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

Fumitoshi Irie, Hedieh Badie-Mahdavi, and Yu Yamaguchi[1]

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.

glycosaminoglycan | conditional knockout | multiple hereditary exostoses

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 100–150 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 glypican-5 and glypican-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.I. and Y.Y. designed research; F.I. and H.B.-M. performed research; F.I. 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.

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

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1117881109/-/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;Ext1$^{flox/flox}$* mice hereafter are designated "Ext1^{CKO}" 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 Ext1^{CKO} 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, Ext1^{CKO} 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*). Ext1^{CKO} mice exhibited no abnormalities in motor functions, reflexes, olfaction, or vision (Table S1). Moreover, there was no difference between control and Ext1^{CKO} mice in visual memory (Table S1) or social memory (Fig. S3*B*). Interestingly, Ext1^{CKO} mice displayed reduced nest-building activity (Fig. S3*A*), which is a phenotype implicated in autism (1, 29), prompting us to examine autism-related behaviors in Ext1^{CKO} mice.

Ext1^{CKO} Mice Recapitulate Three Core Deficits of Autism. Impairment of reciprocal social interaction skills is one of the core characteristics of autism (1). Ext1^{CKO} 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, Ext1^{CKO} mice showed much less interaction (Fig. 1*A* and Movie S2). This impairment in social interaction is not attributable to the impairment of social memory (Fig. S3*B*). 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 Ext1^{CKO} mice seldom chased the intruder (Movie S4). Instead, they frequently showed behaviors suggestive of avoidance, such as freezing and moving away (Fig. 1*B* and Movie S4). Third, the social dominance tube test showed that Ext1^{CKO} mice almost always lose (i.e., retreat out of the tube) in this test (Fig. 1*C* and Movie S6). Together, these results from three independent social paradigms demonstrate a significant impairment in social interaction by Ext1^{CKO} 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 Ext1^{CKO} 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 Ext1^{CKO} 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 Ext1^{CKO} mice (WT: 24.99 ± 1.98 s/min, n = 8; Ext1^{CKO}: 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 Ext1^{CKO} 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 Ext1^{CKO} mice. Nevertheless, Ext1^{CKO} 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, Ext1^{CKO} mice showed a clear tendency to make repeated head-dips into the same hole (Movie S10). The occurrence of this behavior ("stereotyped dip" as defined as in ref. 33) was significantly greater in Ext1^{CKO} mice than in WT mice (Fig. 1*G*), although the total number of head-dips during the session did not differ between

