

Dopamine neuron dependent behaviors mediated by glutamate cotransmission

Susana Mingote^{1,2}, Nao Chuhma^{1,2}, Abigail Kalmbach^{1,2}, Gretchen M. Thomsen¹,
Yvonne Wang¹, Andra Mihali¹, Caroline Sferrazza¹, Ilana Zucker-Scharff¹,
Anna-Claire Siena², Martha G. Welch^{1,3,4}, José Lizardi-Ortiz⁵, David Sulzer^{1,2,5,6},
Holly Moore^{1,7}, Inna Gaisler-Salomon^{1,8}, Stephen Rayport^{1,2*}

¹ Department of Psychiatry, Columbia University, New York, NY 10032, USA

² Department of Molecular Therapeutics, NYS Psychiatric Institute, New York, NY 10032, USA

³ Department of Pediatrics, Columbia University

⁴ Department of Developmental Neuroscience, NYS Psychiatric Institute

⁵ Department of Neurology, Columbia University

⁶ Department of Pharmacology, Columbia University

⁷ Department of Integrative Neuroscience, NYS Psychiatric Institute

⁸ Department of Psychology, University of Haifa, Haifa 3498838, Israel

***Correspondence:**

Stephen Rayport, MD PhD
Columbia Psychiatry/Molecular Therapeutics
1051 Riverside Drive, Unit 62
New York, NY 10032 USA
stephen.rayport@columbia.edu

25 **ABSTRACT**

26 Dopamine neurons in the ventral tegmental area use glutamate as a cotransmitter. To
27 elucidate the behavioral role of the cotransmission, we targeted the glutamate-recycling
28 enzyme glutaminase (gene *GLS1*). In mice with a DAT-driven conditional heterozygous
29 (cHET) reduction of *GLS1* in their dopamine neurons, dopamine neuron survival and
30 transmission were unaffected, while glutamate cotransmission at phasic firing frequencies
31 was reduced, enabling focusing the cotransmission. DAT *GLS1* cHET mice showed
32 normal emotional and motor behaviors, and an unaffected response to acute
33 amphetamine. Strikingly, amphetamine sensitization was reduced and latent inhibition
34 potentiated. These behavioral effects, also seen in global *GLS1* HETs with a schizophrenia
35 resilience phenotype, were not seen in mice with an *Emx1*-driven forebrain reduction
36 affecting most brain glutamatergic neurons. Thus, a reduction in dopamine neuron
37 glutamate cotransmission appears to mediate significant components of the *GLS1* HET
38 schizophrenia resilience phenotype, and glutamate cotransmission appears to be
39 important in attribution of motivational salience.

40 INTRODUCTION

41

42 Dopamine (DA) neurons regulate several aspects of motivated behaviors (Bromberg-
43 Martin et al., 2010; Salamone and Correa, 2012; Schultz, 2013), and are involved in the
44 pathophysiology of neuropsychiatric disorders ranging from drug dependence to
45 schizophrenia (Robinson and Berridge, 2008; Winton-Brown et al., 2014). Like most CNS
46 neurons, DA neurons release multiple neurotransmitters (Trudeau et al., 2014). They
47 release DA with both slower modulatory actions (Tritsch and Sabatini, 2012), as well as
48 faster signaling actions (Ford et al., 2009; Chuhma et al., 2014). They variously release
49 glutamate (GLU) (Hnasko and Edwards, 2012) and GABA (Tritsch et al., 2016) as
50 cotransmitters, conferring both greater dynamic signaling range and heterogeneity in their
51 synaptic actions, as well as differential susceptibility to endogenous and exogenous
52 modulation (Chuhma et al., 2017). Discerning the behavioral role of DA neuron GLU
53 cotransmission has been challenging (Morales and Margolis, 2017).

54

55 DA neuron GLU cotransmission has a crucial neurodevelopmental role. The abrogation of
56 GLU cotransmission via a DA transporter (DAT)-driven conditional knockout (cKO) of
57 vesicular GLU transporter 2 (VGLUT2) (Hnasko et al., 2010; Stuber et al., 2010) impairs
58 survival and axonal arborization of DA neurons *in vitro*, and compromises the
59 development of the mesostriatal DA system *in vivo* leading to a 20% decrease in the
60 number of DA neurons (Fortin et al., 2012). GLU cotransmission also plays an important
61 role in modulating DA release by enhancing packing of DA into vesicles (Hnasko et al.,
62 2010) via vesicular synergy (El Mestikawy et al., 2011). Functionally, DAT VGLUT2 cKO
63 show about a 25% reduction in electrically-evoked DA release and about a 35% reduction
64 in DA content in the nucleus accumbens (NAc) (Hnasko et al., 2010; Fortin et al., 2012).
65 Behaviorally, DAT VGLUT2 cKO show modest deficits in emotional and motor behaviors
66 (Birgner et al., 2010; Fortin et al., 2012), normal reinforcement learning drive by DA neuron
67 activation but decreased response vigor (Wang et al., 2017), a blunted response to
68 psychostimulants (Birgner et al., 2010; Hnasko et al., 2010), and a paradoxical increase in
69 sucrose and cocaine seeking (Alsio et al., 2011). Whether the behavioral phenotypes of
70 DAT VGLUT2 cKO mice are due to the impact of the VGLUT2 deficit on DA neuron
71 development, DA transmission, or GLU synaptic actions is not clear.

72

73 Phasic activity of DA neurons projecting to the NAc encodes the incentive salience of
74 reward-predicting cues and invigorates cue-induced motivated behaviors (Bromberg-
75 Martin et al., 2010; Flagel et al., 2011). At the synaptic level in the striatum, DA neurons
76 make the strongest GLU connections in the NAc shell to cholinergic interneurons (ChIs)
77 (Chuhma et al., 2014; Mingote et al., 2015a). When DA neurons are driven at burst firing
78 frequencies — mimicking their *in vivo* phasic firing — their GLU postsynaptic actions drive
79 synchronized burst-pause sequences in ChIs (Chuhma et al., 2014) that are likely to be
80 important in salience encoding.

81

82 Dysregulated DA neuron firing is thought to disrupt salience processing leading to the
83 development of psychotic symptoms (Kapur, 2003; Winton-Brown et al., 2014). The
84 hyperdopaminergic state associated with positive symptoms of schizophrenia is modeled
85 in rodents by amphetamine sensitization (Peleg-Raibstein et al., 2008), which enhances

86 the motivational salience of drug-associated stimuli (Robinson et al., 2016). Interestingly,
87 amphetamine sensitization as well as gestational MAM treatment, a validated rodent
88 model of schizophrenia, selectively enhance activity of VTA neurons projecting to NAc
89 shell (Lodge and Grace, 2012), a key brain region associated with motivational salience
90 (Ikemoto, 2007) where DA neurons make the strongest GLU connections (Mingote et al.,
91 2015a). Dysregulation in salience processing is also thought to underlie the disruption of
92 latent inhibition (LI) seen in schizophrenia (Weiner, 2003). Disruption of LI is replicated in
93 rodents by amphetamine-induced increases in DA neuron activity (Young et al., 2005), in
94 particular increases in DA neuron phasic firing (Covey et al., 2016). Although DA neuron
95 GLU signals at burst frequencies control NAc shell activity, it remains to be established
96 whether GLU cotransmission is necessary for the expression of behaviors dependent on
97 salience attribution and associated with schizophrenia.

