KO/DOXad vs DTg:X11L-KO/DOXad, $p < 0.01$) (Fig. 4*H*). The recovery of burying behavior was not associated with an increase in general activity (Fig. 4*K*). These data suggest that the X11L protein is required during the developmental stages but not in adults, for normal marble burying behavior. The partial recovery may indicate incomplete expression and/or a cell type specificity of X11L in DTg mice.

To further confirm the role of X11L in developmental stages, we administered DOX (25 μg/ml) in the drinking water of the parents from the mating period through postnatal day 28. This concentration of DOX was sufficient to completely suppress the expression of FLAG-hX11L in DTg:X11L-KO mice (DTg:X11L-KO/DOXdev) (Fig. 4*F*). The expression of FLAG-hX11L did not recover after DOX withdrawal, even after 5 months, in DTg:X11L-KO/DOXdev mice, unlike the case of DOX administration in adults (Fig. 4*F*). The transgenic locus silenced during the developmental stage likely acquires irreversible modifications. The numbers of marbles buried by DTg:X11L-KO/DOXdev mice and

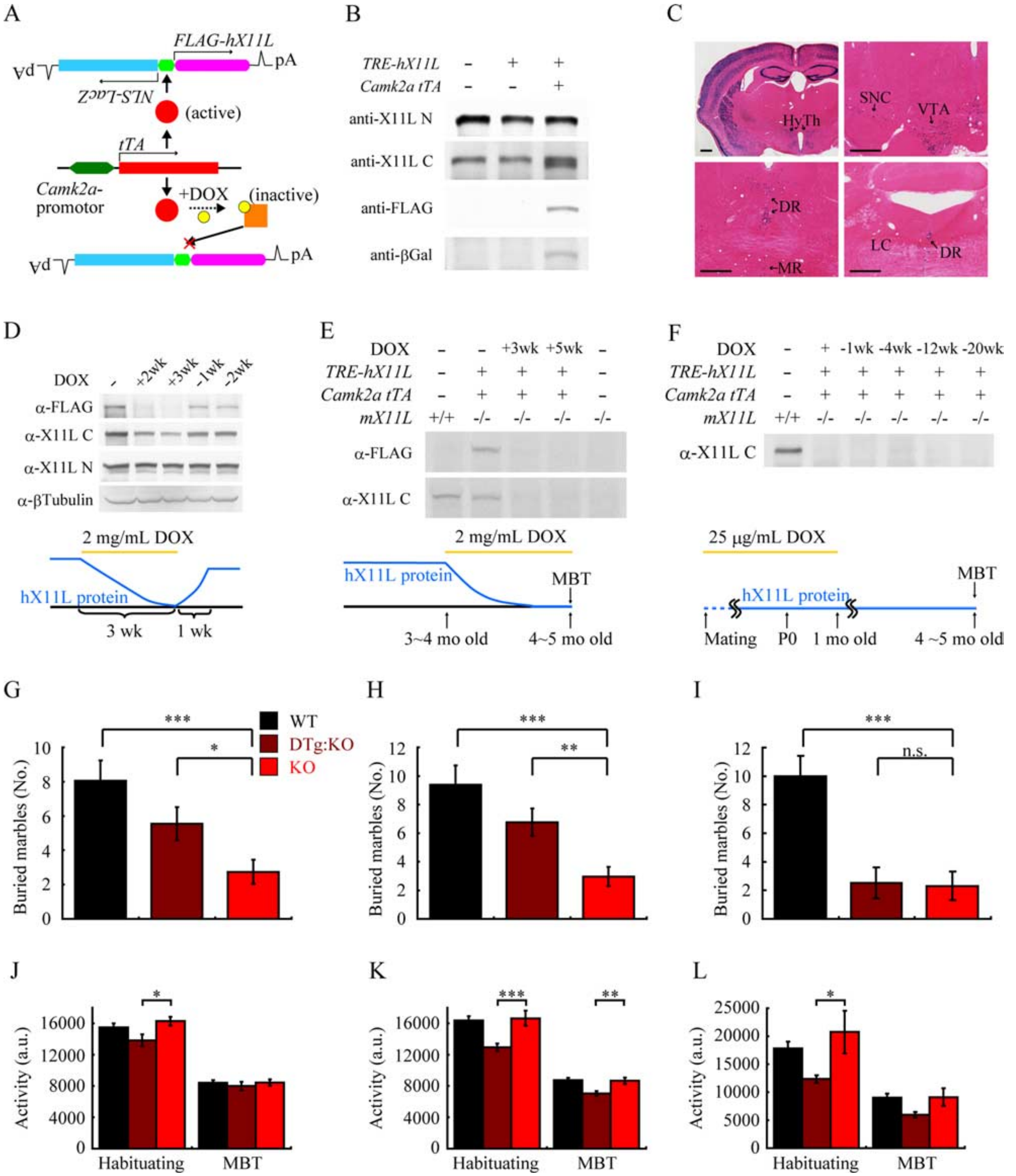

Figure 4. Impairment in marble burying behavior in X11L-KO mice was rescued by the expression of X11L during development, but not in adults. ***A***, The tTA protein is driven by *Camk2a* promoter. The active form of tTA binds to TRE and bidirectionally drives the expression of the *FLAG-hX11L* and *NLS-LacZ* genes. DOX inhibits the expression of both transgenes by binding to tTA and converting it into an inactive conformation. ***B***, Immunoblot of whole brain lysates obtained from transgenic and DTg mice. Anti-X11L N antibody only recognizes the N-terminal region of the mouse X11L protein. Anti-X11L C antibody recognizes the C-terminal region of both human and mouse X11L proteins. ***C***, Expression of the transgenes was detected by LacZ staining. Scale bars, 500 μm. HyTh, hypothalamus; SNC, substantia nigra pars compacta; VTA, ventral tegmental area; DR, dorsal raphe; MR, median raphe; LC, locus ceruleus. ***D***, Immunoblot of whole brain lysates obtained from DTg mice with administration and