Autism-like socio-communicative deficits and stereotypies in mice lacking heparan sulfate

Fumitoshi Irie, Hedieh Badie-Mahdavi, and Yu Yamaguchi1

Genetic Disease Program, Sanford Children’s Health Research Center, Sanford-Burnham Medical Research Institute, La Jolla, CA 92037

Edited by Thomas C. Südhof, Stanford University School of Medicine, Palo Alto, CA, and approved February 13, 2012 (received for review October 31, 2011)

Heparan sulfate regulates diverse cell-surface signaling events, and its roles in the development of the nervous system recently have been increasingly uncovered by studies using genetic models carrying mutations of genes encoding enzymes for its synthesis. On the other hand, the role of heparan sulfate in the physiological function of the adult brain has been poorly characterized, despite several pieces of evidence suggesting its role in the regulation of synaptic function. To address this issue, we eliminated heparan sulfate from postnatal neurons by conditionally inactivating Ext1, the gene encoding an enzyme essential for heparan sulfate synthesis. Resultant conditional mutant mice show no detectable morphological defects in the cytoarchitecture of the brain. Remarkably, these mutant mice recapitulate almost the full range of autistic symptoms, including impairments in social interaction, expression of stereotyped, repetitive behavior, and impairments in ultrasonic vocalization, as well as some associated features. Mapping of neuronal activation by c-Fos immunohistochemistry demonstrates that neuronal activation in response to social stimulation is attenuated in the amygdala in these mice. Electrophysiology in amygdala pyramidal neurons shows an attenuation of excitatory synaptic transmission, presumably because of the reduction in the level of synaptically localized AMPA-type glutamate receptors. Our results demonstrate that heparan sulfate is critical for normal functioning of glutamatergic synapses and that its deficiency mediates socio-communicative deficits and stereotypies characteristic for autism.

Autism, also designated as autism spectrum disorders, is a heterogeneous cognitive syndrome characterized by impairment in reciprocal social interaction, problems with verbal and nonverbal communication, and repetitive behaviors with narrow interests (1). It is a lifelong disorder affecting about one in 100 children (1). There is evidence that genetic factors contribute to the development of autism, but the genetic basis of most autism cases remains unclear and likely involves multigene interactions. It is increasingly evident that autism-susceptibility genes encode diverse molecules with distinct biological functions in neural development and physiology (2). Whether and how mutations in these diverse genes converge on a few common molecular pathways is one of the crucial questions in the field. Analysis of familial autism cases has identified mutations in genes thought to function in the regulation of excitatory synapses (3, 4), suggesting that excitatory synaptic dysfunction is one of the molecular mechanisms of autism (5).

Heparan sulfate (HS) is a highly sulfated linear polysaccharide with a backbone of alternating N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) residues. HS is attached covalently to various core proteins to form HS proteoglycans (HSPGs) that are present on cell surfaces and in extracellular spaces. Through HS moieties, HSPGs bind diverse bioactive molecules, such as growth factors, morphogens, and cell-surface receptors, and regulate numerous biological activities (6). HS synthesis is governed by a series of enzymes, among which Ext1 catalyzes elongation of the linear polymer of alternating GlcA and GlcNAc residues that forms the backbone of HS. Ext1 also is known as one of the causative genes of multiple hereditary exostoses, a genetic disorder characterized by the formation of multiple benign bone tumors and variable accessory symptoms (7).

Roles of HS in neural development have been studied by using animal models that carry mutations in Ext1 and other genes encoding enzymes involved in HS synthesis. These genetic studies revealed that HS is necessary for the specification of certain brain structures, such as the cerebellum and the olfactory bulbs, cortical neurogenesis, and a variety of axon path-finding processes (8–12). Although these studies have established the relevance of HS in neural development, a key unresolved issue concerning HS in the nervous system is the role of HS in the adult brain and its possible relevance to human neurological and mental disorders. Several pieces of evidence suggest a role for HS in synaptic function as well as in higher cognitive function. In adult neurons, HS is enriched in synapses, especially in the postsynaptic membrane of dendritic spines (13, 14). Treatment of hippocampal slices with heparin lyase (heparinase III) has been shown to affect synaptic plasticity (15). Moreover, data from human genetic studies suggest a role for HS and HSPGs in human mental disorders. For instance, there have been reports describing the association of autism and other symptoms of mental impairment with multiple exostoses in patients carrying mutations in HS/HSPG genes (16–20). However, except for two separate cases reported by Li et al. (18) in which frameshift mutations within exons of the Ext1 gene were identified, these early examples involved large-scale deletions or translocations, making it difficult to establish a definitive role for the HS/HSPG genes in the development of autistic symptoms. More recently, genome-wide genetic studies have provided additional insight into the issue. Genetic association has been found between autism and the HS3ST5 gene encoding one of the HS 3-O sulfotransferases in two large cohorts of European ancestry (21). In addition, a genome-wide scan for rare copy number variation (CNV) in 996 autism cases has identified four independent CNVs in the GPC5/GPC6 gene cluster, which encodes the glypicans-5 and glypicans-6 HSPGs in tandem array, on chromosome 13q22 (22). Finally, data from mouse models of autism also suggest the possible connection between autism and HS: Recently it has been shown that the level of HS immunoreactivity is reduced in the brain tissue of BTBR T+tf/J mice (23, 24), a strain that exhibits a host of behaviors recapitulating the major symptoms of autism (25, 26).

To define the role of HS in brain physiology, we generated conditional Ext1-knockout mice targeted to postnatal neurons. These conditional Ext1 mutant mice develop normally without any detectable morphological changes in the brain. Remarkably, these mice recapitulate numerous autism-like behavioral phenotypes encompassing the three core deficits of autism. Results from electrophysiological analyses indicate that removal of HS

Author contributions: F.J. and Y.Y. designed research; F.J. and H.B.-M. performed research; F.J. and Y.Y. analyzed data; and Y.Y. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

1To whom correspondence should be addressed. E-mail: yyamaguchi@sanfordburnham.org.

This article contains supporting information online at www.pnas.orglookup/ulpp/doi:10.1073/pnas.11178811109/DCSupplemental.
compromises glutamatergic synaptic transmission by affecting the synaptic localization of AMPA receptors. Our results demonstrate that HS is required for normal functioning of glutamatergic synapses. Moreover, the development of a constellation of autism-like deficits in these mice suggests that the cellular and molecular conditions resulting from the elimination of neuronal HS recapitulate critical parts of the pathogenic mechanisms of autism.

Results

Neuron-Specific Inactivation of Ext1. To achieve neuron-specific Ext1 inactivation, we crossed mice carrying the conditional Ext1 allele (8) with CaMKII-Cre2834 transgenic mice (27). Resultant CaMKII-Cre2834;Ext1fl/fl mice hereafter are designated “Ext1CKO” mice. Because recombination by the CaMKII-Cre2834 transgene commences after the third postnatal week (27), the effect of Ext1 inactivation on embryonic brain development is essentially bypassed. It has been shown that CaMKII-Cre2834 drives recombination selectively in glutamatergic neurons in the forebrain (28). In agreement, our analysis of Ext1CKO mice confirmed that Ext1 is eliminated selectively from GluA2–pyramidal neurons (Fig. S1 A and B). Biochemical analyses with whole-brain tissue (containing both CaMKII-Cre2834-targeted and nontargeted cell types) demonstrated that both Ext1 protein and HS are reduced significantly in the brain areas where CaMKII-Cre2834 is active, such as the hippocampus and amygdala (Fig. S1 C and D). In contrast, no reduction in the levels of Ext1 protein or HS was detected in the cerebellum, where CaMKII-Cre2834 is not active (27).