98
99 So we sought to temper GLU release at the higher firing frequency of bursts, independent
100 of DA release. For this we targeted phosphate-activated glutaminase (PAG), encoded by
101 *GLS1*, in order to reduce presynaptic glutamate synthesis modestly without affecting DA
102 neuron vesicular dynamics, as well as minimizing effects on DA neuron development. Most
103 presynaptic GLU arises from the action of PAG; once released, GLU is taken up by
104 neighboring astrocytes, metabolized to glutamine, and transferred back to presynaptic
105 terminals where it is converted to GLU by PAG (Marx et al., 2015). This GLU–glutamine
106 cycle is particularly important in sustaining GLU release with higher frequency firing
107 (Billups et al., 2013; Tani et al., 2014). Indeed, deletion (Masson et al., 2006) or
108 heterozygous reduction of *GLS1* (Gaisler-Salomon et al., 2009b) decreases GLU
109 neurotransmission at higher firing frequencies selectively. The global heterozygous *GLS1*
110 reduction impacts several DA dependent behaviors that underpin a schizophrenia
111 resilience phenotype (Gaisler-Salomon et al., 2009b), characterized by an attenuated
112 response to psychostimulant challenge, potentiated latent inhibition, procognitive effects
113 (Hazan and Gaisler-Salomon, 2014), together with CA1 hippocampal hypoactivity inverse
114 to the CA1 hyperactivity seen in patients with schizophrenia (Gaisler-Salomon et al.,
115 2009a; Schobel et al., 2009). Genetic mutations engendering resilience carry strong
116 therapeutic valence as they directly identify therapeutic targets (Mihali et al., 2012).

117
118 Here we show in DAT *GLS1* conditional heterozygous (cHET) mice — with a DAT-driven-
119 *GLS1* reduction — that DA neuron GLU cotransmission is reduced in a frequency
120 dependent manner, without affecting DA neuron development or DA release, and that
121 behaviors that rely on the motivational salience-encoding function of DA neurons are
122 selectively affected, with implications of DA neuron GLU cotransmission for schizophrenia
123 pharmacotherapy.

124 **RESULTS**

125 **Expression of PAG in DA neurons**

126 DA neurons immunoreactive for PAG are found in both the ventral tegmental area (VTA)
127 and substantia nigra pars compacta (SNc) in rat (Kaneko et al., 1990), but this has not
128 been examined in mouse. Moreover, the expression of PAG in DA neurons has never

129 been addressed stereologically. We immunostained ventral midbrain sections containing
130 the VTA and SNc for the DA-synthetic enzyme tyrosine hydroxylase (TH) and for PAG
131 (**Figure 1A; Figure 1—figure supplement 1A**). This revealed TH positive ($^+$) / PAG $^+$, PAG
132 only (TH negative ($^-$) / PAG $^+$), or TH only (TH $^+$ / PAG $^-$) neurons (**Figure 1B**).
133 Stereological counts in P25 mice showed that the three cell populations were present in
134 similar proportions in the VTA and SNc (**Figure 1C**). In contrast, DA neurons expressing
135 VGLUT2 are concentrated in the medial VTA (Yamaguchi et al., 2015).

136
137 Since DA neurons capable of GLU cotransmission express VGLUT2 (Hnasko et al., 2010;
138 Stuber et al., 2010) and the majority of neurotransmitter GLU is produced by PAG, DA
139 neurons expressing VGLUT2 should preferentially express PAG. To determine the number
140 of DA neurons expressing both VGLUT2 and PAG mRNA, we performed a single cell
141 reverse transcription (RT)-PCR analysis in P25-37 mice (**Figure 1D; S1B**). Since DA
142 neurons also corelease GABA (Tritsch et al., 2016), which could derive in part from
143 glutamic acid decarboxylase (GAD) metabolism of GLU (produced by PAG), we also
144 examined the expression of GAD67 mRNA (**Figure 1—figure supplement 1B**). We found
145 that VGLUT2 mRNA was highly concentrated in VTA DA neurons but rarely expressed in
146 SNc DA neurons. Importantly, TH $^+$ / VGLUT2 $^+$ neurons preferentially expressed PAG (9
147 out of 11 TH $^+$ / VGLUT2 $^+$ cells coexpressed PAG; $\chi^2 = 3.6$, $p = 0.035$), further supporting
148 the role of PAG in GLU cotransmission (**Figure 1D**). GAD67 was not found in TH $^+$ / PAG $^+$
149 neurons; while a few DA neurons expressed GAD67 (Kim et al., 2015 supplemental
150 information), a larger sample would be required to assess the role of PAG in GABA
151 cotransmission. Yet, some TH $^-$ / PAG $^+$ neurons in both the VTA (2/6 cells) and SN (6/13
152 cells) were GAD $^+$, identifying them as GABA neurons and suggesting that PAG contributes
153 to GABA synthesis in those neurons (**Figure 1—figure supplement 1B,C**). We also found
154 TH $^-$ / PAG $^+$ VTA neurons that coexpress VGLUT2 (**Figure 1—figure supplement 1B,C**),
155 identifying them as GLU neurons (Hnasko et al., 2012; Yamaguchi et al., 2015). Given that
156 coexpression of VGLUT2 decreases with maturation (Trudeau et al., 2014), we compared
157 the number of TH $^+$ / PAG $^+$ neurons in juvenile (P25) and adult (P60) wild-type mice. The
158 number of PAG $^+$ / TH $^+$ neurons in both the VTA and SNc increased modestly with age
159 (**Figure 1E**). Although DA neurons throughout the ventral midbrain express PAG, only
160 medial DA neurons that also express VGLUT2 are capable of GLU cotransmission, so the
161 impact of a PAG reduction on GLU cotransmission should be further restricted to VGLUT2-
162 expressing DA neurons.

163 **Conditional GLS1 reduction in DA neurons**

164 To address the specific function of DA neuron GLU cotransmission, we bred DAT^{IRESc^{re}}
165 mice (Bäckman et al., 2006) with floxGLS1 mice (Mingote et al., 2015b) to reduce GLS1
166 coexpression selectively (**Figure 2**). DAT and other DA neuron specific gene expression is
167 not affected in the ventral midbrain and striatum of DAT^{IRESc^{re}} HET mice (Bäckman et al.,
168 2006), which we confirmed (**Figure 2—figure supplement 1A**); the acute locomotor
169 response to amphetamine, a drug that targets DAT function, was also not affected (**Figure**
170 **2—figure supplement 1B,C**). We have shown previously that GLS1 expression from the
171 floxGLS1 allele is normal (Mingote et al., 2015b).

172
173 Conditional targeting was verified in DAT GLS1 cKO mice (DAT^{IRESc^{re}/+::GLS1^{lox/lox}}) mice.

