Targeted Tshz3 deletion in corticostriatal circuit components segregates core autistic behaviors
Xavier Caubit, Paolo Gubellini, Pierre L. Roubertoux, Michèle Carlier, Jordan Molitor, Dorian Chabbert, Mehdi Metwaly, Pascal Salin, Ahmed Fatmi, Yasmine Belaidouni, et al.

To cite this version:
Xavier Caubit, Paolo Gubellini, Pierre L. Roubertoux, Michèle Carlier, Jordan Molitor, et al.. Targeted Tshz3 deletion in corticostriatal circuit components segregates core autistic behaviors. Translational Psychiatry, 2022, 12 (1), pp.106. 10.1038/s41398-022-01865-6. hal-03819114

HAL Id: hal-03819114
https://hal.science/hal-03819114v1
Submitted on 18 Oct 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Targeted Tshz3 deletion in corticostriatal circuit components segregates core autistic behaviors

Xavier Caubit1,4, Paolo Gubellini1,4, Pierre L. Roubertoux2,4, Michèle Carlier3,4, Jordan Molitor1, Dorian Chabbert1, Mehdi Metwaly1, Pascal Salim1, Ahmed Fatmi1, Yasmine Belaidouni1, Lucie Brosse1, Lydia Kerkerian-Le Goff1 and Laurent Fasano1

We previously linked TSHZ3 haploinsufficiency to autism spectrum disorder (ASD) and showed that embryonic or postnatal Tshz3 deletion in mice results inbehavioral traits relevant to the two core domains of ASD, namely social interaction deficits and repetitive behaviors. Here, we provide evidence that cortical projection neurons (CPNs) and striatal cholinergic interneurons (SCINs) are two main and complementary players in the TSHZ3-associated ASD syndrome. In the cerebral cortex, TSHZ3 is expressed in CPNs and in a proportion of GABAergic interneurons, but not in cholinergic interneurons or glial cells. In the striatum, TSHZ3 is expressed in all SCINs, while its expression is absent or partial in the other brain cholinergic systems. We then characterized two new conditional knockout (cKO) models generated by crossing Tshz3fl/fl with Emx1-Cre (Emx1-cKO) or Chat-Cre (Chat-cKO) mice to decipher the respective role of CPNs and SCINs. Emx1-cKO mice show altered excitatory synaptic transmission onto CPNs and impaired plasticity at corticostriatal synapses, with neither cortical neuron loss nor abnormal layer distribution. These animals present social interaction deficits but no repetitive patterns of behavior. Chat-cKO mice exhibit no loss of SCINs but changes in the electrophysiological properties of these interneurons, associated with repetitive patterns of behavior without social interaction deficits. Therefore, dysfunction in either CPNs or SCINs segregates with a distinct ASD behavioral trait. These findings provide novel insights onto the implication of the corticostriatal circuitry in ASD by revealing an unexpected neuronal dichotomy in the biological background of the two core behavioral domains of this disorder.

INTRODUCTION

Autism spectrum disorder (ASD) includes a heterogeneous group of neurodevelopmental pathologies the diagnosis of which is based exclusively on behavioral criteria. The two behavioral domains that are selected by the DSM-5 are: (i) deficit in social communication and (ii) restrictive, repetitive patterns of behavior, interests, or activities [1]. These domains also emerge from factor analyses of the 13 available diagnostic instruments in patients [2] and in a model that aligns mouse and patient features [3]. More than 900 genes have been linked to ASD [4], among which >100 impact synaptic functions or interact with genes involved in neuronal development [5]. As a possible neurobiological substrate, clinical and animal studies point to molecular, neurodevelopmental and functional changes of deep-layer cortical projection neurons (CPNs), in particular those of layer 5 (L5) forming the corticostriatal pathway [6–9]. In this context, we have linked heterozygous TSHZ3 gene deletion to a syndrome characterized by neurodevelopmental disorders including autistic behavior, cognitive disabilities and language disturbance, with some patients also showing renal tract abnormalities [10]. TSHZ3 encodes the highly conserved, zinc-finger homeodomain transcription factor TSH3, and has been identified in networks of human neocortical genes highly expressed during late fetal development, which are involved in neurodevelopmental and neuropsychiatric disorders [9, 10]. It is now ranked as a high-confidence risk gene for ASD (https://gene.sfari.org/database/human-gene/TSHZ3#reports-tab). In human and mouse, high TSHZ3 gene or protein expression is detectable in the cortex during pre- and postnatal development [11]. We showed that heterozygous deletion of Tshz3 (Tshz3+/−) and early postnatal conditional knockout (KO) using the Camk2a-Cre promoter (Camk2a-cKO mice) lead to ASD-relevant behavioral deficits paralleled by changes in cortical gene expression and corticostriatal synaptic abnormalities [10, 12]. These data suggest that Tshz3 plays a crucial role in both pre- and postnatal brain development and functioning, and point to CPNs, and in particular to the corticostriatal pathway, as a main player in the Tshz3-linked ASD syndrome. In the mouse striatum, TSHZ3 is not expressed in striatal spiny projection neurons (SSPNs), which represent >90% of striatal neurons, but in a small population of cells that are likely interneurons [10]. We [13] and others [14, 15] identified these cells as being mainly striatal cholinergic interneurons (SCINs), whose implication in ASD has been suggested by some studies [16, 17]. We also showed that the Camk2a-Cre transgene is unexpectedly expressed in the SCIN lineage, where it efficiently elicits the deletion of Tshz3 in

1Aix-Marseille Univ, CNRS, IBDM, UMR7288 Marseille, France. 2Aix-Marseille Univ, INSERM, MMG, UMR1251 Marseille, France. 3Aix-Marseille Univ, CNRS, LPC, UMR7290 Marseille, France. 4These authors contributed equally: Xavier Caubit, Paolo Gubellini, Pierre L. Roubertoux, Michèle Carlier. Email: lydia.kerkerian-le-goff@univ-amu.fr; laurent.fasano@univ-amu.fr

Received: 7 February 2022 Revised: 18 February 2022 Accepted: 24 February 2022 Published online: 15 March 2022

© The Author(s) 2022, corrected publication 2022
Camk2a-Cre mice [13]. Together, these data demonstrate that, within the corticostriatal circuitry, Tshz3 is deficient in both CPNs and SCINs, in Tshz3<sup>+/−</sup>/Tm<sup>−/−</sup> heterozygous [10] as well as in Camk2a-Cre; Emx1-cKO mice [12], which both show the full repertoire of ASD-like behavioral defects. Here, we aimed at investigating the respective contribution of CPNs and SCINs to the pathophysiology of Tshz3-linked ASD using targeted conditional deletion of this gene, and provided evidence for the complementary implication of these two neuronal populations in the ASD-related core features.

Fig. 1 Conditional Tshz3 deletion in CPNs. a Coronal brain sections from control and Emx1-cKO mice immunostained for TSHZ3. Scale bar 250 μm. b Tshz3 mRNA relative expression in the cortex of control and Emx1-cKO mice measured by RT-qPCR (4 cortices per group; *P < 0.05, Mann–Whitney test). c TSHZ3-positive cell density in control and Emx1-cKO mice in cortical layers (cell counts performed using frames of 400 μm width spanning from L1 to L6 in 9 sections from 3 control mice and 18 sections from 3 Emx1-cKO mice; **P < 0.01, Mann–Whitney test) and in the whole striatal surface (cell counts performed in the whole dorsal striatum in 6 sections from 3 control mice and 7 sections from 3 Emx1-cKO mice; *P = 0.1496, Mann–Whitney test). d Representative confocal images showing dendritic spines of GFP-positive L5 neurons from control (Thy1-GFP-M) and Emx1-cKO (Thy1-GFP-M;Emx1-cKO) mice. Scale bar 5 μm. e Density of different classes of dendritic spines in control (1688 spines/1135 μm) and Emx1-cKO (1308 spines/1220 μm) mice. f Coronal brain sections from GAD67-GFP control and Emx1-cKO; GAD67-GFP mice immunostained for TSHZ3. Lower panels are magnifications of the framed areas in the upper images. Scale bars 100 μm. *P < 0.02, **P < 0.001 and ****P < 0.0001, Student's t test. Data in (b) and (c) are expressed as medians with interquartile range; data in (e) are expressed as means ± SEM.
RESULTS