withdrawal of 2 mg/ml DOX in the drinking water. ***E***, Immunoblot of whole brain lysates obtained from DTg:X11L-KO and control mice. Complete depletion of X11L in DTg:X11L-KO mice by 2 mg/ml DOX administration was confirmed. ***F***, Immunoblot of whole brain lysate obtained from DTg:X11L-KO mice after withdrawal from 25 μg/ml DOX administration during the developmental period. ***D–F***, Experimental design and results are schematically represented at the bottom part of each panel. Blue lines indicate X11L protein level. ***G–L***, MBT and concurrent measurement of locomotor activity were performed using DTg:X11L-KO mice with the various DOX administration paradigms. ***G–I***, MBT. The number of marbles buried was counted for each genotype of experimental group: without DOX administration (***G***), with DOX administration during the adult period (***H***), and with DOX administration in the developmental period (***I***) with subsequent withdrawal of DOX administration. ***J–L***, Locomotor activity in MBT and habituating sessions for each experimental group without DOX administration (***J***), the same as ***G***, with DOX in the adult period (***K***), the same as (***H***), and with DOX in the developmental period (***L***), the same as ***I***. Data represent mean ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (WT mice, black column; DTg:X11L-KO mice, brown column; X11L-KO mice, red column).

X11L-KO/DOXdev mice were indistinguishable (4–5 months old, WT/DOXdev, 10.0 ± 1.4, $n = 16$; X11L-KO/DOXdev, 2.3 ± 1.0, $n = 16$; DTg:X11L-KO/DOXdev, 2.5 ± 1.1, $n = 15$; X11L-KO/DOXdev vs DTg:X11L-KO/DOXdev, $p = 0.77$) (Fig. 4*I*). These results further strengthened the notion that X11L has an important role during development.

Locomotor activity in DTg:X11L-KO mice treated with or without DOX was significantly lower than that in X11L-KO mice (Fig. 4*J–L*). Decreased locomotor activity in *Camk2a-tTA* mice was reported by another group (McKinney et al., 2008). This decreased locomotor activity might lead to an underestimation of the degree of the rescued phenotype in our experiments.