As expected from the late onset of Cre expression, Ext1CKO mice grew normally (Fig. S2) and showed no detectable developmental abnormalities in the brain, including neuronal lamination patterns and the morphology of fiber tracts (Fig. S2 C and D). Ext1CKO mice exhibited no abnormalities in motor functions, reflexes, olfaction, or vision (Table S1). Moreover, there was no difference between control and Ext1CKO mice in visual memory (Table S1) or social memory (Fig. S3B). Interestingly, Ext1CKO mice displayed reduced nest-building activity (Fig. S3A), which is a phenotype implicated in autism (1, 29), prompting us to examine autism-related behaviors in Ext1CKO mice.

Ext1CKO Mice Recapitulate Three Core Deficits of Autism. Impairment of reciprocal social interaction skills is one of the core characteristics of autism (1). Ext1CKO mice were subjected to the following three paradigms to assess social behavior. First, social interaction between two siblings of the same genotype after separation was examined by the separation–reunion test. Consistent with a previous report (30), WT mice interacted extensively after reunion (Movie S1). In contrast, Ext1CKO mice showed much less interaction (Fig. S4 and Movie S2). This impairment in social interaction is not attributable to the impairment of social memory (Fig. S3B). Second, the social response to an encounter with an unfamiliar mouse was examined by the resident–intruder test (31). WT mice explored the intruder extensively by sniffing and chasing (Movie S3), but Ext1CKO mice seldom chased the intruder (Movie S4). Instead, they frequently showed behaviors suggestive of avoidance, such as freezing and moving away (Fig. 1B and Movie S4). Third, the social dominance tube test showed that Ext1CKO mice almost always lose (i.e., retreat out of the tube) in this test (Fig. 1C and Movie S5). Together, these results from three independent social paradigms demonstrate a significant impairment in social interaction by Ext1CKO mice.

Abnormal linguistic communication is another key impairment of autism. We analyzed ultrasonic vocalization (USV), which increasingly has been used to model autism-like communication deficits (1), in Ext1CKO mice. When challenged by female odor, WT mice emitted a rapid series of frequency-modulated calls of various types (see Movie S7 for audio playback), as reported previously (32). In contrast, the USVs emitted by Ext1CKO mice were reduced significantly in terms of number, duration, and amplitude of calls (Fig. 1 D–F). The richness and complexity of individual calls also were reduced (see Movie S8 for audio playback). The reduction in the rate of USV was not caused by reduced amounts of time spent sniffing the nest piece, because the duration of this behavior was similar in WT and Ext1CKO mice (WT: 24.99 ± 1.98 s/min, n = 8; Ext1CKO: 27.64 ± 3.18 s/min, n = 8, P = 0.4918, Student’s t test). Overall, these results suggest that vocalization-mediated communication is compromised in Ext1CKO mice.

A third core symptom of autism is stereotypic, repetitive behavior (1). Video monitoring of movements in the home cage revealed no spontaneous stereotyped behavior, such as jumping, circling, paw flapping, or self-grooming, in Ext1CKO mice. Nevertheless, Ext1CKO mice showed clear abnormalities when subjected to the hole-board test. In this test, repetitive head-dips into the same hole are analyzed as a measure of stereotypy (33). WT mice typically explore different holes in a random or successive manner (Movie S9). In contrast, Ext1CKO mice showed a clear tendency to make repeated head-dips (see Movie S10). In addition, the video recordings revealed “stereotyped dips”—behaviors that were significantly greater in Ext1CKO mice than in WT mice (Fig. 1G), although the total number of head-dips during the session did not differ between
groups (WT: 76.82 ± 3.474, n = 11; Ext1\(^{CKO}\): 76.27 ± 5.88 n = 11; \(P = 0.9371\), Student’s \(t\) test). Moreover, Ext1\(^{CKO}\) mice showed a tendency to perform consecutive head-dips of more than four repetitions, a behavior never seen in WT mice (Fig. 1G).

Other Behavioral and Neurological Phenotypes of Ext1\(^{CKO}\) Mice. In addition to the above phenotypes reminiscent of the three core symptoms of autism, Ext1\(^{CKO}\) mice display other behavioral deficits. First, Ext1\(^{CKO}\) mice showed alterations in anxiety-related behaviors. In an elevated plus maze, Ext1\(^{CKO}\) mice spent more than half the session time on the open arms and moved quite freely on them, whereas WT mice remained mostly on the closed arms during the session (Fig. 2A). In the light/dark box test, Ext1\(^{CKO}\) mice spent a much longer time in the brightly illuminated space than did WT mice (Fig. 2B), although the number of transitions between light and dark spaces did not differ between the two genotypes (WT: 5.13 ± 0.58 s, \(n = 8\); Ext1\(^{CKO}\): 6.13 ± 0.64 s, \(n = 8\); \(P = 0.2662\), Student’s \(t\) test). In the open-field test, Ext1\(^{CKO}\) mice spent a significantly longer time in the central area than did WT mice and exhibited higher levels of locomotor activity (Fig. 2C). Together, these results indicate that Ext1\(^{CKO}\) mice have reduced fear of height and open spaces. Second, it was found that Ext1\(^{CKO}\) mice exhibited significantly shorter latency to respond to thermal stimuli (Fig. 2D). Although the relevance of these phenotypes to autism is less clear than the recapitulation of the core symptoms, it is interesting that a lack of fear in response to real dangers, hyperactivity, and odd responses to sensory stimuli are noted as examples of associated features that occasionally are observed in individuals with autism (34).

Mapping of the Location of Neural Activation Deficits in Ext1\(^{CKO}\) Mice. To define the anatomical basis for the autism-like social impairments seen in Ext1\(^{CKO}\) mice, we mapped potential spatial differences in neuronal activation in response to social stimulation using neuronal c-Fos induction as an activity marker (35). In WT mice, stimulation by the separation–reunion paradigm (a protocol similar to that used in the separation–reunion test described above) induced c-Fos expression in various brain regions previously implicated in social behaviors, including the ventral orbitofrontal cortex, piriform cortex, CA3 hippocampus, and basolateral amygdala (36, 37) (Fig. 3A). In the same assays, Ext1\(^{CKO}\) mice showed levels of c-Fos induction in the piriform cortex and CA3 hippocampus equivalent to those seen in WT mice. However, the level of induction was significantly lower in the basolateral and medial amygdala, as well as in the ventral orbitofrontal cortex, which has reciprocal connections with the amygdala that are critical for socio-emotional information processing (Fig. 3 and Table S2) (38). These data suggest that in Ext1\(^{CKO}\) mice functional deficits underlying the behavioral phenotype center mainly in the amygdala system, and we performed the subsequent electrophysiological experiments in the amygdala.