Conditional deletion of Tshz3 in CPNs

High levels of Tshz3 gene or TSHZ3 protein expression are detectable in the mouse cortex during pre- and postnatal development [10, 11]. In the adult cerebral cortex, TSHZ3 is detected in the great majority of CPNs [10]. Here, performing immunostaining for beta-galactosidase (ß-Gal) to report the expression of Tshz3, we show that Tshz3 is also expressed in 26.8 ± 1.3% (n = 20 sections from 3 mice) of cortical GABAergic interneurons, as evidenced using Tshz3lox/lox;GAD67-GFP mice (Fig. S1a); the percentage of dually labeled cells is significantly higher in the deep vs. upper cortical layers (36.5 ± 1.6% vs. 20.0 ± 1.0%, respectively; P < 0.0001, Student’s t test). In contrast, ß-Gal is not detectable in cortical choline acetyltransferase (CHAT) positive neurons (Fig. S1b), Olig2-positive oligodendrocytes (Fig. S1c) and GFAP-positive astrocytes (Fig. S1d, e). To address the role of Tshz3 in CPNs, Tshz3fl/fl ox/ox mice were crossed with Emx1-Cre (empty spiracle homeobox 1) mice (Emx1-cKO). The Emx1-Cre mouse expresses the Cre-recombinase in the progenitors of cortical glutamatergic projection neurons (i.e., CPNs) and glial cells from embryonic day 9 (E9), but neither in those of cortical GABAergic neurons, nor of striatal interneurons, including cholinergic ones [18]. Therefore, in the corticostriatal circuitry of Emx1-cKO mice, Tshz3 should be specifically lost in CPNs. Compared to control, Emx1-cKO mice show a drastic reduction of Tshz3 mRNA levels and of the density of TSHZ3-positive cells in the cerebral cortex, showing the efficacy of the deletion, while the density of striatal cells expressing TSHZ3 is unchanged (Fig. 1a–c). Despite the loss of Tshz3 expression in the vast majority of CPNs, the density of NeuN-positive cells is unchanged (Fig. S2a, b), showing no neuronal loss; in addition, neither the pattern of expression of layer-specific CPN markers, namely CUX1 for L2-4 and BCL11B for L5-6, nor the density of cells expressing these markers is affected (Fig. S2c, d), indicating no major alteration in cortical layering. However, spine density of L5 CPNs from Thy1-GFP-M:Emx1-cKO mice is significantly reduced compared to Thy1-GFP-M control mice (Fig. 1d, e). By crossing Emx1-cKO with GAD67-GFP mice, we show that cortical GABAergic neurons still express TSHZ3 (Fig. 1f), confirming the specificity of Tshz3 deletion in CPNs. To study whether Tshz3 loss in CPNs could indirectly affect cortical GABAergic interneurons, we compared GAD67-GFP control mice (Control-GAD67-GFP) to Emx1-cKO-GAD67-GFP mutant mice. No significant changes in the number of GABAergic interneurons (Control-GAD67-GFP: 140.7 ± 4.9, n = 37 sections from 5 mice; Emx1-cKO-GAD67-GFP: 144.6 ± 6.1, n = 41 sections from 7 mice; P = 0.624, Student’s t test) and in their distribution are found (Fig. S3a, b). CHAT immunostaining on striatal slices in Emx1-cKO mice also shows no significant modification of the density of SCINs (Fig. S3c, d). Overall, these data show that Tshz3 is specifically lost in CPNs of Emx1-cKO mice, with no major consequences on the number and layer distribution of CPNs and GABAergic neurons, but with a significant reduction of L5 CPN dendritic spine density, suggesting altered synaptic communication.
Cortical excitatory synaptic transmission and corticostriatal synaptic plasticity in Emx1-cKO mice

We then examined whether the loss of Tshz3 in CPNs affects their electrophysiological properties and synaptic transmission. We recorded L5 CPNs, which are at the origin of the corticostriatal pathway, in slices from Emx1-cKO mice. They show no significant changes in their membrane properties and excitability compared to control (Fig. S4a–e). Action potential (AP)-dependent glutamate release onto L5 CPNs, evaluated by measuring paired-pulse ratio, is also unaffected (Fig. S4f). However, both NMDA/AMPA ratio (Fig. S4g) and NMDA-induced currents (Fig. S4h) are significantly reduced, suggesting decreased NMDA receptor-mediated signaling in Emx1-cKO mice. The amplitude of AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) is similar in control and Emx1-cKO mice (Fig. S4i), further arguing for the implication of NMDA but not AMPA receptors. Conversely, mEPSC frequency is reduced (Fig. S4i), suggesting decreased AP-independent glutamate release onto L5 CPNs and/or reduced number of active excitatory synapses in Emx1-cKO mice, consistent with the decreased spine density on L5 CPNs (Fig. 1d, e).

SSPNs recorded in slices from Emx1-cKO mice show electrophysiological properties (Fig. S5a–d) and basal corticostriatal synaptic transmission (Fig. S5e–g) similar to control. However, both long-term potentiation (LTP) and long-term depression (LTD)
Fig. 4  Altered electrophysiological properties of SCINs in Chat-cKO mice. a Simplified scheme of the corticostriatal circuitry with the recording patch-clamp pipette placed on a SCIN. TSHZ3-expressing neurons are blue (L1-6, cortical layers 1–6; cc, corpus callosum; st, striatum). b Sample traces obtained from a representative control SCIN: note the prominent voltage sag in response to −200 and −120 pA hyperpolarizing currents, and the AP firing during a + 100 pA depolarizing current (1st line), as well as the sustained and regular firing in cell-attached (CA) and whole-cell (WC) configuration (2nd and 3rd line, respectively). c Sample traces obtained from a representative Chat-cKO SCIN: compared to (b), note the smaller voltage sag as well as the less regular, lower frequency spontaneous firing. b, c The values of voltage sag ratio (VSR) of the response to −120 pA current injection (arrowhead), as well as the frequency and coefficient of variation (CV) of spontaneous firing of these samples, are reported; spikes have been cut; calibration bars are the same in (b) and (c). Compared to control, SCINs from Chat-cKO mice show a significant reduction of mean voltage sag ratio (d) and frequency of spontaneous discharge (e), while the CV of their inter-AP interval is increased (f) meaning that their spontaneous firing is more irregular. The number of recorded SCINs in (d)–(f) is reported in the graphs. g Current–voltage relationship obtained from 51 control and 62 Chat-cKO SCINs, and the linear best fit to calculate input resistance (see Results). *P < 0.05, **P < 0.001, Student’s t test; data in (d–f) are expressed as box and whiskers (25th–75th and 5th–95th percentiles, respectively), where bar = median and cross = mean; data in (g) are expressed as means ± SEM.
at corticostriatal synapses are abolished in Emx1-cKO mice (Fig. 2). These findings suggest that the loss of Tshz3 in CPNs does not impact their electrophysiological properties, but profoundly affects cortical synaptic transmission and corticostriatal synaptic plasticity, confirming a critical role of Tshz3 in the functioning of the corticostriatal circuit.

**Conditional deletion of Tshz3 in cholinergic neurons**

Dual immunodetection of CHAT and β-Gal in Tshz3fl/fl mice was performed to analyze the expression of Tshz3 in brain cholinergic neuron populations. This was preferred to dual immunodetection of CHAT and TSHZ3 since the tissue fixation conditions for obtaining optimal detection of each protein are different, and also because TSHZ3 immunodetection provides weaker labeling and higher background than β-Gal immunodetection. As reported previously [13], virtually all SCINs, both in the dorsal striatum and the nucleus accumbens, express Tshz3 (Fig. S6a, h). In contrast, there are no or a little proportion (<30%) of β-Gal-positive cells within CHAT-positive neurons in the components of the basal forebrain cholinergic system (medial septal nucleus, diagonal band nuclei, nucleus basalis of Meynert and substantia innominata) (Fig. S6a–d, h). SCINs thus represent the major population of Tshz3-expressing cells among the basal forebrain cholinergic neurons.

In addition, there is almost no co-expression of β-Gal and CHAT in the pedunculopontine (Fig. S6e, f, h) and laterodorsal tegmental nuclei (Fig. S6g, h), which are known to provide cholinergic afferents to several brain areas including the striatum [19]. Among the other brainstem nuclei, co-expression ranges from poor to extensive, as illustrated in the parabigeminal nucleus and the oculomotor nucleus, respectively (Fig. S6e, f, h).