Next, we examined the impact of X11L expression on burrowing behavior. We also observed that DTg:X11L-KO mice displaced significantly more food pellets from a tube than X11L-KO mice (5–6 months old, DTg:X11L-KO, 144.7 ± 11.6 g, $n = 14$; X11L-KO, 30.5 ± 9.6 g, $n = 16$; weight of displaced pellets, $p < 0.001$).

Finally, we performed the resident-intruder test using DTg:X11L-KO and X11L-KO mice to confirm whether the expression of X11L can also restore the suppressed social interaction in X11L-KO mice as is the case in the MBT. Resident social interactions with intruder mice were significantly increased in DTg:X11L-KO mice compared with X11L-KO mice (4–5 months old, DTg:X11L-KO $n = 14$, X11L-KO $n = 16$; approach, $F_{(1,28)} = 5.86$, $p < 0.05$; following, $F_{(1,28)} = 4.32$, $p < 0.05$; aggression, $F_{(1,28)} = 0.22$, $p = 0.64$; social interaction, $F_{(1,28)} = 6.86$, $p < 0.05$; Figure 5*A–D*). These data indicate that X11L protein is required not only for normal burying/burrowing behavior but also for normal social behavior, and suggest that social behavior and species-typical behavior may share some common neuronal mechanisms associated with X11L protein.

Monoamine systems are disrupted in X11L-KO mice

Marble burying behavior is affected by anxiolytic drugs, particularly 5-HT agents. Drugs acting on the 5-HT system, such as 5-HT reuptake inhibitors, which increase 5-HT levels at release sites, and some 5-HT agonists, inhibit marble burying behavior without affecting locomotor activity (Njung'e and Handley, 1991b; Shinomiya et al., 2005; Nicolas et al., 2006; Bruins Slot et al., 2008). Some antipsychotics also inhibit marble burying behavior (Broekkamp et al., 1986; Nicolas et al., 2006; Bruins Slot et al., 2008). These reports suggest that monoaminergic activity underlie marble burying behavior. Therefore, we measured the amounts of monoamines and their metabolites in selected brain regions of X11L-KO mice.

Biopsies of the following seven regions were obtained from frozen brain sections: anterior cingulate cortex, striatum, hypothalamus (mainly containing the paraventricular nucleus), somatosensory cortex (mainly containing the barrel field), amygdala, hippocampus (mainly containing the CA3 region), and dorsal and median raphe (Fig. 6*A*). These areas were selected on the basis of high X11L expression, or their known involvement in the behaviors studied here. Although the levels of most monoamines and their metabolites in the X11L-KO mice brains were not significantly different from those in WT mice, 5-HT levels were significantly higher in the hypothalamus and somatosensory cortex (Table 1). Because monoamines are synthesized and broken down in sophisticated biochemical networks, we sought evidence of an altered flux through these networks by analyzing the correlations among monoamines and their metabolites. The correlational relationships in the striatum, hippocampus, and somatosensory cortex of X11L-KO mice were substantially different from those in WT mice, which were reflected in an asymmetrical pattern in the correlation maps (Fig. 6*B*). In contrast, these relationships were highly comparable in the raphe and amygdala (Fig. 6*B*). These results suggest that X11L has an active role in regulating the synthesis and/or breakdown of monoamine neurotransmitters.

