Reduced social interaction and ultrasonic communication in a mouse model of monogenic heritable autism

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Autism spectrum conditions (ASCs) are heritable conditions characterized by impaired reciprocal social interactions, deficits in language acquisition, and repetitive and restricted behaviors and interests. In addition to more complex genetic susceptibilities, even mutation of a single gene can lead to ASC. Several such monogenic heritable ASC forms are caused by loss-of-function mutations in genes encoding regulators of synapse function in neurons, including *NLGN4*. We report that mice with a loss-of-function mutation in the murine *NLGN4* ortholog *Nlgn4*, which encodes the synaptic cell adhesion protein Neuroligin-4, exhibit highly selective deficits in reciprocal social interactions and communication that are reminiscent of ASCs in humans. Our findings indicate that a protein network that regulates the maturation and function of synapses in the brain is at the core of a major ASC susceptibility pathway, and establish Neuroligin-4-deficient mice as genetic models for the exploration of the complex neurobiological disorders in ASCs.

behavior | neuroligin | synaptogenesis

Autism spectrum conditions (ASCs) are common heritable neurodevelopmental conditions that affect ≈0.6% of children. The etiology of ASCs is unknown and, because of the lack of biomarkers, they are diagnosed exclusively on the basis of behavioral criteria. Typical symptoms are social deficits, language impairments, repetitive behaviors, and restricted interests. The genetic contribution to ASCs was first recognized in family studies reporting concordance rates of 60–90% in monozygotic twins, 10% in dizygotic twins, and 5–10% in siblings (1). In 15% of all cases, ASCs can be associated with monogenic heritable syndromes such as Rett syndrome, fragile X syndrome, tuberous sclerosis, or type 1 neurofibromatosis (2). For nonsyndromic cases of ASCs, linkage studies and cytogenetic analyses provided evidence for many autism loci on all chromosomes (2–4).

Recent discoveries of nonsyndromic ASCs caused by gene copy number variations (5, 6) or single gain- or loss-of-function mutations in identified genes (7–9) showed that even nonsyndromic ASCs can be monogenic heritable conditions. Several of these monogenic heritable ASC forms are caused by loss-of-function mutations in the *NLGN3*, *NLGN4*, *NRXN1*, or *SHANK3* genes (6–9). These genes encode Neuroligin-3 (NL-3), Neuroligin-4 (NL-4), and Neurexin-1 (NX-1), which are cell adhesion proteins at nerve cell synapses, and SHANK3, which is a synaptic scaffold protein, indicating that aberrant signaling between nerve cells causes the ASC phenotype in the affected patients. Like all other NLs, NL-3 and -4 form transsynaptic contacts with presynaptically localized NXs. These NL/NX complexes regulate the formation, maturation, and function of synapses by recruiting presynaptic ion channels and signaling proteins and postsynaptic neurotransmitter receptors to nascent synapses (10–13).

We show here that mutant mice lacking the murine ortholog of human NL-4 (NL-4-KOs) exhibit highly selective deficits in reciprocal social interactions and communication reminiscent of ASCs in humans. NL-4-KOs thus represent a genetic animal model of nonsyndromic monogenic heritable ASCs.

Results

Characterization of Murine NL-4 and Generation of NL-4-KO Mice. The full-length mouse NL-4 cDNA (GenBank accession no. EF694290) was obtained on the basis of an EST (GenBank accession no. AF242658) that we identified in BLAST searches with the human NL-4 cDNA sequence. The mouse NL-4 gene (*Nlgn4*) is present in an unmapped clone from the whole mouse genome assembled by Celera (NW_001032912) but otherwise is absent from current mouse genome databases. The NL-4 protein sequence has all typical signatures of NLs, including the signal peptide, esterase domain, transmembrane domain, and PDZ domain-binding motif (Fig. 1). Mouse NL-4 is most homologous to human NL-4 (57%) and NL-4Y (56%), compared with other human NLs (54% for NL-3 and 53% for NL-1 and -2).

The NL-4 protein is specifically expressed in brain and thymus [supporting information (SI) Fig. 5 *A–C*). Levels in the brain are highest in hippocampus, cortex, and septum and lowest in brainstem and cerebellum (SI Fig. 5*C*). During brain development, NL-4 expression increases from low levels during embryonic and early postnatal phases to reach a plateau at 3 weeks after birth when synapse formation is complete (SI Fig. 5*D*). This developmental expression pattern is similar to that of NL-1, -2, and -3 (SI Fig. 5*D*) and indicates that NL-4 is also a synaptic protein. This notion is supported by the observation that NL-4, like all other NLs, is enriched in synaptic membrane fractions

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The authors declare no conflict of interest.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (mouse Neuroligin 4 cDNA sequence, accession no. EF694290).

