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## Targeted Tshz3 deletion in corticostriatal circuit components segregates core autistic behaviors

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1 **Title**

2 **Targeted *Tshz3* deletion in corticostriatal circuit components**  
3 **segregates core autistic behaviors**

4  
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18  
19 **Abstract**

20  
21 We previously linked *TSHZ3* haploinsufficiency to autism spectrum disorder (ASD) and showed that  
22 embryonic or postnatal *Tshz3* deletion in mice results in behavioral traits relevant to the two core domains  
23 of ASD, namely social interaction deficits and repetitive behaviors. Here, we provide evidence that cortical  
24 projection neurons (CPNs) and striatal cholinergic interneurons (SCINs) are two main and complementary  
25 players in the TSHZ3-linked ASD syndrome. We show that in the cerebral cortex, TSHZ3 is expressed in  
26 CPNs and in a proportion of GABA interneurons, while not in cholinergic interneurons or glial cells. TSHZ3-  
27 expressing cells, which are predominantly SCINs in the striatum, represent a low proportion of neurons in  
28 the ascending cholinergic projection system. We then characterized two new conditional knockout (cKO)  
29 models generated by crossing *Tshz3<sup>flox/flox</sup>* with *Emx1-Cre* (*Emx1-cKO*) or *Chat-Cre* (*Chat-cKO*) mice to  
30 decipher the respective role of CPNs and SCINs. *Emx1-cKO* mice show altered excitatory synaptic  
31 transmission onto CPNs and plasticity at corticostriatal synapses, with neither cortical neuron loss nor  
32 impaired layer distribution. These animals present social interaction deficits but no repetitive patterns of  
33 behavior. *Chat-cKO* mice exhibit no loss of SCINs but changes in the electrophysiological properties of  
34 these interneurons, associated with repetitive patterns of behavior without social interaction deficits.  
35 Therefore, dysfunction in either CPNs or SCINs segregates with a distinct ASD behavioral trait. These  
36 findings provide novel insights onto the implication of the corticostriatal circuitry in ASD by revealing an

37 unexpected neuronal dichotomy in the biological background of the two core behavioral domains of this  
38 disorder.

39  
40 **KEYWORDS**

41  
42 Autism spectrum disorder (ASD), cortical projection neurons, sociability, stereotyped behaviors, striatal  
43 cholinergic interneurons.

44  
45 **INTRODUCTION**

46 Autism spectrum disorder (ASD) includes a heterogeneous group of neurodevelopmental pathologies the  
47 diagnosis of which is based exclusively on behavioral criteria. The two behavioral domains that are selected  
48 by the DSM-5 are: i) deficit in social communication and ii) restrictive, repetitive patterns of behavior,  
49 interests, or activities <sup>1</sup>. These domains also emerge from factor analyses of the 13 available diagnostic  
50 instruments in patients <sup>2</sup> and in a model that aligns mouse and patient features <sup>3</sup>. More than 900 genes have  
51 been linked to ASD <sup>4</sup>, among which >100 impact synaptic functions or interact with genes involved in neuronal  
52 development <sup>5</sup>. As a possible neurobiological substrate, clinical and animal studies point to molecular,  
53 neurodevelopmental and functional changes of deep-layer cortical projection neurons (CPNs), in particular  
54 those of layer 5 (L5) forming the corticostriatal pathway <sup>6-9</sup>. In this context, we have linked heterozygous  
55 *TSHZ3* gene deletion to a syndrome characterized by neurodevelopmental disorders including autistic  
56 behavior, cognitive disabilities and language disturbance, with some patients also showing renal tract  
57 abnormalities <sup>10</sup>. *TSHZ3* encodes the highly conserved, zinc-finger homeodomain transcription factor  
58 TSHZ3, and has been identified in networks of human neocortical genes highly expressed during late fetal  
59 development, which are involved in neurodevelopmental and neuropsychiatric disorders <sup>9, 10</sup>. It is now  
60 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  
61 the cortex during pre- and postnatal development <sup>11</sup>. We showed that heterozygous deletion of *Tshz3*  
62 (*Tshz3<sup>+/-lacZ</sup>*) and conditional early postnatal knockout (KO) using the *Camk2a-Cre* promoter (*Camk2a-cKO*  
63 mice) lead to ASD-relevant behavioral deficits paralleled by changes in cortical gene expression and  
64 corticostriatal synaptic abnormalities <sup>10, 12</sup>. These data suggest that *Tshz3* plays a crucial role in both pre-  
65 and postnatal brain development and functioning, and point to CPNs, and in particular to the corticostriatal  
66 pathway, as a main player in the *Tshz3*-linked ASD syndrome. In the mouse striatum, TSHZ3 is not  
67 expressed in striatal spiny projection neurons (SSPNs), which represent >90% of striatal neurons, but in a  
68 small population of cells that are likely interneurons <sup>10</sup>. We <sup>13</sup> and others <sup>14, 15</sup> identified these cells as being  
69 mainly striatal cholinergic interneurons (SCINs), whose implication in ASD has been suggested by some  
70 studies <sup>16, 17</sup>. We also showed that the *Camk2a-Cre* transgene is unexpectedly expressed in the SCIN  
71 lineage, where it efficiently elicits the deletion of *Tshz3* in *Camk2a-Cre* mice <sup>13</sup>. Together, these data  
72 demonstrate that, within the corticostriatal circuitry, *Tshz3* is deficient in CPNs and in SCINs not only in  
73

74 *Tshz3*<sup>+/*lacZ*</sup> heterozygous<sup>10</sup>, but also in *Camk2a*-cKO mice<sup>12</sup>, which both show the full repertoire of ASD-  
75 like behavioral defects. We thus here aimed at investigating the respective contribution of CPNs and SCINs  
76 to the pathophysiology of *Tshz3*-linked ASD using targeted conditional deletion of this gene, and provide  
77 evidence for the complementary implication of these two neuronal populations in the ASD-related core  
78 features.

79  
80  
81

## 82 RESULTS

### 83 Conditional deletion of *Tshz3* in CPNs

84 High levels of *Tshz3* gene or TSHZ3 protein expression are detectable in the mouse cortex during pre- and  
85 postnatal development<sup>10, 11</sup>. In the adult cerebral cortex, TSHZ3 is detected in the great majority of CPNs  
86 (Caubit et al., 2016). Here, performing immunostaining for beta-galactosidase ( $\beta$ -Gal) to report the  
87 expression of *Tshz3*, we show that *Tshz3* is also expressed in part of cortical GABAergic interneurons, as  
88 evidenced using *Tshz3*<sup>+/*lacZ*</sup>;*GAD67-GFP* mice (Fig. S1a). Quantitative analysis indicates that  $29.6 \pm 1.4\%$   
89 of cortical GAD67-expressing neurons co-express  $\beta$ -Gal and that these dually stained neurons are rather  
90 uniformly distributed among superficial ( $43.2 \pm 2.0\%$ ) and deep ( $56.8 \pm 2.0\%$ ) cortical layers (n = 12 sections  
91 from 2 mice). In contrast,  $\beta$ -Gal is not detectable in cortical choline acetyltransferase (CHAT) positive  
92 neurons (Fig. S1b), Olig2-positive oligodendrocytes (Fig. S1c) and GFAP-positive astrocytes (Fig. S1d, e).  
93 To address the role of *Tshz3* in CPNs, *Tshz3*<sup>*fllox/fllox*</sup> mice were crossed with *Emx1-Cre* (*empty spiracle*  
94 *homeobox 1*) mice (*Emx1-cKO*). The *Emx1-Cre* mouse expresses the Cre-recombinase in the progenitors  
95 of cortical glutamatergic projection neurons (i.e., CPNs) and glial cells from embryonic day 9 (E9), but neither  
96 in those of cortical GABAergic neurons, nor of striatal interneurons, including cholinergic ones<sup>18</sup>. Therefore,  
97 in the corticostriatal circuitry of *Emx1-cKO* mice, *Tshz3* function should be specifically lost in CPNs.  
98 Compared to control, *Emx1-cKO* mice show a drastic reduction of *Tshz3* mRNA levels and of the density of  
99 TSHZ3-positive cells in the cerebral cortex, showing the efficacy of the deletion, while the density of striatal  
100 cells expressing TSHZ3 is unchanged (Fig. 1a-c). Despite the loss of *Tshz3* expression in most CPNs, the  
101 density of NeuN-positive cells is unchanged (Fig. S2a, b), showing no neuronal loss; in addition, neither the  
102 pattern of expression of layer-specific CPN markers, namely CUX1 for L2-4 and BCL11B for L5-6, nor the  
103 density of cells expressing these markers is affected (Fig. S2c, d), indicating no major alteration in cortical  
104 layering. However, spine density of L5 CPNs from *Thy1-GFP-M*;*Emx1-cKO* mice is significantly reduced  
105 compared to *Thy1-GFP-M* control mice (Fig. 1d, e). By crossing *Emx1-cKO* with *GAD67-GFP* mice, we  
106 show that cortical GABAergic neurons still express TSHZ3 (Fig. 1f), confirming the specificity of *Tshz3*  
107 deletion in CPNs. To study whether *Tshz3* loss in CPNs could indirectly affect cortical GABAergic  
108 interneurons, we compared *GAD67-GFP* control mice (Control-*GAD67-GFP*) to *Emx1-cKO-GAD67-GFP*  
109 mutant mice. No significant changes in the number of GABAergic interneurons (Control-*GAD67-GFP*: 140.7

110  $\pm 4.9$ ,  $n = 37$  sections from 5 mice; *Emx1-cKO-GAD67-GFP*:  $144.6 \pm 6.1$ ,  $n = 41$  sections from 7 mice;  $P =$   
111  $0.624$ , Student's *t*-test) and in their distribution are found (Fig. S3a, b). CHAT immunostaining on striatal  
112 slices in *Emx1-cKO* mice also shows no significant modification of the density of SCINs (Fig. S3c, d).

113  
114 **Cortical excitatory synaptic transmission and corticostriatal synaptic plasticity in *Emx1-cKO* mice**  
115 L5 CPNs recorded in slices from *Emx1-cKO* mice show no significant changes in their membrane properties  
116 and excitability compared to control (Fig. S4a-e). Action potential (AP)-dependent glutamate release onto  
117 L5 CPNs, evaluated by measuring paired-pulse ratio, is also unaffected (Fig. S4f), while both NMDA/AMPA  
118 ratio (Fig. S4g) and NMDA-induced currents (Fig. S4h) are significantly reduced, suggesting decreased  
119 NMDA receptor-mediated signaling in *Emx1-cKO* mice. The amplitude of AMPA receptor-mediated  
120 miniature excitatory postsynaptic currents (mEPSCs) is similar in control and *Emx1-cKO* mice (Fig. S4i),  
121 further arguing for the implication of NMDA but not AMPA receptors. Conversely, mEPSC frequency is  
122 reduced (Fig. S4i), suggesting decreased AP-independent glutamate release onto L5 CPNs and/or reduced  
123 number of active excitatory synapses in *Emx1-cKO* mice, consistent with the decreased spine density on  
124 L5 CPNs (Fig. 1d, e).

125 SSPNs recorded in slices from *Emx1-cKO* mice show electrophysiological properties (Fig. S4A-D)  
126 and basal corticostriatal synaptic transmission (Fig. S5e-g) similar to control. However, both long-term  
127 potentiation (LTP) and long-term depression (LTD) at corticostriatal synapses are abolished in *Emx1-cKO*  
128 mice (Fig. 2), confirming a critical role of *Tshz3* in the functioning of the corticostriatal circuit.

129  
130 **Conditional deletion of *Tshz3* in cholinergic neurons**

131 Dual immunodetection of CHAT and  $\beta$ -Gal in *Tshz3<sup>+/-lacZ</sup>* mice was performed to analyze the expression of  
132 *Tshz3* in brain cholinergic neuron populations. This was preferred to dual immunodetection of CHAT and  
133 TSHZ3 since the tissue fixation conditions for obtaining optimal detection of each protein are different, and  
134 since TSHZ3 immunodetection provides weaker labeling and higher background than  $\beta$ -Gal  
135 immunodetection. As reported previously<sup>13</sup>, virtually all SCINs express *Tshz3* (Fig. S6a, h). In contrast,  
136 there are no or a little proportion (<30%) of  $\beta$ -Gal-positive cells within CHAT-positive neurons in the  
137 components of the basal forebrain cholinergic system (medial septal nucleus, diagonal band nuclei, *nucleus*  
138 *basalis* of Meynert and *substantia innominata*) (Fig. S6a-d, h). SCINs thus represent the major population  
139 of *Tshz3*-expressing cells among the forebrain cholinergic neurons. In addition, there is almost no co-  
140 expression of  $\beta$ -Gal and CHAT in the pedunculo pontine (Fig. S6e, f, h) and laterodorsal tegmental nuclei  
141 (Fig. S6g, h), which are known to provide cholinergic afferents to several brain areas including the striatum  
142<sup>19</sup>. Among the other brainstem nuclei, co-expression ranges from poor to extensive, as illustrated in the  
143 parabigeminal nucleus and the oculomotor nucleus, respectively (Fig. S6e, f, h).

144 To address the role of *Tshz3* in cholinergic neurons, *Tshz3<sup>flox/flox</sup>* mice were crossed with *Chat-Cre*  
145 mice (*Chat-cKO* model). CHAT is expressed in the brain from early embryonic development and as soon

146 as E18.5 in the striatum<sup>20</sup>. TSHZ3 immunostaining in *Chat-cKO* mice confirms a significant loss of TSHZ3  
147 in SCINs (Fig. 3a, b), which does not affect the number of striatal CHAT-positive cells (Fig. 3c, d). This result  
148 was confirmed using *Chat-Cre;Ai14<sup>Flox/+</sup>* mice (*Chat-Cre;Rosa26-STOP-Tomato*) to visualize SCINs in the  
149 presence or absence of *Tshz3* (Fig. 3e, f).

### 150 151 ***Tshz3* loss and SCIN electrophysiological properties**

152 In acute brain slices, SCINs are easily recognizable among the other striatal neurons due to their larger  
153 soma<sup>21</sup>. Moreover, they are the only autonomously active cells, firing action APs with either a regular,  
154 irregular or bursting pattern<sup>22, 23</sup>. SCINs also show a characteristic depolarizing voltage sag in response to  
155 the injection of negative current pulses due to the activation of the nonspecific *I<sub>h</sub>* cation current mediated by  
156 HCN channels, which largely contributes to the spontaneous AP discharge characterizing these neurons<sup>23-</sup>  
157<sup>25</sup>. To test a possible effect of *Tshz3* loss on these SCIN properties, we measured the mean frequency of  
158 spontaneous AP discharge, its regularity [expressed as the coefficient of variation (CV) of the inter-AP  
159 intervals], and the amplitude of the sag [expressed as voltage sag ratio (VSR)] in SCINs from *Chat-cKO*  
160 mice and control littermates (Fig. 4a-c). We found that SCINs recorded from *Chat-cKO* mice show a  
161 significant reduction of both VSR (Fig. 4d) and spontaneous firing frequency (Fig. 4e), as well as an  
162 increased CV of inter-AP intervals that suggests a less regular discharge activity (Fig. 4f). The resting  
163 membrane potential at steady state is similar between control vs. *Chat-cKO* SCINs ( $46.64 \pm 0.68$  vs.  $45.65$   
164  $\pm 0.64$  mV, 56 vs. 86 SCINs, respectively;  $P = 0.305$ , Student's *t*-test), while the current-voltage relationship  
165 reveals a slight but significant increase of input resistance in *Chat-cKO* SCINs vs. control, calculated as the  
166 slope of the linear best fit (Fig. 4g;  $125.7 \pm 4.5$  vs.  $107.5 \pm 4.0$  M $\Omega$ , respectively;  $F(1,911) = 8.816$ ,  $P =$   
167  $0.0031$ ).