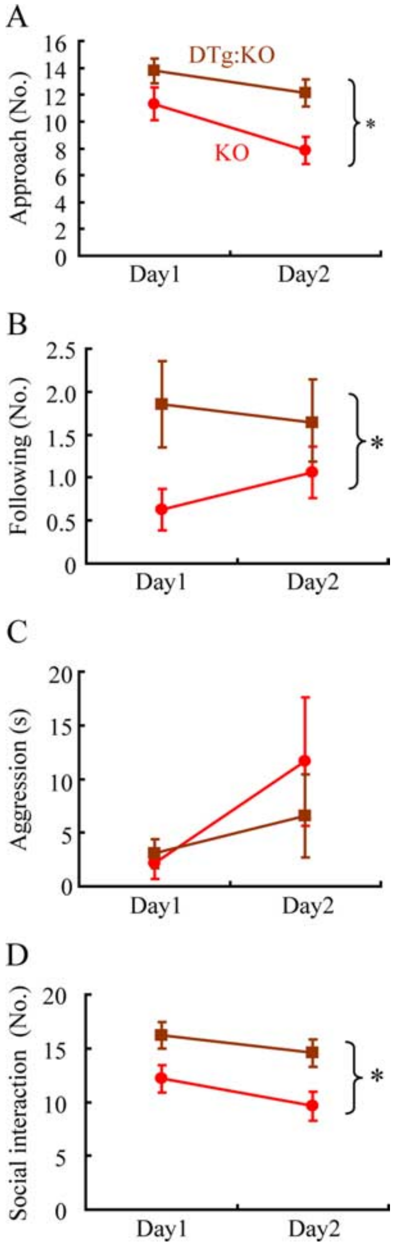

Figure 5. Reduced social interaction in X11L-KO mice was restored by the expression of the X11L protein. The resident-intruder test (DTg:X11L-KO mice, brown square; X11L-KO mice, red circle) was performed for 2 consecutive days using different intruder mice for each trial. ***A***, ***B***, The numbers of approaches (***A***) and following behaviors (***B***) of resident mice against intruder mice were significantly higher in DTg:X11L-KO mice. ***C***, Differences in the amount of time spent by resident mice exhibiting aggressive behavior was not statistically significant between genotypes. ***D***, The summed number of social interactions of resident mice toward intruder mice was significantly increased in DTg:X11L-KO mice compared with X11L-KO mice. Data represent mean ± SE. $^{*}p < 0.05$.

To gain insight into the function of the serotonergic system in X11L-KO mice, we analyzed the hypothermic responses induced by the selective 5-HT1A receptor agonist 8-OH-DPAT, which reflects somatodendritic autoreceptor function in raphe neurons (Goodwin et al., 1985; Bill et al., 1991; Martin et al., 1992; Gross et