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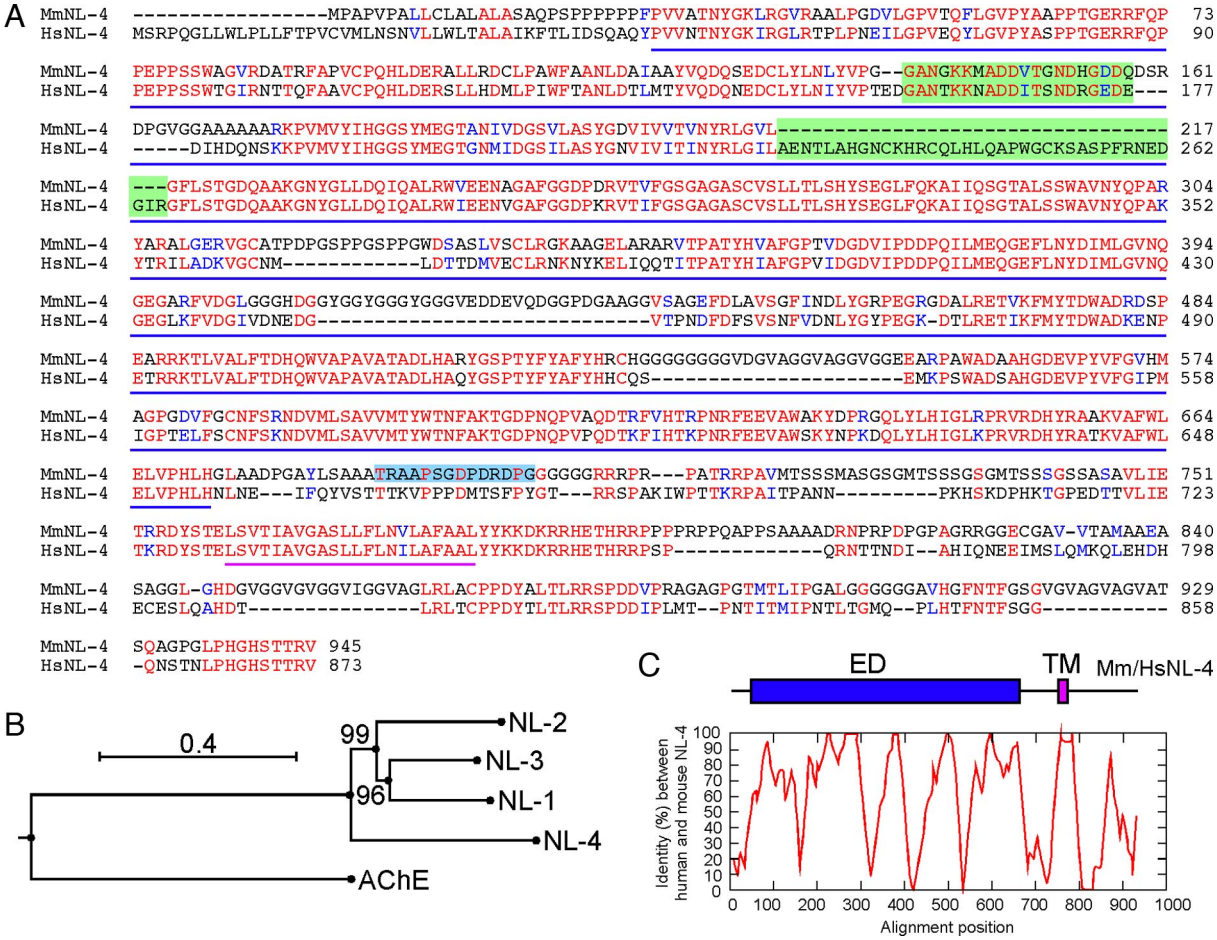

Fig. 1. The mouse NL-4 protein. (*A*) Alignment between mouse and human NL-4 protein sequences. All specific NL signatures, including the signal peptide, the esterase domain (underlined in blue), the transmembrane domain (underlined in purple), and the C-terminal PDZ domain-binding motif are conserved in mouse NL-4. Identical and similar residues are printed red and blue, respectively. Amino acids on green background correspond to alternatively spliced exons in human NL-4. Amino acids on blue background represent the peptide used to generate anti-NL-4 antibodies (SI Fig. 5). (*B*) Phylogenetic tree of the mouse NLs, constructed using the neighbor joining method. The tree has been rooted using the mouse sequence of acetylcholine esterase (AChE). Values at the bifurcation points are bootstraps for 100 samplings. The scale bar indicates 0.4 substitutions per site. (*C*) Pairwise alignment of human and mouse NL-4. Percentage identity between human and mouse NL-4 protein was calculated using 21-aa sliding windows (ED, esterase domain; TM, transmembrane domain).

(LP1, SPM) upon subcellular fractionation of brain homogenates (SI Fig. 5*E*).

NL-4-KOs were generated by BayGenomics by using a 129P2/OlaHsd ES-cell clone with a gene trap insertion 340 bp downstream of the first exon of *Nlgn4* (SI Fig. 6*A*). The insertion leads to the expression of a chimeric mRNA that contains the first coding exon of NL-4, encoding residues 1–138, in-frame with the β-galactosidase sequence. The resulting chimeric protein is nonfunctional, because it contains only a small fragment of the essential esterase domain that cannot bind to NXs. Mutant animals were obtained after germ-line transmission of the ES cells and genotyped by using Southern blot analysis (SI Fig. 6*B*). Homozygous animals were viable and fertile and were obtained with the predicted Mendelian frequency indicative of an autosomal localization of *Nlgn4*. The complete lack of NL-4 expression was confirmed in Western blot analyses (SI Fig. 6*C*). The absence of NL-4 did not modify the expression levels of other NLs (SI Fig. 6*C*) or of a set of NL interaction partners and synaptic proteins we tested (SI Fig. 7). The NL-4 mRNA expression pattern was analyzed in NL-4-KOs using the β-galactosidase expression from the gene-trap insert as readout. We found that NL-4, like all other NLs (13), is expressed throughout the brain gray matter, with highest levels in olfactory bulb, striatum, cortex, and hippocampus (SI Fig. 7*D*).