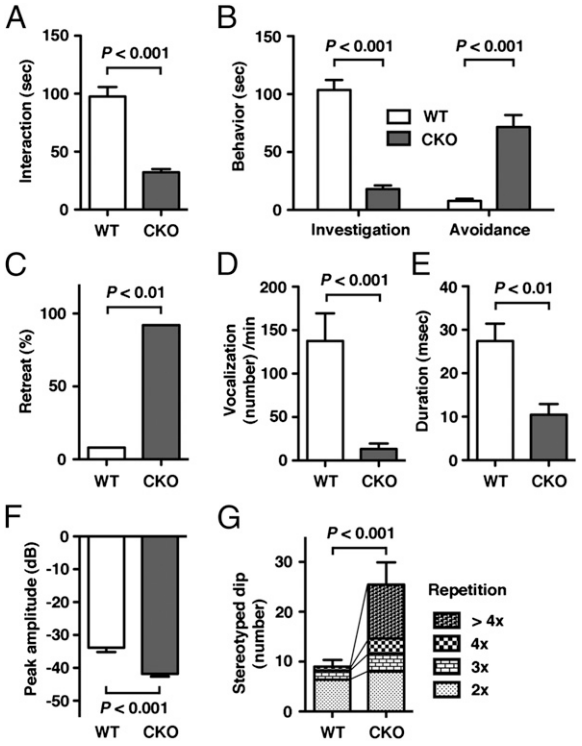

Fig. 1. Autism-like behaviors of Ext1^{CKO} mice. (*A*–*C*) Impairments exhibited by Ext1^{CKO} mice in three social paradigms. (*A*) The separation–reunion test. The bar graph shows time spent in social interaction between littermates of same genotypes [n = 12 WT and 10 Ext1^{CKO} (CKO) mice]. See also Movies S1 and S2. (*B*) The resident–intruder test. The bar graph shows time spent by the resident animal engaged in investigation (e.g., sniffing and following) and avoidance (e.g., moving away and freezing) behaviors, respectively (n = 14 WT and 15 Ext1^{CKO} mice). See also Movies S3 and S4. (*C*) The social dominance tube test. The bar graph shows the percentage of retreats from the tube by each genotype (12 trials). See also Movies S5 and S6. (*D*–*F*) Analysis of USVs emitted in response to female odor. Bar graphs show the number (*D*), duration (*E*), and peak amplitude (*F*) of USVs (n = 9 WT and 8 Ext1^{CKO} mice). Sonograms of USVs and pitch-shifted audio playbacks are available in Movies S7 and S8. (*G*) Stereotyped behavior of Ext1^{CKO} mice in the hole-board test. The graph depicts the number of stereotyped dips, defined in *SI Materials and Methods* (33), and its breakdown in terms of the number of repetitions (n = 11 WT and Ext1^{CKO} mice). See also Movies S9 and S10. Results are mean ± SEM. P values were determined by Student's t test (*A* and *D*–*G*), Bonferroni post hoc test after two-way factorial ANOVA (*B*), and χ^2 test (*C*).

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. 1*G*).

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. 2*A*). In the light/dark box text, Ext1^{CKO} mice spent a much longer time in the brightly illuminated space than did WT mice (Fig. 2*B*), 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. 2*C*). Together, these results indicate that Ext1^{CKO} mice have reduced fear of height and open spaces. Second, it was found that Ext1^{CKO} mice have sensory hypersensitivity. In the hot plate test, Ext1^{CKO} mice exhibited significantly shorter latency to respond to thermal stimuli (Fig. 2*D*). 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 and medial amygdala (36, 37) (Fig. 3*A*). 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 amyg-

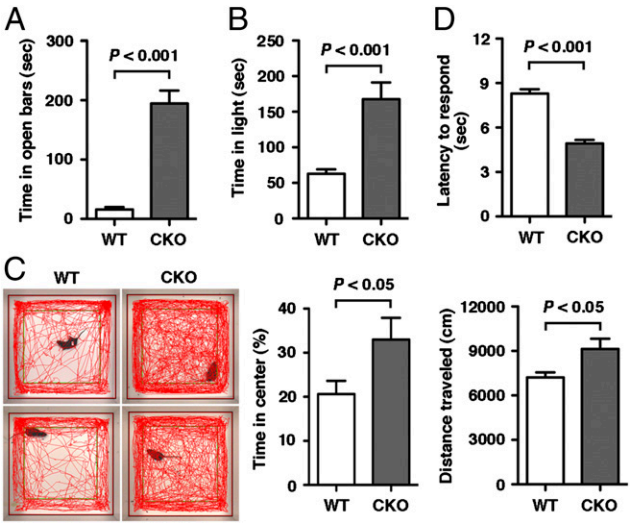

Fig. 2. Additional behavioral and neurological phenotypes of Ext1^{CKO} mice. Ext1^{CKO} mice exhibit alterations in anxiety-related behaviors and sensory hypersensitivity to thermal stimuli. (*A*) Elevated plus maze. The bar graph shows time spent in open arms [n = 16 WT and 14 Ext1^{CKO} (CKO) mice]. (*B*) Light/dark box test. The bar graph shows time spent in the lighted compartment (n = 8 WT and 8 Ext1^{CKO} mice). (*C*) Open-field test. (*Left*) Tracking of four independent (two WT and two Ext1^{CKO}) mice. (*Right*) Bar graphs show time spent in the central area of the open field and the distance traveled during the test. (*D*) Hot plate test for sensory hypersensitivity. The graph shows the latency to respond to thermal stimuli on a hot plate. Results are mean ± SEM. P values were determined by Student's t test.