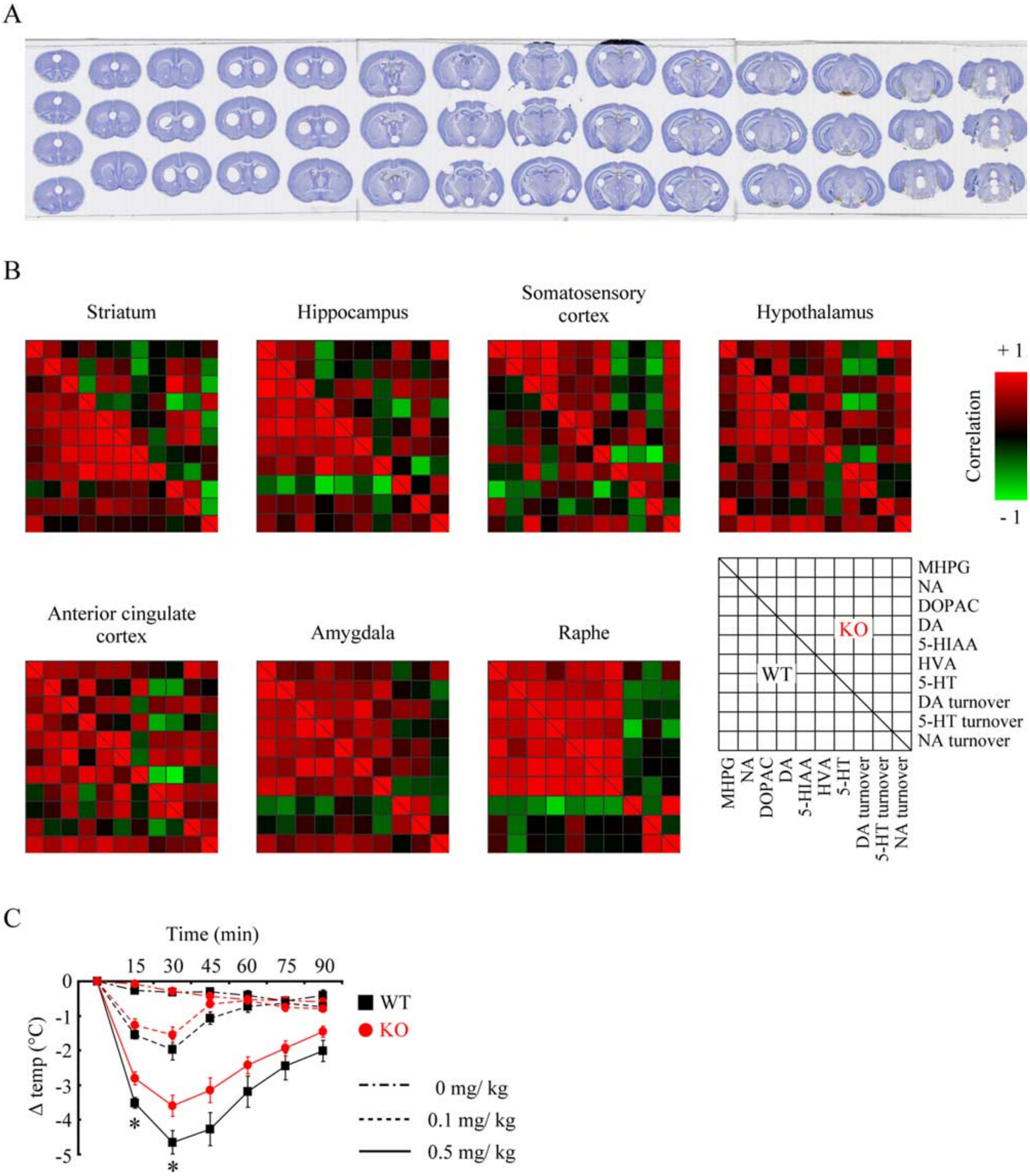

Figure 6. Balance of monoamine systems is disturbed in X11L-KO mice. ***A***, Representative photomicrographs of Nissl-stained brain sections showing locations of biopsies used for neurochemical analyses. ***B***, Correlation maps showing relationships between tissue contents of monoamines, metabolites, and calculated turnover values. Red indicates positive correlation. Green indicates negative correlation. ***C***, Time course and dose–response of 8-OH-DPAT-induced hypothermia in X11L-KO and WT mice. Data represent mean ± SE. *$p < 0.05$ (WT mice, black square; X11L-KO mice, red circle).

al., 2002). Stimulation of 5-HT1A autoreceptors attenuates 5-HT release and induces a hypothermic response. The body temperature of WT mice decreased after 8-OH-DPAT administration (Fig. 6*C*). This hypothermic response was significantly attenuated in X11L-KO mice 15 and 30 min after injection with 0.5 mg/kg 8-OH-DPAT (6–7 months old, WT and X11L-KO mice, $n = 8$ per group; at 15 and 30 min of 0.5 mg/kg dose, $p < 0.05$) (Fig. 6*C*), suggesting that 5-HT1A receptor function or its downstream signaling is attenuated in X11L-KO mice.

Discussion

The main finding of the present study is that X11L-KO mice show a selective deficit in motivated approach behavior, but not in motivated avoidance behavior. That is, X11L-KO mice showed