Excitatory Synaptic Transmission Is Altered in Amygdala Neurons of Ext1\(^{CKO}\) Mice. As noted above, selective loss of Ext1 protein from pyramidal neurons in the amygdala was confirmed (Fig. S1 A and B). We then asked whether there are morphological changes in the amygdala of Ext1\(^{CKO}\) mice. Consistent with the late onset of CaMKII-Cre, we observed no overt abnormalities in the overall morphology of the amygdala or in the morphology of dendritic arbors and spines in pyramidal neurons of the basolateral amygdala.
To examine whether synapses are altered functionally, we performed patch-clamp recording experiments on BLA pyramidal neurons following stimulation of their cortical input, the external capsule. It was found that the input-output curve of compound excitatory postsynaptic currents (EPSCs) is depressed in Ext1\(^{-}\text{CKO}\) mice (Fig. 4A). When the AMPA receptor-mediated response was isolated with GABA\(_{A}\) and NMDA antagonists, the input-output curve of Ext1\(^{-}\text{CKO}\) mice showed a more significant depression (Fig. 4A and B). These results suggest a reduced AMPA receptor-mediated synaptic strength in Ext1\(^{-}\text{CKO}\) BLA neurons. To define the nature of impairment further, we analyzed AMPA receptor-mediated miniature EPSCs (mEPSCs) in BLA pyramidal neurons. The frequency of mEPSCs was reduced in Ext1\(^{-}\text{CKO}\) mice (Fig. 4C and D), suggesting that there is either a decrease in the probability of neurotransmitter release or a decrease in the number of AMPA receptor-containing synapses (39). The amplitude of mEPSCs also was reduced in Ext1\(^{-}\text{CKO}\) BLA neurons (Fig. 4E and F), indicating that AMPA receptor-mediated postsynaptic activity is reduced. On the other hand, there was no difference between WT and Ext1\(^{-}\text{CKO}\) mice in the paired-pulse facilitation response (Fig. 5A and B), indicating normal probability of presynaptic neurotransmitter release in Ext1\(^{-}\text{CKO}\) BLA neurons. Thus, the reduction in mEPSC frequency in Ext1\(^{-}\text{CKO}\) mice represents changes in postsynaptic AMPA receptor activity; these changes are likely to be caused either by a decrease in synaptically expressed AMPA receptors or by a change in channel kinetics of AMPA receptors. Neither the rising nor the decay time of mEPSCs was altered (Fig. 5C), indicating that channel kinetics of AMPA receptors is preserved in Ext1\(^{-}\text{CKO}\) BLA neurons.

To obtain corroborating evidence for the electrophysiological findings, we examined the surface level of AMPA receptors in cultures of Ext1-null primary neurons. Cell-surface biotinylation assay revealed that the level of surface-expressed GluA2 was reduced by 46% in mutant neurons (Fig. 5A). The reduction in surface-expressed GluA2 did not reflect overall reduced expression, because the amount of the total cellular GluA2 was unchanged (Fig. 5B, Total). The surface levels of two other membrane proteins, EphB2 and transferrin receptor, were unchanged, showing the specificity of the effect. Because surface biotinylation assays do not distinguish between synaptic and extrasynaptic AMPA receptors, we further examined GluA2 associated with dendritic spines by live immunostaining. This analysis showed that the intensity of GluA2 immunoreactivity is reduced by 41% in mutant synapses (Fig. 5B). Taken together, these results demonstrate that AMPA receptor-mediated synaptic transmission is compromised in the absence of HS, presumably because of the reduced synaptic expression of AMPA receptors.

**Discussion**

In the present study, we show that ablation of HS expression in excitatory neurons results in a spectrum of behavioral abnormalities similar to those observed in autism. It is particularly remarkable that the similarity encompasses all three core symptoms of autism. Such a high level of phenotypic recapitulation has been described for only a few mouse models with mutations in genes for which the relevance to autism is supported by strong human genetics data, including Nlgn4-null (40) and Cntnap2-null (41) mice and the BTBR T+tf/J mouse, a naturally occurring inbred strain known to recapitulate autistic deficits (25, 26). Although the presence of mutations in Ext1 and other genes involved in HS synthesis remains to be determined in the general autism population, the extensive recapitulation of autism-like deficits in Ext1\(^{-}\text{CKO}\) mice suggests that neuronal HS is functionally involved in the signaling pathway that plays the central role in the development of autism.

It is noteworthy that in Ext1\(^{-}\text{CKO}\) mice the recapitulation of numerous autistic deficits occurred when the knockout was restricted to postnatal excitatory neurons. Although it is not possible to state unequivocally that there are no morphological defects in the brains of Ext1\(^{-}\text{CKO}\) mice, the spatiotemporal specificity of Cre expression and the results of our morphological analysis indicate that functional alteration of synapses, rather than abnormal brain development, is the basis for the behavioral phenotypes seen in Ext1\(^{-}\text{CKO}\) mice. Consistent with this notion, our study also implicates impaired glutamatergic synaptic transmission resulting from the reduced synaptic expression of AMPA receptors as a basis for development of autism-like behavioral deficits. Hypofunction of glutamatergic neurotransmission has been postulated to be a potential mechanism of autism (42, 43). In fact, GluA1-knockout mice exhibit social and anxiety phenotypes that partially overlap with the behavioral phenotypes of Ext1\(^{-}\text{CKO}\) mice (44, 45).

How does HS regulate synaptic expression of AMPA receptors? Unlike its well-established role in regulating secreted morphogens and growth factors, little is known about whether HS controls trafficking and/or surface retention of cell-surface receptors in general. However, it is interesting that AMPA receptors can bind heparin (46). Thus it is possible that AMPA receptors interact with neuronal HS PGs, such as syndecan-2 (13), in the postsynaptic site, and that the interaction modulates surface expression of AMPA receptors in the postsynaptic membrane. Alternatively, HS may regulate AMPA receptors indirectly via modulation of other neuronal molecules. At least, two signaling systems implicated in excitatory synaptic function or viewed as autism-susceptibility genes are known to be modulated by interaction with HS, namely, neuregulin-1/erbB4 (47) and HGF/Met (48). Also interesting is that two autism candidate molecules, neurexin 1 (3) and CNTNAP2 (41, 49), contain laminin G domains,
which potentially can bind HS (50). Thus, strong mutations in the HS synthesis pathway, as modeled in this study, cause the entire spectrum of autistic symptoms by themselves, whereas milder mutations or epigenetic silencing of genes involved in HS synthesis may act as genetic modifiers of other autism candidate genes in human autism. In any event, the development of a remarkable constellation of autistic deficits in Ext1KCO mice suggests that the cellular and molecular conditions resulting from the elimination of neuronal HS closely recapitulate critical parts of the pathogenic mechanisms of human autism. Ext1KCO mice may be useful in dissecting the molecular pathway underlying the disorder.