Selective Deficits of Social Interaction and Social Memory in NL-4-KOs. Because mutations of NL-4 in humans cause ASC, we examined whether the deletion of NL-4 in mice causes specific ASC-related behavioral changes. We initially studied NL-4-KO mice and WT littermates in a battery of tests (SI Table 1) designed to assess vision (visible platform training in Morris water maze; SI Fig. 10*A*), olfaction (buried food finding; SI Fig. 8*A*), taste and anhedonia (sucrose preference; SI Fig. 8*B*), hearing (startle response; SI Fig. 8*C*), sensorimotor gating (prepulse inhibition; SI Fig. 8*D*), locomotor activity and coordination (rota-rod, SI Fig. 9*A*; open field, SI Fig. 9*D*), exploratory behavior (hole board; SI Fig. 9*B*), overall curiosity toward inanimate objects (object preference; SI Fig. 9*C*), anxiety (open field, SI Fig. 9*E*; elevated plus maze, SI Fig. 9*F*), learning and memory (hidden platform training in Morris water maze, SI Fig. 10 *B* and *C*; cued and contextual fear conditioning; SI Fig. 10*E*), and cognitive flexibility (reversal training in Morris water maze; SI Fig. 10*D*). In all these assays, NL-4-KOs were indistinguishable from their WT littermates, indicating that the lack of NL-4 does not cause gross sensory deficits or generalized perturbations of behavior. In addition, NL-4-KOs did not exhibit an altered seizure propensity (SI Fig. 11).

Because deficits in reciprocal social interactions are a key symptom of ASCs, we next studied NL-4-KO and WT mice in

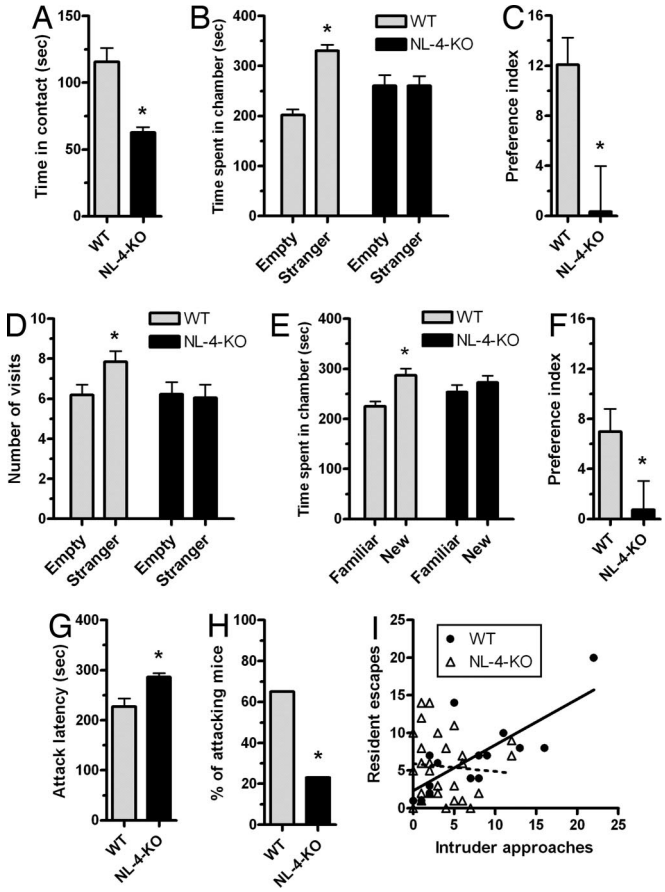

Fig. 2. Selective deficits in social behavior in NL-4-KO mice. (*A*) Social interaction of male mice in pairs of the same genotype (WT, $n = 8$; NL-4-KO, $n = 10$) was observed over 10 min in an open arena. Bars represent total time mice spent in contact. (*B–D*) Evaluation of social interaction of individual WT ($n = 19$) and NL-4-KO mice ($n = 18$) in a tripartite arena. (*B*) Time spent in the compartment with a stranger mouse versus empty compartment. (*C*) Preference index for stranger, calculated as $[time_{stranger}/(time_{stranger} + time_{empty})]\times 100-50$. (*D*) Number of visits to the compartment of the stranger. (*E* and *F*) Social memory of individual WT ($n = 19$) and NL-4-KO mice ($n = 18$) was investigated in a tripartite arena immediately after the social interaction test (*B–D*). (*E*) Time spent in the compartment with previously explored stranger vs. new stranger. (*F*) Preference index for new stranger, calculated as $[time_{new}/(time_{new} + time_{familiar})]\times 100-50$. (*G* and *H*) Evaluation of aggression of WT ($n = 19$) and NL-4-KO mice ($n = 17$) exposed to an intruder. (*G*) Attack latency of the resident. (*H*) Proportion of resident mice exhibiting aggression behavior. Data are presented as mean ± SEM. Asterisks indicate a significant change in NL-4-KO mice ($P = 0.0005$, *A*; $P = 0.0001$, *B*; $P = 0.03$, *C*; $P = 0.04$, *D*; $P = 0.002$, *E*; $P = 0.04$, *F*; $P = 0.004$, *G*; $P = 0.02$, *H*). (*I*) Correlation between intruder approach and resident escape behavior in a modified resident-intruder setting [$n = 16$, WT; $n = 28$, NL-4-KO; $Z = 3.1$, $P = 0.002$ for WT (solid line), $n = 28$, $Z = 0.6$, $P = 0.6$ for NL-4-KO (dashed line) in Spearman correlation analysis].