We previously reported that around 90% of the Tshz3-expressing cells in the striatum are SCINs [13]. Using Tshz3tm1rud, Gad67-GFP mice, we found here that GABAergic neurons constitute 9.7 ± 1.3% (n = 21 sections from 3 mice) of the striatal Tshz3 population. Therefore, in the striatum, Tshz3 is expressed mainly in SCINs and in a very small fraction of GABAergic neurons; the latter are likely to be interneurons, as we previously reported that SSPNs do not express Tshz3 [10]. To address the role of Tshz3 in cholinergic neurons, Tshz3tm1rud mice were crossed with ChatCre mice (Chat-cKO model). CHAT is expressed in the brain from early embryonic development and as soon as E18.5 in the striatum [20]. Chat-cKO mice show a marked decrease in the density of TSHZ3-positive cells, which confirms the loss of Tshz3 in SCINs (Fig. 3a, b). The 20% TSHZ3-positive cells still observed in these mice may include a fraction of SCINs in which the Cre was insufficient and the above-mentioned GABAergic interneurons. The loss of Tshz3 expression in Chat-cKO mice does not affect the number of striatal CHAT-positive cells either in the dorsal striatum (Fig. 3c, d) or in the nucleus accumbens (35.8 ± 1.1 vs. 33.8 ± 1.4 CHAT-positive cells/μm² in control vs. Chat-cKO, respectively; 3 mice per genotype, 15 and 17 sections, respectively; P = 0.285, Student’s t test). This result was confirmed using Chat-Cre;All1fl/fl control mice (Chat-Cre:Rosa26-STOP-Tomato) to visualize SCINs in the presence or absence of Tshz3 (Fig. 3e, f).

**Tshz3 loss and SCIN electrophysiological properties**

We then determined the effect of Tshz3 loss in cholinergic neurons on the electrophysiological properties of SCINs in the dorsal striatum. In acute brain slices, SCINs are easily recognizable among the other striatal neurons due to their larger soma [21]. Moreover, they are the only autonomously active cells, firing APs with either a regular, irregular, or bursting pattern [22, 23]. SCINs also show a characteristic depolarizing voltage sag in response to the injection of negative current pulses due to the activation of the nonspecific Ih cation current mediated by HCN channels, which largely contributes to the spontaneous AP discharge characterizing these neurons [23–25]. To test a possible effect of Tshz3 loss on these SCIN properties, we measured the mean frequency of spontaneous AP discharge, its regularity [expressed as the coefficient of variation (CV) of the inter-AP intervals], and the amplitude of the sag [expressed as voltage sag ratio (VSR)] in SCINs from Chat-cKO mice and control littermates (Fig. 4a–c). We found that SCINs recorded from Chat-cKO mice show a significant reduction of both VSR (Fig. 4d) and spontaneous firing frequency (Fig. 4e), as well as an increased CV of inter-AP intervals that suggests a less regular discharge activity (Fig. 4f). The resting membrane potential at steady state is similar between control vs. Chat-cKO SCINs (46.64 ± 0.68 vs. 45.65 ± 0.64 mV, 56 vs. 86 SCINs, respectively; P = 0.305, Student’s t test), while the current–voltage relationship reveals a slight but significant increase of input resistance in Chat-cKO SCINs vs. control, calculated as the slope of the linear best fit (Fig. 4g: 125.7 ± 4.5 vs. 107.5 ± 4.0 ΩΩΩΩ, respectively; F(1,911) = 8.816, P = 0.0031). Overall, SCINs in Chat-cKO mice have a lower frequency and less regular AP discharge activity possibly due to a reduced Ih, which could impair the physiological cholinergic tone and affect the role these neurons play in modulating striatal function.

**Conditional deletion of Tshz3 in CPNs or in cholinergic neurons segregates the two core behavioral domains of ASD**

As altered physiology of the corticostriatal circuit is postulated to play a central role in the pathophysiology of ASD, we characterized Emx1-cKO and Chat-cKO mice for ASD-relevant phenotype using a battery of behavioral tests [3] after having verified that these mice do not present visual, auditory and olfactory impairment (Fig. S7). They were tested for deficits in social behavior, the first core feature of ASD, as well for stereotyped/repetitive patterns of behavior and for restricted field of interests, which are subcategories of the second ASD core feature. During the habituation phase in the two-chamber test, both Emx1-cKO and Chat-cKO mice show no significant differences in their exploration of the lured boxes as compared to their respective controls (P = 0.14, n² = 0.12, P = 0.84, n² = 0.002, respectively Fig. 5a). However, Emx1-cKO but not Chat-cKO mice show impaired social relationships (Fig. 5). Emx1-cKO mice have less preference than their controls for a conspecific (sociability, Fig. 5b) and for an unfamiliar male (social novelty, Fig. 5c), the interaction between genotype and box content being large in each case, as shown by the effect size that exceeds the typical range of variation (Fig. 5d). Conversely, Chat-cKO but not Emx1-cKO mice present more stereotyped or repetitive patterns of behavior than their controls, as shown by the marble-burying score, time burrowing in a new cage, stereotyped dips on a hole board, and number of leavings in an open field (Fig. 6a–d), with a large effect size (Fig. 6e). Restricted field of interest is impacted neither in Emx1-cKO nor in Chat-cKO mice (Fig. S8a–c).

Finally, since impairment of motor control and learning have been reported in children with ASD [26, 27], we checked Emx1-cKO and Chat-cKO mice for motor and cognitive deficits. Hind paw coordination is impaired in Chat-cKO but not in Emx1-cKO mice (Fig. S8d, e), while spatial learning ability is unaffected in both models (Fig. S8f–i).

**DISCUSSION**

Previous studies showed that haploinsufficiency or postnatal deletion of Tshz3 results in ASD-relevant behavioral deficits and suggested altered function of the corticostriatal circuitry as a possible substrate [10, 12]. The present findings point to SCINs as an additional player in the Tshz3-linked ASD syndrome. They also provide evidence that targeted conditional deletion of Tshz3 in either CPNs (Emx1-cKO) or cholinergic neurons (Chat-cKO) segregates the two core behavioral traits used to diagnose ASD, respectively social behavior deficits and repetitive behavioral patterns, suggesting that alterations in CPNs and in SCINs contribute in a complementary manner to the repertoire of
behavioral deficits linked to Tshz3 deficiency. Restricted field of interest, which defines a sub-category of the second ASD domain, was observed neither in Emx1-cKO nor in Chat-cKO mice. This suggests that the expression of this deficit in the previously characterized models of Tshz3 deletion may involve additional players, such as the cortical and striatal GABAergic interneurons expressing Tshz3 whose specific role remains to be determined, and/or result from the combined dysfunction of CPNs and SCINs due to the loss of Tshz3 in both these neuronal types. Learning ability was impacted neither by Tshz3 postnatal deletion [12], nor in Emx1-cKO and Chat-cKO models.

Among the multiplicity of circuits involved in social behavior, the literature points out the crucial role of the cortex [28, 29]. Here we focused on the sensorimotor cortex and the dorsal striatum as a model circuit that has been characterized in several ASD mouse models. However, we cannot exclude that dysfunction in the prefrontal cortex-nucleus accumbens circuitry may also be implicated in the described ASD-related phenotype, since Tshz3 is expressed in all cortical areas and in SCINs of both the dorsal striatum and the nucleus accumbens. Corticostriatal and striatal circuit dysfunctions are associated with ASD features, both in patients and in mouse models, with CPNs and SSPNs being highly impacted by mutations of ASD-linked genes [7, 8, 10, 12, 30, 31]. There is however increasing evidence incriminating interneuron populations of the cortex and the striatum in ASD [32]. Here, we show that, in the cortex, the ASD-related Tshz3 gene is expressed...
Fig. 6  Repeated patterns of behavior in Chat-cKO but not in Emx1-cKO mice. a Marble-burying, Emx1-cKO, Student’s t(15) = 1.0, P = 0.33; Chat-cKO, t(19) = 3.97, P = 0.001. b Time burrowing, Emx1-cKO, t(15) = 1.16, P = 0.13; Chat-cKO, t(19) = 3.225, P = 0.004. c Stereotyped dips, Emx1-cKO, F_{interaction}(1,15) = 0.08, P = 0.87 (with non-stereotyped dips as covariate, P = 0.76); Chat-cKO, F_{interaction}(1,19) = 32.69, P = 0.00001 (with non-stereotyped dips as covariate, P = 0.24). d Number of leanings, Emx1-cKO, t(15) = 1.51, P = 0.15; Chat-cKO, t(18) = 4.35, P = 0.0003. e Sizes of the difference in Emx1-cKO (η^2 = 0.06, 0.08, 0.13 in (a), (b) and (d), respectively, and partial η^2 = 0.01 in (c) and in Chat-cKO (η^2 = 0.45, 0.35, 0.51 in (a), (b) and (d), respectively, and partial η^2 = 0.63 in (c). Sample size of (a–d) were: 9, 9, 9, and 12 for Emx1-cKO; 8, 8, 9, and 11 for their controls; 12, 12, 12, and 11 for Chat-cKO; 9, 9, 11, and 8 for their controls. Data in (a–d) are expressed as means + SEM. **P < 0.01 ***P < 0.001.
not only in CPNs but also in a third of GABAergic interneurons, while not in cholinergic interneurons. In contrast, in the striatum, Tshz3-expressing cells are for their vast majority cholinergic interneurons [13] and comprise a minority of GABAergic interneurons. To disentangle the role of CPNs from that of interneurons in the ASD symptoms linked to Tshz3 deficiency, we generated and characterized Emx1-cKO mice. We confirmed the specificity of Tshz3 deletion in CPNs within the corticostriatal circuit in this model, Tshz3 expression in cortical and striatal interneurons being maintained. In addition, no change in the numbers and positioning of these interneurons were detected. Interestingly, we found that Emx1-cKO mice specifically exhibit impaired social behavior and that this deficit co-segregates with altered NMDA receptor-mediated transmission in the cortex and loss of plasticity at corticostriatal synapses. Corticostriatal synaptic plasticity has been deeply characterized, but discrepancies concerning its induction protocols and the underlying molecular and cellular mechanisms [33] make it difficult to univocally interpret our results. However, since both LTD and LTP expression require pre- and postsynaptic changes, their disruption in Emx1-cKO mice could be attributable to cortical circuitry defects, such as the observed decrease of NMDA receptor activity in L5 CPNs [34–36]. Our findings are also in line with studies substantiating the involvement of NMDA receptor dysfunction in social deficits associated with ASD in rodent models as well as in patients [37, 38]. Finally, consistent with the literature linking ASD with changes of dendritic spine density [39], we evidence decreased spine density in L5 CPNs of Emx1-cKO mice, as in our previous model [12]. Overall, these data indicate that the loss of Tshz3 in CPNs induces morphofunctional changes in these neurons and deeply affects corticostriatal plasticity, which might result in altered processing of cortical information and account for the observed social behavior deficits.