Materials and Methods

Methods for histology, cell biology, electrophysiology, and behavioral analysis are described in SI Materials and Methods. Mice carrying theloxP-modified Ext1 allele (Ext1fl/fl) were created and maintained on a C57BL/6 background as described previously (8). CaMKII-Cre transgenic mice (line 2834) (27) were obtained from Bernhard Lüischer (Pennsylvania State University, University Park, PA) and backcrossed to C57BL/6 mice for more than eight generations before use in this study. Conditional Ext1-KO knockout mice specific for postnatal neurons (CaMKII-Cre;Ext1fllox/lox), designated Ext1KCO mice in this paper, were generated by crossing these two lines according to a standard breeding scheme (8). Littermates that inherited the incomplete combination of the above alleles were used as WT controls. For the preparation of primary cultures, cortices of Nestin-Cre;Ext1fllox/lox embryos (8) were used as the source of neurons. Animals were kept in a temperature-controlled (22 °C) environment with a 12 h/12 h light/dark cycle throughout their maintenance and behavioral analyses. All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at the Sanford-Burnham Medical Research Institute.

Acknowledgments. We thank Drs. Barbara Ranscht and Dongxian Zhang for advice on electrophysiology; Dr. Amanda Roberts for advice on behavioral assays; Ayame Michino, Misako Okuno, and Saki Lizuka for technical assistance in behavioral analyses; Larkin Slater for animal maintenance and care; and Drs. Elena Pasquale and Takaji Shirasawa for providing reagents. Y.Y. thanks Jim Weston for support during the initial stage of the study and Craig Eaton and Sarah Ziegler of the Multiple Hereditary Exostoses (MHE) Research Foundation for continuous encouragement. This work was supported by National Institutes of Health Grants P01 HD25938 and R21 HD508817, by a Sanford Center Investigator grant, by a Mizutani Foundation grant, by the MHE Coalition, and by the MHE Research Foundation.

NeuroTechnology. Immuno logical parameters (head width, and neck length) were measured by the Bio-Rad 1024 laser-scanning confocal system equipped to detect differences between groups. Cox staining was performed by using a kit from FD NeuroTechnology. For immunohistochemistry, mice that had been stimulated by the reunion paradigm (see separation test below) were perfused 2 h after stimulation, and brains were prepared as described above. Cryosections were incubated with rabbit polyclonal anti-EXT1 (1); mouse monoclonal anti-GluA2 (MAB397; Chemicon); rat monoclonal anti-GFAP (clone: 2.2B10; Calbiochem); guinea pig polyclonal anti-VGlut1 (AB5905; Millipore); and rabbit polyclonal anti-VGAT (AB5062; Millipore). Sections were mounted on a slide glass with ProLong Gold medium (Invitrogen). For double labeling of postsynaptic density 95 (PSD95) protein and gephrin, brains were freshly frozen in isopentane cooled by dry ice. Cryosections were fixed further in methanol at −20°C for 10 min. After blocking, sections were incubated with mouse monoclonal anti-PSD95 antibody (clone: 7E3-1B8; Affinity BioReagents) followed by incubation with the Cy2-conjugated Fab fragment of anti-mouse IgG antibody (Jackson Immunoresearch). Sections were incubated further with mouse monoclonal anti-gephrin antibody (clone GlyR7a; Synaptic Systems) followed by Rhodamine Red-X-conjugated anti-mouse IgG. For c-Fos immunohistochemistry, mice that had been stimulated by the separation–reunion paradigm (see separation–reunion test below) were perfused 2 h after stimulation, and brains were prepared as described above. Cryosections were incubated with rabbit polyclonal anti-c-Fos antibody (sc-52; Santa Cruz) overnight after blocking. Sections were incubated with biotinylated anti-rabbit IgG antibody (Vector Laboratories) for 2 h, followed by incubation with Alexa 488-conjugated avidin (Invitrogen) for 30 min at room temperature. The number of c-Fos+ cells was determined as described (2) and compared across both genotype and treatment groups using the Student’s t test (two-tailed).

Histology. Mice were transcardially perfused with PBS followed by 4% (wt/vol) paraformaldehyde in PBS. The whole brain from these mice was postfixed in 4% (wt/vol) paraformaldehyde overnight and transferred to 10% (wt/vol) and then 30% (wt/vol) sucrose in PBS at 4°C. Brain sections were prepared using a cryostat. For Nissl staining, sections were mounted on a slide glass and then stained with cresyl violet solution (FD NeuroTechnology). For immunohistochemistry, free-floating sections (20 μm thick) were immersed in the blocking solution [1% (wt/vol) BSA, 0.2% Triton X-100 in PBS] for 1 h at room temperature. Sections were incubated overnight at 4°C with the following first antibodies: rabbit polyclonal anti-EXT1 (1); mouse monoclonal anti-GluA2 (MAB397; Chemicon); rat monoclonal anti-GFAP (clone: 2.2B10; Calbiochem); guinea pig polyclonal anti-VGlut1 (AB5905; Millipore); and rabbit polyclonal anti-VGAT (AB5062; Millipore). Sections were then incubated with fluorophore (Cy2, Rhodamine Red-X, Cy5)-conjugated secondary antibodies (Jackson Immunoresearch) for 2 h at room temperature. After washing with PBS, sections were mounted on a slide glass with ProLong Gold medium (Invitrogen). For double labeling of postsynaptic density 95 (PSD95) protein and gephrin, brains were freshly frozen in isopentane cooled by dry ice. Cryosections were fixed further in methanol at −20°C for 10 min. After blocking, sections were incubated with mouse monoclonal anti-PSD95 antibody (clone: 7E3-1B8; Affinity BioReagents) followed by incubation with the Cy2-conjugated Fab fragment of anti-mouse IgG antibody (Jackson Immunoresearch). Sections were incubated further with mouse monoclonal anti-gephrin antibody (clone GlyR7a; Synaptic Systems) followed by Rhodamine Red-X-conjugated anti-mouse IgG. For c-Fos immunohistochemistry, mice that had been stimulated by the separation–reunion paradigm (see separation–reunion test below) were perfused 2 h after stimulation, and brains were prepared as described above. Cryosections were incubated with rabbit polyclonal anti-c-Fos antibody (sc-52; Santa Cruz) overnight after blocking. Sections were incubated with biotinylated anti-rabbit IgG antibody (Vector Laboratories) for 2 h, followed by incubation with Alexa 488-conjugated avidin (Invitrogen) for 30 min at room temperature. The number of c-Fos+ cells was determined as described (2) and compared across both genotype and treatment conditions using two-way ANOVA. Bonferroni post hoc analysis was used to detect differences between groups.