### 168 169 **Conditional deletion of *Tshz3* in CPNs or in cholinergic neurons segregates the two core behavioral** 170 **domains of ASD**

171 For behavioral experiments, only male *Emx1-cKO*, *Chat-cKO* and control littermate mice were used. Neither  
172 *Emx1-cKO* nor *Chat-cKO* mice present visual, auditory and olfactory impairment vs. their respective control  
173 (Fig. S7). They were then tested for deficits in social behavior, the first core feature of ASD, as well for  
174 stereotyped/repetitive patterns of behavior and for restricted field of interests, which are subcategories of  
175 the second ASD core feature.

176 During the habituation phase in the two-chamber test, both *Emx1-cKO* and *Chat-cKO* mice show no  
177 significant differences in their exploration of the lured boxes as compared to their respective controls ( $P =$   
178  $0.14$ ,  $\eta^2 = 0.12$ ,  $P = 0.84$ ,  $\eta^2 = 0.002$ , respectively Fig. 5a). However, *Emx1-cKO* but not *Chat-cKO* mice  
179 show impaired social relationships (Fig. 5). *Emx1-cKO* mice have less preference than their controls for a  
180 conspecific (sociability, Fig. 5b) and for an unfamiliar male (social novelty, Fig. 5c), the interaction between

181 genotype and box content being large in each case, as shown by the effect size that exceeds the typical  
182 range of variation (Fig. 5d).

183 Conversely, *Chat-cKO* but not *Emx1-cKO* mice present more stereotyped or repetitive patterns of  
184 behavior than their controls, as shown by the marble burying score, time burrowing in a new cage,  
185 stereotyped dips on a hole board, and number of leanings in an open field (Fig. 6a-d), with a large effect  
186 size (Fig. 6e). Restricted field of interest is impacted neither in *Emx1-cKO* nor in *Chat-cKO* mice (Fig. S8a-  
187 c). Finally, hind paw coordination is impaired in *Chat-cKO* but not in *Emx1-cKO* mice (Fig. S8d, e), while  
188 spatial learning ability is unaffected in both models (Fig. S8f-i).

189

## 190 **DISCUSSION**

191 Previous studies showed that haploinsufficiency or postnatal deletion of *Tshz3* results in ASD-relevant  
192 behavioral deficits and suggested altered function of the corticostriatal circuitry as a possible substrate<sup>10</sup>.  
193<sup>12</sup>. The present findings point to SCINs as an additional player in the *Tshz3*-linked ASD syndrome. They  
194 also provide evidence that targeted conditional deletion of *Tshz3* in either CPNs (*Emx1-cKO*) or cholinergic  
195 neurons (*Chat-cKO*) segregates the two core behavioral traits used to diagnose ASD, respectively social  
196 behavior deficits and repetitive behavioral patterns, suggesting that alterations in CPNs and in SCINs  
197 contribute in a complementary manner to the repertoire of behavioral deficits linked to *Tshz3* deficiency.  
198 Restricted field of interest, which defines a sub-category of the second ASD domain, was observed neither  
199 in *Emx1-cKO* nor in *Chat-cKO* mice, suggesting that the expression of this deficit in the previously  
200 characterized models of *Tshz3* deletion may involve additional players, and/or result from the combined  
201 dysfunction of CPNs and SCINs due to the loss of *Tshz3* in both these neuronal types. Learning ability was  
202 impacted neither by *Tshz3* postnatal deletion<sup>12</sup>, nor in *Emx1-cKO* and *Chat-cKO* models.

203 Among the multiplicity of circuits involved in social behavior, literature points out the crucial role of  
204 the cortex<sup>26, 27</sup>. In particular, corticostriatal and striatal circuit dysfunctions are associated to ASD features,  
205 both in patients and in mouse models, with CPNs and SSPNs being highly impacted by mutations of ASD-  
206 linked genes<sup>7, 8, 10, 12, 28, 29</sup>. There is however increasing evidence incriminating interneuron populations of  
207 the cortex and the striatum in ASD<sup>30</sup>. Here, we show that, in the cortex, the ASD-related *Tshz3* gene is  
208 expressed not only in CPNs but also in a third of GABA interneurons, while not in cholinergic interneurons.  
209 In contrast, in the striatum, the vast majority of *Tshz3*-expressing cells are cholinergic interneurons<sup>13</sup>. To  
210 disentangle the role of CPNs from that of interneurons in the ASD symptoms linked to *Tshz3* deficiency, we  
211 generated and characterized *Emx1-cKO* mice. We confirmed the specificity of *Tshz3* deletion in CPNs within  
212 the corticostriatal circuit in this model, *Tshz3* expression in cortical and striatal interneurons being  
213 maintained. In addition, no change in the numbers and positioning of these interneurons were detected.  
214 Interestingly, we found that *Emx1-cKO* mice specifically exhibit impaired social behavior, and that this deficit  
215 co-segregates with altered NMDA receptor-mediated transmission in the cortex and disrupted plasticity at  
216 corticostriatal synapses. Corticostriatal synaptic plasticity has been deeply characterized, but discrepancies

217 concerning its induction protocols and the underlying molecular and cellular mechanisms<sup>31</sup> make it difficult  
218 to univocally interpret our results. However, since LTD expression mainly involves presynaptic changes<sup>32</sup>,  
219 its disruption in *Emx1-cKO* mice could be attributable to cortical circuitry defects, such as the observed  
220 decrease of NMDA receptor activity in L5 CPNs that could impair corticostriatal output. LTP expression  
221 mostly depends upon postsynaptic mechanisms<sup>32</sup>, but presynaptic NMDA receptors also play a role<sup>33, 34</sup>.  
222 The lack of changes in SSPNs electrophysiological properties or basal corticostriatal transmission rather  
223 favors a presynaptic hypothesis to explain this loss of LTP. Moreover, our findings are in line with studies  
224 substantiating the involvement of NMDA receptor dysfunction in social deficits associated with ASD in rodent  
225 models as well as in patients<sup>35, 36</sup>. Finally, consistent with the literature linking ASD with changes of dendritic  
226 spine density<sup>37</sup>, we evidence decreased spine density in L5 CPNs of *Emx1-cKO* mice, as in our previous  
227 model<sup>12</sup>. Overall, these data indicate that the loss of *Tshz3* in CPNs induces morphofunctional changes in  
228 these neurons and deeply affects corticostriatal plasticity, which might result in altered processing of cortical  
229 information and account for the observed social behavior deficits.

230 We also investigated the contribution of cholinergic neurons in the pathophysiology of *Tshz3*-linked  
231 ASD. We show that TSHZ3 is expressed in almost 100% of SCINs, while its expression is absent or partial  
232 in the other main brain cholinergic systems. Despite their low number, SCINs have morphofunctional  
233 features that place them as key modulators of striatal microcircuits. They play a crucial role in movement  
234 control, attentional set-shifting, habit-mediated and goal-directed behavior, and selection of appropriate  
235 behavioral responses to changes in environmental contingencies, conferring behavioral flexibility<sup>38-42</sup>.  
236 These interneurons are also involved in basal ganglia-related pathologies such as dystonia, Parkinson's  
237 and Huntington's disease, Tourette's syndrome, obsessive compulsive disorder and drug addiction<sup>43-45</sup>. In  
238 contrast, despite the array of data pointing to basal ganglia and cholinergic transmission abnormalities in  
239 ASD and in ASD models<sup>16, 46-50</sup>, to date there is little evidence showing the specific involvement of SCINs:  
240 the partial depletion of both SCINs and fast-spiking GABAergic interneurons produces stereotypy and  
241 impaired social behavior in male mice<sup>17</sup>, while total elimination of SCINs results in perseverative behavior  
242 that extends to social behavior, rather reminiscent of neuropsychiatric conditions as Tourette's syndrome or  
243 obsessive compulsive disorder<sup>51</sup>. The present work reveals that targeted *Tshz3* deletion in CHAT-  
244 expressing neurons leads to robust stereotyped and repetitive patterns of behavior without impacting social  
245 behavior. Given the literature associating drug-induced stereotypies with abnormalities in striatal cholinergic  
246 signaling<sup>52-54</sup>, and the co-expression of CHAT and TSHZ3 in SCINs but not in brainstem cholinergic neurons  
247 that are known to project to the striatum<sup>19</sup>, this behavioral deficit is likely attributable to SCINs. Whereas  
248 the number of SCIN in *Chat-cKO* mice is unchanged, suggesting that their generation and viability is not  
249 affected, we evidenced modifications in their firing activity and electrophysiological membrane properties.  
250 This finding is an addition to the increasing amount of data stressing the complex implication of SCINs in  
251 health and diseases<sup>55</sup>. How the selective loss of *Tshz3* in SCINs leads to these electrophysiological  
252 changes, what are their molecular bases and what are the consequences on striatal cholinergic signaling



253 still need to be determined. However, SCINs are important modulators of the two populations of SSPNs  
254 forming the "direct" and "indirect" pathways by which the striatum regulates basal ganglia outflow, whose  
255 balanced activity is determinant for appropriate action selection<sup>40, 56</sup>. Thus, the changes in SCIN properties  
256 observed here could alter the way they normally respond to salient stimuli and/or reward-associated cues,  
257 thereby the way they modulate the transfer of cortical information through the striatum<sup>38, 39, 57</sup>, as observed  
258 after targeted deletion of the transcription factor Er81 in SCINs<sup>42</sup>. This could underlie the increased  
259 stereotyped behaviors observed in *Chat-cKO* mice and, possibly, also in *Tshz3<sup>+lacZ</sup>*<sup>10</sup>, as well as in postnatal  
260 *Tshz3* cKO<sup>12</sup> in which we recently showed that *Tshz3* is lost also in SCINs<sup>13</sup>. Finally, *Chat-cKO* mice do not  
261 show basal exploration deficit, similarly to *Emx1-cKO* mice, but present impaired hind paw coordination,  
262 which is in line with motor deficiencies frequently associated with ASD<sup>58</sup> and with a study linking partial  
263 SCIN ablation with motor incoordination<sup>59</sup>. Although TSHZ3 is expressed in about 25% of cholinergic  
264 neurons of the *nucleus basalis* of Meynert and the *substantia innominata*, the similarity of spatial learning  
265 curves of control and *Chat-cKO* mice suggests minor impact of *Tshz3* deletion on the function of the basal  
266 forebrain cholinergic system, which is deeply involved in learning and memory processes<sup>60</sup>.

267 In conclusion, this study shows that the conditional loss of the ASD-related gene *Tshz3* in CPNs  
268 and SCINs does not affect the numbers of these neurons but induces profound changes in their  
269 electrophysiological and synaptic properties, associated with specific ASD-like behavioral defects. To our  
270 knowledge, it represents the first demonstration in mice models that the two behavioral domains used to  
271 diagnose ASD are independent domains that can be underpinned by dysfunction in distinct neuronal  
272 subtypes, in this case two components of the corticostriatal circuitry. These findings may open the road to  
273 domain-specific pharmacological and behavioral therapies.

274

275 **MATERIALS and METHODS**

276

277 **DATA AVAILABILITY**

278

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

284

285

286 **MOUSE STRAINS AND GENOTYPING**

287 The *Tshz3<sup>lacZ</sup>*, *Tshz3<sup>flox/flox</sup>*, *Emx1-Cre*, *Chat-Cre*, *Rosa26-STOP-lacZ* and *Ai14 (Rosa26-STOP-Tomato)*,  
288 *GAD67-GFP* and *Thy1-GFP* mouse lines have been described previously<sup>10, 12, 18, 61-66</sup>. Male heterozygous  
289 Cre mice were crossed with female *Tshz3<sup>flox/flox</sup>* to generate the two *Tshz3* conditional knockout (cKO) mice  
290 models: *Emx1-cKO* and *Chat-cKO*<sup>18, 64</sup>. Littermate *Emx1-Cre<sup>-/-</sup>* and *Chat-Cre<sup>-/-</sup>* mice were used as  
291 respective controls. Animals carrying the *Tshz3<sup>flox</sup>* allele and *Tshz3<sup>A</sup>* allele were genotyped as described  
292 previously<sup>12</sup>. Experimental procedures were in agreement with the recommendations of the European  
293 Communities Council Directive (2010/63/EU). They have been approved by the "Comité National de  
294 Réflexion Ethique sur l'Expérimentation Animale n°14" and the project authorization delivered by the French  
295 Ministry of Higher Education, Research and Innovation. (ID numbers 57-07112012, 2019020811238253-V2  
296 #19022 and 2020031615241974-V5 #25232).

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298

299 **IMMUNOHISTOCHEMISTRY AND HISTOLOGY**

300 All stains were processed on coronal brain sections of postnatal day (P) 28-34 mice. Immunostaining for  
301 TSHZ3 alone was performed on cryostat sections of brains immediately removed after anesthesia (ketamine  
302 + xylazine, 100 + 10 mg/kg, respectively, i.p.) and frozen in dry ice until use. Before incubation with the  
303 antibodies, sections were fixed with 4% paraformaldehyde for 15 min, then washed twice for 5 min in PBS.  
304 For TSHZ3 immunostaining and GFP detection, *GAD67-GFP* mice were anesthetized (see above) and  
305 transcardially perfused with PBS. Brains were immediately dissected out, post-fixed by immersion 2 hours  
306 in 4% paraformaldehyde in PBS, placed in 30% sucrose in PBS overnight and frozen in dry ice until  
307 sectioning. For the other stains, mice were anesthetized (see above) and transcardially perfused with 4%  
308 paraformaldehyde (PFA) in 0.1 M phosphate buffer. Brains were removed and post-fixed in 4% PFA for at  
309 least 2 h before cryostat sectioning (40  $\mu$ m-thick). Brain sections were washed with PBS and blocked in  
310 PBST (0.3% Triton X-100 in PBS) with 5% BSA for 1h at room temperature. Sections were then incubated

311 in primary antibody diluted in blocking solution (PBST, 1% BSA) overnight at 4°C with the following primary  
312 antibodies: mouse anti-NeuN (1:500, Millipore, Mab377), rat anti-BCL11B (1:1,000, Abcam, ab18465), goat  
313 anti-CHAT (1:100, Millipore, AB144P), rabbit anti-β-Galactosidase (1:1,000, Cappel, 599762), goat anti-  
314 CDP/CUX1 (1:200, Santa Cruz Biotechnology, C20, SC6327) and guinea-pig anti-TSHZ3 (1:2,000; ref. <sup>61</sup>).  
315 Sections were then washed with PBS three times and incubated overnight at 4°C in secondary antibodies  
316 diluted 1:1,000 in blocking solution: donkey anti-rabbit Cy3, donkey anti-guinea pig Cy3 and donkey anti-  
317 goat Cy3 (Jackson ImmunoResearch Laboratories) and goat anti-mouse Alexa Fluor 488, goat anti-rat  
318 Alexa Fluor 555 and donkey anti-goat Alexa Fluor 488 (Life Technologies). Sections were counterstained  
319 by 5 min incubation in 300 μM DAPI intermediate solution (1:1,000, Molecular Probes, Cat# B34650).  
320 Section were then washed with PBS three times, mounted on Superfrost Plus slides (Fischer Scientific) and  
321 coverslipped for imaging on a laser scanning confocal microscope (Zeiss LSM780 with Quasar detection  
322 module). Spectral detection bandwidths (nm) were set at 411-473 for DAPI, 498-568 for GFP and 568-638  
323 for Cy3; pinhole was set to 1 Airy unit. Unbiased stereological counting of NeuN, TSHZ3, CUX1, BCL11B,  
324 CHAT and β-Gal positive neurons as well as of GAD-GFP neurons were done from confocal images using  
325 ImageJ software (see Figure legends for frame details). Images were assembled using Photoshop 21.2.3.

