TSHZ3 deletion causes an autism syndrome and defects in cortical projection neurons

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TSHZ3, which encodes a zinc-finger transcription factor, was recently positioned as a hub gene in a module of the genes with the highest expression in the developing human neocortex, but its functions remained unknown. Here we identify TSHZ3 as the critical region for a syndrome associated with heterozygous deletions at 19q12-q13.11, which includes autism spectrum disorder (ASD). In Tshz3-null mice, differentially expressed genes include layer-specific markers of cerebral cortical projection neurons (CPNs), and the human orthologs of these genes are strongly associated with ASD. Furthermore, mice heterozygous for Tshz3 show functional changes at synapses established by CPNs and exhibit core ASD-like behavioral abnormalities. These findings highlight essential roles for Tshz3 in CPN development and function, whose alterations can account for ASD in the newly defined TSHZ3 deletion syndrome.

ASD defines a heterogeneous group of neurodevelopmental disorders that share core behavioral abnormalities, characterized by impairments in social communication and interaction, restricted interests and repetitive behaviors, as defined in DSM-5 (ref. 1). ASD has a large genetic component2, and recent integrative genomic analyses have converged on altered fetal development of glutamatergic projection neurons of the cerebral cortex as a possible substrate3–5.

The neocortex is a highly organized laminar structure. Neurons within each layer adopt specific identities and form appropriate local and long-distance connections. The proper formation of these synaptic connections is instrumental for cognitive and motor abilities, and defects in these developmental processes have been associated with ASD6. The different subtypes of cerebral CPNs, distinguished by their molecular, physiological and connectional properties, have characteristic layer distributions. Neuronal positioning and acquisition of laminar and projectional identity are concomitantly controlled by cell-type-specific and layer-specific transcriptional programs7. Sequence and copy number variations in the genes encoding key transcription factors modulating neuron positioning and identity, such as FEZF2 (also known as FEZL or ZFP312), SATB2, SOX5 and TBR1, have been found in patients with ASD or disabilities frequently associated with ASD, such as developmental and language delays or intellectual disability8–11.

Recent spatiotemporal analysis of the human brain transcriptome has positioned TSHZ3 (encoding teashirt zinc-finger homeobox family member 3; also known as ZNF537) as a hub gene in a module (M8) of the coexpressed genes with the strongest levels of expression in early cortical development, with ‘hub genes’ being defined as genes having the highest degree of connectivity, suggesting functional importance, within the module12. The module notably contains TBR1, FEZF2, FOXG1, SATB2 and EMX1, which have been functionally implicated in the development of CPNs13–21. Linkage analysis implicated 19q12 in autism22,23 and a genome-wide association study (GWAS)24 mentioned TSHZ3 as a potential autism susceptibility gene among 860 candidate genes, but no follow-up study has been performed. These data raise the core question of what role TSHZ3 has in cortical development and in the pathogenesis of neurodevelopmental disorders. We previously provided evidence that Tshz3 is required for the proper

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differentiation and/or survival of a neuronal subpopulation in the developing mouse hindbrain involved in the control of breathing\textsuperscript{25}. As a consequence, \textit{Tshz3-null (Tshz3\textsuperscript{gla2/la2})} mice fail to breathe and die at birth. \textit{Tshz3} is also expressed in the developing and adult mouse cortex\textsuperscript{26}, where its function remains unknown.

Here we identify the gene \textit{TSHZ3} as the minimal region of overlap for 19q12-q13.11 heterozygous deletions found in patients with neurodevelopmental disorders. By combining mouse genetics, RNA–seq analyses, electrophysiology and behavioral testing, we provide strong experimental evidence for a causal relationship between \textit{Tshz3} heterozygosity, functional defects in CPNs and ASD.

**RESULTS**

\textbf{TSHZ3 haploinsufficiency causes neurocognitive impairment}

We identified seven new patients from six unrelated families with 19q12-q13.11 deletions (Fig. 1a). Patients 1, 2 and 5 had large overlapping deletions (2.4, 4.02 and 2.87 Mb, respectively), defining a minimal region of overlap of 0.83 Mb that encompassed a unique protein-coding gene, \textit{TSHZ3} (Fig. 1a and Table 1). Patients 3a, 3b, 6 and 7 had smaller deletions (1.0, 0.46 and 0.05 Mb, respectively), which also overlapped only \textit{TSHZ3}; the 50-kb microdeletion found in patient 7 deleted the second exon and part of the intron of \textit{TSHZ3}. Interestingly, \textit{TSHZ3} was also deleted in seven previously reported cases with 19q12-q13.11 deletions\textsuperscript{27–30}, three of which did not delete the previously described minimal region of overlap at 19q13.11 (Fig. 1a). These three literature cases share characteristic clinical features with the seven cases reported here, including developmental delay (in particular, absence or delay of speech), intellectual disability, autistic features and renal tract abnormalities, but not the microcephaly and ectodermal dysplasia that are unique features associated with \textit{TSHZ3} deletions (Table 1). Notably, among the 22 patients (7 patients in our cohort and 15 previously reported cases) with 19q12-q13.11 deletions (Table 1), those diagnosed with ASD (patients 2, 6 and 7 from the present study, patient 6 from ref. 27 and patient 5 from ref. 28), atypical autism (patient 3a from this study) or ASD-related deficits (patients 1 and 5 from this study) had \textit{TSHZ3} deletions (Fig. 1a and Table 1).

\textbf{Midfetal human deep cortical layer neurons express \textit{TSHZ3}}

\textit{TSHZ3} has been identified as a hub gene in a module (M8) of the coexpressed genes with the highest levels of expression in the midfetal cerebral neocortex\textsuperscript{12}. We performed a new analysis of the original spatiotemporal human brain transcriptome data set, using \textit{TSHZ3} as a seed, to identify the 49 genes whose expression profiles showed the highest correlation with \textit{TSHZ3} in the developing neocortex. This \textit{TSHZ3} network contained 34 ASD candidate genes, of which 6 encode transcription factors that are key regulators of CPN identity and connectivity: TBR1 (refs. 16, 17), FEZF2 (refs. 14, 18), FOXG1 (ref. 13), SATB2 (refs. 19, 21), SOX5 (refs. 31, 32) and MEF2C\textsuperscript{33,34} (Supplementary Fig. 1a,b and Supplementary Table 1). This prompted us to characterize the distribution of \textit{TSHZ3} protein in human midfetal neocortex (20 weeks post-conception), in comparison with the localization of TBR1 and BCL11B (also known as CTIP2), which are markers of corticothalamic and subcerebral projection neurons, respectively\textsuperscript{17,35}. \textit{TSHZ3} was detected at the highest levels in layer 5 (L5), where it colocalized with BCL11B, and in L6 and the subplate, where it colocalized with TBR1 (Fig. 1b). Because coexpression networks have implicated midfetal L5 and L6 CPNs in ASD pathogenesis\textsuperscript{5}, these data support the view that behavioral deficits associated with \textit{TSHZ3} deletion may be related to developmental defects in deep CPNs.