174 PCR screens of genomic DNA showed the non-functional truncated (Δ) GLS1 allele in the
175 ventral midbrain of DAT GLS1 cKO mice, but not in forebrain regions that do not contain
176 DA neurons, the dorsal striatum (dStr), frontal cortex and hippocampus (**Figure 2A**). We
177 used single cell RT-PCR analysis to verify further the GLS1 inactivation in DA neurons
178 (**Figure 2B**). In DAT GLS1 cKO mice, GLS1 mRNA was absent in VTA cells expressing
179 TH mRNA. There was no impact on the number of DA neurons that expressed VGLUT2
180 (3/38 in DAT^{IRESc^{re}/+} vs. 6/30 DAT GLS1 cKO mice, $\chi^2 = 1.2$, $p = 0.27$). To confirm the
181 conditional strategy at the protein level, we examined TH and PAG immunoreactivity in the
182 VTA (**Figure 2C**). In DAT GLS1 cKO mice, all TH⁺ cells were PAG⁻, while neighboring
183 TH⁻ but PAG⁺ cells were seen, demonstrating the specificity of GLS1 targeting. Since
184 heterozygous reduction in GLS1 is sufficient to attenuate GLU transmission at higher-firing
185 frequencies (Gaisler-Salomon et al., 2009b), and to minimize compensatory mechanisms
186 seen in KOs (Bae et al., 2013), we used DAT GLS1 cHET mice (DAT^{IRESc^{re}/+}::GLS1^{lox/+})
187 and DAT^{IRESc^{re}/+} mice as controls (CTRL).

188 **Frequency-dependent attenuation of GLU cotransmission in DAT GLS1 cHETs**

189 To measure DA neuron synaptic transmission, we conditionally expressed
190 channelrhodopsin 2 (ChR2) in DA neurons using Ai32 mice (Madisen et al., 2012), to
191 obtain triple mutant DAT GLS1 cHET::ChR2 (DAT^{IRESc^{re}/+}::GLS1^{lox/+}::Ai32) and double
192 mutant control CTRL::ChR2 (DAT^{IRESc^{re}/+}::Ai32) littermates. We confirmed that the
193 expression of ChR2-EYFP was specific to DA neurons independent of GLS1 genotype
194 (**Figure 3—figure supplement 1A,B**). We also confirmed that TH⁺ / DAT⁻ striatal
195 interneurons (Xenias et al., 2015) do not express ChR2-EYFP (**Figure 3—figure**
196 **supplement 1C**). We then examined the impact of GLS1 deficiency on GLU
197 cotransmission in recordings from cholinergic interneurons (ChIs) and spiny projection
198 neurons (SPNs) in the NAc medial shell, the striatal hotspot for DA neuron GLU
199 transmission (Chuhma et al., 2014; Mingote et al., 2015a) (**Figure 3A**). ChIs and SPNs
200 were identified by soma size and electrophysiological signature, under current clamp
201 (**Figure 3—figure supplement 2A**). We confirmed that the intrinsic membrane properties
202 of ChIs and SPNs did not differ between genotypes (**Figure 3—figure supplement**
203 **2B,C,D,E**).

204
205 We measured DA neuron GLU cotransmission in DAT GLS1 cHET::ChR2 mice (P60-P76)
206 in the NAc shell with single pulse photostimulation (5 msec duration, delivered with a
207 10 sec interval) and burst photostimulation (5 pulses at 20 Hz, delivered with a 30 sec
208 interval) of DA neuron terminals. The burst photostimulation was chosen to mimic *in vivo*
209 phasic firing of DA neurons (Paladini and Roeper, 2014). Single photostimulation-evoked
210 EPSCs in both ChIs and SPNs (**Figure 3B**) were blocked by the AMPA-kainate receptor
211 antagonist CNQX, confirming GLU mediation ($n = 4$ ChIs per genotype; $n = 3$ SPNs per
212 genotype). As reported previously (Chuhma et al., 2014), the amplitude of EPSCs in ChIs
213 (CTRL 51 ± 6.0 pA) was greater than in SPNs (CTRL 21.5 ± 2.2 pA). The amplitude of
214 single-evoked EPSCs was unaffected in cHET mice (ChIs 66.1 ± 6.7 pA; SPNs 21.2 ± 2.3
215 pA) (**Figure 3B**), as were EPSC rise and decay time constants (**Figure 3—figure**
216 **supplement 2F,G**). Burst-induced EPSCs in ChIs and SPNs showed short-term
217 depression in both genotypes that was significantly greater in cHETs (**Figure 3C**). This
218 was particularly evident when EPSC amplitudes were normalized to the first EPSC in the

219 burst, which showed no genotypic difference (**Figure 3C, graphs**). In CTRL mice, EPSCs
220 in ChIs decreased to $48 \pm 6.0\%$ with the second pulse and to $23 \pm 4.2\%$ with the fifth, while
221 in cHET mice EPSCs decreased to $20 \pm 6.3\%$ with the second and to $14 \pm 3.3\%$ with the
222 fifth. The rundown was apparently faster in SPNs (**Figure 3C, bottom traces and graph**); in
223 CTRL mice, EPSCs decreased to $48 \pm 6.0\%$ with the second pulse and to $24 \pm 6.4\%$ with
224 the fifth, while in cHET mice EPSC amplitude decreased to $25 \pm 4.9\%$ with the second,
225 and to $16 \pm 1\%$ with the fifth, which was close to baseline. Observing a more rapid
226 frequency-dependent EPSC depression in cHETs in both ChIs and SPNs, and no
227 differences in their intrinsic properties (**Figure 3—figure supplement 2B,C,D,E**), is
228 consistent with a presynaptic reduction in PAG. The average amplitude and frequency of
229 spontaneous EPSCs, measured in both the SPNs and ChIs, showed no genotypic
230 difference (**Figure 3—figure supplement 2H,I**), indicating that GLU inputs mostly from
231 forebrain regions, as well as signaling through postsynaptic GLU receptors, was
232 unaffected in DAT GLS1 cHETs.

233
234 At the striatal circuit level, DA neuron control of ChI firing in the medial NAc shell (Chuhma
235 et al., 2014) was attenuated in cHET mice (**Figure 3D**). We quantified this using the firing
236 ratio, the firing frequency during train photostimulation (0–0.5 s from the onset of train)
237 divided by the preceding 2 s of baseline firing. There were no genotypic differences in
238 baseline firing frequencies (CTRL 4.7 ± 1.1 Hz; cHET 3.9 ± 0.6 Hz; ANOVA, $F_{(1,33)} = 0.60$,
239 $p = 0.444$). The firing ratio in CTRL mice was 4.3 ± 0.7 compared to 2.1 ± 0.2 in cHET mice,
240 which was significantly reduced (**Figure 3D, right**). In the subsequent half-second window,
241 the firing ratio reversed to below baseline in CTRL (0.6 ± 0.08) and cHETs (0.7 ± 0.11),
242 which did not differ (**Figure 3D, right**). This reduction in firing is mainly mediated by
243 activity-dependent components, and less so by DA D2-mediated inhibition (Chuhma et al.,
244 2014). Color-coded tables with a 50 msec window (**Figure 3E**) clearly show greater burst
245 firing in CTRL than in cHET, but little difference in the post-burst period. Dividing the 0.5 to
246 1 sec interval into 250 ms windows revealed no significant differences (one-way ANOVA:
247 0.5-0.75 period, CTRL 0.6 ± 0.12 vs. cHET 0.8 ± 0.12 , $F_{(1,34)} = 1.98$, $p = 0.168$; 0.75-1
248 period, CTRL 0.5 ± 0.08 vs. cHET 0.8 ± 0.1 , $F_{(1,34)} = 3.510$, $p = 0.070$). Thus, PAG plays an
249 important role in sustaining DA neuron GLU cotransmission at higher firing frequencies
250 and determines their ability to drive ChIs to fire in bursts.