Electrophysiology. Amygdala slices were prepared from 3- to 4-wk-old CaMKII-Cre;Ext1lox/lox (Ext1Cre) and control littermates. Slices (350 μm thick) were cut in cold (2–4°C) oxygenated (95% O2, 5% CO2) artificial cerebrospinal fluid (ACSF) using a slicing vibratome (Vibratome 1000; The Vibratome Co.). The composition of ACSF was (in mM) 130 NaCl, 3.5 KCl, 1.25 NaH2PO4, 1.5 MgSO4, 7H2O, 2 CaCl2·2H2O, 25 NaHCO3, and 10 glucose (pH 7.4, 315–330 mOsm). The slices were equilibrated for 1 h in oxygenated ACSF before recording. A slice then was transferred to a recording chamber and superfused continuously with oxygenated ACSF at a constant rate (2–3 mL/min) at room temperature. Using an Olympus BX61W1 (Olympus America) equipped with infrared filter and Q-imaging camera, we identified pyramidal neurons in the basolateral amygdala. A tungsten bipolar stimulating electrode, which provides cortical inputs to amygdala, was placed in the external capsule. A patch electrode (6–8 MΩ) containing (in mM) 140 K-glucurate, 5 NaCl, 5 MgCl2, 0.2 EGTA, 10 Hpes, 4 NaATP, 0.3 TrisGTP, and 14 phosphocreatine (pH 7.2–7.3) was used to establish whole-cell recording. Compound excitatory postsynaptic currents (EPSCs) were evoked by field stimulation of fibers in the external capsule at 0.1 Hz. The amplitude of EPSCs was recorded and digitized using a MultiClamp 700B amplifier and a Digidata 1440A (Molecular Devices). Using Clampex in the pCLAMP 10 software suite (Molecular Devices), we monitored, compensated, and corrected (70%) the series resistance. Currents were filtered at 1 kHz and digitized at 5 kHz with the holding potential set at −70 mV. To establish synaptic input–output responses of the pyramidal neurons, the external capsule was stimulated at minimal, half-maximal, and maximum intensity using a Master-8 and an ISO-Flex stimulator (A.M.P.I.), and the evoked EPSC amplitude was recorded. Paired pulse facilitation was examined by introducing two stimuli at 50- or 100-msec intervals at 40–50% of maximum intensity. Similar protocols were carried out in the presence of 20 μM bicuculline (Tocris Bioscience) and 50 μM Mtx-2-amino-5-phosphonopantoic acid (AP-V; Tocris Bioscience) to isolate AMPA receptor-mediated input–output response. To measure AMPA receptor-mediated miniature EPSCs, 1 μM tetrodotoxin (Tocris Bioscience) was added to ACSF (containing bicuculline and AP-V), and spontaneous activity was recorded for 5 min. The data were analyzed using Clampfit in pCLAMP 10 (Molecular Devices) and MiniAnalysis (Synaptosoft Inc.) software programs. Statistical differences were analyzed by Student’s t test (two-tailed).

Cell Cultures. Ext1-deficient and wild-type cortical neurons were prepared from embryonic day 16.5 Nestin-Cre;Ext1lox/lox and control embryos, respectively (3). Cerebral cortices dissected from these embryos were dissociated by incubation with papain (0.2 mg/mL; ICN Biomedicals) and DNase (5 μg/mL; Sigma) for 20 min at 37°C. After washing, the cell suspension was plated on culture dishes and incubated in 10% (vol/vol) FCS containing DMEM for 2 h in a CO2 incubator (5% CO2). Nonadherent cells were collected and plated onto 13-mm glass coverslips (4×106 cells per slip for immunocytochemistry) or onto 6-cm culture dishes (5×106 cells per dish, for surface biotinylation experiments) coated with polyethyleneimine (0.5 mg/mL; Sigma) and laminin (5 μg/mL; BD Biosciences). Cultures were maintained in B27-containing Neurobasal medium (Invitrogen) under 5% CO2, 10% O2 at 37°C.

Analysis of AMPA Receptor Cell-Surface Expression. For live immunostaining, neurons on coverslips were incubated with a mouse monoclonal anti-GluA2 antibody (40 μg/mL; MAB397; Millipore), which recognizes the extracellular domain of the receptor subunit, in conditioned medium for 15 min in CO2 incubator. After quick washing with cold PBS, cells were fixed with 4% (wt/vol) paraformaldehyde/4% (wt/vol) sucrose in PBS for 10 min at room temperature. After permeabilization with 0.2% Triton X-100 for 10 min, cells were incubated with rabbit polyclonal MAP2 (Millipore) for 2 h at room temperature. Then cells were incubated with secondary antibodies, Cy2-conjugated anti-mouse IgG and Cy5-conjugated anti-rabbit IgG (Jackson ImmunoResearch), for 1 h. To visualize dendritic spines, cells were stained with rhodamine-
phalloidin (Invitrogen). Fluorescence images were acquired using a Bio-Rad 1024 confocal microscope, and fluorescence intensity was analyzed by using ImageJ software. For surface biotinylation assays, cultures of cortical neurons in 6-cm dishes at 18 d in vitro were incubated with 1 mM sulfo-NHS-biotin (Pierce) in PBS for 10 min in a CO₂ incubator. After washing with PBS, cell lysates were prepared with RIPPA buffer [1% (vol/vol) Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS in PBS] and incubated with streptavidin-agarose (Invitrogen) for 1 h at 4 °C. The surface protein fraction captured by streptavidin-agarose and the total protein fraction from cell lysates were immunoblotted with mouse monoclonal anti-GluA2, rabbit polyclonal EphB2 (a gift from Elena Pasquale, Sanford-Burnham Medical Research Institute, La Jolla, CA), and mouse monoclonal transferrin receptor (clone: H68.4; Invitrogen) and were subjected to densitometric quantification using ImageJ software. The level of surface expression of each protein was normalized to that of each protein in the total cell lysate. Data were analyzed using Student’s t test (two-tailed).

**Immunoblotting and ELISA.** Fresh mouse brains of WT and Ext1Cre mice were cut into coronal slices (1 mm thick) using an acrylic tissue specimen mold (Roboz Surgical Instruments). Tissues corresponding the hippocampus, amygdala (mostly the basolateral amygdala), and cerebellum were dissected from these slices under a stereomicroscope. For immunoblotting, dissected tissues were lysed immediately in SDS sample buffer, and the lysates were immunoblotted with mouse anti-EXT1 (clone: 5A5; Sigma) and mouse α-tubulin (clone: B-5-1-2; Sigma) antibodies. For quantitation of heparan sulfate (HS), tissues dissected as describe above were homogenized in 50 mM Tris-HCl (pH 8.0) containing 1 mM CaCl₂ and 1% (vol/vol) Triton X-100, incubated with Pronase (1 mg/mL; Roche) at 55 °C for 24 h, and clarified by centrifugation. Amounts of HS in the supernatants were determined by using the heparan sulfate ELISA kit (Seikagaku America), and the results were analyzed statistically by two-way factorial ANOVA and Bonferroni post hoc test.

**Behavior Tests.** All behavioral experiments were performed on littermate male WT and Ext1Cre mice in 100% C57BL/6 background at 2–5 mo of age. Experimenters were blinded to genotype during behavioral testing.

**Reflections.** Sensorimotor and postural reflexes were examined according to the method described by Corbo et al. (4).

**Olfaction test.** Mice were habituated to the flavor of a novel food by placing a piece of blueberry cereal bar in their home cages for 3 d before testing. The food was removed in the evening of the third day. On the fourth day, a piece of blueberry cereal bar was buried under 2 cm of bedding in a clean cage into which the test mouse was placed. The time required for the mouse to find the food was measured (5). Data were analyzed using Student’s t test (two-tailed).