**TSHZ3 is expressed in the embryonic mouse neocortex**

The 19q12 region where the human \textit{TSHZ3} gene resides is syntenic with the region on mouse chromosome 7 containing the mouse \textit{Tshz3} gene, and there is a high degree of conservation between \textit{TSHZ3} and \textit{Tshz3}, with 95% amino acid identity for the protein sequences. Similar to humans, at embryonic day (E) 18.5 in the mouse cortex (equivalent to human midfetal development\textsuperscript{36}), \textit{TSHZ3} was detected in postmitotic neurons of L5, L6 and the subplate (Fig. 1c). In the subplate and L6, \textit{TSHZ3} was present in TBR1-positive neurons, and in L5 \textit{TSHZ3} was detected in BCL11B-positive neurons (Fig. 1c). In contrast to humans, \textit{TSHZ3} was also detected in mouse L2 and L3 neurons (Fig. 1c).

\textbf{Tshz3 deletion alters cortical layer marker gene expression}

To identify genes that are regulated by \textit{Tshz3} in the neocortex, we performed RNA–seq using whole mouse cortex isolated from \textit{Tshz3\textsuperscript{gla2/la2}} mutant mice and wild-type controls at E18.5. This analysis identified 243 differentially expressed genes, among which 116 were downregulated and 127 were upregulated (\(P < 0.05\)) in \textit{Tshz3\textsuperscript{gla2/la2}} mice (Supplementary Table 2). To determine whether some of the differentially expressed genes are expressed in neuronal subtypes and might have functional roles in their development, we integrated information from the literature and several public data sources (DeCoN, Allen Brain Atlas, GenePaint, Eurexpress, subplate gene expression atlas). This search yielded 23 genes expressed in the subplate, which contains some of the earliest generated neurons and the first functional synapses of the neocortex\textsuperscript{17}, and 144 markers of CPNs, 62 of which were expressed in all layers and 82 of which had layer specificity (Fig. 2a and Supplementary Table 3). The latter included 52 markers of deep-layer cortical neurons, 12 of which were specific to L5 (subcerebral projection neurons) and 27 of which were specific to L6 (corticothalamic neurons) (Fig. 2b). Interestingly, 11 of 12 (91.6%) of the L5 markers were upregulated and 23 of 27 (85.1%) of the L6 markers were downregulated (Fig. 2b and Supplementary Table 3), indicating that \textit{Tshz3} mutation profoundly alters the gene expression properties of deep-layer cortical neurons. To identify the biological processes in which \textit{TSHZ3} may be involved in the mouse cortex, we performed pathway analysis of the 211 protein-coding genes of the 243 differentially expressed genes using the PANTHER database\textsuperscript{38}. Whereas only 9% of all mouse protein-coding genes (2,070/22,755)
are components of the 150 PANTHER regulatory pathways, 19% of the differentially expressed protein-coding genes (40/211) were involved in as much as 63 of these pathways. More specifically, of the 49 brain and general development pathways encoded by the mouse genome, 39 (79.5%) were represented among differentially expressed genes, with an enrichment for pathways related to neurotransmitter/neuropeptide...
Table 1 Clinical features of individuals with deletions including TSHZ3 and/or the critical region characterizing the 19q13.11 deletion syndrome

| Study | TSHZ3 but not 19q13.11 syndrome critical region | TSHZ3 and 19q13.11 syndrome critical region | 19q13.11 syndrome critical region but not TSHZ3 |
|-------|-----------------------------------------------|---------------------------------------------|-----------------------------------------------|
| Patient | 1 | 2 | 3a | 3b | 5 | 6 | 7 | Patient | 1 | 2 | 3b | 5 | 6 | 7 | Patient | 1 | 2 | 3b | 5 | 6 | 7 |
| Sex | F | M | M | M | M | F | F | M | F | M | M | F | M | F | F | M | F | M | M | F | M | F |
| Deletion size (Mb) | 2.40 | 4.02 | 1.06 | 1.06 | 2.87 | 0.46 | 0.05 | 3.91 | 4.61 | 6.25 | 4.5 | 6.16 | 11 | 8.16 | 4.27 | 3.19 | 2.4 | 1.74 | 2.63 | 2.30 | 1.37 | 2.49 |
| Inheritance | De novo | De novo | Inherited from mother | Inherited from mother | De novo | Inherited from father | De novo | De novo | De novo | Father not tested | De novo | De novo | Father not tested | De novo | De novo | De novo | Father not tested | De novo |
| Phenotypic characteristics | | | | | | | | | | | | | | | | | | | | | | |
| Autistic features/behavioral disorder | | | | | | | | | | | | | | | | | | | | | | |
| ASD | | | | | | | | | | | | | | | | | | | | | | |
| Atypical autism | | | | | | | | | | | | | | | | | | | | | | |
| Intellectual disability | | | | | | | | | | | | | | | | | | | | | | |
| Renal tract anomalies | | | | | | | | | | | | | | | | | | | | | | |
| Pyelocaliceal dilatation | | | | | | | | | | | | | | | | | | | | | | |
| Nephrolithiasis | + | − | + | + | − | − | − | − | + | + | + | + | + | + | − | + | + | + | + | + | + | + | + | + | + |
| Postnatal growth retardation | | | | | | | | | | | | | | | | | | | | | | |
| Birth weight-size defect | | | | | | | | | | | | | | | | | | | | | | |
| Intelectual disability | | | | | | | | | | | | | | | | | | | | | | |
| Coginativential dysfunction | | | | | | | | | | | | | | | | | | | | | | |
| Autism | | | | | | | | | | | | | | | | | | | | | | |
| ASD | | | | | | | | | | | | | | | | | | | | | | |
| Intellectual disability | | | | | | | | | | | | | | | | | | | | | | |
| Renal tract anomalies | | | | | | | | | | | | | | | | | | | | | | |
| Pyelocaliceal dilatation | | | | | | | | | | | | | | | | | | | | | | |
| Nephrolithiasis | + | − | + | + | − | − | − | − | + | + | + | + | + | + | − | + | + | + | + | + | + | + | + | + | + |
| Postnatal growth retardation | | | | | | | | | | | | | | | | | | | | | | |
| Birth weight-size defect | | | | | | | | | | | | | | | | | | | | | | |
| Intellectual disability | | | | | | | | | | | | | | | | | | | | | | |
| Cognitive dyslexicutive trouble, reasoning trouble, limitations in a adaptive behavior | | | | | | | | | | | | | | | | | | | | | | |
| Other neurological features | | | | | | | | | | | | | | | | | | | | | | |
| Memory deficit | | | | | | | | | | | | | | | | | | | | | | |
| Seizures that required antiepileptic drugs | | | | | | | | | | | | | | | | | | | | | | |
| Developmental delay | | | | | | | | | | | | | | | | | | | | | | |
| Speech delay | | | | | | | | | | | | | | | | | | | | | | |
| Absence of speech | + | − | + | + | − | + | + | + | + | + | + | + | + | + | − | + | + | + | + | + | + | + | + | + | + |
| Neonatal feeding difficulty | | | | | | | | | | | | | | | | | | | | | | |
| Fifth-finger clinodactyly | | | | | | | | | | | | | | | | | | | | | | |
| Core clinical features of patients with 19q13.11 deletion syndrome | | | | | | | | | | | | | | | | | | | | | | |
| Aplasia cutis in midline of scalp | | | | | | | | | | | | | | | | | | | | | | |
| Hair, eyebrow, eyelash anomalies | | | | | | | | | | | | | | | | | | | | | | |
| Microcephaly | | | | | | | | | | | | | | | | | | | | | | |
| Male hypoplasia | | | | | | | | | | | | | | | | | | | | | | |