251 252 **Normal DA transmission in DAT GLS1 cHETs**

253 To evaluate the specificity of the reduction in GLU cotransmission in DAT GLS1 cHET
254 mice further, we counted DA neurons by unbiased stereology, at P110 (**Figure 4A**). We
255 found no reduction in the number of DA neurons in the VTA (unilateral counts: CTRL 7548
256 ± 418 , cHET 7310 ± 450) or SNc (CTRL 6595 ± 373 , cHET 6781 ± 518). DA neurons in
257 cHET mice showed no differences in their intrinsic electrophysiological properties (**Figure**
258 **4—figure supplement 1**). Presynaptic DA content and turnover, in the NAc and dStr of
259 adult mice (P71-P110), did not significantly differ between genotypes (**Figure 4B**). We
260 performed fast-scan cyclic voltammetry (FSCV) in DAT GLS1 cHET::ChR2 mice (P71-
261 P85) to determine whether DA release dynamics were affected (**Figure 4C**). We compared
262 DA release evoked by single or burst photostimulation in the NAc medial shell. To
263 challenge DA neuron synapses further, single pulse stimulation was repeated twice
264 followed by a burst, and burst stimulation was repeated twice followed by a single. There

265 were no genotypic differences in DA release with either stimulation pattern (**Figure 4D**).
266 The decay time constant of DA responses did not differ significantly between genotypes
267 with single (CTRL 409 ± 30 ms; cHET 362 ± 26 ms; ANOVA, $F_{(1,23)} = 1.42$, $p = 0.245$) or burst
268 photostimulation (CTRL 540 ± 28 ms; cHET 482 ± 25 ms; ANOVA, $F_{(1,22)} = 2.12$, $p = 0.160$).
269 Thus, the conditional GLS1 reduction does not affect DA neuron DA release in the NAc
270 medial shell, where GLU cotransmission is strongest. Evoked DA release was not affected
271 in the NAc core (**Figure 4—figure supplement 2A,B,C**) nor in the dStr (**Figure 4—figure**
272 **supplement 2D,E,F**), indicating that DA storage and release dynamics throughout the
273 striatum are normal in DAT GLS1 cHETs. The effect sizes for all non-significant F values
274 were small to negligible (partial η^2 : Stereology = 0.014; DA content = 0.004; DA release:
275 range 0.0002 to 0.011). Thus, DA neuron development and DA transmission are
276 unaffected in DAT GLS1 cHETs.

277 **DA neuron dependent behaviors unaffected in DAT GLS1 cHETs**

278 We examined DA neuron dependent behaviors in DAT GLS1 cHETs (P90-120). We
279 assessed motor learning and coordination on the rotarod, which is affected following
280 neurotoxic loss of DA neurons (Rozas et al., 1998; Beeler et al., 2010) and also variably
281 affected in DAT VGLUT2 cKO mice (Fortin et al., 2012) (but see also Birgner et al., 2010;
282 Hnasko et al., 2010). DAT GLS1 cHET mice showed robust motor learning, which did not
283 differ from CTRL mice, on the first training day, when rotarod speed was 20 rpm (**Figure**
284 **5A**), and then accelerated to 30 rpm and 40 rpm on subsequent days. Novelty-induced
285 exploration in the open field was unaffected (**Figure 5B**). Mice used in this experiment
286 belonged to two cohorts that were subsequently used in the amphetamine-induced
287 locomotion and sensitization experiments. The results from the first cohort were replicated
288 in the second cohort; since there was no significant cohort effect (two-way ANOVA for total
289 locomotion in 60 min: cohort, $F_{(1,100)} = 50.9$, $p = 0.64$; cohort X genotype, $F_{(1,100)} = 2.6$,
290 $p = 0.11$), the cohorts were combined.

291 DA neuron loss can have anxiogenic effects (Drui et al., 2013), and DAT VGLUT2 cKO
292 mice showed decreased time spent in the center of the open field, indicative of increased
293 anxiety (Birgner et al., 2010). DAT GLS1 cHET and CTRL mice spent the same time in the
294 center of the open field (DAT GLS1 cHET = 301 ± 23 s; CTRL = 256 ± 43 s; one-way
295 ANOVA, no genotype effect, $F_{(1,102)} = 0.551$, $p = 0.46$). We tested the mice in the elevated
296 plus maze, another test of anxiety. A large cohort of mice (CTRL = 30 mice, cHET mice =
297 37 mice) was tested in an elevated plus maze with short arms. DAT GLS1 cHET and
298 CTRL mice spent the same time in the open arms (CTRL = 31.7 ± 3.8 s, cHET = 25.4 ± 3.5
299 s; one-way ANOVA, no genotype effect, $F_{(1,65)} = 1.11$, $p = 0.30$). A small effect size of 0.022
300 (partial η^2) was detected. So, we tested a second cohort in a more anxiogenic elevated
301 plus maze with longer arms (**Figure 5C**). We found no difference between genotypes in
302 the time spent in the open arms, nor was there a difference between time spent in the
303 open arms per entry (**Figure 5C**), or the time spent in the proximal and distal portions of
304 the longer arms (proximal time, CTRL = 47.4 ± 4.4 s, cHET = 40.9 ± 4.9 s, one-way
305 ANOVA, no genotype effect, $F_{(1,24)} = 0.887$, $p = 0.36$; distal time, CTRL = 43.7 ± 8.5 s,
306 cHET = 51.27 ± 7.90 s, $F_{(1,24)} = 0.41$, $p = 0.53$).

307 DA neurons play a role in fear conditioning (Fernandez Espejo, 2003; Wen et al., 2015).

308 Moreover, stopGLS1 HET mice, with a global GLS1 reduction, show reduced contextual
309 fear conditioning (Gaisler-Salomon et al., 2009b). However, DAT GLS1 cHET mice
310 showed normal tone- and context-dependent fear conditioning (**Figure 5D**).

311 DA neurons are the substrate for psychostimulant-induced behaviors (Lüscher and
312 Malenka, 2011), and DAT VGLUT2 cKO mice show a blunted locomotor response to
313 amphetamine (Birgner et al., 2010) and cocaine (Hnasko et al., 2010). stopGLS1 HET
314 mice also show a reduced response to acute amphetamine (Gaisler-Salomon et al.,
315 2009b), revealing a role of PAG in amphetamine-induced responses. DAT GLS1 cHET
316 mice responded to low (2.5 mg/Kg) and high (5 mg/Kg) doses of amphetamine
317 indistinguishably from CTRL mice (**Figure 5E**).