behavioral paradigms designed to specifically assess social behavior. First, direct contacts between pairs of genotypically identical NL-4-KO or WT mice were analyzed in an open arena. We found that NL-4-KOs spent 45% less time in direct contact with each other than WT controls ($P = 0.0005$; Fig. 2*A*). Second, mice were tested in a tripartite arena consisting of a central compartment connected to two lateral compartments on either side, one of which contained an unfamiliar mouse in a small wire cage, whereas the other contained an identical but empty wire cage. WT mice showed a clear preference for the compartment containing the unfamiliar mouse. They spent 62% more time in and made 27% more visits to the compartment with the unfamiliar mouse compared with the compartment with the empty wire cage ($P = 0.0001$ and $P = 0.04$, respectively; Fig. 2 *B–D*). In contrast, no such preference was observed in NL-4-KO mice. They spent equal time in and made equally frequent visits to the compartment with the unfamiliar mouse as to that with the empty wire cage, resulting in significantly different ratios of time spent in the two compartments (preference index) compared with WT controls ($P = 0.03$; Fig. 2 *B–D*). At the end of the first session, each mouse was tested in a second session to quantify social preference for a new stranger. A second, unfamiliar mouse was placed into the previously empty wire cage such that the test mouse had a choice between the first, already explored mouse and the novel unfamiliar mouse. In this setting, WT mice showed a clear preference for the compartment containing the novel unfamiliar mouse. They spent 28% more time in the compartment with the unfamiliar mouse compared with that with the familiar mouse ($P = 0.002$; Fig. 2 *E* and *F*). Again, no such preference was detectable with NL-4-KOs, which spent roughly equal time in the two compartments, resulting in a significantly different ratio of times spent in the two compartments (preference index) compared with WT controls ($P = 0.04$; Fig. 2 *E* and *F*). Third, we investigated the behavior of NL-4-KO and WT mice in the resident-intruder paradigm. This setting can be used to study aggression of the resident related to territorial behavior. We exposed individually housed resident NL-4-KO or WT mice to an unfamiliar intruder mouse and measured the time spent by the resident until it first attacks the intruder. We found that attack latencies were 28% longer for NL-4-KO residents compared with WT controls ($P = 0.004$; Fig. 2*G*) and the proportion of individual mice attacking at all during the test period was reduced by 63% ($P = 0.02$; Fig. 2*H*). Finally, in a modified version of the resident-intruder setting using an independent cohort of mice, we focused on the correlation between resident (WT or NL-4-KO) escape and intruder approach behavior in a neutral arena. We detected a significant positive correlation between the number of intruder approaches and the number of resident escapes for WT residents (Spearman correlation, $Z = 3.1$, $P = 0.002$), whereas no such correlation was observed if NL-4-KOs were used as residents (Spearman correlation, $Z = 0.6$; $P = 0.6$) (Fig. 2*I*), showing that NL-4-KOs respond atypically to the behavior of a conspecific.

The above data show that NL-4-KOs have selective deficits in reciprocal social behavior reminiscent of the changes in social interactions typically seen in ASC patients. These changes are unlikely to be due to sensory, locomotor, or memory defects, because NL-4-KOs perform normally in tests of olfactory, visual, and auditory abilities (SI Figs. 8 and 10*A*), locomotor activity and coordination (SI Fig. 9 *A*, *B*, *D*, and *E*), and memory (SI Fig. 10).

Reduced Vocalization in NL-4-KOs. Deficits in language acquisition and communication are key symptoms of ASCs. To test whether vocal behavior is impaired in NL-4-KO mice, we recorded ultrasonic vocalizations of individual male NL-4-KO and WT mice upon contact with a female mouse in estrous (14). In this setting, male mice produce bouts of frequency-modulated ultrasound calls of different types (Fig. 3*A*). We found that the latency to start calling was 3.2 times longer in NL-4-KOs compared with WT controls ($P = 0.03$; Fig. 3*C*), and the total number of calls per session was reduced by 48% in NL-4-KO mice compared with WT controls ($P = 0.02$; Fig. 3 *B* and *D*). Given that the acoustic structure of the vocalizations did not differ between NL-4-KO and WT control mice (data not shown), that ventilation frequencies are normal in newborn and adult NL-4-KOs (data not shown), and that sensory deficits could be ruled out (SI Figs. 8 and 10*A*), these results indicate that the NL-4-KOs are either less responsive to the social stimuli eliciting the calling or are otherwise inhibited in their propensity to communicate.