Clinical features of individuals with deletions including TSHZ3 and/or the critical region characterizing 19q13.11 deletion syndrome. Empty cells correspond to data not documented. +, feature present; −, feature absent; ASD, autism spectrum disorder; DD, developmental disorder; EEG, electroencephalogram; F, female; ID, intellectual disability; IUGR, intrauterine growth retardation; M, male; NA, not applicable; ND, not determined; WG, week of gestation.

1ASD diagnosed upon follow-up. 2Postnatal imaging showed bilateral short, echogenic kidneys with the left side contributing 87% of function.
receptor signaling ('metabotropic glutamate receptor', '5HT1-4–type receptor-mediated signaling pathway', etc.). Gene ontology (GO) analysis was performed for the 144 differentially expressed genes categorized as CPN markers, considering separately the 52 deep-CPN-specific genes and the others. GO terms related to neuron and axon development ('axogenesis', 'cell morphogenesis involved in axon development', 'axogenesis', etc.).

**Figure 2**  Tshz3lacZ/lacZ mice show altered gene expression of cortical layer markers at E18.5. (a) Venn diagram identifying the differentially expressed genes common or specific to cortical neuron subtypes (CaPN, callosal projection neurons; CThPN, corticothalamic projection neurons; ScPN, subcerebral projection neurons). (b) Fold changes (FC; log2 scale) of the 52 differentially expressed genes that are preferentially expressed in the L5 and/or L6 layers in Tshz3lacZ/lacZ cortex in comparison to wild-type cortex. An asterisk indicates genes also expressed in the subplate. (c) ISH for selected differentially expressed genes (Fezf2, Gdf10, Ramp3, Hs3st4, Stac2, Col5a1) and CPLX3 immunoreactivity on coronal brain sections. Scale bars, 100 µm. Bar graphs show variation in mRNA levels as determined by qRT–PCR in Tshz3lacZ/lacZ versus wild-type mice. Data are shown as means ± s.e.m. (n = 3 mice per group); *P < 0.05, **P < 0.02, unpaired two-tailed t test. Arrowheads point to CPLX3-positive cells. (d) Human brain and nervous system pathologies associated with orthologs of the 52 Tshz3-regulated differentially expressed genes. Scores: 1, one study; 2, two studies; 3, three or more studies. ADHD, attention deficit/hyperactivity disorder; ALS, amyotrophic lateral sclerosis; ASD, autism spectrum disorder; OCD, obsessive compulsive disorder.
neuron differentiation, ‘neuron projection development’, etc.) were identified among the most significant categories for the deep-CPN-specific genes, specifically. These data suggest regulatory functions for TSHZ3 in cortical circuit development (Supplementary Table 4).

qRT–PCR for 14 differentially expressed genes representative of different expression profiles (Fgf10 for L5, Fezf2 and Nr4a1 for L5 and L6, Ramp3 for L6, Col23a1, Gdf10, Gsg11, Hs3st3b1, Hs3st4, Lgfbp3, Ngfr and Stat2 for L6 and subplate, and Col5a1 and Cplx3 for subplate) validated the RNA–seq data by showing 100% concordance (Fig. 2c and Supplementary Fig. 2a,b). In situ hybridization (ISH) or immunocytochemistry, which gave reliable signal for ten of these selected differentially expressed genes, also confirmed the RNA–seq data and further provided information on the layer specificity of the molecular changes (Fig. 2c and Supplementary Fig. 2b). In addition, ISH for Fgf10, Ngfr, Col5a1 and Lgfbp3 showed spatial caudal–rostral variations for differentially expressed genes, consistent with the gradient of Tshz3 expression (Supplementary Fig. 2b,c). Accordingly, the differentially expressed gene list included previously identified caudal markers (Dkk3, Crym, Tshz2, Bhlhe22 and Ngfr)17, and five additional genes were categorized here as caudal markers on the basis of either a search in gene expression databases (Col5a1, Gdf10, Firt1 and Lgfbp3) or the present ISH data (Fgf10).

**Tshz3 deletion modifies ASD-associated gene expression**

Because 232 of the 243 mouse differentially expressed genes have a non-ambiguous human ortholog, we reasoned that examining their disease association could provide a valuable clue to TSHZ3 function. Extensive PubMed searches for all human orthologs of the differentially expressed genes identified 157 of the 232 genes (67.7%) that are established or putative causal loci for brain and/or nervous system disorders. Interestingly, the great majority of these genes (110/157; 70.1%) have been associated with ASD, with the second most represented disease (20.4%) being schizophrenia (Supplementary Table 5). Among the orthologs of the 52 differentially expressed genes expressed in L5 and L6, the percentages were 61% for association with ASD and 27% for association with schizophrenia (Fig. 2d). Because recent studies associated deep-layer CPNs to neurodevelopmental pathologies, including autism and schizophrenia5,36, our analyses point to TSHZ3 as a nexus in a brain developmental gene network whose defects are associated with these disorders.

**Tshz3 deletion preserves cortical layering and projections**

To investigate potential changes in cortical layering and neuronal density in Tshz3lacZ/lacZ mutants, we analyzed at E18.5 the expression density of classical layer-specific markers for CPNs—SATB2, BCL11B, SOX5, TBR1 and TLE4—which were not among the differentially expressed genes. Tshz3lacZ/lacZ mutants showed normal expression of these five markers (Fig. 3a), indicating that, despite altered molecular identity, cortical layering was unaffected by Tshz3 deletion. No significant differences in cell numbers in L5 and L6 were found in Tshz3lacZ/lacZ mutants in comparison to wild-type mice, when quantifying respectively the numbers of BCL11B-positive (33.94 ± 1.55 versus 33.10 ± 2.23, P = 0.7561) and TBR1-positive (93.45 ± 3.19 versus 87.14 ± 3.22, P = 0.1705) cells (cells/100 μm; n = 28 sections from 3 mice per genotype).

To investigate whether the differential gene expression by deep-layer neurons in the Tshz3 mutant is associated with changes in axon pathfinding, we immunostained coronal sections of E18.5 Tshz3lacZ/lacZ brains for neurofilament and the axonal marker L1-cam and compared them to sections from wild-type brains. We found that Tshz3lacZ/lacZ brains displayed no gross defects in major axon tracts (Fig. 3b,c).