318 For all these behavioral experiments, effect sizes were negligible for nonsignificant F
319 values (partial η^2 : Rotarod genotype effect = 0.0002 and interaction = 0.0085; Open Field
320 genotype effect = 0.0064 and interaction = 0.0032; Center Time genotype effect = 0.0053,
321 Elevated Plus Maze genotype effect = 0.0002, Context Fear Conditioning genotype effect
322 = 0.013, Acute Amphetamine genotype effect = 0.0010 and interaction = 0.0054). Tone
323 fear conditioning did show a medium effect size (partial $\eta^2 = 0.067$), but a significant
324 genotypic effect was not seen in a replication experiment (**Figure 6F**). Thus attenuation of
325 phasic GLU cotransmission does not affect motor performance, exploratory behaviors,
326 anxiety regulation, fear conditioning or responses to acute amphetamine, revealing that
327 several DA neuron VGLUT2-dependent and PAG-dependent behaviors were normal in
328 DAT GLS1 cHET mice.

329 **Reduced amphetamine sensitization and potentiated latent inhibition in DAT GLS1** 330 **cHET mice**

331 stopGLS1 HET mice manifest a schizophrenia resilience phenotype characterized
332 behaviorally by reduced amphetamine sensitization and potentiated LI (**Figure 6—figure**
333 **supplement 1**, and Gaisler-Salomon et al., 2009b); as do Δ GLS1 HET mice, with a global
334 GLS1 reduction, generated by breeding floxGLS1 mice with mice expressing cre under the
335 control of the ubiquitous tamoxifen-inducible ROSA26 promoter (**Figure 6—figure**
336 **supplements 2 and 3**). The activity of DA neurons projecting to the NAc shell, the majority
337 of which are capable of GLU cotransmission, play a crucial role in both amphetamine
338 sensitization and LI (Ikemoto, 2007; Nelson et al., 2011), so we asked whether DAT GLS1
339 cHETs display similar behavioral phenotypes.

340 We tested DAT GLS1 cHET mice (P90-P120) for amphetamine sensitization, following the
341 protocol schematized in **Figure 6A**. Two cohorts were tested, since there was no
342 difference between the cohorts (ANOVA cohort effect: CTRL veh, $F_{(1,17)} = 0.37$, $p = 0.872$;
343 cHET Veh, $F_{(1,15)} = 0.49$, $p = 0.494$; CTRL Amph, $F_{(1,19)} = 0.94$, $p = 0.346$; cHET Amph,
344 $F_{(1,19)} = 3.752$, $p = 0.068$) they were combined. With daily amphetamine injections
345 (2.5 mg/kg) over 5 days, CTRL mice showed an increase in drug-induced
346 hyperlocomotion, characteristic of a sensitized response (**Figure 6B**), while cHET mice
347 showed no increase in hyperlocomotion. Ten days later, all mice were tested, first with a
348 vehicle challenge (Day 18) and then with amphetamine (2.5 mg/kg; Day 19). The vehicle
349 challenge revealed a modest but significant conditioned response in the Amph-treated
350 groups. During the amphetamine challenge, amphetamine-treated CTRL mice showed a

351 significant sensitized response, while amphetamine-treated cHET mice showed a
352 significant but smaller sensitized response (**Figure 6B**, gray area). Further comparison of
353 the locomotor response during the 90 min post-amphetamine (**Figure 6C**) showed no
354 difference between vehicle-treated cHET and CTRL mice, but a significantly smaller
355 sensitized response in amphetamine-treated cHET mice compared to amphetamine-
356 treated CTRL mice. Thus attenuating phasic GLU cotransmission blocks the induction of
357 amphetamine sensitization and reduces the expression of sensitization, after a withdrawal
358 period.

359 LI is characterized by an attenuated response to a conditioned stimulus (CS) presented
360 without reinforcement prior to being paired with an unconditioned stimulus (US) (Weiner,
361 2003). LI is potentiated by neurotoxin-induced loss of DA neurons projecting to the NAc
362 shell (Joseph et al., 2000; Nelson et al., 2011), which would affect GLU cotransmission.
363 We asked whether DAT GLS1 cHETs show potentiated LI, using the protocol schematized
364 in **Figure 6D**. On Day 1, mice (P90-120) were assigned either to a preexposure (PE)
365 group that received 20 tone exposures prior to tone (CS) - shock (US) pairing, or to a non-
366 preexposure (NPE) group that received only the CS-US pairing. The number of CS pre-
367 exposures was limited so as not to elicit LI in the PE group, enabling detection of
368 potentiated LI. On Day 2, freezing to context was tested in the same chamber; there was
369 no genotypic difference between the NPE and PE groups (CTRL NPE = 20 ± 4.5 s; cHET
370 NPE = 35 ± 5.4 s; CTRL PE = 39.9 ± 5.9 s; cHET PE = 36.8 ± 5.7 s; two-way ANOVA;
371 genotype factor, $F_{(1,32)} = 1.00$, $p = 0.323$; preexposure factor, $F_{(1,32)} = 3.46$, $p = 0.074$;
372 interaction, $F_{(1,32)} = 2.358$, $p = 0.134$). On Day 3, mice were put in a different context and
373 presented with the CS. Less freezing during CS presentation in PE compared to NPE
374 groups reflects potentiation of LI. During the 3 min before CS presentation, both CTRL and
375 cHET mice showed less than 20% freezing, and there was no difference between the NPE
376 and PE groups (**Figure 6E**). During CS presentation, CTRL mice showed increased
377 freezing with no difference between the NPE and PE groups (**Figure 6E**, left graph),
378 revealing the learned tone-fear association and no LI. In contrast, the cHET PE group
379 showed less freezing in comparison to the NPE group, revealing potentiated LI (**Figure 6E**,
380 right graph). Importantly, when analyzing the total freezing during the CS presentation and
381 comparing responses between genotypes directly, the cHET NPE group did not differ from
382 the CTRL NPE group, showing that aversive associative learning *per se* was not affected
383 in cHETs (**Figure 6F**), replicating previous findings (**Figure 5D**). Thus, the restricted GLS1
384 reduction in DA neurons is sufficient to reduce amphetamine sensitization and potentiate
385 LI.

386 **Normal amphetamine sensitization and no potentiation of latent inhibition in EMX1** 387 **GLS1 cHET mice**

388 It is striking that the behavioral phenotypes seen in GLS1 HETs were engendered by the
389 restricted GLS1 reduction in DA neurons, and apparently do not depend on GLS1
390 reductions in forebrain where GLS1 and PAG are highly expressed (Kaneko, 2000;
391 Gaisler-Salomon et al., 2012). To verify this, we made a forebrain-restricted GLS1
392 reduction by breeding EMX1^{IRESc^{re}} mice with floxGLS1 mice to generate EMX1 GLS1
393 cHET progeny (**Figure 6G** and **Figure 6—figure supplement 4**). EMX1 GLS1 cHETs
394 (P85-107) did not differ from CTRL mice in their novelty-induced locomotion in the open