326 Cell counts were performed in the dorsal striatum (excluding the nucleus accumbens) and in the  
327 surrounding motor and sensorimotor cortex on sections spanning from bregma 0 to +1.18 mm, AP. The  
328 whole surface was analyzed for the striatum. For the cortex, counts were performed in frames of 400-μm  
329 width either spanning the total thickness of the cortex (NeuN), the thickness of specific layers or divided into  
330 10 bins of equal size for the analysis of the distribution of Gad67GFP-positive cells. For the different  
331 cholinergic nuclei, the analyses were performed on sections spanning from bregma +0.62 to +0.38 mm for  
332 ms and hdb, -0.34 to -0.8 for si and nbm, +3.8 to -4.16 for 3N, -4.16 to -4.6 for pbg and pptg and -4.72 to -  
333 5.2 for ldtg.

334

335

### 336 **MORPHOMETRIC AND DENDRITIC SPINE ANALYSIS OF L5 CPNS**

337 We used transgenic mouse lines (P28) expressing *Thy1-GFP* (green fluorescent protein) in L5 CPNs <sup>66</sup>.  
338 *Thy1-GFP-M; Emx1-cKO* were obtained by crossing *Emx1-Cre*; *Tshz3<sup>flox/flox</sup>* males with *Tshz3<sup>flox/flox</sup>* females  
339 heterozygous for *Thy1-GFP*. Analysis of spine density and morphology was performed on stacks from 100  
340 μm-thick vibratome sections (1 μm z-step) on 4 littermate pairs using a Zeiss LSM780 (Oberkochen,  
341 Germany) laser scanning confocal microscope (63X objective NA 1.4, 0.03 μm/pixel, voxel size 0.033 μm<sup>2</sup>  
342 x 0.37 μm). Spine counts were obtained from second or third order basal dendritic branches of randomly  
343 selected L5 CPNs. Dendrites from 5 to 7 cells were analyzed per animal, providing a cumulated dendrite  
344 length > 750 μm for each genotype. Spine identification and density measures were done using  
345 NeuronStudio <sup>67</sup>.

346

347  
348 **RT-qPCR**  
349 Total RNA from control and *Tshz3* mutant (P28) cerebral cortex was prepared using RNeasy Plus Universal  
350 Mini Kit gDNA eliminator (*Qiagen*<sup>TM</sup>) and first strand cDNA was synthesized using iScript Reverse  
351 Transcription Supermix kit (Bio-RAD<sup>TM</sup>). Real-time quantitative PCR (RT-qPCR) was performed on a CFX96  
352 qPCR detection system (Bio-RAD<sup>TM</sup>) using *SYBR*<sup>®</sup> *GreenER*<sup>TM</sup> qPCR SuperMixes (*Life Technologies*<sup>TM</sup>).  
353 RT-qPCR conditions: 40 cycles of 95 °C for 15s and 60 °C for 60 s. Analyses were performed in triplicate.  
354 Transcript levels were first normalized to the housekeeping gene *Gapdh*. Primer sequences used for RT-  
355 qPCR: *Gapdh* Forward: 5' GTCTCCTGCGACTTCAACAGCA 3'; *Gapdh* Reverse: 5'  
356 ACCACCCTGTTGCTGTAGCCGT 3'. *Tshz3* Forward: 5' CACTCCTTCCAGCATCTCTGAG 3'; *Tshz3*  
357 Reverse: 5' TAGCAGGTGCTGAGGATTCCAG 3'.

358  
359  
360 **ELECTROPHYSIOLOGY**  
361 Electrophysiological data were obtained from 57 *Emx1-cKO* and 44 *Emx1-Cre*<sup>-/-</sup> control littermates, and from  
362 16 *Chat-cKO* and 16 *Chat-Cre*<sup>-/-</sup> control littermates, aged P21-28. Procedures were similar to those  
363 described previously<sup>10, 12, 68</sup>. Briefly, acute coronal slices (250 μm-thick) containing cortex and striatum were  
364 cut using a S1000 Vibratome (Leica) in ice-cold solution containing (in mM): 110 choline, 2.5 KCl, 1.25  
365 NaH<sub>2</sub>PO<sub>4</sub>, 7 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 25 NaHCO<sub>3</sub>, 7 glucose, pH 7.4. Slices were kept at room temperature in  
366 oxygenated artificial cerebrospinal fluid (ACSF), whose composition was (in mM): 126 NaCl, 2.5 KCl, 1.2  
367 MgCl<sub>2</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 2.4 CaCl<sub>2</sub>, 11 glucose and 25 NaHCO<sub>3</sub>, pH 7.4. Electrophysiological recordings were  
368 performed in oxygenated artificial cerebrospinal fluid (ACSF) at 34-35 °C, flowing at ~2 ml/min. L5 CPNs of  
369 the primary motor and somatosensory cortex, and SSPNs and SCINs of the dorsolateral striatum were  
370 identified by infrared video microscopy and by their electrophysiological properties<sup>69, 70</sup>. They were recorded  
371 by whole-cell patch-clamp using borosilicate micropipettes (5-6 MΩ) filled with an internal solution containing  
372 (in mM): 125 K-gluconate, 10 NaCl, 1 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 0.5 BAPTA, 19 HEPES, 0.3 Na-GTP, and 1 Mg-ATP,  
373 pH 7.3 (except for NMDA/AMPA ratio experiments, see below). Electrophysiological data were acquired by  
374 an AxoPatch 200B amplifier and pClamp 10.7 software (Molecular Devices, Wokingham, UK). Series and  
375 input resistance were continuously monitored by sending 5 mV pulses, and neurons showing ≥ 20% change  
376 in these parameters were discarded from the analysis.

377  
378 **Characterization of CPNs, SSPNs and synaptic transmission**  
379 A stimulating bipolar electrode was placed either in the cortex at the level of L4 to activate local fibers and  
380 evoke excitatory postsynaptic currents (EPSCs) in L5 CPNs, or in the *corpus callosum* to activate  
381 corticostriatal fibers and evoke EPSCs in SSPNs<sup>12</sup>. Glutamatergic EPSCs were recorded in the presence  
382 of 50 μM picrotoxin at a holding potential of -60 mV (CPNs) or -80 mV (SSPNs). Spontaneous miniature

383 EPSCs (mEPSCs) were recorded in the presence of 50  $\mu$ M picrotoxin and 1  $\mu$ M tetrodotoxin. Current-  
384 voltage (I-V) relationship was obtained in current-clamp mode by injecting hyperpolarizing and depolarizing  
385 current steps ( $\Delta I = \pm 50$  pA, 800 ms), and input resistance was calculated by linear regression analysis, i.e.  
386 as the slope of the linear best fit of the I-V relationship of each recorded neuron. Rheobase was measured  
387 as the minimal injected current (+5 pA increments) capable of eliciting an action potential (AP). For paired-  
388 pulse ratio (PPR), EPSC amplitude was measured on 6 averaged traces at each inter-pulse interval. For  
389 analyzing mEPSCs, the detection threshold (around 3-4 pA) was set to twice the noise after trace filtering  
390 (Boxcar low-pass), and only cells exhibiting stable activity and baseline were considered. For NMDA/AMPA  
391 ratio experiments, the internal solution contained (in mM): 140 CsCl, 10 NaCl, 0.1 CaCl<sub>2</sub>, 10 HEPES, 1  
392 EGTA, 2 Mg-ATP and 0.5 Na-GTP, pH 7.3. The AMPA component of the EPSC was measured at the peak  
393 at a holding potential of -60 mV, while the NMDA component was measured at +40 mV and 40 ms after the  
394 stimulation artifact, when the AMPA component is negligible, as previously described<sup>12</sup>. Tonic NMDA  
395 currents were elicited by bath application of 50  $\mu$ M NMDA for 60 s, after a stable baseline of at least 120 s;  
396 their amplitude was measured by averaging the current values of a 5 s window around the negative peak,  
397 compared to baseline; only neurons that were capable of returning to their baseline after washout were  
398 considered. EPSC amplitude for monitoring corticostriatal long-term depression and potentiation (LTD and  
399 LTP, respectively) was measured on averaged traces (6 per minute) to obtain time-course plots and to  
400 compare this parameter before (baseline) and after induction protocols. The induction protocol for  
401 corticostriatal LTD consisted of 3 trains at 100 Hz, 3 s duration, 20 s interval, at half intensity compared to  
402 baseline<sup>71</sup>. LTP induction protocol was identical but, during each train, neurons were depolarized to -10 mV  
403 to allow strong activation of NMDA receptors<sup>10, 12, 72</sup>. For a review about corticostriatal LTD and LTP see<sup>32</sup>.

404

#### 405 **Characterization of SCINs**

406 The resting membrane potential (RMP) was measured at the steady state between two consecutive APs.  
407 The current-voltage relationship was calculated from the membrane response at the end of current steps  
408 from -200 to -20 pA (20 pA steps lasting 800 ms). The voltage sag ratio (VSR) was calculated from the  
409 response to a -120 pA current step as the peak voltage drop (sag) against the voltage at the end of the  
410 current pulse<sup>73, 74</sup>. Such relatively small current step was chosen because, with larger steps, the sag  
411 amplitude was extremely variable between different SCINs. Spontaneous AP firing was analyzed in terms  
412 of discharge frequency (expressed in Hz) and regularity; to quantify this latter parameter, we calculated the  
413 coefficient of variation (CV) of the inter-AP intervals. Note that spontaneous AP firing was analyzed only  
414 from cell-attached recordings, which were done before switching to whole-cell; in some cases, spontaneous  
415 firing was not detectable in cell-attached configuration, thus the number of samples for AP firing analyses  
416 is smaller than the whole number of recorded SCINs.

417

418

## 419 **BEHAVIORAL ANALYSIS**

### 420 **Housing conditions**

421 Experiments were conducted blind for the genotypes in P71-87 male *Emx1-cKO* and *Chat-cKO* mice and  
422 their respective *Emx1-Cre<sup>-/-</sup>* and *Chat-Cre<sup>-/-</sup>* control littermates. We used males and not female mice  
423 because the ambulatory activity of females is impacted by the estrous cycle phases in rodents <sup>75</sup> and may  
424 bias the results of repetitive behavior measures that are partly dependent on motor activity.

425 Mice used in studies on social behavior are generally reared in groups of variable size and more  
426 rarely in isolation. The choice of our rearing strategy was based on the fact that the measures of social  
427 behavior in adult mice depends on the characteristics of the previous interactions that the observed male  
428 has experienced with its peers <sup>3, 76-78</sup>. In the rearing in group strategy, the social behaviors directed towards  
429 the tested male can vary according to the genotypes, the androgen levels and the neurotransmitter profiles  
430 of the individuals in the groups <sup>79</sup>. Consequently, the social behavior measured in an individual is the  
431 resultant of the individual social ability plus a component corresponding to the interactions of the individual  
432 with the other members of the group; this effect varies with the size of the group. In addition, behavioral  
433 “contamination” resulting in an impairment of sociability in wild-type mice by cohabitant KO modeling ASD  
434 was described <sup>78</sup>. Such undesirable effect plus the heterogeneity of the measures in mice reared in group  
435 should contribute to avoid this strategy for testing social behavior. On the other hand, maintaining the mice  
436 socially deprived generates a specific set of agonistic reactions that prevents the measures of social  
437 abilities. To circumvent such biases, we have developed an alternative solution for years: each tested male  
438 is housed with one female mouse belonging to a single inbred strain <sup>79</sup>. Here, a cKO or a control male mouse  
439 was reared and maintained with CBA/H/Gnc female mice <sup>3</sup>. Housing was done in transparent 35 x 20 x 18  
440 cm cages with 1-liter poplar woodchip bedding and weekly renewed enrichment (cardboard shelter). The  
441 light (07:00-19:00) was 60 lux on the ground of the cages. The temperature was  $21.5 \pm 0.5^{\circ}\text{C}$ . Behavioral  
442 tests were performed in a dedicated room, the housing cage having been transferred one hour before the  
443 beginning of the observations.

444

### 445 **Assessment of sensory function**

446 Visual, auditory and olfactory integrity is required to ensure the validity of the behavioral data. These  
447 sensorial capacities were tested according to previously described protocols <sup>3, 10, 12, 80</sup>.

448 *Visual capacities.* The mouse was raised, taken by the tail, and a thin stick was approached to its eyes,  
449 without touching the vibrissae. Raising the head was scored 1 and grasping or trying to grasp the pen was  
450 scored 2. The test was administered five times and the sum of the scores recorded. Swimming towards a  
451 distant shelf in the Morris Water Maze provided an additional assessment of the visual abilities.

452 *Auditory capacities* were measured using the Preyer’s response. It consists in a pinna twitching and going  
453 flat backwards against the head as reaction to sound. It is correlated with the average evoked auditory  
454 potential and can be considered as an indicator of auditory acuity <sup>81-83</sup>. Mice emit vocalizations (less than

455 20 kHz) and ultrasounds (above 20 kHz) in the presence of a conspecific male. For this reason, we  
456 evaluated the responses to stimulations in the ultrasound bandwidth ( $50 \pm 0.008$  kHz and  $35 \pm 0.010$  kHz)  
457 using commercial dog whistles. The mice received 5 stimulations with each sound. We scored 1 for ear  
458 twitching and 2 for a pinna going flat backwards against the head.

459 *Olfactory ability* to detect an odor was evaluated by an increased time in sniffing a new odor using an  
460 olfactory habituation/dishabituation test. Non-social aromas and social odors (urines from C57BL/6J and  
461 SWR male mice) were presented individually to each mouse<sup>84</sup>. The trial was renewed the following day.  
462 The individual score was the median time spent.

463

#### 464 **ASD core features**

465 Behaviors modeling the ASD domains as defined by DSM-5 were assessed. The tests were selected based  
466 on their strong robustness (reliability from 0.77 to 0.92) and on their high loading scores in a factor analysis  
467 <sup>3</sup>.

468 *Deficit in social behavior.* A two-chamber test derived from Moy et al., 2004<sup>85</sup> was used to assess sociability  
469 and interest in social novelty. The setup and the protocol were detailed previously<sup>3, 10, 12</sup>. We used a 550 x  
470 550 mm Plexiglas box split in a 150 x 550 mm empty chamber and a 400 x 550 mm chamber containing the  
471 two boxes (43 mm diameter, distant from 340 mm) in which the mice or the lure were placed. *Sociability* is  
472 operationally defined as the higher number of visits towards the box containing a conspecific versus the one  
473 containing a lure (an adult mouse-sized oblong grey pebble), and the *interest in social novelty* as the higher  
474 number of visits towards a novel conspecific than towards the familiar one. Loss of social interest and poor  
475 interest in social novelty are expected in mice models of ASD. Briefly, the test consisted in a three-period  
476 observation, each lasting 10 min: 1) habituation (the two boxes containing lures), 2) sociability (one box  
477 containing a lure and the other a C57BL/6J male) and 3) interest in social novelty (one box containing the  
478 same C57BL/6J and the other a new SWR male). The behaviors were video-recorded (Viewpoint-Behavior  
479 technologies) and the number of nose pokes towards the boxes was counted as measure of the number of  
480 visits<sup>86</sup>.