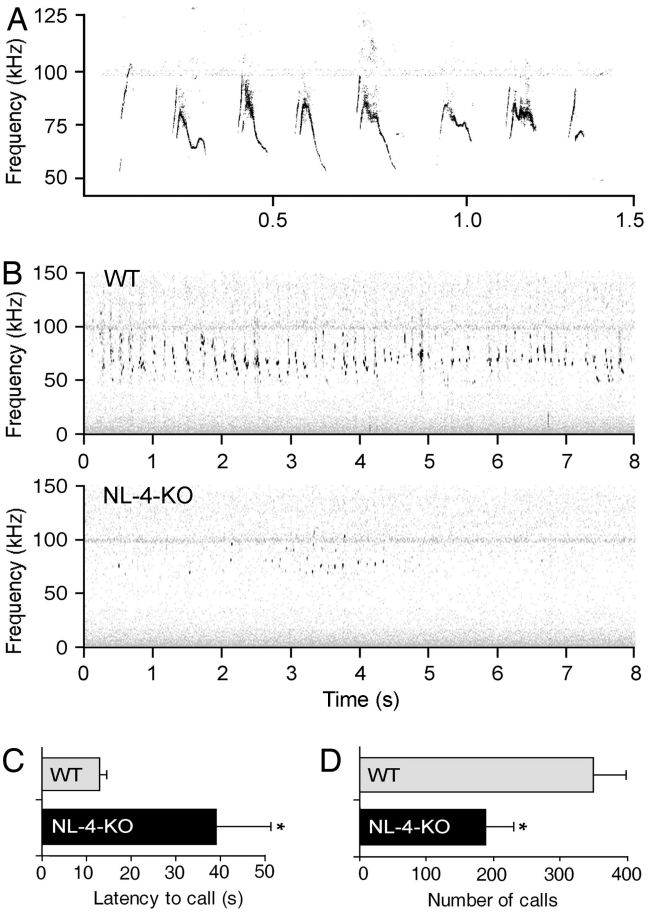

Fig. 3. Reduced ultrasound vocalization in NL-4-KO mice. Ultrasonic vocalizations of individual male WT and NL-4-KO mice were measured upon contact of the male test mouse with an unfamiliar female mouse in estrous. (*A*) Frequency spectrogram of typical ultrasonic vocalizations of a male WT mouse. (*B*) Frequency spectrograms of ultrasonic vocalizations of a male WT (*Upper*) and a male NL-4-KO (*Lower*) mouse, indicating the reduced number of vocalizations in NL-4-KOs. (*C*) Quantitative analysis of the latency between the time the female mouse was put into the arena of the male test mouse and the first ultrasonic call of the male in WT ($n = 20$) and NL-4-KO ($n = 16$) mice. Data are presented as mean ± SEM. The asterisk indicates a significant increase in latency in NL-4-KO mice ($P = 0.03$). (*D*) Quantitative analysis of the number of ultrasonic calls made by male WT ($n = 20$) and NL-4-KO ($n = 16$) mice during a 3-min session. Data are presented as mean ± SEM. The asterisk indicates a significant reduction in the number of calls made by NL-4-KO mice ($P = 0.02$).

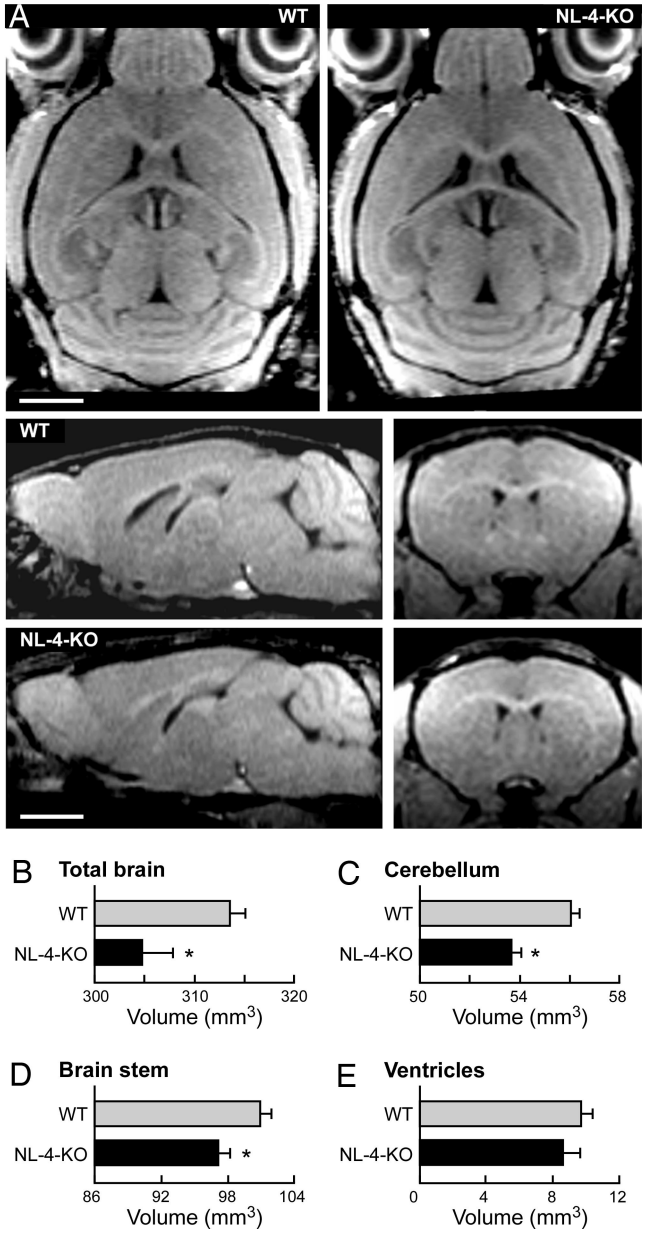

Fig. 4. Decrease of brain volume in NL-4-KO mice. (*A*) Representative MRI sections of the brains of WT and NL-4-KO animals. (*B–E*) Results of volumetric analysis of selected brain regions in WT ($n = 10$) and NL-4-KO mice ($n = 10$). (*B*) Total brain, (*C*) cerebellum, (*D*) brainstem, and (*E*) ventricles. Data are presented as mean ± SEM. Asterisks indicate a significant change in NL-4-KO mice ($P = 0.004$, *B*; $P = 0.0005$, *C*; $P = 0.01$, *D*).