395 field (**Figure 6H**) and amphetamine sensitization (**Figure 6I**). The effect size for the
396 nonsignificant drug treatment X time X genotype interaction was negligible (partial η^2 :
397 0.005). EMX1 GLS1 cHETs (P80-96) did not show potentiation of LI (ES for nonsignificant
398 PE X genotype interaction = 0.016) (**Figure 6J**). To confirm in EMX1 GLS1 cHETs that the
399 limited number of pre-exposures did not elicit LI and yet was sufficient to reveal
400 potentiation of LI, we tested for clozapine-induced potentiation of LI (Gaisler-Salomon et
401 al., 2009b) (**Figure 6—figure supplement 5A**). In both CTRL and EMX1 GLS1 cHETs,
402 clozapine treatment on Day 1, prior to testing potentiated LI in the PE groups (**Figure 6—**
403 **figure supplement 5B**), had no effect in the NPE groups showing that it did not affect
404 learning. Similar clozapine effects were seen in Δ GLS1 HET and DAT GLS1 cHET mice
405 (**Figure 6—figure supplement 5C**). The lack of further potentiation of LI in Δ GLS1 HET
406 and DAT GLS1 cHET mice suggests that clozapine treatment and GLS1 deficiency in DA
407 neurons each either induce maximal potentiation of LI, or involve shared mechanisms so
408 that GLS1 deficiency occludes clozapine-induced potentiation of LI. In summary, our
409 results argue that reducing GLS1 in DA neurons is not only sufficient but also necessary
410 for the reduction of amphetamine sensitization and potentiation of LI.

411 **DISCUSSION**

412 Here we show that a conditional heterozygous reduction of GLS1 in DA neurons
413 selectively attenuates GLU cotransmission at phasic firing frequencies without directly
414 affecting DA transmission, enabling a focus on the role of DA neuron GLU cotransmission.
415 The conditional GLS1 reduction in DAT GLS1 cHETs is extremely restricted as it affects
416 only those DA neurons that express GLS1 and also VGLUT2 (about one third of VTA
417 neurons and one tenth of SN neurons) and are thus capable of GLU cotransmission. The
418 conditional GLS1 reduction attenuates DA neuron excitatory drive in a frequency-
419 dependent manner, further adding to its restricted impact, and revealing a crucial role of
420 PAG in GLU cotransmission. Strikingly, this modest GLS1 heterozygous reduction
421 profoundly affects two DA neuron dependent behaviors, namely psychostimulant
422 sensitization and LI (**Table 1**), suggesting that phasic GLU cotransmission regulates
423 attribution of motivational salience. The affected behaviors are components of the
424 schizophrenia resilience profile of global GLS1 HETs and align with the actions of
425 antipsychotic drugs, revealing that potential therapeutic effects of PAG inhibition may be
426 mediated by attenuated DA neuron GLU cotransmission.

427 **PAG in DA neurons supports GLU cotransmission during sustained firing**

428 A stereological analysis of PAG expression in DA neurons revealed that about half of DA
429 neurons express PAG in both the VTA and SNc, in contrast to VGLUT2 expression, which
430 is mostly restricted to DA neurons in the VTA (Yamaguchi et al., 2015). The function of
431 PAG in SNc DA neurons incapable of GLU cotransmission is still uncertain, although we
432 show that a minor reduction of PAG expression in those neurons had no impact on their
433 survival or intrinsic physiology, nor did it affect DA transmission in the dStr or motor
434 behaviors controlled by the dStr. In contrast, DA neurons capable of GLU cotransmission
435 (TH^+ / $VGLUT2^+$ cells) preferentially express PAG, and a reduction of PAG in those VTA
436 DA neurons was sufficient to attenuate phasic GLU cotransmission in the NAc shell and
437 impact behaviors controlled by the NAc, revealing the important role of PAG in DA neuron

438 GLU cotransmission.

439 The reduction in PAG activity of about 20% seen in stopGLS1 HET brain slices (El Hage et
440 al., 2012) is associated with about a 15% reduction in GLU content (Gaisler-Salomon et
441 al., 2009b) that presumably reflects a presynaptic diminution, since the highest
442 concentrations of GLU are intracellular (Danbolt, 2001). Decreases in presynaptic GLU
443 lead to decreases in vesicular GLU content and synaptic efficacy (Ishikawa et al., 2002). In
444 DAT GLS1 cHETs, the first EPSC elicited by burst photostimulation was unaffected, as
445 was observed in cultured GLS1 KO neurons (Masson et al., 2006), indicating that the
446 readily releasable vesicle pool is replete. Smaller subsequent responses may reflect either
447 diminished filling of the recycling pool (Alabi and Tsien, 2012), or decreased probability of
448 release of vesicles with diminished GLU content (Iwasaki and Takahashi, 2001).

449 PAG expression in VTA DA neurons is weak to moderate relative to other brain regions
450 (Kaneko, 2000). The fact that a heterozygous GLS1 reduction in DA neurons is sufficient
451 to decrease synaptic efficacy indicates that PAG levels are not only lower but rate limiting.
452 Single cell RT-PCR studies show that DA neurons also have low VGLUT2 mRNA copy
453 numbers (Trudeau et al., 2014). Lower VGLUT2 expression would place further demands
454 on the GLU-glutamine cycle to sustain synaptic transmission during periods of high
455 activity, given that vesicular loading depends both on cytosolic GLU concentration and
456 vesicular transporter number (Wilson et al., 2005). This indicates that the DA neuron GLU
457 cotransmission in DAT GLS1 cHETS is highly dependent on PAG activity, and suggests
458 that the global reduction in PAG activity in global GLS1 HETs affects DA neuron GLU
459 cotransmission preferentially.

460 **Role DA neuron glutamate cotransmission**

461 Discerning the behavioral role of DA neuron GLU cotransmission has been challenging
462 because of the impact of knocking out VGLUT2 in DA neurons on DA function. In DAT
463 VGLUT2 cKOs, DA neuron function is affected profoundly due to the developmental role of
464 VGLUT2 in DA neurons (Fortin et al., 2012). In DAT GLS1 cHETs, DA neuron DA
465 functions appear normal; GLS1 reduction affects neither the survival of DA neurons nor
466 their intrinsic electrophysiological properties. VGLUT2 also plays an important role in
467 vesicular DA uptake (Hnasko et al., 2010), but there was no impact of GLS1 deficiency on
468 DA content or release, even when DA terminals were stimulated repeatedly to increase the
469 demand on DA release. Since DAT GLS1 cHET DA neurons show normal GLU
470 cotransmission with low-frequency activity, our results suggest that modestly reduced
471 presynaptic GLU is sufficient for the maintenance of normal vesicular DA dynamics in
472 adulthood. Alternately, DA neuron GLU release may arise from segregated release sites
473 (Zhang et al., 2015), so reduced vesicular GLU filling would not affect DA release. In the
474 absence of a direct effect on synaptic DA transmission, finding that GLU signaling with
475 high-frequency activity was affected selectively in DAT GLS1 cHETs allowed us to focus
476 on the function of GLU cotransmission.