481 *Repeated patterns of behavior.* We selected four measures that were highly loaded on the “repetitive  
482 patterns of behavior” factor in a factor analysis<sup>3</sup>: *marble burying* and *time burrowing* in a new cage, *number*  
483 *of stereotyped dips* in a hole-board device, and *number of leanings* in an open field. The protocols used  
484 have been previously detailed<sup>3, 10, 12</sup>. The *marble burying* and *time burrowing* tests quantify perseverating  
485 behavior<sup>87, 88</sup>. *Marble burying* consists in scoring the amount of marbles buried by each mouse in a 30 min  
486 session, using a 40 x 18 cm cage with 45 cm-thick litter and containing 20 marbles (9 mm diameter) on the  
487 surface of 70 mm-thick dust-free sawdust. Completely buried, 2/3 buried and 1/2 buried marbles were  
488 scored 3, 2 and 1, respectively. The *time burrowing* test leans on spontaneous digging and pushing behavior  
489 that rodents display when placed into a new home cage. The length of time each mouse spent digging plus  
490 pushing was measured. The *number of stereotyped dips* was counted in a hole-board device, consisting in

491 a 40 x 40 cm board with 16 equidistant holes (3.5 cm diameter) each equipped with photo-beams for  
492 detecting head dipping. Exploratory head dipping occurs when a rodent is placed on a surface with holes:  
493 the mouse puts its head once into one hole of the board. Head dipping is considered stereotyped when the  
494 head dips at least twice in the same hole within 2 s. The open field behavior was measured in a circular  
495 open field (100 cm diameter and 45 cm high walls) brightly lighted (210 lux on the ground). The ground was  
496 virtually divided in three concentric zones of equal surface. The distances walked and the times spent in the  
497 open field in the zones were automatically measured via the Viewpoint-Behavior technologies system  
498 (<http://www.viewpoint.fr/news.php>). The observation lasted 20 min. The number of leanings (rearing while  
499 leaning) on the walls of the structure was previously validated as a measure of repetitive behavior<sup>3, 89</sup>. The  
500 number of zones crossed is a measure of the narrowness of the field of interest. The total distance walked  
501 during the observation period served as covariate for the comparison between cKO mice and their  
502 respective controls<sup>3</sup>.

503

#### 504 **Additional behavioral measures**

505 Motor abnormalities and intellectual disability are not included among the ASD core features while having a  
506 noticeable but incomplete prevalence in ASD patients ( $\leq 79\%$  and  $\sim 45\%$ , respectively<sup>58</sup>). In this connection,  
507 two additional tests were conducted.

508 *Hind paw coordination.* A mouse was first trained to cross a smooth bar (50 x 5 x 5 cm) with large platforms  
509 on each extremity. The trained mouse was then placed on the central platform (3 x 5 cm) of a notched bar  
510 (100 cm) formed of 1.5 cm deep carvings regularly spaced (2 cm). The task consisted in ten bar crossings  
511 from the central to an extremity platform. The experimenters on each side of the setup counted the left and  
512 right hind paw slips according to<sup>90</sup>.

513 *Spatial learning.* The Morris water maze provides measures of the ability of rodents to solve spatial learning  
514 problems, namely the ability to find a submerged resting platform concealed beneath opaque water. The  
515 platform is a glass cylinder (66 mm diameter, 9 mm beneath the surface of the water) positioned 23 cm from  
516 the edge of a 100 cm diameter circular tank filled with water at  $26 \pm 1^\circ\text{C}$  and the light at 70 lux on the surface.  
517 Each mouse performed 7 blocks of 4 trials each: one block on day 1, and two blocks daily (one in the  
518 morning and one in the afternoon) for 3 successive days. A trial was stopped after 90 s if the mouse failed  
519 to reach the platform. We considered that the mouse had reached the platform when it stayed on the  
520 platform for 5 s at least. We presented a small metal shelf to the mouse 5 cm above the platform at the end  
521 of each trial of the first block (shaping). The mouse climbed on it and was transferred in a cage with dry  
522 sawdust for 120 s. We had previously assigned 4 virtual cardinal points to the tank, each being the starting  
523 point for a trial. The starting point for each trial was chosen randomly and within a block the mouse never  
524 started more than once from the same virtual cardinal point. We measured 1) the time to reach the hidden  
525 platform and 2) the cumulative distance to the center of the platform during swimming. The second measure  
526 eliminates possible bias resulting from floating during the trial. The time to reach the platform and the



527 distance were automatically measured by a video tracking setup (Viewpoint-Behavior technologies), each  
528 over the 7 blocks. Strains can achieve different performance levels between blocks, but without a cumulative  
529 reduction in the time to reach the platform, which is the criterion to identify learning process. We computed  
530 the slopes of the learning curves, a negative slope indicating learning behavior<sup>91</sup>. The strategy was used  
531 for both the time to reach the hidden platform and the cumulative distance to the center of the platform. The  
532 probe-test procedure, conducted after removing the platform, was done 24 h after block 7 to meet the  
533 requirements for reference memory<sup>92</sup> and lasted 90 s. The mouse was placed in the center of the tank, and  
534 we measured the time of first crossing the virtual annulus corresponding to the location of the platform. To  
535 check whether the differences in the time to reach the platform were due to vision and/or swimming abilities  
536 rather than learning ability, we also tested groups of naïve *Emx1-cKO* and *ChAT-cKO* mice, and their  
537 respective control, to the visible platform version of the test, in which the platform is 5 mm above non-  
538 opacified water.

539

## 540 **STATISTICS**

### 541 **Immunohistochemistry**

542 Data were analyzed by Prism 7.05 (GraphPad Software, USA). Sample sizes, tests used, and *P* values are  
543 reported in Figure legends. The significance threshold was set at  $P < 0.05$ .

544

### 545 **RT-qPCR**

546 Statistical analysis for was performed by unpaired Student's *t*-tests using the qbasePLUS software version  
547 2 (Biogazelle). The significance threshold was set at  $P < 0.05$ .

548

### 549 **Electrophysiology**

550 Statistical analysis was performed by Prism 7.05 (GraphPad Software, USA). Student's *t*-test or two-tailed  
551 Mann-Whitney test was used for comparing two data sets when passing or not D'Agostino & Pearson's  
552 normality test, respectively. Two-way ANOVA was used to analyze the influence of 2 categorical variables.  
553 2-samples Kolmogorov-Smirnov test was used to compare cumulative distributions. Sample sizes (*n*)  
554 reported in Figure legends refer to the number of recorded neurons. The significance threshold was set at  
555  $P < 0.05$ . Tests used, *P* values and sample sizes are indicated in the Figure.

556

### 557 **Behavior**

558 Data were processed by *Statistical Package for the Social Sciences* [SPSS software, version 25<sup>93</sup>]. The  
559 same statistical designs were used to compare *Emx1-cKO* and *ChAT-cKO* mice to their respective controls.  
560 Non-parametric statistics were chosen when the assumption of normality was rejected.

561 *Impairment of social behavior.* To analyze data from each social phase of the two-chamber test (sociability  
562 and interest for social novelty), a mixed design analysis of covariance (ANCOVA) was used including the

563 genotype as fixed factor, the box content as repeated measure, with measure of activity during habituation  
564 as covariate. A significant interaction between genotype and box content indicates that social behavior  
565 differs between the cKO and its control group.

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

573 *Sensorial abilities.* Comparison of the visual and auditory capacities of the cKO and their respective controls  
574 were conducted using a Student's *t*-test. Mixed repeated measures ANOVA, with genotype as fixed factor  
575 and 15 odors as repeated measures, was used to compare cKO and their respective controls for olfactory  
576 capacities.

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

586 *Effect size.* Effect sizes are expressed as  $\eta^2$  or as partial  $\eta^2$  with 95% confidence interval<sup>93, 94</sup>

587

588

### 589 **Ethic Statement**

590 The animal study was reviewed and approved by the "Comité National de Réflexion Ethique sur  
591 l'Expérimentation Animale n°14" and the project authorization delivered by the French Ministry of Higher  
592 Education, Research and Innovation. (ID numbers 57-07112012, 2019020811238253-V2 #19022 and  
593 2020031615241974-V5 #25232) and were in agreement with the European Communities Council Directive  
594 (2010/63/EU).

595

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604

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611

#### 612 **Author Contribution**

613 X.C., J.M. and P.S. performed the histological experiments and the quantitative analyses; M.C. and P.L.R.  
614 conducted the behavioral experiments and analyzed the resulting data; Y.B., L.B., J.M. and D.C. performed  
615 patch-clamp experiments and P.G. analyzed electrophysiological data; M.M. performed dendritic spine  
616 imaging and counting; A.F. performed RT-qPCR; X.C. and J.M. generated and maintained transgenic  
617 mouse lines; X.C., L.F., P.G. and L.K.-L.G. conceived the project, supervised the work and wrote the paper  
618 with the contribution of M.C. and P.L.R; all authors read and approved the final manuscript.

619

#### 620 **Competing Financial Interest**

621 The authors declare no competing interests or potential conflicts of interest.

622

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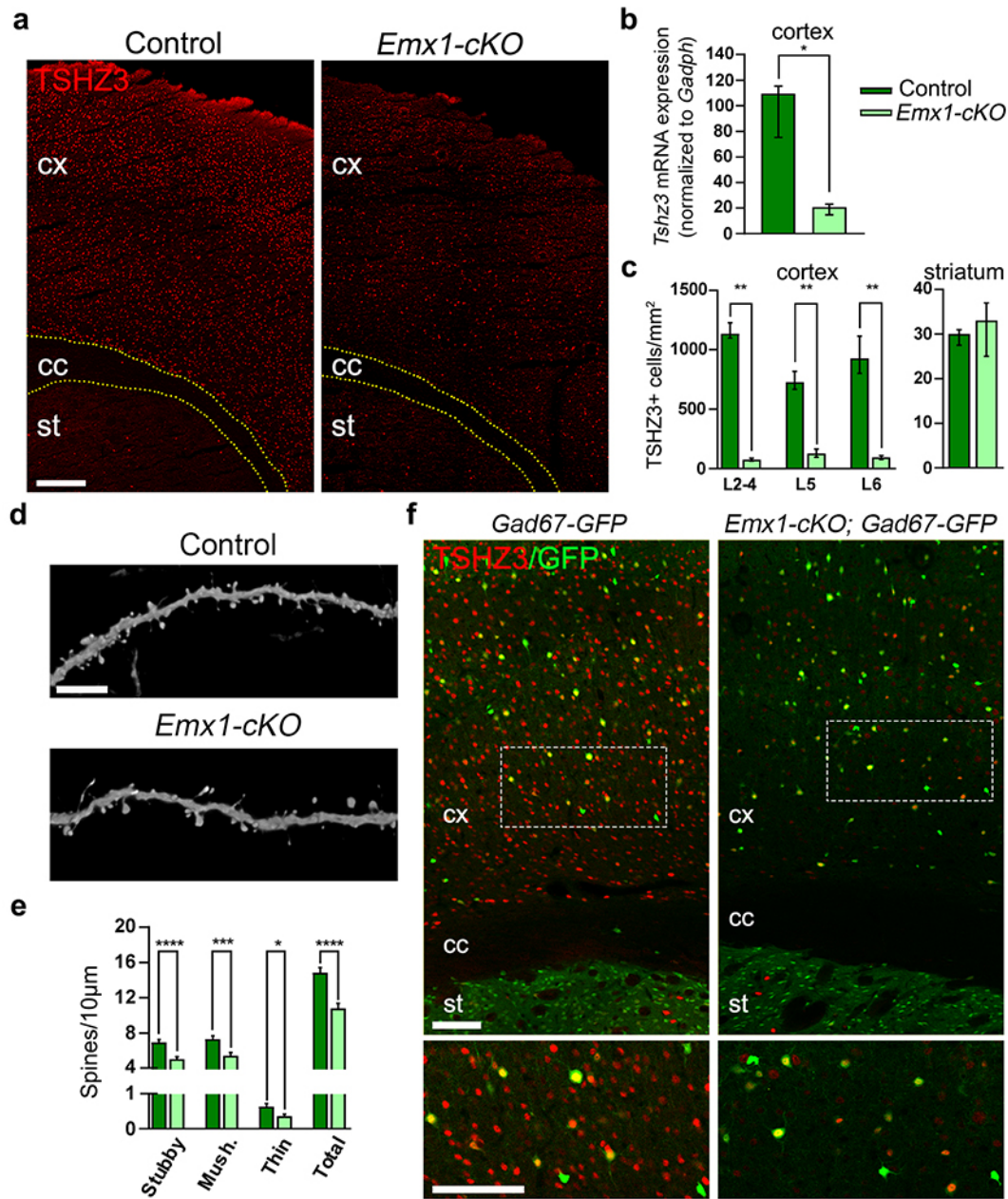
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1 **FIGURES**

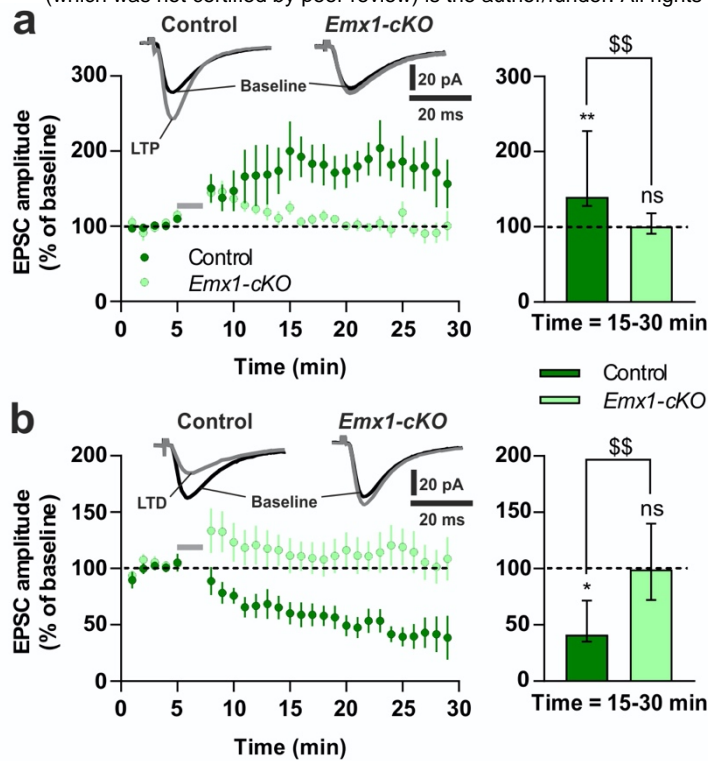
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4 **Figure 1. Conditional *Tshz3* deletion in CPNs.** **a** Coronal brain sections from control and *Emx1-cKO*  
5 mice immunostained for TSHZ3. Scale bar 250 μm. **b** *Tshz3* mRNA relative expression in the cortex of  
6 control and *Emx1-cKO* mice measured by RT-qPCR (4 cortices per group; \**P* < 0.05, Mann-Whitney  
7 test). **c** TSHZ3-positive cell density in control and *Emx1-cKO* mice in cortical layers (cell counts  
8 performed using frames of 400 μm width spanning from L1 to L6 in 9 sections from 3 control mice and  
9 18 sections from 3 *Emx1-cKO* mice; \*\**P* < 0.01, Mann-Whitney test) and in the whole striatal surface  
10 (cell counts performed in the whole dorsal striatum in 6 sections from 3 control mice and 7 sections from  
11 3 *Emx1-cKO* mice; *P* = 0.1496, Mann-Whitney test). **d** Representative confocal images showing  
12 dendritic spines of GFP-positive L5 neurons from control (*Thy1-GFP-M*) and *Emx1-cKO* (*Thy1-GFP-M*;  
13 *Emx1-cKO*) mice. Scale bar 5 μm. **e** Density of different classes of dendritic spines in control (1688  
14 spines/1135 μm) and *Emx1-cKO* (1308 spines/1220 μm) mice. **f** Coronal brain sections from *GAD67-*  
15 *GFP* control and *Emx1-cKO-GAD67-GFP* mice immunostained for TSHZ3. Lower panels are  
16 magnifications of the framed areas in the upper images. Scale bars 100 μm. \**P* < 0.02, \*\*\**P* < 0.001 and  
17 \*\*\*\**P* < 0.0001, Student's *t*-test. Data in **b** and **c** are expressed as medians with interquartile range; data  
18 in **e** are expressed as means + SEM.