We also investigated the contribution of cholinergic neurons in the pathophysiology of Tshz3-linked ASD. We show that TSHZ3 is expressed in virtually 100% of SCNs of both the dorsal striatum and the nucleus accumbens, while its expression is absent or partial in the other main brain cholinergic systems. Despite their low number, SCNs have morphofunctional features that place them as key modulators of striatal microcircuits. They play a crucial role in movement control, attentional set-shifting, habit-mediated and goal-directed behavior, and selection of appropriate behavioral responses to changes in environmental contingencies, conferring behavioral flexibility [40–44]. These interneurons are also involved in basal ganglia-related pathologies such as dystonia, Parkinson’s and Huntington’s disease, Tourette’s syndrome, obsessive compulsive disorder and drug addiction [45–50]. In contrast, despite the array of data pointing to basal ganglia and to cholinergic transmission abnormalities in ASD and in ASD models [16, 51–55], to date there is little evidence showing the specific involvement of SCNs: the partial depletion of both SCNs and fast-spiking GABAergic interneurons produces stereotypy and impaired social behavior in male mice [17], while total elimination of SCNs results in perseverative behavior that extends to social behavior, rather reminiscent of neuropsychiatric conditions as Tourette’s syndrome or obsessive-compulsive disorder [56]. The present work reveals that targeted Tshz3 deletion in CHAT-expressing neurons leads to robust stereotyped and repetitive patterns of behavior without impacting social behavior. Given the literature associating drug-induced stereotypes with abnormalities in striatal cholinergic signaling [57–59], and the co-expression of CHAT and TSHZ3 in SCNs but not in brainstem cholinergic neurons that are known to project to the striatum [19], this behavioral deficit is likely attributable to SCNs. The lack of social behavior impairment is surprising, as altered striatal physiology is assumed to be a central node mediating repetitive motor behaviors and also a range of ASD-associated behaviors, including social deficit [30]. However, the studies examining the specific involvement of SCNs in several neurodevelopmental disorders have associated altered sociability with the depletion of this interneuron population [17, 56, 60], which is not observed in Chat-cKO mice. Whereas the number of SCNs in these mice is unchanged, suggesting that their generation and viability are not affected, we evidenced modifications in their firing activity and electrophysiological membrane properties. This finding is an addition to the increasing amount of data stressing the complex implication of SCNs in health and diseases [61]. How the selective loss of Tshz3 in SCNs leads to these electrophysiological changes, what are their molecular bases and what are the consequences on striatal cholinergic signaling still need to be determined. However, SCNs are important modulators of the two populations of SPNs forming the “direct” and “indirect” pathways by which the striatum regulates basal ganglia outflow, whose balanced activity is determinant for appropriate action selection [42, 62]. Thus, the changes in SCN properties observed here could alter the way they normally respond to salient stimuli and/or reward-associated cues, thereby the way they modulate the transfer of cortical information through the striatum [40, 41, 63], as observed for example after targeted deletion of the transcription factor Er81 in SCNs [44]. This could underlie the increased stereotyped behaviors observed in Chat-cKO mice and, possibly, also in Tshz3-/-mice [10], as well as in CamK2a-cKO [12] in which we recently showed that Tshz3 is lost also in SCNs [13]. Finally, Chat-cKO mice do not show basal exploration deficit, similarly to Emx1-cKO mice, but present impaired hind paw coordination, which is in line with motor deficiencies frequently associated with ASD [64] and with a study linking partial SCN ablation with motor incoordination [65]. Although TSHZ3 is expressed in about 25% of cholinergic neurons of the nucleus basalis of Meynert and the substantia innominata, the similarity of spatial learning curves of control and Chat-cKO mice suggests minor impact of Tshz3 deletion on the function of the basal forebrain cholinergic system, which is deeply involved in learning and memory processes [66]. In conclusion, this study shows that the conditional loss of the ASD-related gene Tshz3 in CPNs and SCNs does not affect the numbers of these neurons but induces changes in their electrophysiological and synaptic properties, paralleled by specific ASD-like behavioral defects. It provides new experimental evidence that the two behavioral domains used to diagnose ASD are independent domains that can be triggered by dysfunction in distinct neuronal subtypes. These findings may open the road to domain-specific pharmacological and behavioral therapies.

MATERIALS AND METHODS

Mouse strains and genotyping

The Tshz3lox/lox, Tshz3lox/lox, Emx1-Cre, Chat-Cre, Rosa26-STOP-lacZ and Ai14 (Rosa26-STOP-Tomato), GAD67-GFP, and Thy1-GFP mouse lines have been described previously [10, 12, 18, 67–72]. Male heterozygous Cre mice were crossed with female Tshz3lox/lox to generate the two Tshz3 conditional knockout (cKO) mice models: Emx1-cKO and Chat-cKO [18, 70], Littermate Emx1-Cre+/- and Chat-Cre+/- mice were used as respective controls. Animals carrying the Tshz3lox allele and Tshz3lox allele were genotyped as described previously [12]. Experimental procedures were in agreement with the recommendations of the European Communities Council Directive (2010/63/EU). They have been approved by the “Comité National de Rèflexion Ethique sur l’Expérimentation Animale n°14” and the project authorization delivered by the French Ministry of Higher Education, Research and Innovation. (ID numbers 57-0711021, 2019020811238253-V2 #19022 and 2020031615241974-VS #2532). No randomization was used and no animals or samples were excluded from the different analyses performed.

Immunohistochemistry and histology

All stains were processed on coronal brain sections of postnatal day (P) 28–34 mice. Immunostaining for TSHZ3 alone was performed on cryostat...
sections of brains immediately removed after anesthesia (ketamine + xylazine, 100 + 10 mg/kg, respectively, i.p.) and frozen in dry ice until use. Before incubation with the antibodies, sections were fixed with 4% paraformaldehyde (PFA) for 15 min, then washed twice for 5 min in PBS. For Tshz3 immunostaining and GFP detection, GAPDH and control littersates, and from 16 Chat-kO and 16 Chat-Cre / - / control littersates, aged P21–28. No binding was done. Procedures were similar to those described previously [10, 12, 74]. Briefly, acute coronal slices (250 µm-) containing cortex and stratum were cut using a 5100 Vibrotome (Leica) in ice-cold solution containing (in mM): 110 choline, 2.5 KCl, 1.25 NaH2PO4, 7 MgCl2, 0.5 CaCl2, 25 NaHCO3, 7 glucose, pH 7.4. Slices were kept at room temperature in oxygenated artificial cerebrospinal fluid (ACSF), whose composition was (in mM): 126 NaCl, 2.5 KCl, 1.25 MgCl2, 1.2 NaH2PO4, 24 CaCl2, 11 glucose and 25 NaHCO3, pH 7.4. Electrophysiological recordings were performed in oxygenated artificial cerebrospinal fluid (ACSF) at 34–35°C flowing at ~2 ml/min. L5 CPNs of the primary motor and somatosensory cortex, and SPNs and SCNs of the dorsolateral striatum were identified by infrared video microscopy and by their electrophysiological properties [75, 76]. They were recorded by whole-cell patch-clamp using borosilicate micropipettes (5–6 MΩ) filled with an internal solution containing (in mM): 125 K-glutamate, 1 NaCl, 1 CaCl2, 1 MgCl2, 0.5 BaCl2, 2.5 Mg-ATP, 1 Na-GTP, and 1 Mg-ATP, pH 7.3 (except for NMDA/AMPA ratio experiments, see below). Electrophysiological data were acquired by an AxoPatch 2008 amplifier and pClamp 10.7 software (Molecular Devices, Wokingham, UK). Series and input resistance were continuously monitored by sending 5 mV pulses, and neurons showing ≥20% change in these parameters were discarded from the analysis.