**Visual placing test.** Mice were lifted by the base of the tail to a height of 15 cm and were lowered to a wire-mesh grid within 1 s. The visual placing response was characterized by observing how close the test mouse was to the grid when it extended its forelimbs. Scoring of the response was as follows: 0 for no extension of forelimbs; 1 for extension when the nose contacted the grid; 2 for extension when vibrissa contacted the grid; 3 for extension before vibrissa contact (extension at 18–25 mm above the grid); and 4 for early vigorous extension (extension at 25 mm or more above the grid) (http://www.brc.riken.go.jp/lab/bpmp/SOPs/Classification_by_Platform/GSC_ENU_Mutagenesis/modified_shirpa_v1.xml). Data were analyzed using Student’s t test (two-tailed).

**Hot plate tests.** Mice were placed on a hot plate (model LE7406; LSI Letica) heated at 55 °C. The latency to jump, lick or shake limbs was measured (4) and analyzed using Student’s t test (two-tailed).

**Cage-top hang test.** Mice were tested for their ability to hang from a wire cage lid (4). Mice were placed on the lid, and the lid was turned upside down. The latency to falling was measured and analyzed using Student’s t test (two-tailed).

**Rotarod test.** Mice were placed on the drum of a Rotarod apparatus (Model ENV-575M; Med Associates Inc.). The rotation was started at 4 rpm and increased to 40 rpm over a 5-min period (4). The mean latency to falling in three trials (with 30-min intervals between trials) was measured and analyzed using Student’s t test (two-tailed).

**Open-field test.** Mice were placed in the center of an open-field apparatus (30 cm wide × 30 cm long × 20 cm high) and allowed to move freely for 30 min. Activity was videorecorded and analyzed by a computerized mouse tracking system (SMART System; San Diego Instruments). Total distance traveled and time spent in center area were analyzed using Student’s t test (two-tailed).

**Separation–reunion test.** Sociability between mice of the same genotype was assessed by the separation–reunion test (6). After weaning, two mice of the same genotype from the same litter were housed together in a cage. On the day before testing, the mice were transferred to two separate cages and isolated for 12 h. On the next morning, one of the two mice was chosen randomly as the stimulus mouse, and the other was designated as the test mouse. In the test, the stimulus mouse was placed in the cage of the test mouse, and their behaviors were videorecorded for 5 min. The video was scored in terms of the time spent in various social activities (sniffing, grooming, social mounting, following). Data were analyzed using Student’s t test (two-tailed).

**Resident–intruder test.** Social response of Ext1Cre and control WT mice to an unfamiliar mouse (‘intruder’) was assessed by the resident–intruder test (7). Ext1Cre and control WT mice were housed individually before the test; C57BL/6 mice serving as intruders were group-housed from weaning. In the test, an intruder mouse was introduced into the home cage of a test (‘resident’) mouse. For 5 min, behaviors of the resident mouse were videorecorded and examined in terms of the time spent in investigation of (approaching, social sniffing, genital sniffing, following) and avoidance of (walking away, running away, freezing) the intruder mouse (7). Data were analyzed by two-way factorial ANOVA and Bonferroni post hoc test.

**Social dominant tube test.** The tube test assesses social dominance between two mice of different genotypes (8). In a test (‘bout’), two mice were positioned in opposite ends of a cylindrical tube (3-cm diameter, 30 cm long) and released simultaneously. The animal was considered to have lost a bout when it retreated out of the tube. The percentage of losses in the total number of trials was calculated for each genotype and analyzed using a χ² test.

**Ultrasonic vocalization.** Ultrasonic vocalization of test animals was recorded and analyzed by using a system from Avisoft Bioacoustics. For recording, mice were placed singly in a cage without bedding (20 cm wide × 30 cm long × 14 cm high), and an ultrasound microphone (CM16; Avisoft Bioacoustics) was set 5 cm above the top of the cage. After 5-min habituation, a small piece of bedding material taken from the nest of a female C57BL/6 mouse was placed on the floor of the cage directly under the microphone. Ultrasonic vocalization from the test mouse was recorded using an UltraSoundGate 116–200 acquisition system and the Avisoft-Recorder version 3.3 (sampling rate: 250 kHz; format: 16 bit). Simultaneously, the behavior of the mouse was videorecorded from the top of the cage to determine the time spent in sniffing the nest piece. Vocalization during a 1-min period after the mouse began to sniff the nest piece was analyzed with Avisoft-SASLab Pro version 4.4. The sonograms were produced by the following parameters: Fourier transformation length, 256; frame size, 100%; overlap of temporal resolution, 50%; Hamming window. Quantitative analysis of the number, duration, and peak amplitude of individual ultrasonic calls was performed by automated measurement with SA SLab Pro (Avisoft Bioacoustics). Sounds under 30 kHz were removed...
manually from the data. Quantitative data were analyzed using Student’s t test (two-tailed).

**Hole-board test.** The hole-board apparatus consists of a white Plexiglas board (40 × 40 cm) with 16 equally spaced holes (3-cm diameter) forming four columns and four rows; the board is placed 5 cm from the bottom of an open Plexiglas box. In the test, an individual mouse was placed in the center of the hole-board, and its head-dipping behavior was video-recorded during a 10-min test session. The analysis of head-dipping behavior was performed using the scoring system by Makanjuola et al. (9). Here an “exploratory dip” was defined as any head-dip into a hole different from the previous one. A “stereotyped dip” was a dip into the same hole as the previous dip. For each trial, the sequence of holes into which a mouse dipped its nose was recorded, and each dip was scored as an exploratory or a stereotyped dip, as described above. The number of stereotyped dips and the total number of dips were determined for each trial. Data were analyzed using Student’s t test (two-tailed).

**Novel-object recognition test.** Visual memory of Ext1 CKO and control mice was assessed by the novel-object recognition test (10). Mice first were habituated to the open-field box for 10 min/d for 3 d. On the fourth day, mice were given a training session. For this session, two objects of different shape and color (LEGO blocks) were placed in the open-field box. An individual mouse was placed in the open-field box and allowed to explore for 10 min. The time spent exploring each object was recorded. Test sessions to examine memory retention were performed at 2 h, 1 d, and 4 d after the training session. In these sessions, one of the objects used in the training session was replaced with a novel object (a different LEGO block). Mice were allowed to explore the objects for 5 min, and the time spent in exploring each object again was recorded in each test session. Data were analyzed using two-way repeated ANOVA and Bonferroni post hoc test.