**Tshz3 haploinsufficiency alters neocortical gene expression**

Heterozygous Tshz3 deletion that genetically mimics the TSHZ3 patient condition is expected to provide data relevant to processes underlying the human syndrome. Tshz3lacZ/+ heterozygous mice show decreased neonatal viability25,39 (100% lethal on a C57BL/6J background and 50% lethal on a CD1 (Crl:CD1 (ICR); Charles River) or CBA/H NGC background), but otherwise they remain poorly characterized. Consistent with the association of Tshz3 deletion with renal tract defects (100% of Tshz3lacZ/lacZ homozygous mice had bilateral hydroureter39, about one-fourth of heterozygous embryos from E16.5 onward presented unilateral hydroureter (26.8%; 19/71).
We tested whether such heterozygosity altered the expression of the differentially expressed genes identified at E18.5 in Tshz3lacZ/lacZ cortex. We addressed this question using qRT–PCR for the 14 differentially expressed genes previously selected for the validation of RNA–seq data. In Tshz3lacZ/lacZ mice at E18.5, significant up- or downregulation in comparison to wild-type embryos was found for ten of the genes, as in Tshz3lacZ/lacZ mutants: Col5a1, Col23a1, Cpxl3, Fgfl10, Gsg11, Hst3t3b1, Hst3t6, Igbf3p, Ngrf and Ramp3 (Fig. 4a). At postnatal day (P) 5, the expression of five genes was also modified in Tshz3lacZ/lacZ mice in comparison to wild-type mice, similarly to Tshz3lacZ/lacZ mutants: Cpxl3, Fgfl10, Gsg11, Igbf3p and Ramp3 (Fig. 4a). At P20, two were differentially expressed in Tshz3lacZ/lacZ mice in comparison to wild-type mice as in Tshz3lacZ/lacZ mutants (Nrt4a1 and Ramp3), while four were inversely regulated (Col5a1, Col23a1, Gdf10 and Igbf3p) (Fig. 4a). These results indicate that Tshz3 haploinsufficiency induces a complex temporal dynamic of molecular changes from embryonic to postnatal stages, presumably affecting the maturation and/or differentiation of cortical neurons.

**Tshz3 haploinsufficiency alters synaptic function**
Defects in the corticostriatal circuit have been implicated in ASD-like behaviors40. In the mouse, corticostriatal projection neurons are mostly located in L5 and their axons reach the striatum at P3–P4 (ref. 41), where they start forming synapses from P10 onward. Their main targets are the dendritic spines of medium-sized spiny neurons (MSNs), which constitute more than 90% of the whole striatal population and are projection neurons. In wild-type postnatal brains, TSHZ3 was expressed in corticostriatal neurons (Fig. 4b) but not in MSNs; the few striatal TSHZ3-positive neurons were not positive for BCL11B, which is enriched in MSNs (Fig. 4b)12, and are thus likely interneurons.

We used the corticostriatal circuit as a model system to investigate the presence and functionality of CPNs in Tshz3lacZ/lacZ mice. Dual retrograde tract tracing from the dorsal striatum and from the thalamus using two different cholera toxin subunit B–conjugated fluorophores demonstrated that the corticostriatal projections from L5 CPNs and the corticothalamic projections from L6 CPNs were present in Tshz3lacZ/lacZ brains as in wild-type mice (Fig. 4c).

To analyze corticostriatal synaptic transmission, we performed slice electrophysiological recordings of MSNs in the dorsolateral striatum (Fig. 5a). The resting membrane potential, action potential discharge, input resistance and current–voltage relationship of MSNs were similar in wild-type and Tshz3lacZ/lacZ mice (Supplementary Fig. 3). However, the paired-pulse ratio (PPR) of AMPA receptor–mediated excitatory postsynaptic currents (EPSCs), evoked by electrical stimulation of corticostriatal fibers, was lower in MSNs from Tshz3lacZ/lacZ mice (Fig. 5b). This suggests increased probability of action potential–dependent glutamate release from the CPNs of heterozygous mice. Spontaneous miniature EPSCs (mEPSCs) recorded in the presence of tetrodotoxin were similar in wild-type and Tshz3lacZ/lacZ mice, in terms of both frequency (Fig. 5c) and amplitude (Fig. 5d). These data suggest, respectively, that action potential–independent glutamate release from CPNs is not affected by heterozygous Tshz3 loss and that the sensitivity of AMPA receptors located on striatal MSNs is unchanged. Accordingly, the AMPA/NMDA receptor ratio was similar in Tshz3lacZ/lacZ and wild-type mice (Fig. 5e). Finally, we observed that corticostriatal long-term potentiation (LTP) was present in both wild-type and heterozygous mice but was significantly enhanced in the latter (Fig. 5f).

**Tshz3 haploinsufficiency results in autism-like behavior**
Unless otherwise mentioned, Tshz3lacZ/lacZ male mice on a CBA/H/Gcnc × CD1 F1 background were used for behavioral studies. We verified that these mice did not show visual, auditory or olfactory deficits (Supplementary Fig. 4). We then investigated whether Tshz3 haploinsufficiency resulted in ASD-like traits by measuring the two core features, that is, impairment of social interactions and stereotyped repetitive behaviors with restricted interests, that serve to diagnose ASD (DSM-5)1. The first criterion was evaluated using a two-chamber device13 and a protocol we adapted84 from the three-chamber test15,46. Wild-type and Tshz3lacZ/lacZ mice did not significantly differ in exploration of empty boxes during the habituation stage (Fig. 6b). Unlike wild-type mice, Tshz3lacZ/lacZ mice did not interact more frequently with a conspecific than with an empty box containing a lure (sociability) (Fig. 6c) and they did not display more interaction with a ‘novel’ than with a ‘familiar’ conspecific (preference for social novelty) (Fig. 6d). Similar results were obtained when examining mice with the CD1 background and using the three-chamber test15,46 (Supplementary Fig. 5), which confirmed the robustness of the impact of gene haploinsufficiency on social functioning. The second criterion (restricted, repetitive patterns of behavior) was assessed in three independent tasks. The marble-burying test showed a repetitive pushing and digging activity in the Tshz3lacZ/lacZ mice (Fig. 6e).
In the present study, we identified the potential–dependent glutamate release from corticostriatal synapses (traces show samples of two consecutive EPSCs normalized to EPSC 1). (in the ratio is similar for these analyses indicate that when it was used as a covariate (for Fig. 6f mice performed a higher number of stereotyped dips (similar to that for wild-type mice. But when considering separately (values are normalized to baseline; *P < 0.001 versus baseline, **P < 0.001 (Bonferroni post-test)), suggesting increased action potential–dependent glutamate release from corticostriatal synapses (traces show samples of two consecutive EPSCs normalized to EPSC 1). (c) mEPSC frequency is similar for Tshz3lacZ and wild-type mice (left graph: interevent interval, P > 0.05 (two-sample Kolmogorov–Smirnov test), 5-ms bins; right histogram: average frequency, P > 0.05 (Mann–Whitney test); traces show samples of mEPSCs). (d) mEPSC amplitude is similar for Tshz3lacZ and wild-type mice (left graph: P > 0.05 (two-sample Kolmogorov–Smirnov test), 1-pA bins; right histogram: P > 0.05 (Mann–Whitney test)). (e) AMPA/NMDA ratio is similar for Tshz3lacZ and wild-type mice. These results suggest that heterozygous Tshz3 loss affects neither action potential–dependent glutamate release from corticostriatal synapses nor ionotropic glutamate receptor sensitivity on striatal MSNs. (f) Although corticostriatal LTP is induced in both wild-type and Tshz3lacZ mice, this form of synaptic plasticity is significantly enhanced in mutants. The left graph shows the time course of EPSC amplitude (the gray bar represents the LTP induction protocol, and the right histogram shows the average EPSC amplitude after LTP induction (values are normalized to baseline; *P < 0.001 versus baseline, **P < 0.001 (Mann–Whitney test); traces depict sample EPSCs before (black) and after (gray) the induction of LTP in the two groups). Data are expressed as means ± s.e.m. Sample size (n) refers to the number of recorded MSNs.