Characterization of CPNs, SPNs, and synaptic transmission
A stimulating bipolar electrode was placed either in the cortex at the level of L4 to activate local fibers mainly arising from L2 and 3 and evoke excitatory postsynaptic currents (EPSCs) in L5 CPNs, or in the corpus callosum to activate corticostriatal fibers and evoke EPSCs in SPNs [12]. We did not distinguish the two main L5 CPN subtypes, i.e., intratelencephalic and pyramidal tract neurons, because they do not differ in the electrophysiological properties analyzed here [12, 77]. Glutamatergic EPSCs were recorded in the presence of 50 µM picrotixin and 1 µM tetrodotoxin. Current–voltage (I–V) relationship was obtained in current-clamp mode by injecting hyperpolarizing and depolarizing current steps (ΔI = ± 50 pA, 800 ms), and input resistance was calculated by linear regression analysis, i.e., as the slope of the linear best fit of the I–V relationship of each recorded neuron. Rheobase was measured as the minimal injected current (±5 pA increments) capable of eliciting an action potential (AP). For paired-pulse ratio (PPR), EPSC amplitude was measured on 6 averaged traces at each inter-pulse interval. For analyzing mEPSCs, the detection threshold (around 3–4 pA) was set to twice the noise after trace filtering (Boxcar low-pass), and only cells exhibiting stable activity and baseline were considered. For NMDA/AMPA ratio experiments, the internal solution contained (in mM): 140 CsCl, 10 NaCl, 0.1 CaCl2, 10 HEPES, 1 EGTA, 2 Mg-ATP and 0.5 Na-GTP, pH 7.3. The AMPA component of the EPSC was measured at the peak at a holding potential of ~60 mV, while the NMDA component was measured at ~40 mV and 40 ms after the stimulation artifact, when the AMPA component is negligible, as previously described [12]. Tonic NMDA currents were elicited by bath application of 50 µM NMDA for 60 s, after a stable baseline of at least 120 s; their amplitude was measured by averaging the current values of a 5 s window surrounding the negative peak, compared to baseline; only neurons that were capable of returning to their baseline after washout were considered. EPSC amplitude to potentiate monitoring corticostriatal long-term depression and potentiation (LTD and LTP, respectively) was measured on averaged traces (6 per minute) to obtain time-course plots and to compare this parameter before (baseline) and after induction protocols. The induction protocol for corticostriatal LTD consisted of 3 trains at 100 Hz, 3 s duration, 20 s interval, at half intensity compared to baseline [78]. LTD induction protocol was identical but, during each train, neurons were depolarized to −10 mV to allow strong activation of NMDA receptors [10, 12, 79]. For a review about corticostriatal LTD and LTP see [36].

Characterization of SCNs
The resting membrane potential (RMP) was measured at the steady state between two consecutive APs. The current–voltage relationship was
calculated from the membrane response at the end of current steps from –200 to –20 pA (20 pA steps lasting 800 ms). The voltage sag ratio (VSR) was calculated from the response to a –120 pA current step as the peak voltage drop (sag) against the voltage at the end of the current pulse [80, 81]. Such relatively small current step was chosen because, with larger steps, the sag amplitude was extremely variable between different SCNs. Spontaneous AP firing was analyzed in terms of average firing rate (expressed in Hz) and regularity; to quantify this latter parameter, we calculated the coefficient of variation (CV) of the inter-AP intervals. Note that spontaneous AP firing was analyzed only from cell-attached recordings, which were done before switching to whole-cell; in some cases, spontaneous firing was not detectable in cell-attached configuration, thus the number of samples for AP firing analyses is smaller than the whole number of recorded SCNs.

Behavioral analysis

Housing conditions. Experiments were conducted blind for the genotypes in P7-17 male Emx1-CreKO and Chat-kKO mice and their respective Emx1-Cre+ and Chat-Cre+ control littermates. We used males and not female mice because the ambulatory activity of females is impacted by the estrous cycle phases in rodents [82] and may bias the results of repetitive behavior measures that are partly dependent on motor activity.

Mice used in studies on social behavior are generally reared in groups of variable size and more rarely in isolation. The choice of our rearing strategy was based on the fact that the measures of social behavior in adult mice depends on the characteristics of the previous interaction that the observed male has experienced with its peers [3, 83–85]. In the rearing in group strategy, the social behaviors directed towards the tested male can vary according to the genotypes, the androgen levels and the neurotransmitter profiles of the individuals in the groups [86]. Consequently, the social behavior measured in an individual is the resultant of the individual social ability plus a component corresponding to the interactions of the individual with the other members of the group; this effect varies with the size of the group. In addition, behavioral “contamination” resulting in an impairment of sociability in wild-type mice by cohabitant KO modeling ASD was described [85]. Such undesirable effect plus the heterogeneity of the measures in mice reared in the group should contribute to avoid this strategy for testing social behavior. On the other hand, maintaining the mice socially deprived generates a specific set of agonistic reactions that prevent the measures of social abilities. To circumvent such biases, we have developed an alternative solution for years: each tested male is housed with one female mouse belonging to a single inbred strain [86]. Here, a kKO or a control male mouse was reared and maintained with CBA/H/Gnc female mice [3]. Housing was done in transparent 35 × 20 × 18 cm cages with 1-liter poplar woodchip bedding and weekly renewed enrichment (cardboard shelter).

Social abilities. To circumvent such biases, we have developed an appropriate set of agonistic reactions that prevent the measures of social abilities. On the other hand, maintaining the mice socially deprived generates a specific set of agonistic reactions that prevent the measures of social abilities. To circumvent such biases, we have developed an alternative solution for years: each tested male is housed with one female mouse belonging to a single inbred strain [86]. Here, a kKO or a control male mouse was reared and maintained with CBA/H/Gnc female mice [3]. Housing was done in transparent 35 × 20 × 18 cm cages with 1-liter poplar woodchip bedding and weekly renewed enrichment (cardboard shelter).

Visual capacities: The mouse was raised, taken by the tail, and a thin stick was approached to its eyes, without touching the vibrissae. Raising the head was scored 1 and grasping or trying to grasp the pen was scored 2. The test was administered five times and the sum of the scores recorded. Swimming towards a distant shelf in the Morris Water Maze was calculated from the response to a 120 pA current step as the peak voltage drop (sag) against the voltage at the end of the current pulse [80, 81]. Such relatively small current step was chosen because, with larger steps, the sag amplitude was extremely variable between different SCNs. Spontaneous AP firing was analyzed in terms of average firing rate (expressed in Hz) and regularity; to quantify this latter parameter, we calculated the coefficient of variation (CV) of the inter-AP intervals. Note that spontaneous AP firing was analyzed only from cell-attached recordings, which were done before switching to whole-cell; in some cases, spontaneous firing was not detectable in cell-attached configuration, thus the number of samples for AP firing analyses is smaller than the whole number of recorded SCNs.

Olfactory ability to detect an odor was evaluated by an increased time in sniffing a new odor using an olfactory habituation/dishabituation test.
Spatial learning: The Morris water maze provides measures of the ability of rodents to solve spatial learning problems, namely the ability to find a submerged resting platform concealing opaque water. The platform is a glass cylinder (66 mm diameter, 9 mm beneath the surface of the water) positioned 23 cm from the edge of a 100 cm diameter circular tank filled with water at 26 ± 1 °C and the light at 70 lux on the surface. Each mouse performed 7 blocks of 4 trials each: one block on day 1, and two blocks daily (one in the morning and one in the afternoon) for 3 successive days. A trial was stopped after 90 s if the mouse failed to reach the platform. We considered that the mouse had reached the platform when it stayed on the platform for 5 s at least. We presented a small metal shelf to the mouse 5 cm above the platform at the end of each trial of the first block (exploring). The mouse climbed on it and was returned to its cage with dry sawdust for 120 s. We had previously assigned 4 virtual cardinal points to the tank, each being the starting point for a trial. The starting point for each trial was chosen randomly and within a block the mouse never started more than once from the same virtual cardinal point. We measured (1) the time to reach the hidden platform and (2) the starting point for each trial was chosen randomly and within a block the mouse never started more than once from the same virtual cardinal point. The significant threshold was set at P < 0.05.

RT-qPCR. Statistical analysis was performed by unpaired Student’s t test using the qbasePLUS software version 2 (Biogazelle). The significance threshold was set at P < 0.05.