**Social memory test.** Social memory of Ext1 CKO and control mice was assessed by a paradigm using repeated pairings with the same ovariectomized mouse (11). Mice to be tested were housed individually for 10 d before testing to permit establishment of home-cage territory. Ovariectomized “stimulus” mice were prepared from group-housed female CD-1 mice 3–4 mo of age. In the test, a stimulus mouse was introduced into the home cage of a test mouse, and time that the test mouse spent investigating the stimulus mouse (sniffing, close following) was measured for 1 min. Then the stimulus mouse was removed and placed in a holding cage for 10 min. This sequence was repeated four times. On the fifth trial, a different stimulus female was presented to the male, and the time spent investigating the new stimulus mouse was measured. Data were analyzed by two-way repeated ANOVA and Bonferroni post hoc test.

**Elevated plus maze.** The maze consists of two open, unprotected and two closed, protected arms (each arm measures 5 × 30 cm; the wall of the closed arms is 15 cm high); mice have free access to both arms. The entire X-shaped apparatus was elevated 90 cm from the floor. An individual mouse was released in the center of the maze facing one of the closed arms. The movement of the mouse was video-recorded from the top of the maze for 5 min. The time spent in the open and closed arms was determined by the SMART system and analyzed using Student’s t test (two-tailed).

**Light/dark box test.** The box consists of open (light) and closed (dark) compartments, each 22 wide × 22 long × 20 cm high, with a 6 × 6 cm opening for transition between them. Mice were placed in the center of the open compartment and allowed to explore the box freely for 5 min. The time spent in each compartment and the number of transitions between them were determined. Data were analyzed using Student’s t test (two-tailed).

---

Characterization of CaMKII-Cre2834–mediated Ext1 inactivation. (A and B) Selective loss of Ext1 protein from excitatory neurons. We previously showed that CaMKII-Cre2834 drives neuron-specific inactivation of Ext1 in the hippocampus (1), consistent with the original report (2, 3). Here we performed an analysis of Ext1 inactivation in the amygdala, because electrophysiological experiments were performed in the amygdala based on the results of the c-Fos activity mapping (Fig. 3). (A) Sections of the basolateral amygdala were double-labeled with antibodies against Ext1 (green) and GluA2 (red). In the Ext1CKO (CKO) amygdala, Ext1 immunoreactivity is lost from GluA2+ pyramidal neurons (asterisks), but immunoreactivity in GluA2− cells (closed arrowheads), which presumably are nonpyramidal neurons and glial cells, is unaffected. (Scale bar, 20 μm.) (B) Sections of the basolateral amygdala were triple-labeled with antibodies against Ext1 (green), GFAP (blue), and GluA2 (red). In the WT amygdala, Ext1 protein is expressed both in GluA2+ pyramidal neurons (asterisks) and GluA2− multipolar cells (closed arrowheads). GFAP staining demonstrates that these multipolar cells are astrocytes. In the Ext1CKO amygdala, Ext1 immunoreactivity in astrocytes is not affected, but immunoreactivity in GluA2+ neurons is lost (asterisks). (Scale bar, 20 μm.) (C) Immunoblotting analysis of Ext1 protein in different brain regions. Tissue lysates were prepared from the hippocampus, amygdala, and cerebellum of WT and Ext1CKO mice as described in SI Materials and Methods and were immunoblotted with anti-Ext1 and anti–α-tubulin antibodies. Note that the level of Ext1 protein is decreased in the hippocampus and amygdala of Ext1CKO mice. In contrast, the level of Ext1 protein is not affected in the cerebellum, where CaMKII-Cre2634 does not induce recombination (2). (D) Quantitation of H5 in different brain regions. The amount of H5 in the respective brain regions was determined by ELISA. Results are mean ± SEM (n = 3 WT and 3 Ext1CKO mice). P values were determined by Bonferroni post hoc test after two-way factorial ANOVA. The level of H5 is decreased significantly in the hippocampus and amygdala of Ext1CKO mice, but the level in the cerebellum is unchanged.

Fig. S2. Overall characterization of Ext1CKO mouse brain. (A and B) The body (A) and brain (B) of 4-mo-old WT (Left) and Ext1CKO (CKO) (Right) mice. (C) Nissl staining of brain sections of 4-mo-old WT (Left) and Ext1CKO (Right) mice. (Scale bar, 2 mm.) (D) Photomicrographs (from Top to Bottom) of the hippocampus, cerebral cortex, amygdala, and cerebellum. (Scale bars, 200 μm.) Note that there are no discernible differences between WT and Ext1CKO mice in body size, brain anatomy, or cellular organization of various brain regions.

Fig. S3. Nest-building activity and social memory of Ext1CKO mice. (A) Impaired nest-building activity of Ext1CKO (CKO) mice. (Left) Representative pictures of nests built by three WT and three Ext1CKO mice 12 h after the mouse was placed in a new cage with a fresh cotton pad. (Right) Quantitative analysis of nest height, a parameter of nest-building activity. Results are mean ± SEM (n = 9 WT and 8 Ext1CKO mice). P values were determined by Student’s t test. (B) Intact social memory of Ext1CKO mice. Social memory of male WT and Ext1CKO mice was examined during repeated pairings with ovariectomized mice. WT mice show a decline in the amount of time spent investigating the same ovariectomized female during repeated pairing trials (trials #1–4). The time spent in investigation reverted to the level of the first trial when a different ovariectomized female was introduced (trial #5). Although Ext1CKO mice generally spent less time in investigation (likely because they are less social than WT mice), they showed the same pattern of decline and reversion during repeated pairings and introduction of a different female, indicating that social memory is not compromised in Ext1CKO mice. Results are mean ± SEM (n = 12 WT and 12 Ext1CKO mice). P values were determined by Bonferroni post hoc test after two-way repeated ANOVA.
**Fig. S4.** Morphology of basolateral amygdala pyramidal neurons in Ext1^CKO^ mice. (A) Golgi–Cox staining of neurons in the basolateral amygdala of WT (Left) and Ext1^CKO^ (CKO) (Right) mice. (Upper) Overall morphology of pyramidal neurons (Scale bar, 100 μm.) (Lower) Magnified views of dendrites bearing dendritic spines. (Scale bar, 10 μm.) (B) Quantitation of density, size (head diameter), and length of dendritic spines. No difference was observed in any of these parameters. Results are mean ± SEM (n = 40 WT and 40 Ext1^CKO^ mice). P values were determined by Student’s t test. n.s., not statistically significant.

**Fig. S5.** Normal density of excitatory and inhibitory synapses in the basolateral amygdala of Ext1^CKO^ (CKO) mice. (A) (Upper) Immunohistochemistry of presynaptic markers. Sections of the basolateral amygdala were stained for vesicular glutamate transporter (VGlut, green) and vesicular GABA transporter (VGAT, red), which are localized in excitatory and inhibitory presynaptic terminals, respectively. (Scale bar, 10 μm.) (Lower) Quantitation of the number of VGlut^+^ (Left) and vesicular inhibitory amino acid transporter (ViAAT, VGA^+^) (Right) puncta. (B) Immunohistochemistry with postsynaptic markers. (Upper) Sections of the basolateral amygdala were stained for PSD95 (green) and gephyrin (red), which are postsynaptic markers of excitatory and inhibitory synapses, respectively. (Scale bar, 10 μm.) (Lower) Quantitation of the number of PSD95^+^ and gephyrin^+^ puncta. Results are mean ± SEM (n = 30 WT and 30 Ext1^CKO^ mice). P values were determined by Student’s t test. n.s., not statistically significant.
Fig. S6. Electrophysiological analysis of amygdala neurons in Ext1CKO mice. (A) Input–output curves of compound synaptic responses in the cortical input to the basolateral amygdala \([n = 11\) WT and \(11\) Ext1CKO (CKO) mice]. (B) Paired-pulse facilitation of basolateral amygdala pyramidal neurons. (Upper) Representative traces at 50-ms and 100-ms intervals. (Lower) Quantitation of paired-pulse facilitation, calculated as the ratio of the second to the first EPSC amplitude \((n = 8\) WT and \(8\) Ext1CKO mice). (C) Rising and decay time in AMPA-mediated miniature EPSCs \((n = 8\) WT and \(7\) Ext1CKO mice). Results are mean ± SEM. \(P\) values were determined by Student’s \(t\) test. n.s., not statistically significant.