In the hole-board, Tshz3lacZ mice made a total number of nose dips similar to that for wild-type mice. But when considering separately exploratory and stereotyped mice as previously defined47,48, Tshz3lacZ mice performed a higher number of stereotyped dips (Fig. 6h,g). In the open field, while the total distance walked was similar for the two genotypes (Fig. 6h), the number of zone crossings was lower in the Tshz3lacZ genotype (Fig. 6i), indicating a reduced field of interest. All the differences were characterized by an effect size that was large enough to be considered as in the range of the pathological variation49. Because anxiety has high prevalence in ASD50, we also measured anxiety-like behavior. Tshz3lacZ mice avoided the central zone of the open field (Fig. 6j) and, in an elevated plus maze, while traveling a total distance similar to that of wild-type mice (Fig. 6k), they traveled less in the open arms (Fig. 6l), which indicates increased anxiety-like behavior. Body mass was lower in Tshz3lacZ than in wild-type mice, but the difference did not reach significance at the age at which the experiment was performed ((35.25 ± 2.10 g versus 36.58 ± 1.96 g) for Fig. 6b–d and (36.01 ± 2.08 g versus 37.18 ± 1.81 g) for Fig. 6e–l) and it never influenced the different behaviors described. Reduced penetrance may explain inheritance of the deletion from unaffected parents. The autistic features presented by the patient who has the smallest (50-kb) deletion involving only the TSHZ3 gene lead us to consider TSHZ3 as the gene of interest responsible for the neurocognitive phenotype of these patients. Thus, our data validate GWAS identification of the TSHZ3 gene24 as an ASD risk gene. A possible explanation for the scarcity of studies that have associated TSHZ3 with ASD could be that TSHZ3 is a dosage-sensitive gene whose haploinsufficiency is linked to high lethality during development. In mice, Tshz3 heterozygosity is 50% lethal on the CD1 background and 100% lethal on the C57BL/6J background39. We thus speculate that TSHZ3 mutations define an ASD subtype characterized by the clinical association of autistic features with other syndromic features, especially genitourinary tract defects. Notably, such an association could help identify additional patients with mutations in TSHZ3. The link between TSHZ3 deletion and this newly reported syndrome is strengthened by the mouse studies. For instance, Tshz3 is expressed at key stages of the developing metanephros and neocortex in mouse as TSHZ3 is in human, and Tshz3 mutation in the mouse recapitulates features of the human phenotype: hydrourer and ASD-relevant behavioral abnormalities. Interestingly, mutations in several genes have been associated with complex phenotype including both ASD features and renal tract abnormalities25-35. There is strong evidence for spatiotemporal convergence among groups of disease-related mutations, all known to lead to ASD, in mid-fetal L5 and L6 glutamatergic CPNs3. Interestingly, our coexpression network analysis of the developing human neocortex using TSHZ3 as a seed identified 34 ASD candidate genes among the 49 genes with the highest correlation to TSHZ3, including TBR1, FEZF2 and SOX5.

**DISCUSSION**

In the present study, we identified the TSHZ3 gene as the smallest region of overlap of 19q12–q13.11 deletions in a new cohort of seven patients and in seven previously reported subjects27–30. The characteristic clinical features encompass neurodevelopmental disorders, including autistic traits, speech disturbance and intellectual disability, as well as renal tract abnormalities. Most deletions occurred de novo, supporting their association with the new syndrome described. Reduced penetrance may explain inheritance of the deletion from unaffected parents. The autistic features presented by the patient who has the smallest (50-kb) deletion involving only the TSHZ3 gene lead us to consider TSHZ3 as the gene of interest responsible for the neurocognitive phenotype of these patients. Thus, our data validate GWAS identification of the TSHZ3 gene24 as an ASD risk gene. A possible explanation for the scarcity of studies that have associated TSHZ3 with ASD could be that TSHZ3 is a dosage-sensitive gene whose haploinsufficiency is linked to high lethality during development. In mice, Tshz3 heterozygosity is 50% lethal on the CD1 background and 100% lethal on the C57BL/6J background39. We thus speculate that TSHZ3 mutations define an ASD subtype characterized by the clinical association of autistic features with other syndromic features, especially genitourinary tract defects. Notably, such an association could help identify additional patients with mutations in TSHZ3. The link between TSHZ3 deletion and this newly reported syndrome is strengthened by the mouse studies. For instance, Tshz3 is expressed at key stages of the developing metanephros and neocortex in mouse as TSHZ3 is in human, and Tshz3 mutation in the mouse recapitulates features of the human phenotype: hydrourer and ASD-relevant behavioral abnormalities. Interestingly, mutations in several genes have been associated with complex phenotype including both ASD features and renal tract abnormalities25-35. There is strong evidence for spatiotemporal convergence among groups of disease-related mutations, all known to lead to ASD, in mid-fetal L5 and L6 glutamatergic CPNs3. Interestingly, our coexpression network analysis of the developing human neocortex using TSHZ3 as a seed identified 34 ASD candidate genes among the 49 genes with the highest correlation to TSHZ3, including TBR1, FEZF2 and SOX5.
Moreover, 5 of the 49 TSHZ3-connected genes in the human module are differentially expressed in Tshz3lacZ/+ mice and have been associated with ASD: FEZF2, KLHL11, PRDM8, SLA and SLC44A5 (Supplementary Fig. 1a). These data provide support for a hub position of TSHZ3 in a cortical transcriptional regulatory network associated with ASD.

Our study in the mouse, showing gene expression variation in the cerebral cortex, enrichment of ASD-related genes in orthologs of differentially expressed genes, functional alteration in neural circuits formed by CPNs and behavioral abnormalities associated with Tshz3 deficiency, provide some clues to the link between TSHZ3 deletions and ASD.