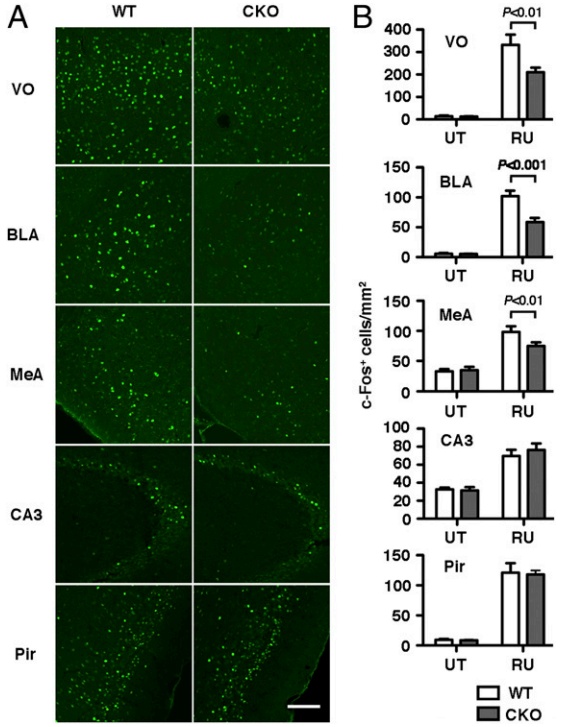

Fig. 3. Mapping of the site of neural activation deficits by c-Fos immunohistochemistry. (*A*) Analysis of c-Fos induction in various brain regions of WT and Ext1^{CKO} (CKO) mice after the separation–reunion paradigm. BLA, basolateral amygdala; CA3, hippocampal CA3 field; MeA, medial amygdala; Pir, piriform cortex; VO, ventral orbitofrontal cortex. (Scale bar, 100 μm.) (*B*) Quantitative analysis of c-Fos induction. The number of c-Fos–immunoreactive cells per square millimeter was determined in respective brain regions. RU, mice stimulated by the separation–reunion paradigm (n = 8 WT and 8 Ext1^{CKO} mice); UT, unstimulated mice (n = 7 WT and 7 Ext1^{CKO} mice). Open bars, WT mice; shaded bars, Ext1^{CKO} mice. Results are mean ± SEM. P values were determined by Bonferroni post hoc tests after two-way factorial ANOVA.

dala (BLA) (Fig. S4). Also, there were no detectable differences in the density of synapses in the region (Fig. S5).

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^{CKO} mice (Fig. S6*A*). When the AMPA receptor-mediated response was isolated with $GABA_A$ and NMDA antagonists, the input–output curve of Ext1^{CKO} mice showed a more significant depression (Fig. 4 *A* and *B*). These results suggest a reduced AMPA receptor-mediated synaptic strength in Ext1^{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^{CKO} mice (Fig. 4 *C* 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^{CKO} BLA neurons (Fig. 4 *E* and *F*), indicating that AMPA receptor-mediated postsynaptic activity is reduced. On the other hand, there was no difference between WT and Ext1^{CKO} mice in the paired-pulse facilitation response (Fig. S6*B*), indicating normal probability of presynaptic neurotransmitter release in Ext1^{CKO} BLA neurons. Thus, the reduction in mEPSC frequency in Ext1^{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. S6*C*), indicating that channel kinetics of AMPA receptors is preserved in Ext1^{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. S7*A*). The reduction in surface-expressed GluA2 did not reflect overall reduced expression, because the amount of the total cellular GluA2 was unchanged (Fig. S7*A*, 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. S7*B*). 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.