477 DAT GLS1 cHET mice do not show several behavioral phenotypes of DAT VGLUT2 cKOs,
478 such as decreased novelty-induced locomotion, motor deficits on the rotarod, an anxiety
479 phenotype, or blunted responses to psychostimulants (Birgner et al., 2010; Hnasko et al.,
480 2010; Fortin et al., 2012). Presumably the behaviors not affected by a mild disruption in

481 GLU cotransmission, are sensitive to manipulations that affect both DA and GLU
482 transmission, such as in DAT VGLUT2 cKO mice. Strikingly, the subtle activity-dependent
483 reduction in DA neuron GLU cotransmission in DAT GLS1 cHETs had major effects on
484 amphetamine sensitization and LI, arguing that DA neuron GLU cotransmission is a key
485 regulator of these behaviors. DA signaling increases with psychostimulant sensitization
486 (Vezina, 2004; Bocklisch et al., 2013; Covey et al., 2014). While DA neuron DA signaling
487 was not affected in DAT GLS1 cHETs, changes in DA signaling with repeated
488 psychostimulant administration are likely, although attenuated due to reduced DA neuron
489 GLU cotransmission. DA neuron excitatory connections to SPNs in the NAc core are
490 modestly but significantly increased weeks after chronic psychostimulant (Ishikawa et al.,
491 2013); psychostimulant-induced plasticity may be even stronger at DA neuron excitatory
492 connections to ChIs in the NAc shell (Chuhma et al., 2014). At the VTA-NAc circuit level,
493 reducing GLU cotransmission may attenuate increases in DA neuron activity associated
494 with sensitization (Bocklisch et al., 2013). While subsequent circuit effects impacted by
495 reduced GLU cotransmission involve DA signaling, we show here for the first time that the
496 attenuation of GLU cotransmission in the absence of developmental alterations and direct
497 effects on DA transmission has strong and selective behavioral effects, revealing a new
498 mechanism through which DA neurons control behavior.

499 **Implications for salience and schizophrenia-resilience**

500 DA neuron activity mediates both amphetamine sensitization and LI by encoding
501 motivational salience of relevant events (Young et al., 2005; Bromberg-Martin et al., 2010;
502 Robinson et al., 2016). Our results suggest that DA neuron GLU signaling plays a key role
503 in salience attribution. In amphetamine sensitization, increases in DA neuron firing are
504 restricted to medial VTA DA neurons (Lodge and Grace, 2012), the majority of which are
505 capable of GLU cotransmission (Yamaguchi et al., 2015). Recent evidence suggests that
506 all abused drugs increase DA neuron activity to strengthen the motivational salience of
507 drug exposure or associated events (Covey et al., 2014). DAT GLS1 cHET mice do not
508 show sensitization to amphetamine with repeated administration, and after a withdrawal
509 period show reduced expression of sensitization. Similar results were found in mice with a
510 conditional NR1 deletion in their DA neurons that resulted in a dramatic reduction in phasic
511 firing (Zweifel et al., 2009). While the development of sensitization was unaffected in DAT
512 NR1 cKO mice, the mice showed reduced expression of sensitization weeks after
513 withdrawal (Zweifel et al., 2008). Taken together, several lines of evidence suggest that
514 phasic DA neuron GLU signals facilitate sensitization by determining how rapidly and
515 efficiently pathological levels of salience are attributed to drug exposure. In contrast, DAT
516 NR1 cKO showed a reduction in conditioned responses to context not seen in the present
517 study, suggesting that the abrogation of both DA and GLU phasic transmission must be
518 affected to impact the development of drug-induced conditioned responses.

519 In LI, it is thought that the activity of DA neurons in the NAc updates the salience of a
520 preexposed stimulus during the conditioning phase by integrating previous with current
521 behavioral experiences (Young et al., 2005). Thus, the potentiation of LI seen in DAT
522 GLS1 cHET mice represents a failure of DA neurons to increase the salience of the
523 inconsequential preexposed stimulus under changed reinforcement contingencies during
524 conditioning. The temporal precision of the DA neuron GLU signal makes it particularly

525 suitable for updating salience. In the NAc medial shell, a structure known to regulate
526 motivational salience (Ikemoto, 2007), DA neuron GLU connections to ChIs drive them to
527 fire in bursts (Chuhma et al., 2014). Direct optogenetic excitation of ChIs in the NAc
528 shell — as would result from DA neuron GLU actions — does not drive reinforcement
529 learning on its own but instead modulates learning (Lee et al., 2016), and GLU
530 cotransmission is not required for self-administration reinforced by DA neuron activation
531 (Wang et al., 2017). Instead, our behavioral results suggest that DA neuron GLU signals
532 modulate learning by regulating the attribution of motivational salience to relevant events
533 via their direct control over ChI activity.

534 In the context of schizophrenia, the behaviors affected in DAT GLS1 cHETs align with the
535 schizophrenia resilience phenotype of stopGLS1 HET (Gaisler-Salomon et al., 2009b), as
536 well as Δ GLS1 HET mice, both with a global GLS1 heterozygous reduction. Several other
537 phenotypes of GLS1 HETs, a blunted locomotor response to novelty, diminished sensitivity
538 to acute amphetamine or reduced contextual fear conditioning were not seen in DAT GLS1
539 cHETs and so apparently do not depend on GLU cotransmission. Furthermore, none of
540 these behavioral deficits were recapitulated in EMX1 cHETs, with a forebrain-restricted
541 GLS1 reduction, demonstrating that PAG in DA neurons is necessary for amphetamine
542 sensitization and potentiation of LI, and reinforcing the likelihood that DA neuron GLU
543 cotransmission is particularly sensitive to PAG reduction.

544 Modeling resilience in mice using transgenic approaches offers a direct path to
545 intervention, as resilience mutations point directly to therapeutic targets (Mihali et al.,
546 2012). Supported by the recent demonstration of VGLUT2 expression — and thus of GLU
547 cotransmission — in primate DA neurons (Root et al., 2016), the therapeutic potential of
548 PAG inhibition as a pharmacotherapy for schizophrenia (Mingote et al., 2015b) may
549 involve tempering DA neuron GLU cotransmission. Finally, our findings put forward the
550 possibility that an increase in GLU cotransmission in the NAc may contribute to the
551 pathophysiology of schizophrenia, in particular to aberrant salience leading to psychosis.
552 Increased activity in the midbrain and NAc has been associated with aberrant salience
553 attribution to irrelevant stimuli in patients with psychosis or individuals at high risk
554 (Romaniuk et al., 2010; Roiser et al., 2013), while increased NAc activity does not
555 correlate with increased dopamine synthesis capacity (Roiser et al., 2013). This
556 inconsistency would be reconciled if increased activity in the VTA and NAc in SCZ is
557 associated with a pathological increase in GLU cotransmission with less of an increase in
558 DA transmission.