Fig. S7. Decreased surface expression of AMPA receptors in Ext1-deficient neurons. (A) Analysis of GluA2 by surface biotinylation assays. (Left) The fraction of cell-surface proteins isolated by surface biotinylation (Surface) and the total cell fraction (Total) were immunoblotted for GluA2, EphB2, and transferrin receptor (TfR). (Right) The bar graph represents the relative level of expression. Results are mean ± SEM from three independent experiments. (B) Analysis of surface GluA2 by live immunocytochemistry. (Left) Neurons were triple-stained for surface-expressed GluA2 (green) by live staining, by F-actin (red) to visualize dendritic spines, and by MAP2 (blue) to visualize dendritic shafts. A gallery of images of four independent spines is shown. (Scale bar, 1 \(\mu m\).) (Left) The bar graph represents the relative level of GluA2 immunoreactivity on the synaptic surface. Results are mean ± SEM \([n = 205\) WT and \(265\) Ext1-deficient (KO) neurons]. \(P\) values were determined by Student’s \(t\) test.

Table S1. Basic physical and behavioral assessment of Ext1CKO mice

<table>
<thead>
<tr>
<th>Assessment</th>
<th>WT (mean ± SEM)</th>
<th>Ext1CKO (mean ± SEM)</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical assessment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>29.03 ± 0.64</td>
<td>28.52 ± 0.47</td>
<td>(P = 0.5415^*)</td>
</tr>
<tr>
<td>Sensorimotor reflexes</td>
<td>Normal</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Postural reflexes</td>
<td>Normal</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Motor activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cage top hang (s)</td>
<td>56.30 ± 1.42</td>
<td>55.78 ± 1.26</td>
<td>(P = 0.7932^*)</td>
</tr>
<tr>
<td>Rotarod (s)</td>
<td>149.78 ± 19.60</td>
<td>141.13 ± 24.33</td>
<td>(P = 0.7836^*)</td>
</tr>
<tr>
<td>Sensory</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olfaction (sec)</td>
<td>144.33 ± 19.60</td>
<td>151.87 ± 29.10</td>
<td>(P = 0.8293^*)</td>
</tr>
<tr>
<td>Visual placing (score)</td>
<td>2.78 ± 0.15</td>
<td>2.63 ± 0.18</td>
<td>(P = 0.5209^*)</td>
</tr>
<tr>
<td>Novel object recognition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Training trial</td>
<td>50.49 ± 1.78</td>
<td>49.61 ± 3.63</td>
<td>(P &gt; 0.05^†)</td>
</tr>
<tr>
<td>Retention trial −2 h</td>
<td>59.79 ± 2.98</td>
<td>60.63 ± 2.44</td>
<td>(P &gt; 0.05^†)</td>
</tr>
<tr>
<td>−1 d</td>
<td>72.50 ± 2.55</td>
<td>68.07 ± 3.10</td>
<td>(P &gt; 0.05^†)</td>
</tr>
<tr>
<td>−4 d (sec)</td>
<td>58.90 ± 3.37</td>
<td>57.05 ± 2.14</td>
<td>(P &gt; 0.05^†)</td>
</tr>
</tbody>
</table>

\(^*\)\(P\) values were calculated by Student’s \(t\) test \((n = 9\) WT and \(8\) 4- to 5-mo-old cKO mice).  
\(^†\)\(P\) values were calculated by Bonferroni post test after two-way ANOVA \((F[1,21] = 0.06, \(P = 0.8114\) between genotypes). \((n = 12\) WT and \(11\) 4- to 5-mo-old cKO mice).
Table S2. Statistical results (two-way ANOVA) of the c-Fos induction experiment by separation–reunion paradigm

<table>
<thead>
<tr>
<th>Area</th>
<th>Genotype</th>
<th>Treatment</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventral orbitofrontal cortex</td>
<td>$F_{[1,26]} = 6.71, P = 0.0151$</td>
<td>$F_{[1,26]} = 148.69, P &lt; 0.0001$</td>
<td>$F_{[1,26]} = 6.69, P = 0.0156$</td>
</tr>
<tr>
<td>Basolateral amygdala</td>
<td>$F_{[1,26]} = 16.95, P = 0.0003$</td>
<td>$F_{[1,26]} = 199.70, P &lt; 0.0001$</td>
<td>$F_{[1,26]} = 16.95, P = 0.0003$</td>
</tr>
<tr>
<td>Medial amygdala</td>
<td>$F_{[1,26]} = 6.10, P = 0.0204$</td>
<td>$F_{[1,26]} = 156.61, P &lt; 0.0001$</td>
<td>$F_{[1,26]} = 6.55, P = 0.0167$</td>
</tr>
<tr>
<td>Hippocampal CA3 field</td>
<td>$F_{[1,26]} = 1.46, P = 0.2380$</td>
<td>$F_{[1,26]} = 398.26, P &lt; 0.0001$</td>
<td>$F_{[1,26]} = 0.71, P = 0.4085$</td>
</tr>
<tr>
<td>Piriform cortex</td>
<td>$F_{[1,26]} = 0.30, P = 0.5902$</td>
<td>$F_{[1,26]} = 590.09, P &lt; 0.0001$</td>
<td>$F_{[1,26]} = 0.16, P = 0.6966$</td>
</tr>
</tbody>
</table>

Movie S1. Behavior of a pair of WT mice in the separation–reunion test. Viewers are requested not to upload any movies in the online SI to other publicly accessible sites.

Movie S2. Behavior of a pair of Ext1CKO mice in the separation–reunion test.


Movie S5. A social dominance tube test with a WT–WT pair.

Movie S6. A social dominance tube test with an Ext1^CKO–WT pair.

Movie S7. Audio playback of ultrasonic vocalizations emitted by a WT male during sniffing a female’s nest piece. Recorded data were converted from a sample rate of 250 kHz to 22.05 kHz for human listening.

Movie S8. Audio playback of ultrasonic vocalizations emitted by an Ext1^CKO male during sniffing a female's nest piece. Recorded data were converted from a sample rate of 250 kHz to 22.05 kHz for human listening.

Movie S10. Behavior of an Ext1CKO mouse during the hole-board test.