Electrophysiology. Statistical analysis was performed by Prism 7.05 (GraphPad Software, USA). Two-tailed Student’s t test or Mann–Whitney test was used for comparing two data sets when passing or not D’Agostino & Pearson’s normality test, respectively. Sample sizes, tests used, and P values are reported in Figure legends. The significance threshold was set at P < 0.05.

Behavior. Data were processed by Statistical Package for the Social Sciences (SPSS software, version 25 [1000]). The same statistical designs were used to compare Emx1-cKO and ChAT-cKO mice to their respective controls. Non-parametric statistics were chosen when the assumption of normality was rejected.

Impairment of social behavior: To analyze data from each social phase of the two-chamber test (sociability and interest for social novelty), a mixed-design analysis of covariance (ANCOVA) was used including the genotype as a fixed factor, the box content as repeated measure, with a measure of activity during habituation as covariate. A significant interaction between genotype and box content indicates that social behavior differs between the cKO and its control group.

Repetitive patterns of behavior and motor performance: The difference between two independent groups (cKO and its control group) was tested by an unpaired two-sample Student’s t test in each case where it was not necessary to include a covariate in the statistical design (i.e., stereotyped behavior: marble-burrowing score, time burrowing, number of cleanings; motor behavior: number of hind paw slips). For measures of stereotyped dips, on which the activity level could have an impact, an analysis of covariance (ANCOVA) was performed, using the genotype as a fixed factor (cKO vs. respective control) and non-stereotyped dips as covariate.

Sensory abilities: Comparison of the visual and auditory capacities of the cKO and their respective controls were conducted using a Student’s t test. Mixed repeated measures ANOVA, with genotype as a fixed factor and 15 odors as repeated measures, was used to compare cKO and their respective controls for olfactory capacities.

Spatial learning: The statistical design was the same for the time to reach the platform and the cumulative distance to the center of the platform in the Morris water maze test. Differences between the 7 blocks were tested either with Friedman’s ANOVA, a non-parametric version of one-way repeated measures ANOVA, or with two-way repeated measures mixed ANOVA design, with blocks as repeated measures variable and cKO vs. control as between-group variable. Learning may be deduced from within-block statistical difference and reduced time to reach the platform from one bloc to the next. The slope of the median values of the four trials in each of the seven blocks was calculated for each mouse. The median slopes for the cKO and their respective controls, as well as the time to reach the virtual platform (probe test) and the visible platform, were compared with a Student’s t test.

Effect size: Effect sizes are expressed as η² or as partial η² with 95% confidence interval [100, 101].

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request. Raw data (FastQ files) from the sequencing experiment (triplicates from wild-type and Tshz3-mutant striatum) and raw abundance measurements for genes (read counts) for each sample are available from Gene Expression Omnibus (GEO) under accession GSE157658, which should be quoted in any manuscript discussing the data.

REFERENCES

1. American Psychiatric Association. Diagnostic and statistical manual of mental disorders, DSM-5: 5th edn. Washington, DC: American Psychiatric Association; 2013.
2. Shuster J, Perry A, Belbko J, Toplak ME. Review of factor analytic studies examining symptoms of autism spectrum disorders. J Autism Dev Disord. 2014;44:90–110.
3. Roubertoux PL, Tordjman S, Caubit X, di Cristopharo J, Ghata A, Fasano L, et al. Construct validity and cross validity of a test battery modeling autism spectrum disorder (ASD) in mice. Behav Genet. 2020;50:26–40.
4. Banerjee-Basu S, Packer A. SPARi Gene: an evolving database for the autism research community. Dis Models Mech. 2010;3:133–5.
5. Bourgon T, Currat B. Current knowledge on the genetics of autism and propositions for future research. C R Biol. 2016;339:300–7.
6. Delmonte S, Gallagher L, O’Hanlon E, McGrath J, Balsters JH. Functional and structural connectivity of frontostriatal circuitry in autism spectrum disorder. Front Hum Neurosci. 2013;7:430.
7. Shepherd GM. Corticostriatal connectivity and its role in disease. Nat Rev: Neurosci. 2013;14:278–91.
8. Li W, Pozzo-Miller L. Dysfunction of the corticostriatal pathway in autism spectrum disorders. J Neurosci Res. 2020;98:2130–47.
9. Li M, Santee G, Inamura Kawasawa Y, Evgrafov O, Gulden FO, Pochareddy S, et al. Integrative functional genomic analysis of human brain development and neurodevelopmental disorders. Science. 2016;352:eaad7615.
10. Caubit X, Gubellini P, Andreux J, Roubertoux PL, Metwally M, Jacq B, et al. TSHZ3 deletion causes an autism syndrome and defects in cortical projection neurons. Nat Genet. 2016;48:1359–69.
11. Caubit X, Tiveron MC, Cremer H, Fasano L. Expression patterns of the three Teashirt-related genes define specific boundaries in the developing and postnatal mouse forebrain. J Comp Neurol. 2005;486:76–88.

12. Chabbert D, Caubit X, Roubertoux PL, Carlier M, Habermann B, Jacq B, et al. Postnatal Thz3 depletion drives altered corticostriatal function and autism spectrum disorder-like behavior. Biol Psychiatry. 2019;86:274–85.

13. Caubit X, Arbeille E, Chabbert D, Desplat F, Ferezak I, Fatmi A, et al. Camk2a-Cre and Thz3a expression in mouse striatal cholinergic interneurons: implications for autism spectrum disorder. Front Genet. 2021;12:683959.

14. Munoz-Manchado AB, Bengtsson Gonzales C, Zelis A, Munguba D, Kienlen FM, de Rivero H, et al. Molecular diversity and specializations among the cells of the adult mouse brain. Cell. 2018;174:1015-30; e1016

15. Kavart G, Kimchi T. Acetylcholine elevation relieves cognitive rigidity and social deficiency in a mouse model of autism. Neuropsychopharmacology. 2014;39:831–40.

16. Smith R, Chuang H, Rundquist S, Maat-Schieman ML, Colgan L, Englund E, et al. Cholinergic neuronal deficit without cell loss in Huntington’s disease. Hum Mol Genet. 2006;15:3119–31.

17. McKinley JW, Shi J, Zwikovska I, Hur M, Tamford U, Sudarsana Devi SP, et al. Dopamine deficiency reduces striatal cholinergic neuron function in models of Parkinson’s disease. Neuron. 2019;103:1056–72; e1056

18. Abdukevoumu N, Hernandez-Flores T, Garcia-Munoz M, Arubothun G. Cholinergic modulation of striatal microcircuits. Eur J Neurosci. 2019;49:604–22.

19. Martin-Ruiz CM, Lee M, Perry RH, Baumann M, Court JA, Perry EK. Molecular analysis of nicotinic receptor expression in autism. Brain Res Mol Brain Res. 2004;123:81–90.

20. Perry EK, Lee ML, Martin-Ruiz CM, Court JA, Volsen SG, Merritt J, et al. Cholinergic activity in autism: abnormalities in the cerebral cortex and basal forebrain. Am J Psychiatry. 2001;158:1058–66.

21. Deutsch SI, Urbanio MR, Neumann SA, Burket JA, Katz E. Cholinergic abnormalities in autism: Is there a rationale for selective nicotinic agonist interventions? Clin Neuropsychopharmacol. 2010;33:114–20.

22. Ogren PO, Mohler EG, Prior M, Palencia CA, Rozman S. Acetylcholine activity in selective striatal regions supports behavioral flexibility. Neurobiol Learn Mem. 2009;91:13–22.

23. Eissa N, Azimullah S, Jayaprakash P, Jayaraj RL, Reiner D, Ojha SK, et al. The role of acetylcholine modulation in autism: An analysis of nicotinic receptor expression in autism. Autism. 2019;10:981–97.

24. MacGowan AP, Smith Y. Cholinergic interneurons in the dorsal and ventral striatum: anatomical and functional considerations in normal and diseased conditions. Ann N Y Acad Sci. 2015;1349:1–45.

25. Eissa N, Palone E, Schlegel D, Herzog MC. A competitive model for striatal cholinergic synaptic activity in autism: The role of acetylcholine in autism. Neuropsychopharmacology. 2018;43:406–15.

26. Lai MC, Lombardo MV, Baron-Cohen S. Autism. Lancet. 2014;383:896–910.
65. Xu M, Kobets A, Du JC, Lennington I, Li L, Banasz M, et al. Targeted ablation of cholinergic interneurons in the dorsolateral striatum produces behavioral manifestations of Tourette syndrome. Proc Natl Acad Sci USA. 2015;112:893–8.