Reduced Brain Volume in NL-4-KOs. A consistent finding in histopathological and structural imaging studies on children with ASCs is an increase in brain volume. However, no such alterations are reliably detectable in adult ASC patients or in patients with *NLGN4* mutations (8). In addition, analyses of individual brain regions of ASC patients provided suggestive evidence of neuronal loss in cerebellum, thickened frontal cortices, decreased corpus callosum volume, reduced brainstem size, and other less well characterized morphological alterations (15). To test whether related changes occur upon deletion of NL-4 in mice, we analyzed the volume of the whole brain and selected brain regions in NL-4-KO and WT mice by MRI volumetry (Fig. 4*A*). We observed small but significant reductions in the volume of the total brain (1.5% reduction, $P = 0.004$), cerebellum (4.1% reduction, $P = 0.0005$), and brainstem (3.8% reduction, $P = 0.01$) in NL-4-KOs compared with WT controls, whereas ventricular volumes were similar in the two genotypes (Fig. 4 *B–E*). These findings are compatible with a selective loss of gray matter in the altered brain regions of NL-4-KOs and congruent with data obtained in adult ASC patients.

Discussion

Loss-of-function mutations in the human *NLGN4* gene can cause monogenic heritable autism or Asperger syndrome (8, 9) and mental retardation (9). The present study shows that an analogous loss-of-function mutation in the orthologous mouse *Nlgn4* gene leads to a selective perturbation of social behavior and vocalization. Adult male NL-4-KO mice exhibit a reduced interest in conspecific mice (Fig. 2) and reduced ultrasonic vocalization upon contact with a female (Fig. 3), whereas sensory ability, locomotor and exploratory activity, anxiety behavior,

and learning and memory are not detectably altered. Thus, NL-4-KOs specifically and selectively mimic two of the three cardinal symptoms of ASCs in humans, i.e., reduced or aberrant social interactions and impaired communication. In addition, we detected reductions in the brain size of adult NL-4-KOs (Fig. 4) that corroborate reports of reduced brain size in adult ASC patients (15). These observations establish the NL-4-KO as a genetic animal model of monogenic heritable ASC. We did not detect abnormal repetitive behavior patterns in NL-4-KO mice, which are typically observed in human ASC patients, indicating that NL-4-KOs do not model all characteristic ASC symptoms. This is not unexpected, because not all human ASC patients with NL-4 mutations exhibit repetitive behavior patterns (8), and many of the more nuanced aspects of ASCs are difficult or even impossible to assess in animal models.

In the past, all reports on genetic mouse models of ASCs except one were related to syndromic human genetic diseases frequently associated with ASCs, including Rett syndrome, fragile X syndrome, tuberous sclerosis, and neurofibromatosis type 1. The corresponding mouse models with mutations in MeCP2, FMR1, TSC1/TSC2, or NF1 exhibit certain behavioral and morphological changes that are reminiscent of ASCs (16–25). The same is true for mice in which a limited number of cortical and hippocampal neurons lack the lipid phosphatase PTEN (26), which is functionally linked to TSC2 and mutated in some individuals with ASC (27, 28). Animal models designed to mimic the contribution of environmental factors such as certain viral infections or drugs to ASCs are less well defined. This is mainly due to pleiotropic effects of the corresponding treatments, for example, in a valproic acid rat model of autism (29). The key disadvantage of the syndromic and environmental animal models of ASCs mentioned above is that ASC-related phenotypic changes are invariably associated with multiple additional neurological and behavioral changes not typically seen in ASCs. The same applies to the corresponding human conditions, which are not even always associated with ASC features. In contrast, patients lacking NL-4 have rather specific ASC symptoms without overt or complex comorbidity (8), and NL-4-KOs exhibit highly selective ASC-like behavioral deficits (Figs. 2 and 3) and only very mild morphological changes in brain size congruent with respective changes seen in many adult ASC patients (Fig. 4), but otherwise no alterations in brain cytoarchitecture (data not shown) or behavior (SI Figs. 8–11). Thus, in terms of ASC specificity, the NL-4-KO model is an ideal tool for further studies on the biological basis of ASCs. Our data on mutant mice show that the *Nlgn4* mutation alone, without the contribution of confounding external factors, causes ASC-related symptoms in mice, and the same is likely to be the case in human ASC patients with *NLGN4* mutations.

Very recently, the phenotype of NL-3 mutant mice carrying a mutation implicated in monogenic heritable autism (R451C; ref. 8) was described (30). NL-3^{R451C} mice exhibit deficits in social interaction that are similar to but less selective than the behavioral phenotype of NL-4-KO described here (Fig. 2 *A–F*), as NL-3^{R451C} mice also exhibit other behavioral changes such as enhanced spatial learning, which is not seen in NL-4-KOs and could indirectly affect social behavior. Aggressive behavior and vocalization, which are specifically perturbed in NL-4-KOs (Figs. 2 *G–I* and 3), were not tested in NL-3^{R451C} mutant mice. The behavioral changes in NL-3^{R451C} mutant mice are associated with a selective increase in inhibitory synaptic transmission, indicating that a perturbation of synaptic function may contribute to the behavioral deficits seen in these mice.

Currently, no efficient therapies for ASCs are available. However, the fact that neurological symptoms in mutant mouse models of Rett and fragile X syndrome can be reversed (31, 32) indicates that ASC-related conditions might ultimately be curable. Whether the NL-4-KO model described here or the NL-3^{R451C} model described earlier (30) are of general relevance for ASCs in humans and can thus be used systematically for the development of novel diagnostic and therapeutic strategies cannot yet be decided. Mutations in *NLGN* genes are rare among ASC patients (33–36) and, in several of the identified monogenic heritable ASC cases, the genes involved cannot be linked directly to NL function (5, 6). However, the fact that not only mutations of *NLGN4* or *NLGN3* but also mutations of *NRXN1* or *SHANK3* can cause monogenic heritable ASCs indicates that a protein network that regulates the maturation and function of synapses in the brain is at the core of a major ASC susceptibility pathway, which can be studied with unique specificity using the NL-4-KO described here.