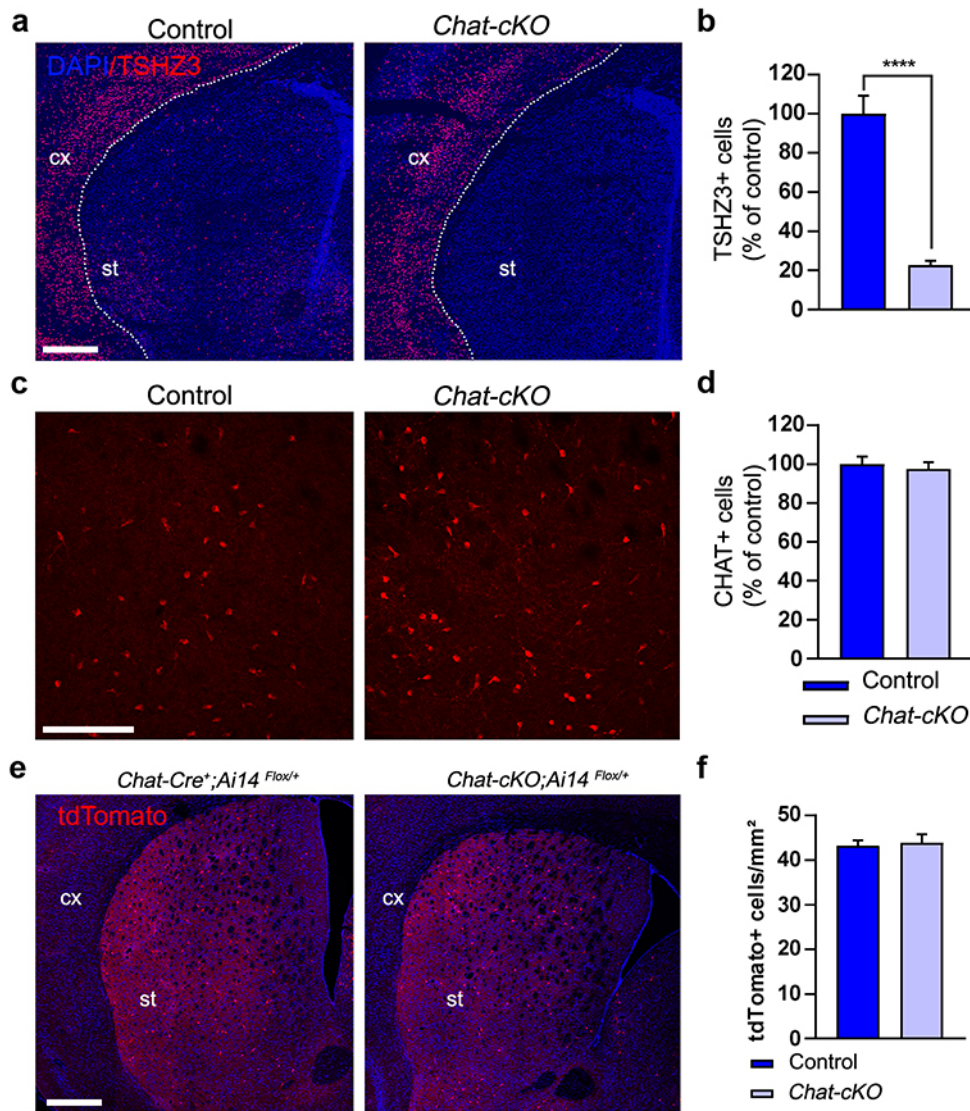
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**Figure 2. Impaired corticostriatal synaptic plasticity in *Emx1-cKO* mice.** LTP **a** and LTD **b** are lost in *Emx1-cKO* mice. Left graphs: time-course (normalized EPSC amplitude expressed as means  $\pm$  SEM; grey bars represent induction protocols; 2-way ANOVA from 15 to 30 min; LTP:  $F(1,211) = 44.8$ ,  $P < 0.0001$ ; LTD:  $F(1,216) = 153.2$ ,  $P < 0.0001$ ). Traces show EPSCs before (black) and after (grey) LTP and LTD induction protocols. Right graphs: EPSC amplitude at 15-30 min (medians with interquartile range; Wilcoxon matched-pairs signed rank test vs. baseline:  $*P < 0.05$ ,  $**P < 0.01$ , ns = non-significant; Mann-Whitney test:  $^{**}P < 0.01$ ). Data obtained from 17 SSPNs of control and 14 of *Emx1-cKO* mice.

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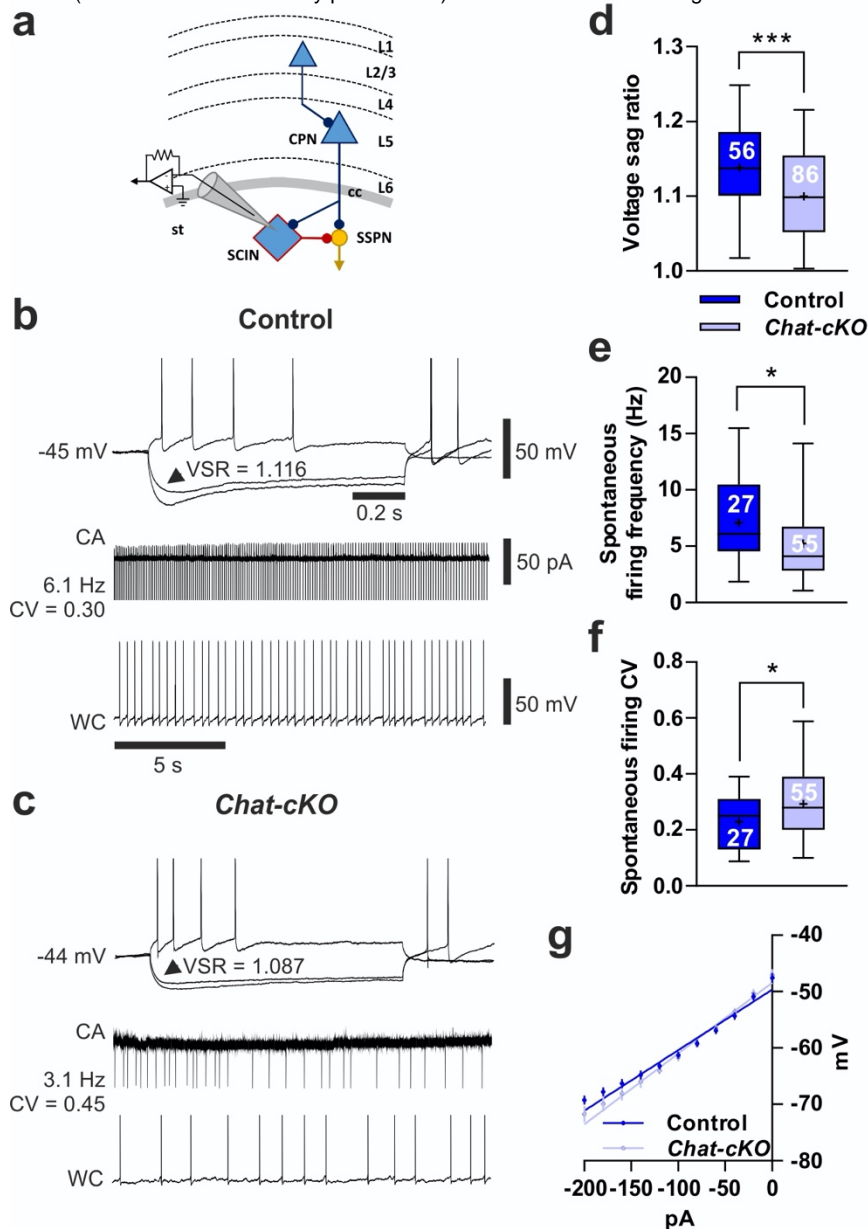


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**Figure 3. Conditional *Tshz3* deletion in cholinergic neurons.** **a** Coronal brain sections from control and *Chat-cKO* mice immunostained for TSHZ3 and counterstained with DAPI. Scale bar 500  $\mu$ m. **b** Number of TSHZ3-positive cells in the striatum of control and *Chat-cKO* mice; results are expressed as percent of mean control value (15 sections from 3 control mice; 11 sections from 3 *Chat-cKO* mice; \*\*\*\* $P$  < 0.0001, Student's  $t$ -test). **c** Coronal brain sections from control and *Chat-cKO* mice stained for CHAT. Scale bar 200  $\mu$ m. **d** Number of CHAT-positive SCINs in the striatum of control and *Chat-cKO* mice; results are expressed as percent of mean control value (40 sections from 9 control mice; 53 sections from 11 *Chat-cKO* mice;  $P$  = 0.6373, Student's  $t$ -test). **e** Representative images showing tdTomato fluorescence detection (red) in SCINs of *Chat-Cre*;Ai14<sup>Flox/+</sup> control and *Chat-cKO*;Ai14<sup>Flox/+</sup> mutant mice (coronal sections). cx, cerebral cortex; st, striatum. Nuclei are counterstained with DAPI. Scale bar 500  $\mu$ m. **f** Density of tdTomato-positive cells in the striatum of *Chat-Cre*;Ai14<sup>Flox/+</sup> control and *Chat-cKO*;Ai14<sup>Flox/+</sup> mutant mice (14 sections from 3 control mice; 12 sections from 3 *Chat-cKO*;Ai14<sup>Flox/+</sup> mice;  $P$  = 0.6777, Student's  $t$ -test). Data in **b**, **d** and **f** are expressed as means + SEM.

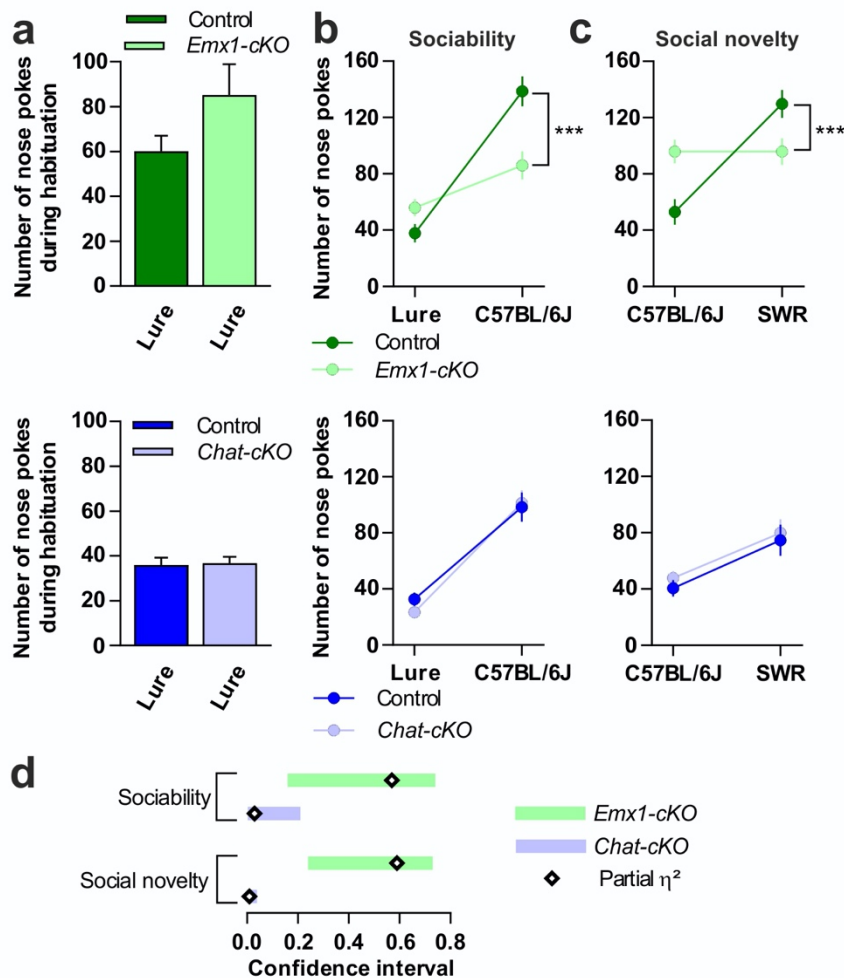
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 47 **Figure 4. Altered electrophysiological properties of SCINs in *Chat-cKO* mice.** **a** Simplified scheme  
 48 of the corticostriatal circuitry with the recording patch-clamp pipette placed on a SCIN. TSHZ3-  
 49 expressing neurons are blue (L1-6, cortical layers 1-6; cc, corpus callosum; st, striatum). **b** Sample  
 50 traces obtained from a representative control SCIN: note the prominent voltage sag in response to -200  
 51 and -120 pA hyperpolarizing currents, and the AP firing during a +100 pA depolarizing current (1<sup>st</sup> line),  
 52 as well as the sustained and regular firing in cell-attached (CA) and whole-cell (WC) configuration (2<sup>nd</sup>  
 53 and 3<sup>rd</sup> line, respectively). **c** Sample traces obtained from a representative *Chat-cKO* SCIN: compared  
 54 to **b**, note the smaller voltage sag as well as the less regular, lower frequency spontaneous firing. (b, c)  
 55 The values of voltage sag ratio (VSR) of the response to -120 pA current injection (arrowhead), as well  
 56 as the frequency and coefficient of variation (CV) of spontaneous firing of these samples, are reported;  
 57 spikes have been cut; calibration bars are the same in b and c. Compared to control, SCINs from *Chat-*  
 58 *cKO* mice show a significant reduction of mean voltage sag ratio (**d**) and frequency of spontaneous  
 59 discharge **e**, while the CV of their inter-AP interval is increased (**f**) meaning that their spontaneous firing  
 60 is more irregular. The number of recorded SCINs in d-f is reported in the graphs. **g** Current-voltage  
 61 relationship obtained from 51 control and 62 *Chat-cKO* SCINs, and the linear best fit to calculate input  
 62 resistance (see Results). \* $P < 0.05$ , \*\*\* $P < 0.001$ , Student's *t*-test; data in d-f are expressed as box and  
 63 whiskers (25th-75th and 5th-95th percentiles, respectively), where bar = median and cross = mean; data  
 64 in g are expressed as means  $\pm$  SEM.  
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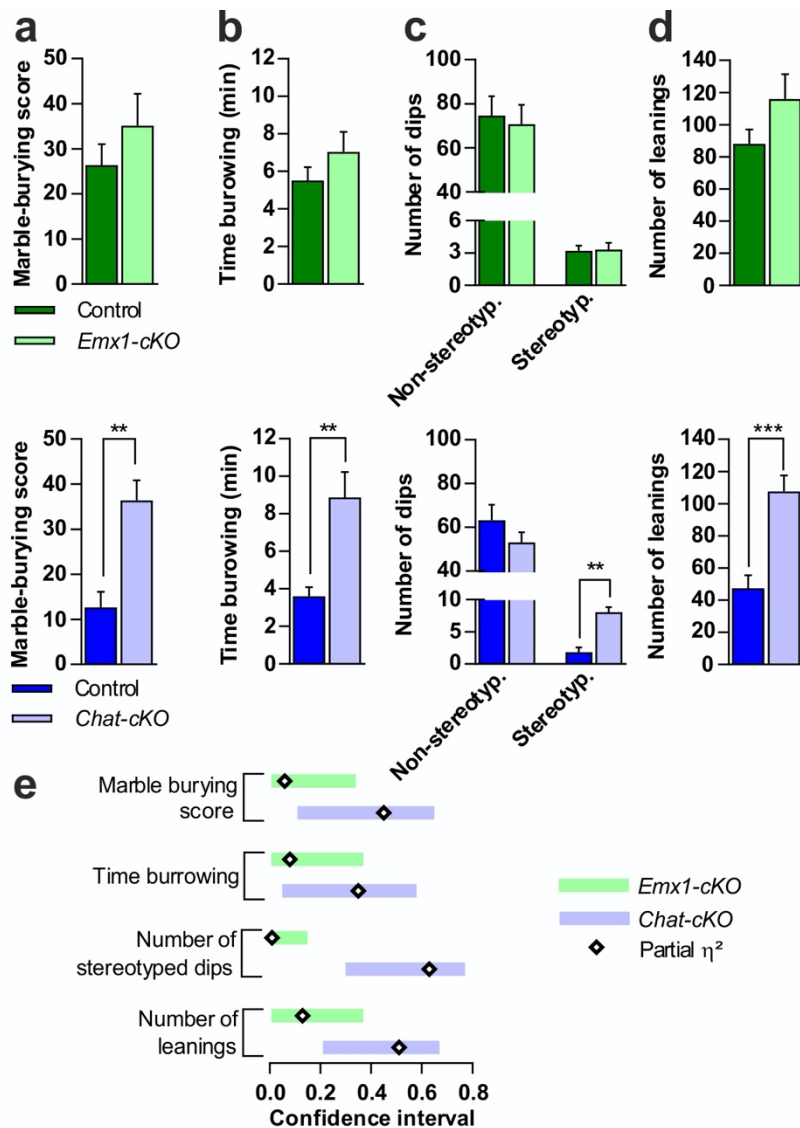