66. Blake MG, Boccia MM. Basal forebrain cholinergic system and memory. Curr Top Behav Neurosci. 2018;37:23–73.

67. Caubit X, Lye CM, Martin E, Core N, Long DA, Vola C, et al. Treashtr 3 is necessary for urethral smooth muscle differentiation downstream of SHH and BMP4. Development. 2008;135:3301–10.

68. Mao X, Fujiwara Y, Orkin SH. Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. Proc Natl Acad Sci USA. 1999;96:5037–42.

69. Madsen L, Zwingmann TA, Sunkin SM, Oh SW, Zarivala HA, Gu H, et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci. 2010;13:133–40.

70. Rossi J, Balthasar N, Olson D, Scott M, Berglund E, Lee CE, et al. Melanocortin-4 receptors expressed by cholinergic neurons regulate energy balance and glucose homeostasis. Cell Metab. 2011;13:195–204.

71. Tamamaki N, Yanagawa Y, Tomioka R, Miyazaki J, Obata K, Kaneko T. Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. J Comp Neurol. 2003;467:60–79.

72. Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, et al. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron. 2000;28:41–51.

73. Rodriguez A, Ehlenberger DS, Dickstein DL, Hof PR, Wearne SL. Automated three-dimensional detection and shape classification of dendritic spines from fluorescence microscopy images. PLOS ONE. 2008;3:e1997.

74. Chassain C, Melon C, Salin P, Vitale F, Couraud S, Durif F, et al. Metabolic, synaptic and behavioral impact of 5-week chronic deep brain stimulation in hemiparkinsonian rats. J Neurochemistry. 2016;136:1004–16.

75. Hattox AM, Nelson SB. Layer V neurons in mouse cortex projecting to different targets have distinct physiological properties. J Neurophysiol. 2007;98:3330–40.

76. Jiang ZG, NR MA. Membrane properties and synaptic responses of rat striatal neurones in vitro. J Physiol. 1991;443:533–53.

77. Chen L, Daniels S, Kim Y, Chu HY. Cell type-specific decrease of the intrinsic excitability of motor cortical pyramidal neurons in Parkinsonism. J Neurosci. 2021;41:5533–65.

78. Calabresi P, Maj R, Pisani A, Mercuri NB, Bernardi G. Long-term synaptic depression in the striatum: physiological and pharmacological characterization. J Neurosci. 1992;12:4224–33.

79. Calabresi P, Pisani A, Mercuri NB, Bernardi G. Long-term potentiation in the striatum is unmasked by removing the voltage-dependent magnesium block of nmda receptor channels. Eur J Neurosci. 1992;4:929–35.

80. Haghdoust H, Janahmadi M, Behzadi G. Physiological role of dendrotoxin-sensitive K+ channels in the rat cerebellar Purkinje neurons. Physiol Res. 2007;56:807–13.

81. Maisano X, Litvina E, Tagliatela S, Aaron GB, Grabel LB, Naegeli JR. Differentiation and functional incorporation of embryonic stem cell-derived GABAergic interneurons in the dentate gyrus of mice with temporal lobe epilepsy. J Neurosci. 2012;32:46–61.

82. Beau Yon de Jonage-Canonico M, Roubertoux PL, Lenoir V, Carlier M, Kerdelhué C. Long-term synaptic depression of dendritic spines from factorially structured to quantitative trait loci mapping. Eur J Pharm. 2005;526:172–85.

83. Roubertoux PL, Ghata A, Carlier M. Measuring preweaning sensorial and motor development in the mouse. Curr Protoc Mouse Biol. 2018;8:54–78.

84. Ehret G, Romand R. Development of tone response thresholds, latencies and tuning in the mouse inferior colliculus. Brain Res Dev Brain Res. 1992;67:217–26.

85. Francis RL. The Preyer reflex audiogram of several rodents, and its relation to the “absolute” audiogram in the rat. J Aud Res. 1979;19:217–33.

86. Willott JF. The auditory psychobiology of the mouse. C.C. Thomas; 1983.

87. Roubertoux PL, Ghata A, Carlier M. Measuring preweaning sensorial and motor development in the mouse. Curr Protoc Mouse Biol. 2018;8:54–78.

88. Nyugne J, Handley SL. Effects of 5-HT uptake inhibitors, agonists and antagonists on the burying of harmless objects by mice; a putative test for anxiolytic agents. Br J Pharm. 1991;104:105–12.

89. Thomas A, Burant A, Bui N, Graham D, Yuva-Paylor LA, Paylor R. Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. Psychopharmacology (Berl). 2009;204:361–73.

90. Moy SS, Nadler JJ, Pavez A, Barbaro RP, Johns JM, Magnuson TR, et al. Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. Genes Brain Behav. 2004;3:287–302.

91. Roubertoux PL, Carlier M, Tordjman S. Organism models of autism spectrum disorders. Springer New York; 2015. 487 pp.

92. Feuerhahn S, Galanopoulou A, Vayenas DC, Mitropoulos N, Katsaros D, Koutsoukas E, et al. Targeted ablation of cholinergic interneurons in the dorsolateral striatum produces behavioral manifestations of Tourette syndrome. Proc Natl Acad Sci USA. 2015;112:893–8.
Correction: Targeted Tshz3 deletion in corticostriatal circuit components segregates core autistic behaviors

Xavier Caubit, Paolo Gubellini, Pierre L. Roubertoux, Michèle Carlier, Jordan Molitor, Dorian Chabbert, Mehdi Metwaly, Pascal Salin, Ahmed Fatmi, Yasmine Belaidouni, Lucie Brosse, Lydia Kerkerian-Le Goff and Laurent Fasano

© The Author(s) 2022

Translational Psychiatry (2022) 12:211 ; https://doi.org/10.1038/s41398-022-01925-x

Correction to: Translational Psychiatry https://doi.org/10.1038/s41398-022-01865-6, published online 15 March 2022

The original version of this article unfortunately contained a mistake. The figure legends of the supplemental figures were missing. The missing legends can be found below. The original article has been corrected.

Fig. S1. TSHZ3 expression in interneurons and glial cells in the cerebral cortex. (a–e) Coronal brain sections. a Tshz3 expression as β-Gal staining in Tshz3<sup>fl/fl</sup>; GAD67-GFP mouse brain. The two images on the right are magnifications of the framed areas in A. Scale bars 100 μm. b Double immunofluorescence staining for β-Gal and CHAT. The framed areas in (b) are magnified on the right. Scale bars 100 μm. c Double immunofluorescence staining for Olig2 and β-Gal (left) and detail of the framed area (right). Scale bars 100 μm. d, e Double immunofluorescence staining for GFAP and β-Gal. Scale bars 100 μm (d) and 50 μm (e). Nuclei in c–e are counterstained with DAPI. cc, corpus callosum; cx, cerebral cortex; st, striatum.

Fig. S2. Cortical layering is preserved in Emx1-cKO mouse brain. a Coronal brain sections from Emx1-cKO and control mice immunostained for NeuN detection. Scale bar 250 μm. b Number of NeuN-positive cells counted in frames of 400 μm width spanning the entire cortical thickness of control and Emx1-cKO mice. No genotype difference is found (11 sections from 3 mice per genotype; P = 0.9488, Student’s t-test). c Coronal brain sections from Emx1-cKO and control mice immunostained for CUX1 and BCL11B. Nuclei are counterstained with DAPI. Scale bar 100 μm; cc corpus callosum, st striatum, L layer. d Number of CUX1-positive cells in L2-4 and of BCL11B-positive cells in L5 and L6 in control and Emx1-cKO mice. No genotype difference is found (BCL11B-positive cells: 14 sections from 3 control mice and 18 sections from 3 Emx1-cKO mice; CUX1-positive cells: 28 sections from 4 control mice and 21 sections from 4 Emx1-cKO mice; counts were performed in cortical frames of 400 μm width; P = 0.3207 (L2/3), P = 0.4007 (L5) and P = 0.1180 (L6), Student’s t-test). Data are expressed as means ± SEM.

Fig. S3. Loss of Tshz3 in Emx1-cKO mice does not affect the numbers of cortical GABAergic and striatal cholinergic interneurons. Representative images a and quantitative analysis b showing the distribution of GAD67-GFP-positive cells in the cerebral cortex in coronal brain sections from GAD67-GFP control and Emx1-cKO-GAD67-GFP mice. Scale bar in A 250 μm. Data in b are expressed as percent of total GFP-positive cells per bin (37 sections from 5 control mice; 41 sections from 7 Emx1-cKO mice; F<sub>genotype</sub> (1100) = 0.00006, P = 0.994, F<sub>interaction</sub> (9100) = 0.381, P = 0.942, 2-way ANOVA). Images of CHAT immunostaining c and analysis of the density of CHAT-positive cells d in coronal brain sections at striatal level of control and Emx1-cKO mice. Scale bar 100 μm (18 sections from 3 control and 3 Emx1-cKO mice, respectively; P = 0.465, Student’s t-test). Data in b are expressed as median with interquartile range; data in d as means ± SEM.