NLs and NXs are cell adhesion proteins. They form a transsynaptic adhesion system that is responsible for the recruitment of presynaptic Ca^{2+}-channels and postsynaptic receptors to maturing synapses *in vivo* (12, 13). SHANK3 is a postsynaptic scaffold protein that plays a role in the formation of glutamatergic postsynaptic spines and serves as a linker between postsynaptic receptors and components of the cytoskeleton (37–41). The simultaneous deletion of all α-NXs or the triple deletion of NL-1, -2, and -3 cause profound deficits in synaptic transmission (12, 13). Deletion of NL-3 alone causes a partial deficit in glutamatergic synaptic transmission in the brainstem (13), and the same is true for the deletion of NL-1 (42), whereas NL-2 function is required selectively for GABAergic synaptic transmission *in vivo* (13, 42). The phenotypic consequences of NL-4 deletion have not yet been studied in detail, but preliminary data indicate a selective deficit in glutamatergic synaptic transmission after NL-4 loss (data not shown). Based on these findings, and in view of the selective increase of inhibitory synaptic transmission in NL-3^{R451C} mutant mice (30) and that several other ASC susceptibility genes are directly related to glutamatergic or general synaptic transmission (3, 5–9, 43), we support the notion that ASCs are ultimately disorders of synapses, i.e., synaptopathies.

In light of the present findings, future studies on ASCs should include the modulation of synaptic function as a possible focus for functional analyses and the development of new therapeutic strategies. The NL-4-KO mice described here, which exhibit a more selective ASC-like phenotype than the NL-3^{R451C} mice (30) and genetically model the most frequently observed monogenic heritable forms of ASCs in humans (8, 9), can serve as useful tools in this context. In addition, the NL-4-KO model can be used to assess the role of environmental risk factors or preventive measures in the etiology of ASCs by determining the consequences of presumed deleterious (e.g., stress, deprivation, drugs) or beneficial measures (e.g., enriched environment, late weaning) on the development of ASC-like behavioral changes in the mutant mice.

Materials and Methods

Analysis of NL-4 cDNA and Generation of NL-4-KOs. For information on methods and materials used for the cloning of mouse NL-4 cDNA (GenBank accession no. EF694290) and the generation of NL 4-KOs (BayGenomics) see *SI Materials and Methods*.

Protein Analysis and Antibodies. For information on protein analysis methods and antibodies used see *SI Materials and Methods*.

Behavioral Testing. Male WT and NL-4-KO mice (littermates) at 3 months of age were tested in a battery of behavioral tests in the following order: (*i*) elevated plus maze, (*ii*) open field, (*iii*) hole board, (*iv*) rota-rod, (*v*) prepulse inhibition, (*vi*) social interaction and memory, (*vii*) social interaction in pairs, (*viii*) buried food finding, (*ix*) sucrose preference, (*x*) Morris water maze with reversal task, (*xi*) ultrasound vocalization recording, (*xii*) resident-intruder test, (*xiii*) contextual and cued fear conditioning, and (*xiv*) chemical seizures threshold measurement. For details on behavioral tests and statistical analysis see *SI Materials and Methods*.

MRI Volumetry. For information on MRI volumetry methods used see *SI Materials and Methods*.