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69 **Figure 5. Sociability and social novelty deficits in *Emx1-cKO* but not in *Chat-cKO* mice.** a Nose  
 70 pokes during habituation, used as covariate for mixed-design ANCOVAs in b and c. b Sociability  
 71 measured as the number of nose pokes against a C57BL/6J male mouse or a lure. *Emx1-cKO* mice (n  
 72 = 9) vs. control (n = 8):  $F_{interaction}(1,14) = 18.59, P < 0.001$ . *Chat-cKO* mice (n = 12) vs. control (n = 9):  
 73  $F_{interaction}(1,18) = 0.55, P = 0.47$ . c Interest in social novelty measured as the number of nose pokes  
 74 against the same C57BL/6J or a SWR mouse. *Emx1-cKO* vs. control:  $F_{interaction}(1,14) = 19.70, P < 0.001$ .  
 75 *Chat-cKO* vs. control:  $F_{interaction}(1,18) = 0.02, P = 0.89$ . d Sizes of the difference for *Emx1-cKO* (partial  
 76  $\eta^2 = 0.57$  and  $0.59$  for b and c, respectively) and *Chat-cKO* mice (partial  $\eta^2 = 0.03$  and  $0.001$ ,  
 77 respectively) vs. their respective control. Data in a-c are expressed as means  $\pm$  SEM. \*\*\* $P < 0.001$ .

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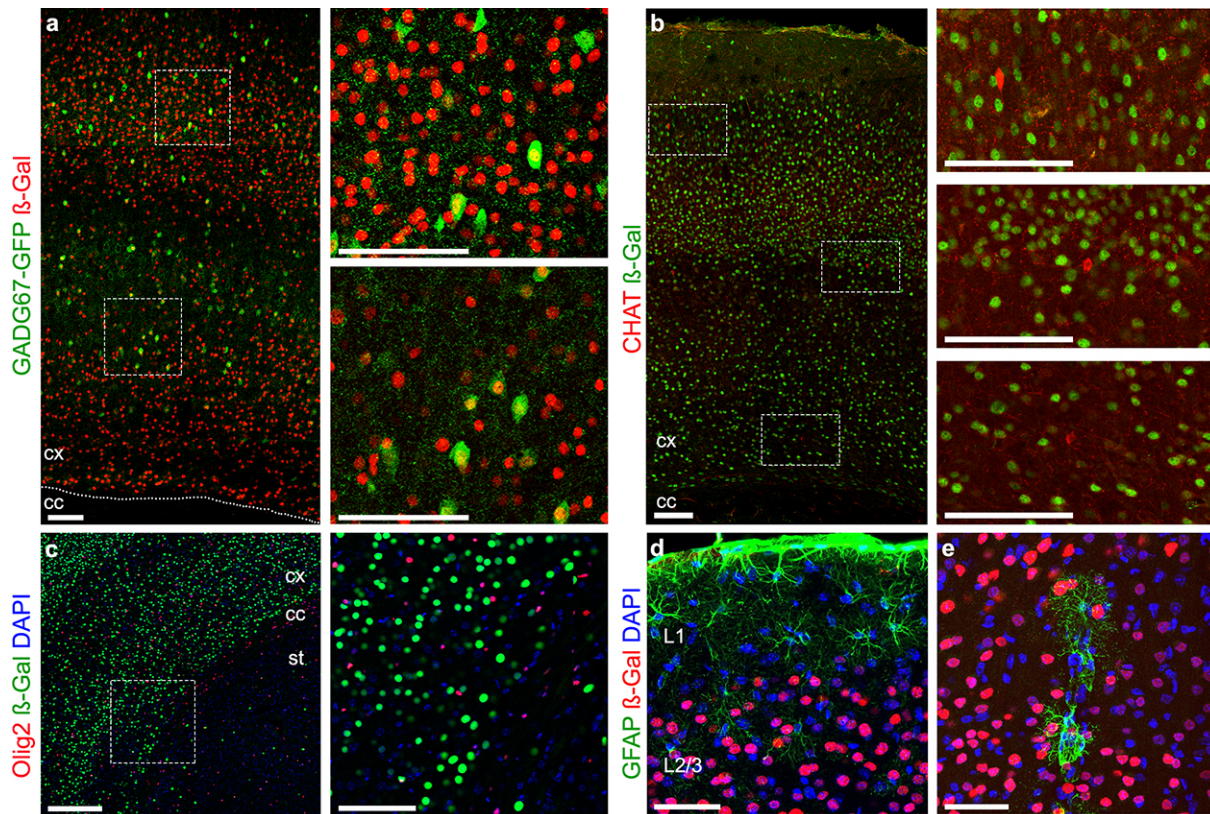
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82 **Figure 6. Repeated patterns of behavior in *Chat-cKO* but not in *Emx1-cKO* mice.** **a** Marble-burying, *Emx1-cKO*, Student's- $t(15) = 1.0$ ,  $P = 0.33$ ; *Chat-cKO*,  $t(19) = 3.97$ ,  $P = 0.001$ . **b** Time burrowing, *Emx1-cKO*,  $t(15) = 1.16$ ,  $P = 0.13$ ; *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* ( $\eta^2 = 0.06$ ,  $0.08$ ,  $0.13$  in **a**, **b** and **d**, respectively, and partial  $\eta^2 = 0.01$  in **c**) and in *Chat-cKO* ( $\eta^2 = 0.45$ ,  $0.35$ ,  $0.51$  in **a**, **b** and **d**, respectively, and partial  $\eta^2 = 0.63$  in **c**). Sample size of **a**, **b**, **c** and **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. \*\* $P < 0.01$  \*\*\* $P < 0.001$ .

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1 **Supplementary Figures**

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4 **Figure S1. TSHZ3 expression in interneurons and glial cells in the cerebral cortex. (a-e)** Coronal

5 brain sections. **a** *Tshz3* expression as β-Gal staining in *Tshz3<sup>+lacZ</sup>; GAD67-GFP* mouse brain. The two

6 images on the right are magnifications of the framed areas in A. Scale bars 100 μm. **b** Double

7 immunofluorescence staining for β-Gal and CHAT. The framed areas in (b) are magnified on the right.

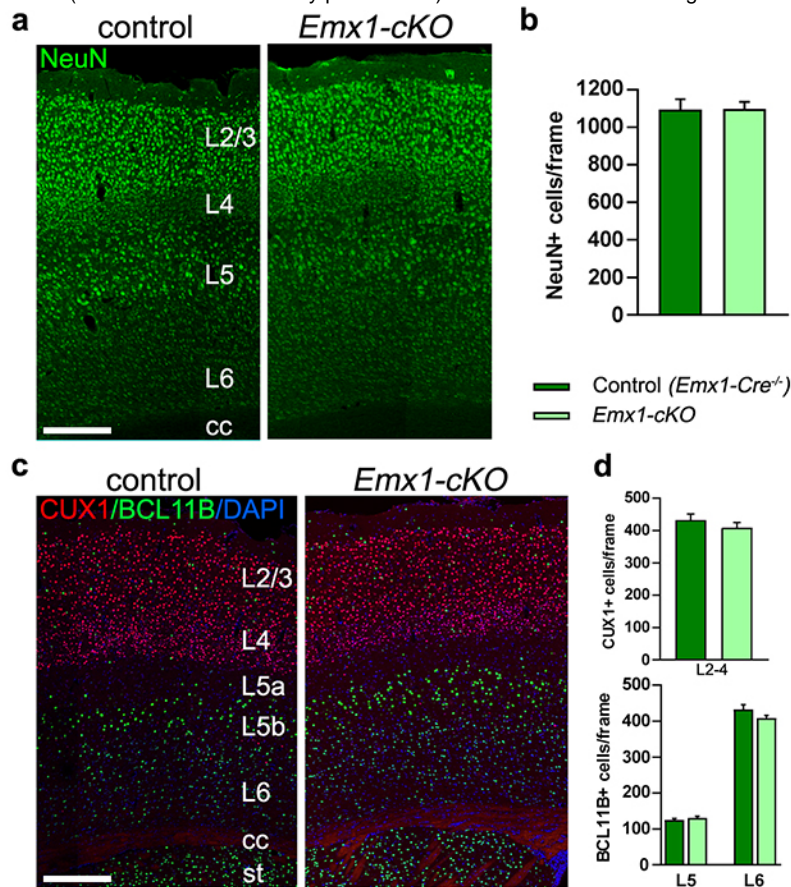
8 Scale bars 100 μm. **c** Double immunofluorescence staining for Olig2 and β-Gal (left) and detail of the

9 framed area (right). Scale bars 100 μm. **(d, e)** Double immunofluorescence staining for GFAP and β-Gal.

10 Scale bars 100 μm (d) and 50 μm (e). Nuclei in c-e are counterstained with DAPI. cc, corpus callosum;

11 cx, cerebral cortex; st, striatum.

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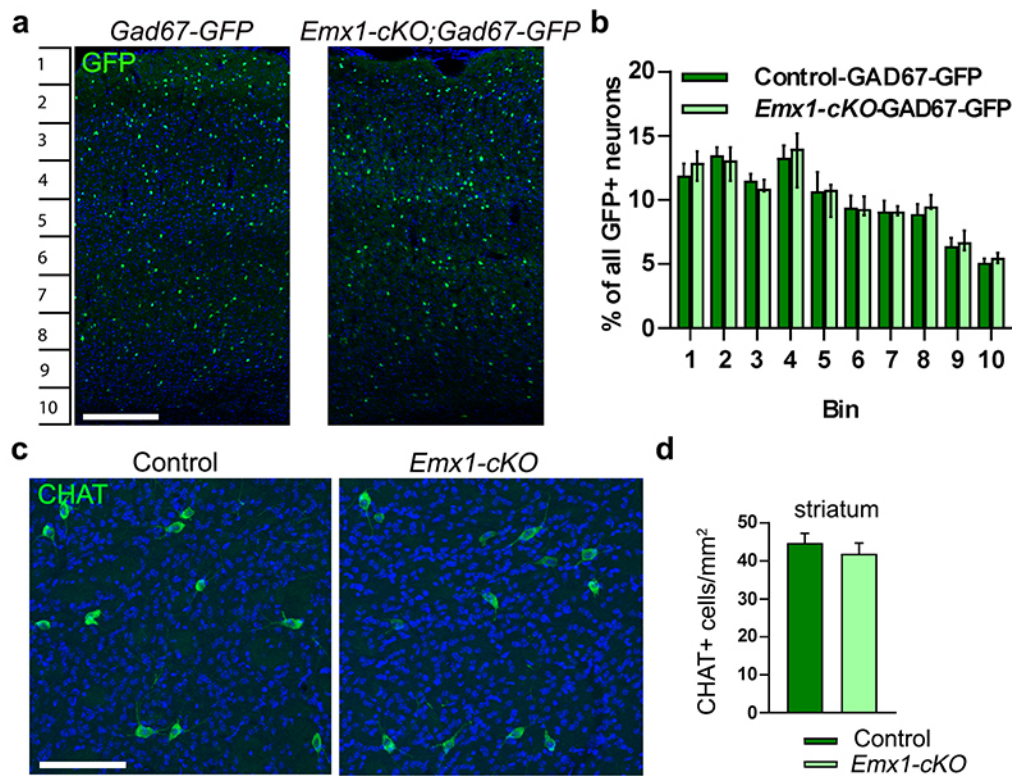


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**Figure S2. Cortical layering is preserved in *Emx1-cKO* mouse brain.** **a** Coronal brain sections from *Emx1-cKO* and control mice immunostained for NeuN detection. Scale bar 250  $\mu$ m. **b** Number of NeuN-positive cells counted in frames of 400  $\mu$ m 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  $\mu$ m; 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; countings were performed in cortical frames of 400  $\mu$ m 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.