During prenatal development, we found that Tshz3 function is dispensable for cortical layering but required for normal expression of marker genes of the subplate and deep-layer cortical neurons, with enrichment for GO terms related to neuron and axon development when considering differentially expressed genes categorized as deep-layer enrichment for GO terms related to neuron and axon development marker genes of the subplate and deep-layer cortical neurons, with differentially expressed genes, functional alteration in neural circuits formed by CPNs. This is consistent with the PANTHER analysis of differentially expressed genes in these mutants showing enrichment for pathways related to neurotransmitter/neuropeptide receptor signaling. Perinatally in the mouse, axons from L5 CPNs of all cortical areas project toward the spinal cord and, from P0 to P6, form a similar set of collaterals. Then, from P6–P14, axon branches are selectively eliminated in an area-specific manner. While defects in collateral formation, selective branch elimination and/or synapse refinement might occur in Tshz3 mutants, the early neonatal lethality of Tshz3lacZ/+ makes it currently difficult to test this hypothesis. In the subplate, a highly dynamic sector of the developing neocortex, we observed altered expression of Fezf2 and Ngfr, which are critical for corticofugal connectivity and for giving information to thalamocortical incoming connections, respectively. It is noteworthy that genes expressed in a subplate-specific manner during development show statistically significant enrichment for association with ASD and schizophrenia.

Interestingly, Tshz3lacZ/+ mice, which closely model the condition of patients with heterozygous TSHZ3 deletions, also show altered gene expression in the embryonic and postnatal cortex, and their...
behavioral phenotype includes the two clinical traits characterizing ASD according to DSM-5 (ref. 1): they display both poor sociability and poor interest in social novelty, not imputable to sensory deficits, and fulfill the restricted field of interest and repetitive behavior condition. The altered gene expression in the postnatal cortex of Tshz3+/− mice suggests that postnatal defects in CPN development or function can contribute to the ASD-like phenotype linked to Tshz3 heterozygosity. There is substantial evidence associating the corticostriatal circuitry with ASD 60–67 and changes in corticostriatal function, although heterogeneous, have been evidenced in mouse ASD models, such as knockouts for Shank3 (ref. 58) or Ngnl1 (neurogligin-1) 59 and in 16p11±/− mice. Here we provide evidence for altered corticostriatal synaptic transmission (increased action potential–dependent glutamate release) and plasticity (enhanced LTP) in Tshz3+/− mice, suggesting increased functional corticostriatal connectivity, consistent with functional imaging data from patients with ASD 57. The results from our mouse model thus reinforce the gene–phenotype relationship between TSHZ3 haploinsufficiency and autistic features we evidenced in patients with TSHZ3 deletion.

This study, from human to rodent model, identifies TSHZ3/Tshz3 as a new gene linked to ASD, essential for CPN development and function. Its deletion affects the cortical expression of a number of genes related to ASD and induces ASD-relevant deficits, associated with functional changes at synapses formed by deep-layer CPNs without obvious alterations in neuron viability, layering and pathfinding. Our data point to TSHZ3 as a key member of a transcriptional regulatory network whose alteration at different nodes (such as TBR1, FEZF2 and SATB2) can lead to a convergence of brain phenotypes centering on ASD and to murine Tshz3 mutants as new candidate animal models of ASD.

URLs. Affymetrix Whole-Genome Human SNP Array 6, http://www.affymetrix.com/; Mouse Genome Informatics (MGI) GO term finder, http://www.informatics.jax.org/gotools/MGI_Term_Finder.html; Viewpoint: Behavior Technology, http://www.viewpoint.fr/en/.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Data have been deposited in the Gene Expression Omnibus ( GEO) under accession GSE85512. Raw data (FastQ files) from the sequencing experiment (triplicates from wild-type and Tshz3-mutant cortices) and raw abundance measurements for genes (read counts) for each sample are available from GEO. Series record GSE85512 provides access to all of our data and is the accession that should be quoted in any manuscript discussing the data.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

X.C., P.G., P.L.R., L.H.-A., N.S., A.N.G., L.K.-L.G. and L.F. designed the study. X.C., P.G., P.L.R., J.A., A.N.G., B.J., M.M., L.H.-A., K.Y.K., P.S. and Y.Z. performed experiments. J.A., A.L., E.R., M.S., C.V.-D., J.-M.C., M.-P.L., E.A., B.D., J.-F.L., A.S.W. and D.B. contributed clinical samples and clinical data. A.F., X.C. and L.F. prepared RNA samples, A.F. performed qRT–PCR, and D.S. and E.D. produced RNA-seq and performed bioinformatics analysis of them (MGX-Montpellier GenomiX). X.C., P.G., P.L.R., L.H.-A., P.L.R., M.C., A.N.G., B.J., K.Y.K., N.S., L.K.-L.G. and L.F. analyzed data. X.C., P.G., P.L.R., L.H.-A., N.S., A.N.G., A.S.W., L.K.-L.G. and L.F. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Consent and human ethics approval. All subjects or their legal representatives gave written informed consent for the study. The present work used only unlinked anonymized data and was performed in accordance with the Declaration of Helsinki protocols and approved by the French, Swedish and US ethics committees. The clinical cytogenetic sample consisted of patients referred to France, Sweden or USA from regional pediatrics, other health specialists and/or genetics centers. DNA from subjects was extracted from peripheral blood lymphocytes by standard extraction procedures.

Identification and mapping of deletions in 19q12-q31.11. DNA concentration was measured with a NanoDrop spectrophotometer. The Agilent comparative genomic hybridization (CGH) 60K array was used for patients 1 and 2, the 44K array was used for patients 5 and 7, and the 180K array was used for patients 3a, 3b and their parents (Agilent Technologies). Genomic DNA from females or males was used as a reference in sex-matched hybridization, and results were analyzed with CGH Analytics, CytoGenomics and Feature Extraction software (Agilent Technologies) and CytoSure Interpret software (Oxford Gene Technology). To confirm deletion and check parents’ genome for carrier status (patients 1, 2, 4, 5 and 7), qRT–PCR was performed using LightCycler480 SYBR Green 1 Master chemistry on an LC480 apparatus (Roche), and the data were analyzed using LightCycler480 software. The Affymetrix Genome-Wide Human SNP Array 6.0 was used for patient 6. Copy number analysis was performed using Affymetrix Genotyping Console software.

Mouse strains. In all the experiments, wild-type littermates were used as the control. The Tshz3lacZ mouse line has been described previously39. Experimental procedures were approved by the Comité National de Réflexion Éthique sur l’Expérimentation Animale 14” (57-07112012) and were in agreement with the recommendations of the European Communities Council Directive (2010/63/EU).