Table 1. The amount of monoamines and metabolites

Monoamines and metabolites	Genotypes	Anterior cingulate cortex	Striatum	Hypothalamus	Somatosensory cortex	Amygdala	Hippocampus	Raphe
5-HT	WT	1681 ± 144	2444 ± 165	4039 ± 170	917 ± 45	5403 ± 342	3566 ± 318	9779 ± 697
	KO	1491 ± 97	2709 ± 61	5021 ± 245**	1130 ± 78*	4819 ± 287	3464 ± 188	9069 ± 908
5-HIAA	WT	2300 ± 219	2500 ± 221	3939 ± 221	758 ± 23	3663 ± 235	4661 ± 381	11,438 ± 933
	KO	2148 ± 64	2620 ± 79	3964 ± 212	759 ± 54	3247 ± 209	4121 ± 211	10,006 ± 855
NA	WT	3420 ± 214	73 ± 11	13,750 ± 607	2275 ± 78	3539 ± 188	2713 ± 217	5644 ± 493
	KO	3129 ± 134	117 ± 27	15,455 ± 654	2562 ± 150	3142 ± 141	2584 ± 93	5988 ± 619
MHPG	WT	513 ± 40	29 ± 7	577 ± 67	169 ± 7	573 ± 39	277 ± 28	1042 ± 78
	KO	449 ± 37	42 ± 8	553 ± 51	168 ± 8	452 ± 22*	245 ± 18	990 ± 118
DA	WT	460 ± 37	10,9843 ± 8939	2431 ± 133	90 ± 4	5345 ± 451	219 ± 30	1156 ± 99
	KO	394 ± 21	12,0274 ± 3273	2605 ± 157	94 ± 9	4227 ± 401	189 ± 12	1138 ± 117
DOPAC	WT	502 ± 46	18,005 ± 2298	854 ± 49	146 ± 12	2040 ± 152	265 ± 29	639 ± 41
	KO	420 ± 22	21,580 ± 1520	826 ± 28	141 ± 11	1654 ± 186	237 ± 14	594 ± 52
HVA	WT	1113 ± 148	14,317 ± 1178	1293 ± 97	192 ± 17	2145 ± 169	289 ± 20	1300 ± 70
	KO	889 ± 63	14,513 ± 312	1215 ± 65	153 ± 17	1647 ± 198	273 ± 21	1233 ± 111
5-HT	WT	1.27 ± 0.07	0.94 ± 0.05	0.90 ± 0.05	0.77 ± 0.04	0.63 ± 0.03	1.22 ± 0.07	1.08 ± 0.04
Turnover	KO	1.37 ± 0.10	0.89 ± 0.03	0.74 ± 0.05*	0.63 ± 0.06	0.62 ± 0.03	1.12 ± 0.08	1.03 ± 0.04
NE	WT	0.14 ± 0.01	0.36 ± 0.06	0.04 ± 0.004	0.07 ± 0.003	0.15 ± 0.004	0.09 ± 0.003	0.17 ± 0.01
Turnover	KO	0.13 ± 0.01	0.36 ± 0.04	0.03 ± 0.003	0.06 ± 0.001*	0.13 ± 0.01	0.09 ± 0.005	0.16 ± 0.01
DA	WT	3.16 ± 0.53	0.26 ± 0.02	0.77 ± 0.03	3.30 ± 0.29	0.69 ± 0.02	2.36 ± 0.18	1.47 ± 0.05
Turnover	KO	2.92 ± 0.20	0.27 ± 0.02	0.69 ± 0.04	2.82 ± 0.20	0.69 ± 0.06	2.44 ± 0.24	1.44 ± 0.10

Data shown are mean ± SE (pg/mg protein).

significantly attenuated ethological responses to attractive stimuli and withdrew during approach-avoidance conflict, such as that encountered during competitive feeding conditions and social interaction. Additionally, attenuated marble-burying, burrowing and reduced social behavior in X11L-KO mice was rescued by expression of X11L protein during development. Together, these findings suggest that X11L is involved in the maturation of neuronal circuits regulating conflict resolution-related behaviors. We propose that X11L-KO mice provide a novel model for analyzing emotional behavior, which differs from the existing models of generalized anxiety-like and depression-like behaviors (Finn et al., 2003; Cryan and Mombereau, 2004; Crawley, 2007).