Fig. S4. Electrophysiological characterization of L5 CPNs and basal cortical synaptic transmission. a Simplified scheme of the corticostriatal circuitry with the recording patch-clamp pipette placed on a L5 CPN and the stimulating electrode placed in L4. TSHZ3-expressing neurons are blue (L1-6 cortical layers 1–6, cc corpus callosum, st striatum). b Current-voltage relationship recorded from CPNs of Emx1-cKO mice and littermate controls show similar slopes and input resistance (148.9 ± 13.3 vs. 151.3 ± 11.6 MΩ, respectively; n = 21 and n = 28, respectively; P > 0.05, Student’s t-test). Resting membrane potential (RMP; n = 28-38) and d rheobase (n = 11–21) do not significantly differ between control and Emx1-cKO CPNs (P > 0.05 for both; Student’s t-test and Mann-Whitney test, respectively). e The number of action potentials (APs) emitted by control (n = 10) and Emx1-cKO (n = 15) CPNs in response to increasing current injections is similar (2-way ANOVA: genotype F(1,135) = 3.068, P = 0.0821; interaction F(5,138) = 0.9349, P = 0.4605; multiple t-tests: P > 0.05). The trace shows an example of AP firing during a 100 pA, 500 ms current step. f Paired-pulse ratio (PPR) is not significantly different between control (n = 19) and Emx1-cKO (n = 14) CPNs (2-way ANOVA: genotype F(1,155) = 0.901, P = 0.344; interaction F(4,155) = 1.431, P = 0.2263). The trace shows an example of paired EPSCs recorded from the same CPN at ±40 and −60 mV, respectively. The tonic inward currents induced by bath application of NMDA (50 μM, 60 s) are significantly smaller in CPNs from Emx1-cKO mice compared to control (n = 15 and n = 14, respectively; *P < 0.05, Student’s t-test). The trace shows a sample response of a CPN (sEPSCs have been cut) to NMDA bath application (grey bar).
distribution of mEPSC inter-event intervals is significantly different between control (n = 12) and Emx1-cKO (n = 11) CPNs (P < 0.0001, 2-samples Kolmogorov-Smirnov test), as well as their median frequency (inset) (**P < 0.001, Mann-Whitney test). Conversely, both the distribution and the median values of mEPSC amplitude are similar in control and Emx1-cKO CPNs (P > 0.05, 2-samples Kolmogorov-Smirnov test and Mann-Whitney test). Cumulative plots represent mean values (light and dark green) and SEM (grey). Traces show sample mEPSCs recorded from control and Emx1-cKO CPNs. Data in b, c, e-h and in i (cumulative plots) are expressed as means ± SEM; data in d and in l (insets) are expressed as medians with interquartile range.

Fig. S5. Electrophysiological characterization of SSPNs and basal corticostriatal synaptic transmission. a Simplified scheme of the corticostriatal circuitry with the recording patch-clamp pipette placed on a SSPN and the stimulating electrode placed on the cc. TSHZ3-expressing neurons are blue (L1-6, cortical layers 1-6; cc, corpus callosum; st, striatum). b Current-voltage relationship recorded from SSPNs of control and Emx1-cKO mice provide similar slopes and input resistance (97.4 ± 2.3 vs. 93.0 ± 2.1 MΩ, respectively; n = 7 and n = 15, respectively; P = 0.1862, Mann-Whitney test). c Resting membrane potential (RMP) and d rheobase are not significantly different between control (n = 7) and Emx1-cKO (n = 15) SSPNs (P > 0.05, Mann-Whitney test). e NMDA/AMPA ratio is similar between control (n = 11) and Emx1-cKO (n = 12) SSPNs (P > 0.05, Mann-Whitney test); traces in e show an example of a NMDA receptor- and an AMPA receptor-mediated EPSC recorded from the same SSPN at +40 and −60 mV, respectively. f Paires-pulse ratio (PPR) is similar between control (n = 18) and Emx1-cKO (n = 24) SSPNs (2-way ANOVA: genotype F(1,162) = 0.1135, P = 0.7367; interaction F(4,162) = 0.8429, P = 0.4999). The trace shows an example of paired EPSCs (40 ms inter-pulse interval). g The distribution of mEPSC inter-event intervals is significantly different between control (n = 8) and Emx1-cKO (n = 7) SSPNs (P < 0.001, 2-samples Kolmogorov-Smirnov test), but their median frequency (inset) is similar (P > 0.05, Mann-Whitney test). Both the distribution and the median value of mEPSC amplitude are not significantly different between control and Emx1-cKO SSPNs (P > 0.05, 2-samples Kolmogorov-Smirnov test and Mann-Whitney test). Cumulative plots represent average values (light and dark green) and SEM (grey). Traces show sample mEPSCs recorded from control and Emx1-cKO SSPNs. Data in b, f and g (cumulative plots) are expressed as means ± SEM; data in c-e and g insets are expressed as medians with interquartile range.

Fig. S6. TSHZ3 expression in the main brain cholinergic systems. Forebrain (a–d) and brainstem (e–g) coronal sections stained for β-Gal and CHAT. (b, d, f) Higher-power images of framed regions in a, c and e, respectively. In Quantification of β-Gal-positive cells within the CHAT-positive population in brain structures containing cholinergic neurons. aq, aqueduct; hdb, nucleus of the horizontal limb of the diagonal band; gp globus pallidus, ldtg laterodorsal tegmental nucleus, ms medial septal nucleus, nac nucleus accumbens, nbm nucleus basalis of Meynert, pbg parabigeminal nucleus, ppgt pedunculopontine tegmental nucleus, st substantia innominata, st striatum, 3N oculomotor nucleus, 4V 4th ventricle. Nuclei were counterstained with DAPI. Data are expressed as medians with interquartile range; they were obtained from 6 (3N), 7 (hdb), 9 (ms) 12 (pbg, si), 16 (ldtg), 17 (nac), 19 (st), 24 (ppgt) and 40 (nbm) sections from 3 (hdb, ldtg, ms, pbg and ppgt), 4 (si and 3N), 6 (nac), 7 (st) and 8 (nbm) mice, respectively.

Fig. S7. Visual, auditory and olfactory capacities in Emx1-cKO and Chat-cKO mice compared with their respective littermate controls. Ten mice per genotype were used in each screening. a Visual capacity differs neither in Emx1-cKO mice compared to their controls (Student’s t < 1, df = 18, non-significant (NS)), nor in Chat-cKO compared to their controls (Student’s t < 1, df = 18, NS). b Auditory capacities differ neither in Emx1-cKO mice compared to their controls (Student’s t = 1.2, df = 18, NS), nor in Chat-cKO mice compared to their controls (Student’s t < 1, df = 18, NS). c Time spent scenting non-social (water, violet, vanilla) and social (C57BL/6J, SWR) odors were analyzed with two mixed ANOVAs (Emx1-cKO and Chat-cKO vs. their respective control, and 15 odors as repeated measures). The genotype factor was not significant (F < 1, df = 1,18) in both cases. Emx1-cKO, Chat-cKO and their respective control spent more time sniffing social than non-social odors, as shown by comparing time sniffing vanilla 3 vs. C57BL/6J urine 1, the size of the differences being similar in each case for the KO and the control group (Emx1-cKO and Chat-cKO, P = 0.1862, Mann-Whitney test). d Framed regions in (40 ms inter-pulse interval). e Event intervals is signifi-
indicating that the differences can be disregarded. h Cumulative distance from the hidden platform during the blocks. Learning was analyzed with parametric statistics (two-way mixed ANOVA with blocks as repeated-measures and cKO vs. control as between group variable). Emx1-cKO mice (n = 10) and their control (n = 12) learn equally (F = 63.18, df = 6120, P = 7E-35, partial η² = 0.76; interaction between blocks and groups (F < 1), with linear trend (F = 209.77, df = 120, P = 4E-12, partial η² = 0.91)) and the slopes are identical (Student's t = 0.76, df = 20, P = 0.46, η² = 0.03). Chat-cKO mice (n = 10) and their control (n = 11) also learn equally (F = 71.44, df = 6114, P = 2E-36, partial η² = 0.79; interaction between blocks and groups (F < 1), with linear trend (F = 196.94, df = 1,19, P = 1E-11, partial η² = 0.91)). The slopes are identical (Student's t = 0.03, df = 19, P = 0.98, η² = 0.00004). i The confidence intervals of the effect size for the learning slopes includes zero for both Emx1-cKO and Chat-cKO vs. their respective controls, indicating that the learning slopes do not differ in the two groups. Data are expressed as means ± SEM (a, b, d and h), or as medians with interquartile range f. ***P < 0.001.