Histology. Fluorescence immunocytochemistry. Human fetal brains at 19 and 20 weeks after conception were obtained from the Human Fetal Tissue Repository of the Albert Einstein College of Medicine under guidelines approved by the Yale Institutional Review Board. They were fixed by immersion in 4% formaldehyde in PBS and cryoprotected overnight in PBS/20% sucrose, embedded in OCT and sections were incubated overnight at 4 °C with primary antibodies. L.1-cam and neurofilament immuno- staining was performed on 100-µm vibratome sections (Leica) for retrograde tracing, P28 mice under xylazine/ketamine anesthesia received stereotaxic injections of 0.3 µl of cholera toxin subunit B (CT-B, 1 mg/ml; Thermo Fisher Scientific) conjugated with Alexa Fluor 488 in the striatum (A: +1 mm L: +1.8 mm DV: −2.9 mm from dura) and conjugated with Alexa Fluor 647 in the thalamus (A: −1.3 mm L: +1.15 mm DV: −3.5 mm from dura) using Bregma coordinates45. This allowed retrograde labeling of, respectively, L5 CPNs (striatal injection) and L6 CPNs (thalamic injection). At 10 d after injection, sections were processed for the dilutions used are detailed in Supplementary Table 7. Images were acquired using a laser scanning confocal microscope (LSM780, Carl Zeiss) and processed using Adobe Photoshop.

Retrograde tracing. P28 mice under xylazine/ketamine anesthesia received stereotaxic injections of 0.3 µl of cholera toxin subunit B (CT-B, 1 mg/ml; Thermo Fisher Scientific) conjugated with Alexa Fluor 488 in the striatum (A: +1 mm L: +1.8 mm DV: −2.9 mm from dura) and conjugated with Alexa Fluor 647 in the thalamus (A: −1.3 mm L: +1.15 mm DV: −3.5 mm from dura) using Bregma coordinates45. This allowed retrograde labeling of, respectively, L5 CPNs (striatal injection) and L6 CPNs (thalamic injection). At 10 d after injection, sections were processed for the dilutions used are detailed in Supplementary Table 7. Images were acquired using a laser scanning confocal microscope (LSM780, Carl Zeiss) and processed using Adobe Photoshop.

Building of the TSHZ3 gene coexpression network. The coexpression network of TSHZ3 was created using a published microarray data set, including samples from 1,340 tissue samples collected from 16 brain regions of 57 developing and adult healthy donors. A full description of tissue acquisition and processing, data generation, validation and analyses has been provided12. We used TSHZ3 as a seed and selected the 49 genes with the highest correlation to TSHZ3. The network was created using VisANT, with nodes representing genes and edges representing pairwise correlation between genes. We set the cutoff for correlation to >0.7 for edges; that is, only genes with correlation greater than 0.7 were connected.

Molecular analyses. RNA–seq analysis. Three independent replicates, each containing cortices from 3–4 embryos from multiple litters, were prepared from wild-type and Tshz3-mutant neocortex at E18.5. RNA–seq libraries were constructed from 1 µg of total RNA with the TruSeq stranded mRNA sample preparation kit (low-throughput protocol) from Illumina. After poly(A)-based mRNA enrichment (using poly(T) oligonucleotide attached to magnetic beads) and mRNA fragmentation (using divalent cations under elevated temperature), RNA fragments were copied into first-strand cDNA using reverse transcriptase and random primers; the second strand of cDNA was synthesized subsequently. These cDNA fragments were added with a single A base and then ligated with the adaptor. The products were purified and enriched with 15 cycles of PCR. The final cDNA libraries were validated with a DNA 1000 Labchip on a Bioanalyzer (Agilent Technologies) and quantified with a KAPA qPCR kit. For one sequencing lane, six libraries were pooled in equal proportions, as denatured with sodium hydroxide and diluted to 7 pM before clustering. Clustering and 50 nt single-read sequencing were performed according to the manufacturer’s instructions.

Image analysis and base calling were performed using HiSeq Control software and Real-Time Analysis component. Data quality was assessed using FastQC from the Babraham Institute and the Illumina software SAQ (Sequence Analysis Viewer). Demultiplexing was performed using Illumina’s sequencing analysis software (CASA 1.8.2). TopHat 2.0.9, a splice junction mapper66 (using Bowtie 2.1.0; ref. 67), was used to align RNA–seq reads to the mouse genome (mm10) with a set of gene model annotations (genes.gtf downloaded from UCSC on 6 March 2013). Final read alignments having more than three mismatches were discarded. Then, counting was performed with HTSeq count 0.5.3p9 (union mode). The data are from a strand-specific assay, and the read had to be mapped to the opposite strand of the gene. Before statistical analysis, genes with fewer than 15 reads (cumulating all the analyzed samples) were filtered and thus removed. Differentially expressed genes were identified using the Bioconductor package DESeq2 1.2.5, and the package edgeR 3.4.0, as genes with adjusted P < 0.05, according to the false discovery rate method from Benjamini and Hochberg.

Database and bioinformatics analyses. To provide insight into the analyses of the 243 differentially expressed genes, relevant information was extracted from several literature, disease or molecular databases: PubMed, SFARI, OMIM and PANTHER. The first three databases were used to characterize the brain and nervous system diseases associated with the human orthologs of the mouse differentially expressed genes. Extensive PubMed searches on each of these genes were performed between September 2015 and April 2016, and all clinical brain/nervous system disorders observed in patients harboring mutations in each of the 232 orthologs of mouse differentially expressed genes were noted (Supplementary Table 5). All 806 genes contained in the human module of the SFARI autism database68 were extracted, and the SFARI genes common to the orthologs of mouse differentially expressed genes were conserved (Supplementary Table 5). Finally, among the 232 human orthologs of the mouse differentially expressed genes, all genes for which a clear genotype-phenotype relationship is described in the human genetic OMIM database were also noted (Supplementary Table 5).

PANTHER database version 9.0 was used to characterize the regulatory pathways in which some of the differentially expressed genes are engaged (Supplementary Table 4). Finally, a GO term enrichment analysis was performed using the MGIA GO term finder to functionally compare, within the mouse differentially expressed genes, those specific to the cortical L5 and L6 layers to those expressed in layers L2–L6 (Supplementary Table 4).

qRT–PCR. Total RNA from wild-type and Tshz3-mutant brains at E18.5 was prepared using RNeasy Plus Universal Mini Kit gDNA eliminator (Qiagen),
and first-strand cDNA was synthesized using the Script Reverse Transcription Supermix kit (Bio-Rad). Real-time PCR was performed on a CFX96 qPCR detection system (Bio-Rad) with SYBR GreenER qPCR SuperMixes (Life Technologies). The qPCR conditions included 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Analyses were performed in triplicate. Transcript levels were first normalized to those of the housekeeping gene Gapdh and were then normalized to their respective control group. The primer sequences used for SYBR qPCR are listed in Supplementary Table 8. Statistical analysis was performed by unpaired t test using qbasePLUS software version 2 (Biogazelle). A P value < 0.05 was considered to be significant.