X11L-KO mice lost more body weight than WT mice, specifically under a competitive feeding regimen with WT mice. This was unlikely due to physical defects or changes in general activity, but is likely related to the suppression of a social interaction-induced behavioral response. The results from the resident-intruder test support this notion. That is, X11L-KO mice exhibited reduced exploratory behavior toward intruder mice. The social behavior of one mouse toward another mouse probably reflects the net result of conflicts from coexisting incentives, including those that might induce approach (e.g., investigation of intruder mice and approach to housemates that have a food pellet) and those that might induce avoidance (e.g., threat from novel intruders, avoiding competition with housemates, and generalized anxiety). Anxiety-like behaviors, which are often expressed as avoidance, are thought to be closely associated with decreased social interaction (File, 1992; Sankoorikal et al., 2006; Crawley, 2007; Moy et al., 2007). Interestingly, X11L-KO mice did not show anxiety-like behavior in standard behavioral tests, such as the elevated plus maze, light and dark transition, and open field tests. Thus, low levels of social interaction in X11L-KO mice appear to be independent of "traditional" anxiety-like behaviors that are expressed as avoidance of aversive stimuli.

X11L-KO mice showed prominent defective ethological stimuli-responses, such as reduced burying, digging and burrowing behaviors, whereas they showed no phenotypes in many of the most common behavioral tests (e.g., locomotor activity, circadian rhythm, open field, elevated plus maze, light and dark transition, tail suspension, prepulse inhibition and some learning/memory tests) commonly applied in the study of behavioral genetics. These data highlight the importance of testing species-typical behaviors to better understand brain functions. So-called ethological tests likely have strong potential to extract brain function and mechanisms. Burrowing behavior is highly sensitive to dysfunctions of the hippocampus induced by cytotoxic lesion, scrapie infection and in AD model mice (Deacon et al., 2001, 2002, 2008). Marble burying behavior is correlated very well with activity in the serotonin system and is proposed to have predictive validity as a model of obsessive-compulsive disorder (OCD)-like behavior as drugs used to treat OCD inhibit marble burying behavior (Njung'e and Handley, 1991b; Shinomiya et al., 2005; Nicolas et al., 2006; Bruins Slot et al., 2008; Kobayashi et al., 2008). Thus, species-typical ethological responses may provide the most sensitive parameters to estimate the therapeutic effect using animal models.

Transgene rescue experiments using the *Camk2a-tTA* mouse line suggested that X11L is involved in the development of neuronal circuits regulating emotional and conflict-related behaviors. We found that monoamine concentrations in forebrain regions of X11L-KO mice were imbalanced. Monoamine signaling is critically involved in developing neuronal circuits that modulate emotion in adults (Reif and Lesch, 2003). The development of 5-HT function in the postnatal period determines adult emotional behavior (Andersen et al., 2002; Gross et al., 2002; Ansorge et al., 2004, 2008; Lo Iacono and Gross, 2008). Catecholamine levels during early life also influence adult emotional behavior (Estelles et al., 2007; Gray et al., 2007). Maturation and/or refinement of forebrain circuits during postnatal weeks 2 through 5 via somatodendritic 5HT1A receptor interactions is considered critical for programming anxiety-related behaviors (Gross et al., 2002; Lo Iacono and Gross, 2008). X11L expression begins during development, is strongly localized to the somatodendritic compartment of forebrain region (Nakajima et al., 2001; Ho et al., 2003), and binds many molecules involved in regulating neuronal activity. Therefore, X11L may function as a neuronal mediator in the development of emotional circuits via molecules relating to monoaminergic systems.

Alternatively, or additionally, the behavioral deficits observed in X11L-KO mice in adulthood may reflect ongoing neurochemical perturbations. In the present study, the statistically strongest neurochemical difference was increased 5-HT content in the hypothalamus. Previous studies show that decreased hypothalamic 5-HT levels are associated with increased aggression in rats [Vergnes et al., (1988); see Popova (2006) for discussion of the role of 5-HT in aggression]. By the same token, the subordinate behavior of X11L-KO mice reported here may relate to increased 5-HT levels and/or altered 5-HT metabolism. It should be noted, however, that monoamine turnover rates were marginally lower in X11L-KO mice in some regions studied, in addition to monoamine-metabolite imbalances being evident. Thus, the possibility that other neurochemical changes underlie the behavioral deficits observed here cannot be ruled out. There is undoubtedly a close relationship between activity in monoaminergic systems, as exemplified by studies of 5-HT1A and 5-HT1B receptor KO mice, which show increases in both 5-HT and dopamine turnover (Ase et al., 2000). Indeed, all three monoaminergic systems are targeted in the treatment of anxiety-related disorders and social phobias in the clinical setting, and so much further research is necessary to elucidate the detailed interactions between these systems that bring about the sophisticated behaviors studied here.