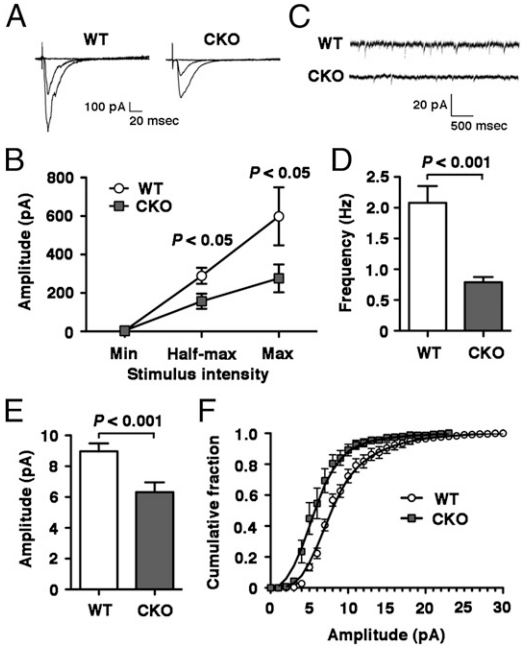

Fig. 4. Reduced excitatory synaptic transmission in BLA pyramidal neurons of Ext1^{CKO} mice. (*A* and *B*) AMPA-mediated synaptic input–output response following stimulation of the external capsule [*n* = 6 WT and 6 Ext1^{CK} (CKO) mice]. (*A*) Representative traces indicate three responses of various intensities: minimal (Min), half-maximum (Half-max), and maximum (Max). (*B*) AMPA-mediated synaptic input-output curves. (*C*–*F*) Analysis of AMPA-mediated mEPSCs. (*C*) Representative mEPSC recorded in pyramidal neurons of BLA. (*D*) Frequency (*n* = 7 WT and 12 Ext1^{CKO} mice). (*E*) Amplitude (*n* = 7 WT and 7 Ext1^{CKO} mice). (*F*) Cumulative fraction of mEPSC amplitude (*n* = 7 WT and 7 Ext1^{CKO} mice). The amplitudes at 0.5 cumulative fraction for WT and Ext1^{CKO} mice are 8.2 ± 0.15 pA and 5.8 ± 0.12 pA, respectively. Results are mean ± SEM. *P* values were determined by Student's *t* test.

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^{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^{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 brain of Ext1^{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^{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^{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 HSPGs, 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 $Ext1^{CKO}$ 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. $Ext1^{CKO}$ 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 the loxP-modified *Ext1* allele ($Ext1^{flox}$) were created and maintained on a C57BL/6 background as described previously (8). CaMKII-Cre transgenic mice (line 2834) (27) were obtained from Bernhard Lüscher (Pennsylvania State University, University Park, PA) and backcrossed to C57BL/6 mice for more than eight generations before use in this study. Conditional *Ext1*-knockout mice specific for postnatal neurons (*CaMKII-Cre;*$Ext1^{flox/flox}$), designated $Ext1^{CKO}$ 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;*$Ext1^{flox/flox}$ 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 Iizuka for technical assistance in behavioral analyses; Larkin Slater for animal maintenance and care; and Drs. Elena Pasquale and Takuji 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 HD050817, by a Sanford Center Investigator grant, by a Mizutani Foundation grant, by the MHE Coalition, and by the MHE Research Foundation.