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1. Fombonne E (2005) Epidemiology of autistic disorder and other pervasive developmental disorders. *J Clin Psychiatry* 66 Suppl 10:3–8.
2. Freitag CM (2007) The genetics of autistic disorders and its clinical relevance: a review of the literature. *Mol Psychiatry* 12:2–22.
3. Persico AM, Bourgeron T (2006) Searching for ways out of the autism maze: genetic, epigenetic and environmental clues. *Trends Neurosci* 29:349–358.
4. Vorstman JA *et al.* (2006) Identification of novel autism candidate regions through analysis of reported cytogenetic abnormalities associated with autism. *Mol Psychiatry* 11:18–28.
5. Sebat J *et al.* (2007) Strong association of *de novo* copy number mutations with autism. *Science* 316:445–449.
6. The Autism Genome Project Consortium (2007) Mapping autism risk loci using genetic linkage and chromosomal rearrangements. *Nat Genet* 39:319–328.
7. 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.
8. Jamain S *et al.* (2003) Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. *Nat Genet* 34:27–29.
9. Laumonnier F *et al.* (2004) X-linked mental retardation and autism are associated with a mutation in the NLGN4 gene, a member of the neuroligin family. *Am J Hum Genet* 74:552–557.
10. Chih B, Engelman H, Scheiffele P (2005) Control of excitatory and inhibitory synapse formation by neuroligins. *Science* 307:1324–1328.
11. Graf ER, Zhang X, Jin SX, Linhoff MW, Craig AM (2004) Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. *Cell* 119:1013–1026.
12. Missler M *et al.* (2003) Alpha-neurexins couple Ca^{2+} channels to synaptic vesicle exocytosis. *Nature* 423:939–948.
13. Varoqueaux F *et al.* (2006) Neuroligins determine synapse maturation and function. *Neuron* 51:741–754.
14. Holy TE, Guo Z (2005) Ultrasonic songs of male mice. *PLoS Biol* 3:e386.
15. Penn HE (2006) Neurobiological correlates of autism: a review of recent research. *Child Neuropsychol* 12:57–79.
16. Chen L, Toth M (2001) Fragile X mice develop sensory hyperreactivity to auditory stimuli. *Neuroscience* 103:1043–1050.
17. Mineur YS, Huynh LX, Crusio WE (2006) Social behavior deficits in the Fmr1 mutant mouse. *Behav Brain Res* 168:172–175.
18. 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.
19. Moretti P *et al.* (2006) Learning and memory and synaptic plasticity are impaired in a mouse model of Rett syndrome. *J Neurosci* 26:319–327.
20. Onda H *et al.* (2002) Tsc2 null murine neuroepithelial cells are a model for human tuber giant cells, and show activation of an mTOR pathway. *Mol Cell Neurosci* 21:561–574.
21. Silva AJ *et al.* (1997) A mouse model for the learning and memory deficits associated with neurofibromatosis type I *Nat Genet* 15:281–284.
22. Tavazoie SF, Alvarez VA, Ridenour DA, Kwiatkowski DJ, Sabatini BL (2005) Regulation of neuronal morphology and function by the tumor suppressors Tsc1 and Tsc2. *Nat Neurosci* 8:1727–1734.
23. The Dutch-Belgian Fragile X Consortium (1994) Fmr1 knockout mice: a model to study fragile X mental retardation. *Cell* 78:23–33.
24. Uhlmann EJ *et al.* (2002) Heterozygosity for the tuberous sclerosis complex (TSC) gene products results in increased astrocyte numbers and decreased p27-Kip1 expression in TSC2+/- cells. *Oncogene* 21:4050–4059.
25. Uhlmann EJ *et al.* (2002) Astrocyte-specific TSC1 conditional knockout mice exhibit abnormal neuronal organization and seizures. *Ann Neurol* 52:285–296.
26. Kwon CH *et al.* (2006) Pten regulates neuronal arborization and social interaction in mice. *Neuron* 50:377–388.
27. Butler MG *et al.* (2005) Subset of individuals with autism spectrum disorders and extreme macrocephaly associated with germ-line PTEN tumour suppressor gene mutations. *J Med Genet* 42:318–321.
28. Goffin A, Hoefsloot LH, Bosgoed E, Swillen A, Fryns JP (2001) PTEN mutation in a family with Cowden syndrome and autism. *Am J Med Genet* 105:521–524.
29. Markram K, Rinaldi T, Mendola DL, Sandi C, Markram H (2007) Abnormal fear conditioning and amygdala processing in an animal model of autism. *Neuropsychopharmacology*, in press.
30. Tabuchi K *et al.* (2007) A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice. *Science* 318:71–76.
31. Guy J, Gan J, Selfridge J, Cobb S, Bird A (2007) Reversal of neurological defects in a mouse model of Rett syndrome. *Science* 315:1143–1147.
32. Hayashi ML *et al.* (2007) Inhibition of p21-activated kinase rescues symptoms of fragile X syndrome in mice. *Proc Natl Acad Sci USA* 104:11489–11494.
33. Blasi F *et al.* (2006) Absence of coding mutations in the X-linked genes neuroligin 3 and neuroligin 4 in individuals with autism from the IMGSAC collection. *Am J Med Genet B Neuropsychiatr Genet* 141:220–221.
34. Gauthier J *et al.* (2005) NLGN3/NLGN4 gene mutations are not responsible for autism in the Quebec population. *Am J Med Genet B Neuropsychiatr Genet* 132:74–75.
35. Vincent JB *et al.* (2004) Mutation screening of X-chromosomal neuroligin genes: no mutations in 196 autism probands. *Am J Med Genet B Neuropsychiatr Genet* 129:82–84.
36. Ylisaukko-oja T *et al.* (2005) Analysis of four neuroligin genes as candidates for autism. *Eur J Hum Genet* 13:1285–1292.
37. Boeckers TM *et al.* (1999) Proline-rich synapse-associated protein-1/cortactin binding protein 1 (ProSAP1/CortBP1) is a PDZ-domain protein highly enriched in the postsynaptic density. *J Neurosci* 19:6506–6518.
38. Kreienkamp HJ, Zitzer H, Gundelfinger ED, Richter D, Bockers TM (2000) The calcium-independent receptor for alpha-latrotoxin from human and rodent brains interacts with members of the ProSAP/SSTRIP/Shank family of multidomain proteins. *J Biol Chem* 275:32387–32390.
39. Naisbitt S *et al.* (1999) Shank, a novel family of postsynaptic density proteins that binds to the NMDA receptor/PSD-95/GKAP complex and cortactin. *Neuron* 23:569–582.
40. Sala C *et al.* (2001) Regulation of dendritic spine morphology and synaptic function by Shank and Homer. *Neuron* 31:115–130.
41. Zitzer H, Honck HH, Bachner D, Richter D, Kreienkamp HJ (1999) Somatostatin receptor interacting protein defines a novel family of multidomain proteins present in human and rodent brain. *J Biol Chem* 274:32997–33001.
42. Chubykin AA *et al.* (2007) Activity-dependent validation of excitatory versus inhibitory synapses by neuroligin-1 versus neuroligin-2. *Neuron* 54:919–931.
43. Belmonte MK, Bourgeron T (2006) Fragile X syndrome and autism at the intersection of genetic and neural networks. *Nat Neurosci* 9:1221–1225.