How X11L affects monoaminergic systems remains to be determined. 8-OH-DPAT (5-HT1A receptor specific antagonist)-induced hypothermia was attenuated in the X11L-KO mice. The 5-HT1A receptor is a G_i-type G-protein coupled receptor. Its activation opens G-protein-coupled inwardly rectifying K^+ channels (GIRKs) by binding liberated $G_\beta\gamma$ proteins from 5-HT1A receptor, resulting in cell hyperpolarization and inhibition of 5-HT release (Andrade and Nicoll, 1987; Williams et al., 1988; Lüscher et al., 1997). Functional coupling of 5-HT1A receptors to GIRKs does not occur until postnatal day 14 (Béïque et al., 2004). GIRKs are also downstream regulators of several other G-protein coupled receptors such as dopaminergic (D2), adrenergic (α2), and muscarinic (M2) autoreceptors, and they act to regulate cellular excitability and coupling (North, 1989; Hille, 1992; Yamada et al., 1998). GIRKs containing the GIRK2 subunit are control hubs for the hypothermic response induced by various G-protein coupled receptor agonists (Costa et al., 2005). The GIRK2 subunit is widely distributed throughout the brain and strongly localized to the somatodendritic compartment (Murer et al., 1997; Schein et al., 1998; Inanobe et al., 1999; Saenz del Burgo et al., 2008). Additionally, GIRK3 and GIRK2c, which is a splice variant of GIRK2, subunits have a PDZ-binding motif (-ESKV) at their C-terminal and their trafficking is regulated by PDZ-containing adaptor proteins (Inanobe et al., 1999; Ma et al., 2002; Lunn et al., 2007). In contrast, X11L regulates the intracellular localization of its binding protein and also has coat protein properties (Hill et al., 2003; Sano et al., 2006a). Therefore, it is possible that X11L regulates the intracellular localization of GIRK2, and the functional attenuation of GIRKs, by ultimately altering neuronal excitability, may disturb monoamine balance and reduce the hypothermic response in X11L-KO mice. Indeed, we have observed specific binding between X11L and GIRK2c by coimmunoprecipitation analyses using a cell culture system (YS, HS, and SI, unpublished data).

Other mechanisms may underlie the role of X11L in neurochemical activity and behavior. X11L regulates APP metabolism (Sano et al., 2006a) and interacts with many other types of neuronal/synaptic machinery (Okamoto and Südhof, 1997; Tomita et al., 1999; Biederer and Südhof, 2000; Gotthardt et al., 2000; Lau et al., 2000; Kitano et al., 2002; Araki et al., 2003; Hill et al., 2003; Kimura et al., 2004). The unique behavioral abnormalities in X11L-KO mice may be due to the combined dysfunction of interacting molecules and the compensatory effect of X11, which has binding partners and cellular functions similar to those of X11L (Ho et al., 2006; Miller et al., 2006; Rogelj et al., 2006; Sano et al., 2006a). Interestingly, compared with WT mice, X11-KO mice exhibit a vigorous and rapid escape response when the sides and body of the animal are stroked (Mori et al., 2002). Comparative studies using these mice and mice with conditional double mutations in limited subsets of neurons might help to better understand the mechanisms that underlie emotional control.

Finally, the X11L gene, namely *APBA2*, is located at the 15q11-q12 locus. The close association between abnormalities in the 15q11-q13 region and autism is frequently reported (Cook et al., 1997, 1998; Gillberg, 1998; Schroer et al., 1998; Shao et al., 2002; Muhle et al., 2004). Autism is a neurodevelopmental disorder characterized by impaired social interaction and communication. It is possible that X11L dysfunction during the developmental stage relates to the pathogenesis of developmental disorders with mental retardation such as autism, and X11L may have an important role in the development of emotionality and sociability in humans.

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