1. Silverman JL, Yang M, Lord C, Crawley JN (2010) Behavioural phenotyping assays for mouse models of autism. *Nat Rev Neurosci* 11:490–502.
2. Geschwind DH (2008) Autism: Many genes, common pathways? *Cell* 135:391–395.
3. Yan J, et al. (2008) Neurexin 1α structural variants associated with autism. *Neurosci Lett* 438:368–370.
4. Durand CM, et al. (2007) Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. *Nat Genet* 39:25–27.
5. Zoghbi HY (2003) Postnatal neurodevelopmental disorders: Meeting at the synapse? *Science* 302:826–830.
6. Bishop JR, Schuksz M, Esko JD (2007) Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature* 446:1030–1037.
7. Stieber JR, Dormans JP (2005) Manifestations of hereditary multiple exostoses. *J Am Acad Orthop Surg* 13:110–120.
8. Inatani M, Irie F, Plump AS, Tessier-Lavigne M, Yamaguchi Y (2003) Mammalian brain morphogenesis and midline axon guidance require heparan sulfate. *Science* 302:1044–1046.
9. Matsumoto Y, Irie F, Inatani M, Tessier-Lavigne M, Yamaguchi Y (2007) Netrin-1/DCC signaling in commissural axon guidance requires cell-autonomous expression of heparan sulfate. *J Neurosci* 27:4342–4350.
10. Pratt T, Conway CD, Tian NM, Price DJ, Mason JO (2006) Heparan sulphation patterns generated by specific heparan sulfotransferase enzymes direct distinct aspects of retinal axon guidance at the optic chiasm. *J Neurosci* 26:6911–6923.
11. Conway CD, et al. (2011) Heparan sulfate sugar modifications mediate the functions of slits and other factors needed for mouse forebrain commissure development. *J Neurosci* 31:1955–1970.
12. Kantor DB, et al. (2004) Semaphorin 5A is a bifunctional axon guidance cue regulated by heparan and chondroitin sulfate proteoglycans. *Neuron* 44:961–975.
13. Ethell IM, Yamaguchi Y (1999) Cell surface heparan sulfate proteoglycan syndecan-2 induces the maturation of dendritic spines in rat hippocampal neurons. *J Cell Biol* 144: 575–586.
14. Hsueh YP, Sheng M (1999) Regulated expression and subcellular localization of syndecan heparan sulfate proteoglycans and the syndecan-binding protein CASK/LIN-2 during rat brain development. *J Neurosci* 19:7415–7425.
15. Lauri SE, et al. (1999) Regulatory role and molecular interactions of a cell-surface heparan sulfate proteoglycan (N-syndecan) in hippocampal long-term potentiation. *J Neurosci* 19:1226–1235.
16. Bolton P, et al. (1995) Autism, mental retardation, multiple exostoses and short stature in a female with 46,X,t(X;8)(p22.13;q22.1). *Psychiatr Genet* 5:51–55.
17. Ishikawa-Brush Y, et al. (1997) Autism and multiple exostoses associated with an X;8 translocation occurring within the GRPR gene and 3′ to the SDC2 gene. *Hum Mol Genet* 6:1241–1250.
18. Li H, Yamagata T, Mori M, Momoi MY (2002) Association of autism in two patients with hereditary multiple exostoses caused by novel deletion mutations of EXT1. *J Hum Genet* 47:262–265.
19. Wuyts W, et al. (2002) Multiple exostoses, mental retardation, hypertrichosis, and brain abnormalities in a boy with a de novo 8q24 submicroscopic interstitial deletion. *Am J Med Genet* 113:326–332.
20. Swarr DT, et al. (2010) Potocki-Shaffer syndrome: Comprehensive clinical assessment, review of the literature, and proposals for medical management. *Am J Med Genet A* 152A:565–572.
21. Wang K, et al. (2009) Common genetic variants on 5p14.1 associate with autism spectrum disorders. *Nature* 459:528–533.
22. Pinto D, et al. (2010) Functional impact of global rare copy number variation in autism spectrum disorders. *Nature* 466:368–372.
23. Mercier F, Kwon YC, Douet V (2012) Hippocampus/amygdala alterations, loss of heparan sulfates, fractones and ventricle wall reduction in adult BTBR T+ tf/J mice, animal model for autism. *Neurosci Lett* 506:208–213.
24. Meyza KZ, Blanchard DC, Pearson BL, Pobbe RL, Blanchard RJ (2012) Fractone-associated N-sulfated heparan sulfate shows reduced quantity in BTBR T+tf/J mice: A strong model of autism. *Behav Brain Res* 228:247–253.
25. McFarlane HG, et al. (2008) Autism-like behavioral phenotypes in BTBR T+tf/J mice. *Genes Brain Behav* 7:152–163.