**Ex vivo electrophysiology.** Tshz3lacZ/+ and wild-type F1 littermates obtained by crossing CD1 Tshz3lacZ/+ males with CBA/H GNC females were used for electrophysiological recordings. Corticostriatal slices (250 µm) were obtained from brains at P21–P28 as described previously69. MSNs of the dorsolateral striatum were identified by infrared videomicroscopy and by their electrophysiological properties70, and they were recorded by borosilicate micropipettes (whole-cell patch-clamp, 5–6 MΩ) filled with an internal solution containing (in mM) 125 K-glucuronate, 10 NaCl, 1 CaCl2, 2 MgCl2, 0.5 BAPTA, 19 HEPES, 1 Mg-ATP and 0.3 Na-GTP, pH 7.3. For NMDA/AMPA ratio experiments71, the solution contained (in mM) 140 CsCl, 10 NaCl, 0.1 CaCl2, 10 HEPES, 1 EGTA, 2 Mg-ATP and 0.5 Na-GTP. All recordings were performed in the presence of 50 µM picrotoxin. A tungsten bipolar electrode was placed in the dorsal corpus callosum to evoke EPSCs, while spontaneous mEPSCs were recorded in the presence of 1 µM tetrodotoxin. The LTP induction protocol consisted of three stimulation trains (100 Hz) of 3 s in duration during which the neuron was depolarized to −10 mV, with each train separated by a 20-s interval. Electrophysiological data were obtained by an AxoPatch 200b amplifier (Molecular Devices) and analyzed offline by Clampfit 10.2 (Molecular Devices) and MiniAnalysis 6.0 (Synaptosoft). Statistical analysis was performed by Prism 5 (GraphPad) software. Sample sizes, P values and statistical tests are indicated in Figure 5 and Supplementary Figure 3.

**Behavioral testing.** Unless otherwise mentioned, Tshz3lacZ/+ and wild-type mice were F1 littermates obtained by crossing CD1 Tshz3lacZ/+ males with CBA/H GNC females. Only males were subjected to the experimental tasks, at 80–90 d of age. Each male was singly housed with a female in a physically enriched environment from weaning until testing. Interest in social interactions was evaluated using a two-chamber social approach, restricted field of interest and repetitive behavior, as described in ref. 44, and anxiety-like behavior was tested using the open field and elevated plus maze. All the behavioral evaluations were scheduled between 10 a.m. and 3 p.m., with low light (60–70 lux on the ground), except open field (150 lux), by experimenters blinded to genotype.

We performed parametric analysis with SPSS version 19. ANOVA for repeated measures was used for sociability and preference for social novelty, with paired t tests used for partial comparisons. Student’s t test served for comparisons in the other tasks. ANCOVA served to partial out weight or general activity. The size of the effect was calculated for each inferential result49.

**Sociability and social novelty preference test.** Sociability (number of interactions toward a conspecific) and interest in social novelty (increased number of interactions toward an unknown conspecific, i.e., a new arrival) were measured in Tshz3lacZ/+ and wild-type male mice according to the principles of the three-chamber test55,68 but in a different two-chamber setup45,44, with opaque walls, as shown in Figure 6a. Behavior was video-recorded via a camera located 170 cm above the setup (Viewpoint: Behavior Technologies). After 5 min of habituation in the smaller compartment, the assay consisted of three successive sessions of 10 min each, during which the tested mouse was allowed to explore the entire device and the numbers of nose pokes on two pencil boxes were counted. In the first session (habituation), the two boxes were empty. In the second session (sociability), one box contained a stranger C57BL/6 (B6) male and the other a lure (a black pebble 37 mm long and 12 mm thick). In the third session (social novelty), the C57BL/6 (B6) mouse was left in its box and the lure was replaced by a stranger SWR male. Before the second and third sessions, the tested mouse was gently pushed into the smaller compartment while positioning the B6 and SWR conspecifics.

Social interactions were also assessed in a separate series of experiments in Tshz3lacZ/+ and wild-type mice on the CD1 background, using the three-chamber test according to previously reported protocols56,66 but without automation. Experiments were conducted in male mice, which were housed 3–5 per cage from weaning to behavioral testing.

**Open-field exploration test.** Mice were placed at the periphery of a white cylinder (100 cm in diameter), divided into three virtual concentric zones of equal surface (150 lx on the ground). The total distance walked, the number of zone crossings and the time spent in the center of the arena were video-recorded for 20 min and used, respectively, to assess ambulatory activity, field of interest and anxiety-like behavior.

**Hole-board test.** Reduced field of interest with repetitive behavior was evaluated using an automated hole-board. The apparatus consisted of a grey vinyl plastic board (40 × 40 cm) with 16 equidistant holes (3.5 cm in diameter) forming four rows and four columns. Photobeams crossing the holes allowed automatic counts of nose pokes for each hole. The board was located in the center of a room (60 lx). The mouse was always placed in the same corner of the board and allowed to explore for 10 min. We measured the total number of nose dips according to refs. 47,48, distinguishing exploratory dips from stereotyped dips for each mouse.

**Marble-burying test.** This test relates to pushing and digging behavior and provides a measure of repetitive and perseverating behavior72. The number of covered marbles depends on the frequency of pushing and digging episodes. Cages (40 × 40 × 18 cm) are filled with litter (5 cm thick). After 10 min of habituation in a new cage, the tested mouse was restricted to a corner of the cage with a mobile partition, while 20 marbles (1 cm in diameter) in four evenly spaced rows of 5 marbles each were placed on top of the bedding. The partition was removed and the mouse was left alone for 30 min. Buried marbles were defined as those completely covered by the litter and were scored as 3; a score of 2 corresponded to two-thirds of the marble being buried and a score of 1 corresponded to one-half of the marble being buried.

**Elevated plus maze.** The elevated plus maze provides a measure of anxiety-like behavior based on the avoidance of a condition generating anxiety. We used a plus-shaped device with two open and two closed arms, elevated 80 cm from the floor. Each mouse was placed in the central area of the maze (6 × 6 cm), with its head toward the enclosed arm, and allowed to move freely for 15 min. The distance traveled (cm) in the opened and closed arms was measured by a video tracking system.

**Sensory functions.** We examined Tshz3lacZ/+ and wild-type mice for vision, hearing and smell, as the results obtained in different tasks depend on the integrity of these functions. The mice were subjected to sensorial controls within 2 weeks after the last experimental testing.

**Auditory performance.** Preyer response was used to detect potential auditory impairment. It consisted in pinna twitching and going flat backward against the head as a reaction to sound. The response was validated as an indicator of auditory acuity by measuring the associated averaged evoked auditory potential73,74. We evaluated responses to stimulations in the ultrasound bandwidth. Mice, placed in a soundproof chamber, received sounds from two dog whistles (10 cm from the ear). The first produced sounds of 50 ± 0.008 kHz and the second produced sounds of 35 ± 0.010 kHz. The mice received five stimulations from each whistle at -3 min intervals. The Preyer response was scored 1 for a partial response (ear startling) and 2 for a full response (pinna going flat backward against the head).

**Olfactory capacities.** The olfactory habituation/dishabituation test was performed according to the previously described classical protocol75 that measures the capacity to detect and discriminate different odors. Several odors were presented to the mouse on a cotton tip: neutral (water), non-social (synthetic violet and vanilla aromas that were sugar free) and social (urines from...
B6 and SWR male mice). Each odor was presented three times for 2 min, and the time spent in sniffing the cotton tip was recorded. The median of three consecutive trials was calculated for each odor and for each mouse, and the score of each group was the mean of the individual median scores. The testing room was ventilated, and only one mouse was present during the trials.

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