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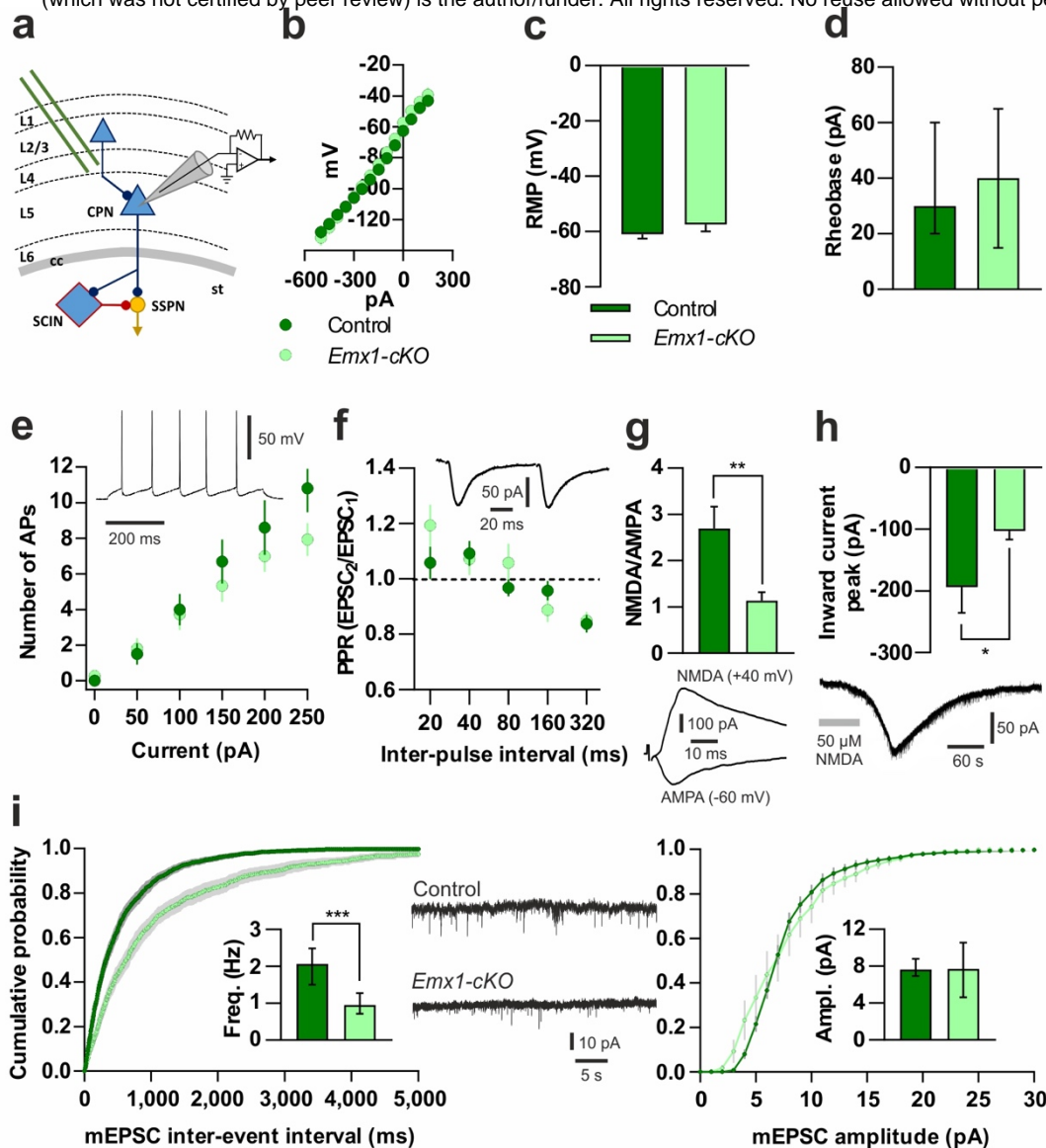
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31 **Figure S3. Loss of *Tshz3* in *Emx1-cKO* mice does not affect the numbers of cortical GABAergic**  
32 **and striatal cholinergic interneurons.** Representative images **a** and quantitative analysis **b** showing  
33 the distribution of GAD67-GFP-positive cells in the cerebral cortex in coronal brain sections from  
34 *GAD67-GFP* control and *Emx1-cKO-GAD67-GFP* mice. Scale bar in **A** 250  $\mu$ m. Data in **b** are expressed  
35 as percent of total GFP-positive cells per bin (37 sections from 5 control mice; 41 sections from 7 *Emx1-*  
36 *cKO* mice;  $F_{genotype}(1,100) = 0.00006$ ,  $P = 0.994$ ,  $F_{interaction}(9,100) = 0.381$ ,  $P = 0.942$ , 2-way ANOVA).  
37 Images of CHAT immunostaining **c** and analysis of the density of CHAT-positive cells **d** in coronal brain  
38 sections at striatal level of control and *Emx1-cKO* mice. Scale bar 100  $\mu$ m (18 sections from 3 control  
39 and 3 *Emx1-cKO* mice, respectively;  $P = 0.465$ , Student's *t*-test). Data in **b** are expressed as median  
40 with interquartile range; data in **d** as means + SEM.

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45 **Figure S4. Electrophysiological characterization of L5 CPNs and basal cortical synaptic**

46 **transmission. a** Simplified scheme of the corticostriatal circuitry with the recording patch-clamp pipette

47 placed on a L5 CPN and the stimulating electrode placed in L4. TSHZ3-expressing neurons are blue

48 (L1-6, cortical layers 1-6; cc, corpus callosum; st, striatum). **b** Current-voltage relationship recorded from

49 CPNs of *Emx1-cKO* mice and littermate controls show similar slopes and input resistance ( $148.9 \pm 13.3$

50 vs.  $151.3 \pm 11.6$  M $\Omega$ , respectively;  $n = 21$  and  $n = 28$ , respectively;  $P > 0.05$ , Student's *t*-test). **c** Resting

51 membrane potential (RMP;  $n = 28-38$ ) and **d** rheobase ( $n = 11-21$ ) do not significantly differ between

52 control and *Emx1-cKO* CPNs ( $P > 0.05$  for both; Student's *t*-test and Mann-Whitney test, respectively).

53 **e** The number of action potentials (APs) emitted by control ( $n = 10$ ) and *Emx1-cKO* ( $n = 15$ ) CPNs in

54 response to increasing current injections is similar (2-way ANOVA: genotype  $F(1,138) = 3.068$ ,  $P =$

55  $0.0821$ ; interaction  $F(5,138) = 0.9349$ ,  $P = 0.4605$ ; multiple *t*-tests:  $P > 0.05$ ). The trace shows an

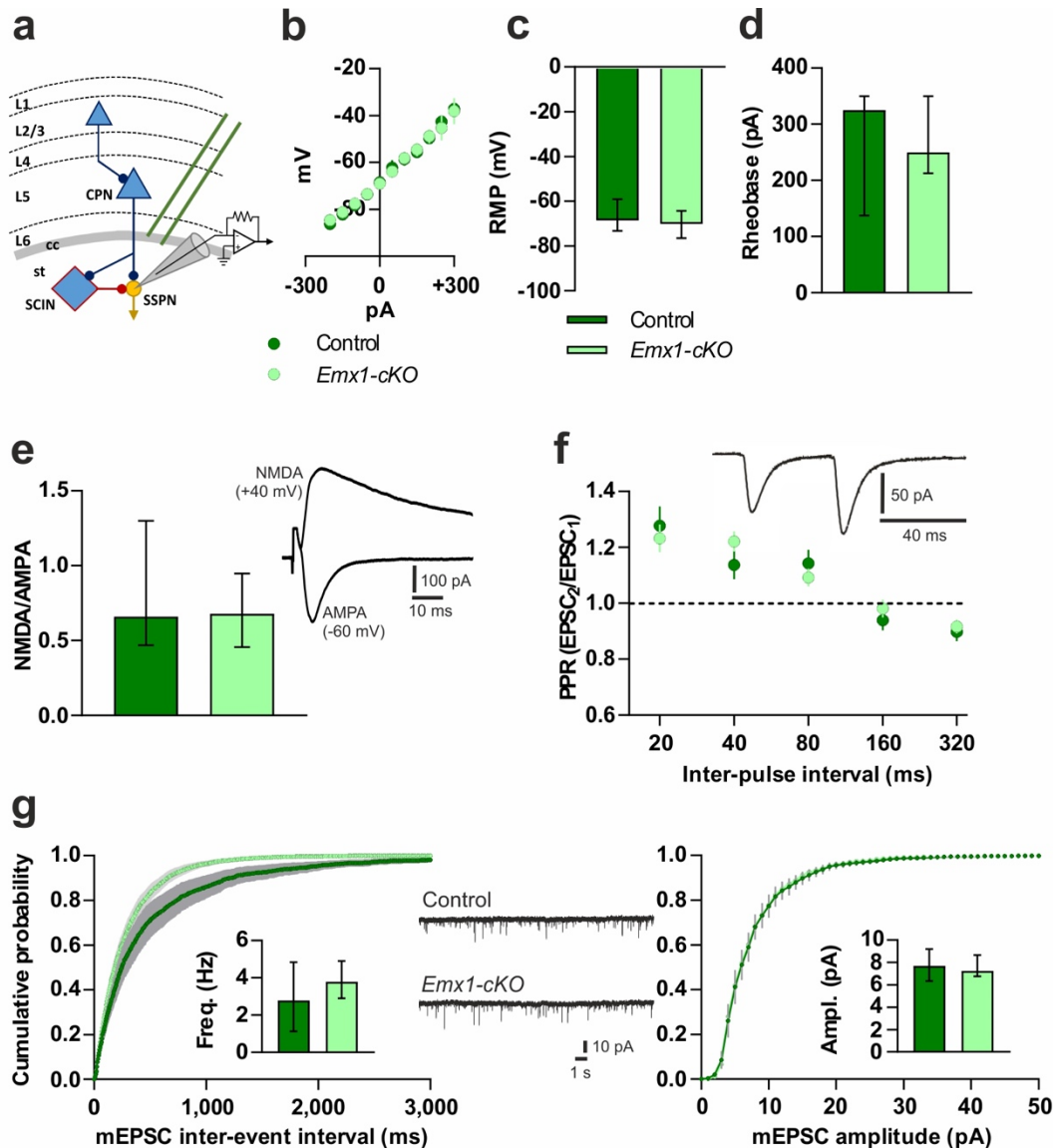
56 example of AP firing during a 100 pA, 500 ms current step. **f** Paired-pulse ratio (PPR) is not significantly

57 different between control ( $n = 19$ ) and *Emx1-cKO* ( $n = 14$ ) CPNs (2-way ANOVA: genotype  $F(1,155) =$

58 0.901,  $P = 0.344$ ; interaction  $F(4,155) = 1.431$ ,  $P = 0.2263$ ). The trace shows an example of paired  
59 EPSCs (80 ms inter-pulse interval). **g** NMDA/AMPA ratio is significantly decreased in CPNs of *Emx1-*  
60 *cKO* mice compared to control ( $n = 15$  for each genotype,  $**P < 0.01$ , Student's *t*-test). Traces show an  
61 example of a NMDA- and an AMPA receptor-mediated EPSC recorded from the same CPN at +40 and  
62 -60 mV, respectively. **h** The tonic inward currents induced by bath application of NMDA (50  $\mu$ M, 60 s)  
63 are significantly smaller in CPNs from *Emx1-cKO* mice compared to control ( $n = 15$  and  $n = 14$ ,  
64 respectively;  $*P < 0.05$ , Student's *t*-test). The trace shows a sample response of a CPN (sEPSCs have  
65 been cut) to NMDA bath application (grey bar). **i** The distribution of mEPSC inter-event intervals is  
66 significantly different between control ( $n = 12$ ) and *Emx1-cKO* ( $n = 11$ ) CPNs ( $P < 0.0001$ , 2-samples  
67 Kolmogorov-Smirnov test), as well as their median frequency (inset) ( $***P < 0.001$ , Mann-Whitney test).  
68 Conversely, both the distribution and the median values of mEPSC amplitude are similar in control and  
69 *Emx1-cKO* CPNs ( $P > 0.05$ , 2-samples Kolmogorov-Smirnov test and Mann-Whitney test). Cumulative  
70 plots represent mean values (light and dark green) and SEM (grey). Traces show sample mEPSCs  
71 recorded from control and *Emx1-cKO* CPNs. Data in **b**, **c**, **e-h** and in **i** (cumulative plots) are expressed  
72 as means  $\pm$  SEM; data in **d** and in **i** (insets) are expressed as medians with interquartile range.

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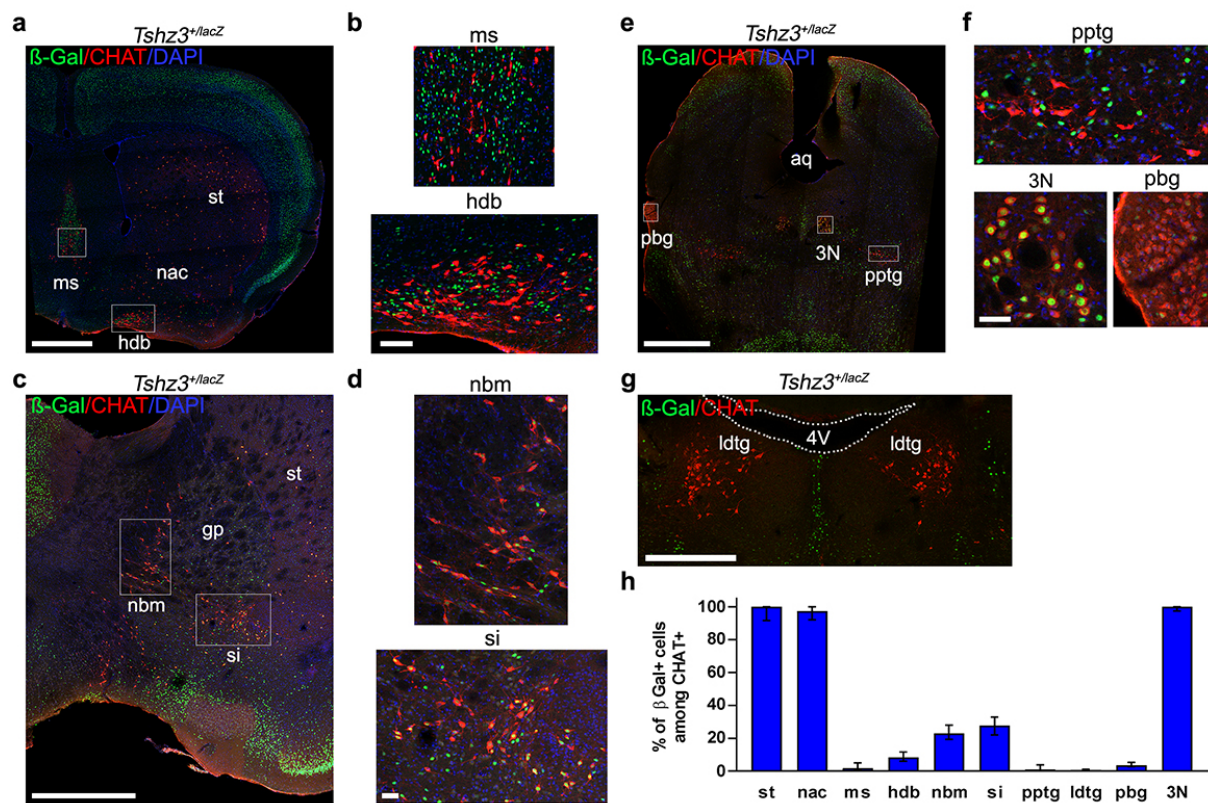
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77 **Figure S5. Electrophysiological characterization of SSPNs and basal corticostriatal synaptic**  
 78 **transmission. a** Simplified scheme of the corticostriatal circuitry with the recording patch-clamp pipette  
 79 placed on a SSPN and the stimulating electrode placed on the cc. TSHZ3-expressing neurons are blue  
 80 (L1-6, cortical layers 1-6; cc, corpus callosum; st, striatum). **b** Current-voltage relationship recorded from  
 81 SSPNs of control and *Emx1-cKO* mice provide similar slopes and input resistance ( $97.4 \pm 2.3$  vs.  $93.0$   
 82  $\pm 2.1$  M $\Omega$ , respectively;  $n = 7$  and  $n = 15$ , respectively;  $P = 0.1862$ , Mann-Whitney test). **c** Resting  
 83 membrane potential (RMP) and **d** rheobase are not significantly different between control ( $n = 7$ ) and  
 84 *Emx1-cKO* ( $n = 15$ ) SSPNs ( $P > 0.05$ , Mann-Whitney test). **e** NMDA/AMPA ratio is similar between  
 85 control ( $n = 11$ ) and *Emx1-cKO* ( $n = 12$ ) SSPNs ( $P > 0.05$ , Mann-Whitney test); traces in **e** show an  
 86 example of an NMDA receptor- and an AMPA receptor-mediated EPSC recorded from the same SSPN  
 87 at +40 and -60 mV, respectively. **f** Paired-pulse ratio (PPR) is similar between control ( $n = 18$ ) and  
 88 *Emx1-cKO* ( $n = 24$ ) SSPNs (2-way ANOVA: genotype  $F(1,162) = 0.1135$ ,  $P = 0.7367$ ; interaction  
 89  $F(4,162) = 0.8429$ ,  $P = 0.4999$ ). The trace shows an example of paired EPSCs (40 ms inter-pulse

90 interval). **g** The distribution of mEPSC inter-event intervals is significantly different between control (n =  
91 8) and *Emx1-cKO* (n = 7) SSPNs ( $P < 0.001$ , 2-samples Kolmogorov-Smirnov test), but their median  
92 frequency (inset) is similar ( $P > 0.05$ , Mann-Whitney test). Both the distribution and the median value of  
93 mEPSC amplitude are not significantly different between control and *Emx1-cKO* SSPNs ( $P > 0.05$ , 2-  
94 samples Kolmogorov-Smirnov test and Mann-Whitney test). Cumulative plots represent average values  
95 (light and dark green) and SEM (grey). Traces show sample mEPSCs recorded from control and *Emx1-*  
96 *cKO* SSPNs. Data in **b**, **f** and **g** (cumulative plots) are expressed as means  $\pm$  SEM; data in **c-e** and **g**  
97 insets are expressed as medians with interquartile range.