26. Scattoni ML, Ricceri L, Crawley JN (2011) Unusual repertoire of vocalizations in adult BTBR T+tf/J mice during three types of social encounters. *Genes Brain Behav* 10:44–56.
27. Schweizer C, et al. (2003) The γ 2 subunit of $GABA(_A)$ receptors is required for maintenance of receptors at mature synapses. *Mol Cell Neurosci* 24:442–450.
28. Earnheart JC, et al. (2007) GABAergic control of adult hippocampal neurogenesis in relation to behavior indicative of trait anxiety and depression states. *J Neurosci* 27:3845–3854.
29. Moretti P, Bouwknecht JA, Teague R, Paylor R, Zoghbi HY (2005) Abnormalities of social interactions and home-cage behavior in a mouse model of Rett syndrome. *Hum Mol Genet* 14:205–220.
30. Panksepp JB, et al. (2007) Affiliative behavior, ultrasonic communication and social reward are influenced by genetic variation in adolescent mice. *PLoS ONE* 2:e351.
31. Thurmond JB (1975) Technique for producing and measuring territorial aggression using laboratory mice. *Physiol Behav* 14:879–881.
32. Holy TE, Guo Z (2005) Ultrasonic songs of male mice. *PLoS Biol* 3:e386.
33. Makanjuola ROA, Hill G, Maben I, Dow RC, Ashcroft GW (1977) An automated method for studying exploratory and stereotyped behavior in rats. *Psychopharmacology (Berl)* 52:271–277.
34. American Psychiatric Association (2000) Diagnostic and Statistical Manual of Mental Disorders. (DSM-IV-TR), (American Psychiatric Association, Washington, DC), 4th Ed, pp 70–75.
35. Sagar SM, Sharp FR, Curran T (1988) Expression of c-fos protein in brain: Metabolic mapping at the cellular level. *Science* 240:1328–1331.
36. Ferguson JN, Aldag JM, Insel TR, Young LJ (2001) Oxytocin in the medial amygdala is essential for social recognition in the mouse. *J Neurosci* 21:8278–8285.
37. Scearce-Levie K, et al. (2008) Abnormal social behaviors in mice lacking Fgf17. *Genes Brain Behav* 7:344–354.
38. Bachevalier J, Loveland KA (2006) The orbitofrontal-amygdala circuit and self-regulation of social-emotional behavior in autism. *Neurosci Biobehav Rev* 30:97–117.
39. Béïque JC, et al. (2006) Synapse-specific regulation of AMPA receptor function by PSD-95. *Proc Natl Acad Sci USA* 103:19535–19540.
40. Jamain S, et al. (2008) Reduced social interaction and ultrasonic communication in a mouse model of monogenic heritable autism. *Proc Natl Acad Sci USA* 105:1710–1715.
41. Peñagarikano O, et al. (2011) Absence of CNTNAP2 leads to epilepsy, neuronal migration abnormalities, and core autism-related deficits. *Cell* 147:235–246.
42. Carlsson ML (1998) Hypothesis: Is infantile autism a hypoglutamatergic disorder? Relevance of glutamate-serotonin interactions for pharmacotherapy. *J Neural Transm* 105:525–535.
43. Purcell AE, Jeon OH, Zimmerman AW, Blue ME, Pevsner J (2001) Postmortem brain abnormalities of the glutamate neurotransmitter system in autism. *Neurology* 57:1618–1628.
44. Vekovischeva OY, et al. (2004) Reduced aggression in AMPA-type glutamate receptor GluR-A subunit-deficient mice. *Genes Brain Behav* 3:253–265.
45. Wiedholz LM, et al. (2008) Mice lacking the AMPA GluR1 receptor exhibit striatal hyperdopaminergia and 'schizophrenia-related' behaviors. *Mol Psychiatry* 13:631–640.
46. Hall RA, et al. (1996) Effects of heparin on the properties of solubilized and reconstituted rat brain AMPA receptors. *Neurosci Lett* 217:179–183.
47. Li Q, Loeb JA (2001) Neuregulin-heparan-sulfate proteoglycan interactions produce sustained erbB receptor activation required for the induction of acetylcholine receptors in muscle. *J Biol Chem* 276:38068–38075.
48. Derksen PW, et al. (2002) Cell surface proteoglycan syndecan-1 mediates hepatocyte growth factor binding and promotes Met signaling in multiple myeloma. *Blood* 99:1405–1410.
49. Alarcón M, et al. (2008) Linkage, association, and gene-expression analyses identify CNTNAP2 as an autism-susceptibility gene. *Am J Hum Genet* 82:150–159.
50. Rudenko G, Hohenester E, Muller YA (2001) LG/LNS domains: Multiple functions—One business end? *Trends Biochem Sci* 26:363–368.