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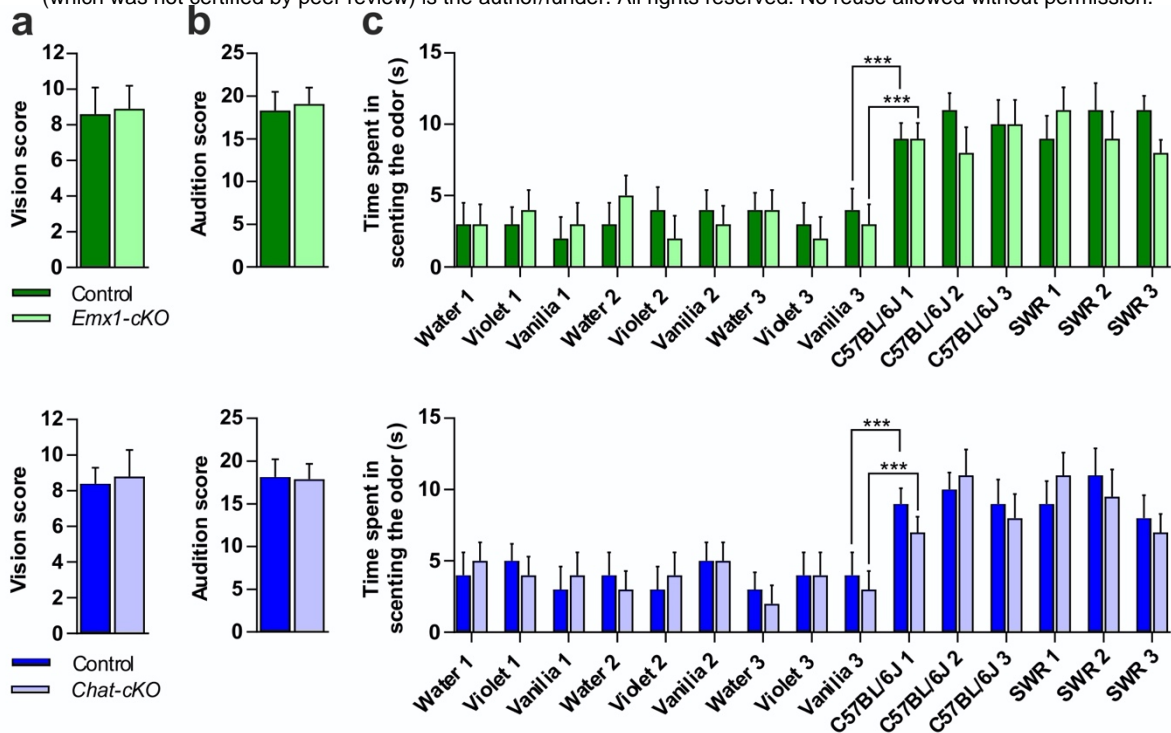


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102 **Fig. S6. TSHZ3 expression in the main brain cholinergic systems.** Forebrain (a-d) and brainstem  
 103 (e-g) coronal sections stained for  $\beta$ -Gal and CHAT. (b, d, f) Higher-power images of framed regions in  
 104 a, c and e, respectively. h Quantification of  $\beta$ -Gal-positive cells within the CHAT-positive population in  
 105 brain structures containing cholinergic neurons. aq, aqueduct; hdb, nucleus of the horizontal limb of the  
 106 diagonal band; gp, globus pallidus; ldtg, laterodorsal tegmental nucleus; ms, medial septal nucleus; nac,  
 107 nucleus accumbens; nbm, nucleus basalis of Meynert; pbg, parabigeminal nucleus; pptg,  
 108 pedunclopontine tegmental nucleus; si, substantia innominata; st, striatum; 3N, oculomotor nucleus;  
 109 4V, 4<sup>th</sup> ventricle. Nuclei were counterstained with DAPI. Data are expressed as medians with  
 110 interquartile range; they were obtained from 6 (3N), 7 (hdb), 9 (ms) 12 (pbg, si), 16 (ldtg), 17 (nac), 19  
 111 (st), 24 (pptg) and 40 (nbm) sections from 3 (hdb, ldtg, ms, pbg and pptg), 4 (si and 3N), 6 (nac), 7 (st)  
 112 and 8 (nbm) mice, respectively.

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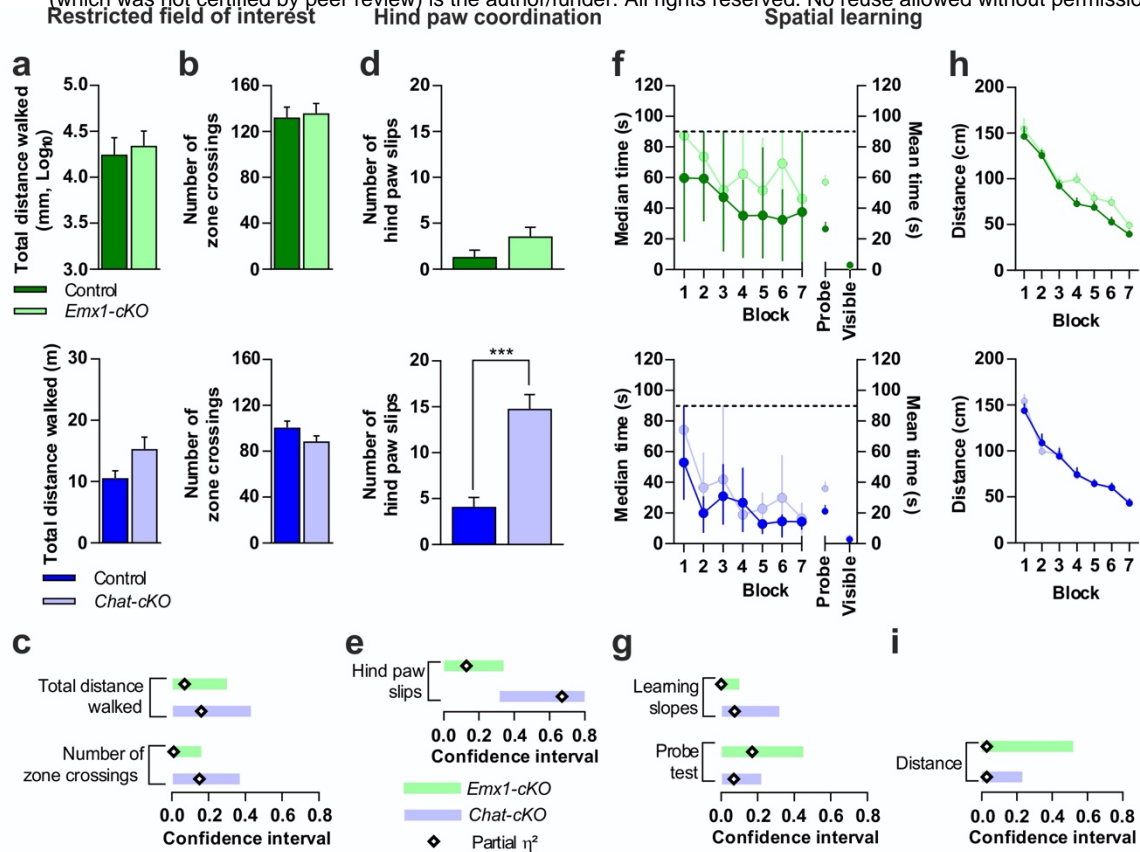
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**Figure S7. Visual, auditory and olfactory capacities in *Emx1-cKO* and *Chat-cKO* mice compared with their respective littermate controls.** Ten mice per genotype were used in each screening. **a** Visual capacity differs neither in *Emx1-cKO* mice 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 control littermate: paired Student's  $t = 4.5$ ,  $df = 9$ , and  $t = 3.78$ ,  $df = 9$ , respectively;  $P < 0.001$ ; sizes of the differences:  $\eta^2 = 0.57$  and  $0.51$ , respectively; *Chat-cKO* and control littermate: paired Student's  $t = 5.7$ ,  $df = 9$ , and  $t = 4.9$ ,  $df = 9$ , respectively;  $P < 0.001$ ; sizes of the differences:  $\eta^2 = 0.49$  and  $0.40$ , respectively). Data are expressed as means + SEM. \*\*\* $P < 0.001$ .



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135 **Figure S8. Restricted field of interest, hind paw coordination and spatial learning in *Emx1-cKO***

136 **vs. littermate control mice and *Chat-cKO* vs. littermate control mice.** **a-c** The narrowness of the

137 field of interest, expressed as the number of zone crossing in the open field **b** with the total distance

138 walked **a** as covariate, is impacted neither in *Emx1-cKO* (n = 9) nor in *Chat-cKO* mice (n = 12) compared

139 to their respective control (n = 8 and n = 8, respectively). **c** The partial  $\eta^2$  are very low and their

140 confidence intervals includes zero. **d-e** Hind paw coordination. *Chat-cKO* mice (n = 9) exhibit a high

141 deficit when compared to their control (n = 9) (Student's  $t = 5.72$ ,  $df = 16$ ,  $P = 0.00003$ ). On the opposite,

142 *Emx1-cKO* mice (n = 10) do not differ from their control (n = 8) (Student's  $t = 1.76$ ,  $df = 16$ ,  $P = 0.10$ ). **e**

143 The effect size of the difference in *Chat-cKO* ( $\eta^2 = 0.67$ ) exceeds the limit of impairment (0.30), whereas

144 it is not considered in *Emx1-cKO* mice because its confidence interval encompassed zero. **(f- i)** Spatial

145 learning in the Morris water maze. Time to reach the visible platform **f** is similar both in *Emx1-cKO* mice

146 (n = 12) and their control (n = 11) and in *Chat-cKO* mice (n = 10) and their control (n = 13) (Student's  $t = 0.90$ ,

147  $df = 21$ ,  $P = 0.38$  and Student's  $t = 1.28$ ,  $df = 22$ ,  $P = 0.21$ , respectively), showing that different

148 learning performances cannot be attributed to motor or sensorial abilities. Non-parametric statistics were

149 used in the hidden platform version when the assumption of normality of the distributions was rejected.

150 We examined the learning slopes with the Friedman's test for non-parametric ANOVA with repeated

151 values. The four groups of mice learned across blocks 1 to 7. *Emx1-cKO* and their control learn equally

152 (Friedman's test for non-parametric ANOVA with repeated values:  $\chi^2 = 21.42$ ,  $df = 6$ ,  $P = 0.002$  and  $\chi^2 =$

153  $19.22$ ,  $df = 6$ ,  $P = 0.004$ , respectively), with similar slopes (Student's  $t = 0.01$ ,  $df = 22$ ,  $P = 0.99$ ). *Chat-*

154 *cKO* and their control also learned across blocks 1 to 7 with similar trends ( $\chi^2 = 24.41$ ,  $df = 6$ ,  $P = 0.0004$



155 and  $\chi^2 = 30.67$ ,  $df = 6$ ,  $P = 0.00002$ , respectively) and similar slopes (Student's  $t = 1.30$ ,  $df = 21$ ,  $P =$   
156  $0.21$ ). In the probe test version, the Student's  $t$  in *Emx1-cKO* vs. control and *Chat-cKO* vs. controls are,  
157 respectively: Student's  $t = 2.22$ ,  $df = 22$ ,  $P = 0.04$  and Student's  $t = 1.14$ ,  $df = 21$ ,  $P = 0.27$ . Dotted lines  
158 represent the 90 s cutoff. Dots indicating the visible platform values overlap. **g** The confidence intervals  
159 of the effect size for the learning slopes ( $\eta^2 = 0.002$  for *Emx1-cKO* vs. control and  $\eta^2 = 0.07$  for *Chat-*  
160 *cKO* vs. control) include zero, indicating that the difference of the learning slope can be disregarded.  
161 The confidence intervals of the effect size for the probe test ( $\eta^2 = 0.17$  for *Emx1-cKO* vs. control and  $\eta^2$   
162  $= 0.05$  for *Chat-cKO* vs. controls) encompassed zero, indicating that the differences can be disregarded.  
163 **h** Cumulative distance from the hidden platform during the blocks. Learning was analyzed with  
164 parametric statistics (two-way mixed ANOVA with blocks as repeated-measures and cKO vs. control as  
165 between group variable). *Emx1-cKO* mice ( $n = 10$ ) and their control ( $n = 12$ ) learn equally ( $F = 63.18$ ,  $df$   
166  $= 6, 120$ ,  $P = 7E-35$ , partial  $\eta^2 = 0.76$ ; interaction between blocks and groups ( $F < 1$ ), with linear trend ( $F$   
167  $= 209.77$ ,  $df = 1, 20$ ,  $P = 4E-12$ , partial  $\eta^2 = 0.91$ ) and the slopes are identical (Student's  $t = 0.76$ ,  $df =$   
168  $20$ ,  $P = 0.46$ ,  $\eta^2 = 0.03$ ). *Chat-cKO* mice ( $n = 10$ ) and their control ( $n = 11$ ) also learn equally ( $F = 71.44$ ,  
169  $df = 6, 114$ ,  $P = 2E-36$ , partial  $\eta^2 = 0.79$ ; interaction between blocks and groups ( $F < 1$ ), with linear trend  
170 ( $F = 196.94$ ,  $df = 1, 19$ ,  $P = 1E-11$ , partial  $\eta^2 = 0.91$ ). The slopes are identical (Student's  $t = 0.03$ ,  $df =$   
171  $19$ ,  $P = 0.98$ ,  $\eta^2 = 0.00004$ ). **i** The confidence intervals of the effect size for the learning slopes includes  
172 zero for both *Emx1-cKO* and *Chat-cKO* vs. their respective controls, indicating that the learning slopes  
173 do not differ in the two groups. Data are expressed as means + SEM (**a**, **b**, **d** and **h**), or as medians with  
174 interquartile range **f**. \*\*\* $P < 0.001$ .

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