559 **MATERIALS AND METHODS**

560 **Experimental animals**

561 Mice were handled in accordance with guidelines of the National Institutes of Health *Guide*
562 *for the Care and Use of Laboratory Animals*, under protocols approved by the Institutional
563 Animal Care and Use Committees of Columbia University and New York State Psychiatric
564 Institute. We used stopGLS1 (JIMSR Cat# JAX:017956, RRID:IMSR_JAX:017956,
565 Gaisler-Salomon et al., 2009b) and floxGLS1 mice (IMSR Cat# JAX:017894,
566 RRID:IMSR_JAX:017894, Mingote et al., 2015b), both on a 129SVE-F background, and

567 DAT^{IREScree} (IMSR Cat# JAX:006660, RRID:IMSR_JAX:006660), EMX1^{IREScree} (IMSR Cat#
568 JAX:005628, RRID:IMSR_JAX:005628) and Rosa26^{creERT2} mice (IMSR Cat# JAX:008463,
569 RRID:IMSR_JAX:008463) on a C57BL/6 background. These mice were used to generate
570 DAT GLS1 cHET or cKO mice, EMX1 GLS1 cHET or cKO mice, and ΔGLS1 HET mice, all
571 on a mixed 129SVE-F and C57BL/6 background. Inducible Rosa26^{creERT2::GLS1^{lox/+}} mice
572 were used to produce a global heterozygous GLS1 deletion in adulthood by administration
573 of tamoxifen. Tamoxifen (Sigma-Aldrich, T5648) was dissolved in a peanut oil/ethanol (9:1
574 mixture) at 25 mg/ml, solubilized by vortexing for 5 minutes and warming to 37 °C for
575 several hours. Mice received 0.2 mL i.p. (5 mg tamoxifen) daily for 5 successive days.
576 Tamoxifen-treated Rosa26^{creERT2::GLS1^{Δ/+}} mice were then crossed with wild-type C57BL/6
577 mice (JAX, strain 000664) to generate ΔGLS1 HETs.

578 Immunohistochemistry

579 Mice were anesthetized with ketamine (90 mg/kg) + xylazine (7 mg/kg) and perfused with
580 cold PBS followed by 4% paraformaldehyde (PFA), the brains removed, post-fixed
581 overnight in 4% PFA, and cut at 50 μm with a vibrating microtome (Leica VT1200S).
582 Coronal slices were collected into a cryoprotectant solution (30% glycerol, 30% ethylene
583 glycol in 0.1 M Tris HCl [pH 7.4]) and kept at -20 °C until processing. Sections were
584 washed in PBS (100 mM; pH 7.4) and incubated in glycine (100 mM) for 30 min to quench
585 aldehydes. Non-specific binding was blocked with 10% normal goat serum (NGS;
586 Millipore) in 0.1% PBS Triton X-100 for 2 hours (PBS-T). Primary antibodies used were
587 anti-TH (1:10,000 dilution, mouse monoclonal, Millipore Cat# MAB318 Lot#
588 RRID:AB_2201528), anti-PAG (1:10,000 dilution, rabbit polyclonal, Norman Curthoys,
589 Colorado State), and anti-GFP (1:2000 dilution; rabbit polyclonal, Millipore Cat# AB3080
590 Lot# RRID:AB_11211640). Secondary antibodies were: anti-rabbit Alexa Fluor 488 (1:200
591 dilution, ThermoFisher Scientific Cat# A-21206 Lot# RRID:AB_2535792) and anti-mouse
592 Alexa Fluor 594 (ThermoFisher Scientific Cat# A-21203 Lot# RRID:AB_2535789). Primary
593 antibodies in 0.02% PBS-T and 2% NGS were applied for 24 h at 4°C. Sections were then
594 washed with PBS and secondary antibodies applied for 45 min in 0.02% PBS-T at room
595 temperature. Sections were mounted on slides and cover slipped with Prolong Gold
596 aqueous medium (ThermoFisher Scientific) and stored at 4 °C. Fluorescence images were
597 acquired with a Fluoview FV1000 (Olympus) or A1 (Nikon) confocal laser scanning
598 microscope, or a Axiovert 35M (Zeiss) epifluorescence microscope.

599 Stereological analysis of DA neuron number

600 The SNc and VTA were delineated based on low-magnification images of TH
601 immunostaining. Stereological counts were made of DA neurons using the Optical
602 Fractionator Probe in Stereo Investigator (MBF Bioscience) at regular predetermined
603 intervals (grid size: x = 170 μm, y = 120 μm) with an unbiased counting frame (x = 55μm, y
604 = 33.6 μm; dissector height, z = 33.6 μm). The actual mounted section thickness averaged
605 24 μm (50% shrinkage from the unprocessed section thickness).

606 Single-cell reverse transcription PCR

607 Sampling was done from acute ventral midbrain slices. Mice (male or female WT or DAT
608 GLS1 cKO and littermate control mice) were decapitated and brains quickly removed in

609 ice-cold high-glucose artificial cerebrospinal fluid (aCSF; in mM: 75 NaCl, 2.5 KCl, 26
610 NaHCO₃, 1.25 NaH₂PO₄, 0.7 CaCl₂, 2 MgCl₂ and 100 glucose, adjusted to pH 7.4). 300
611 μm coronal midbrain sections were cut on a vibrating microtome (Leica VT1200S).
612 Sections were preincubated for at least one hour at room temperature in high sucrose
613 aCSF saturated with carbogen (95% O₂ 5% CO₂), then mounted in a chamber on the
614 stage of an upright microscope (Olympus BX61WI) continuously perfused with standard
615 aCSF (in mM: 125 NaCl, 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂ and 25
616 glucose, pH 7.4; perfusion 1 ml/min) saturated with 95% O₂ 5% CO₂. Sampling was done
617 from the VTA and SN, using the medial lemniscus as the dividing boundary. Glass pipettes
618 for sampling were fabricated from thin wall glass capillaries (Harvard Apparatus), which
619 were cleaned with water and ethanol and then treated at 200 °C for 4 hours to inactivate
620 RNase. Pipettes were filled with 5 μl DEPC treated water. Whole cell recordings were
621 made using digitally enhanced DIC optics, at room temperature (21-23 °C). The cytosol of
622 single neurons was aspirated using a glass pipette. In most cases, the nucleus was
623 aspirated along with the cytosol. The sampled single-cell cytosol was ejected in a 0.2 ml
624 PCR tube with a sample mixture of 0.5 μl dithiothreitol (DTT; 0.1 M, Invitrogen), 0.5 μl
625 RNase inhibitor (RNaseOUT, 40 U/ml, Invitrogen), 1 μl random hexamers (50 μM, Applied
626 Biosciences) and 5 μl DEPC treated water. Sampling was done and the tubes with sample
627 mixture were kept on ice until reverse transcription. The sample mixture was treated at 70
628 °C for 10 min. The second mixture (4 μl x5) was added to the sample mixture. First strand
629 buffer (Invitrogen), 0.5 μl RNase inhibitor, 1 μl dNTP mix (10 mM, Invitrogen), 1.5 μl DTT,
630 and 1 μl reverse transcriptase (SuperScript III, 200 U/μl, Invitrogen). Reverse transcription
631 was done at 50 °C for 50 min, and stopped by raising the temperature to 85 °C for 5 min.
632 Subsequently, 0.5 μl RNase (2 u/μL, Invitrogen) was added to each tube and incubated at
633 37 °C for 20 min to eliminate RNA contamination. The cDNA produced by reverse
634 transcription was frozen at -80 °C pending PCR analysis. After reverse transcription, cDNA
635 was amplified by nested PCR. First round PCR primers spanned at least one intron to
636 preclude amplification of genomic DNA. TH and GAD67 primer sequences for both first
637 and second round PCR were obtained from Liss *et al.* (1999); VGLUT2 primer sequences
638 for the second round were obtained from Mendez *et al.* (2008). VGLUT2 first round
639 primers and GLS1 primers for both the first and second round PCR were custom designed,
640 with the following sequences (5' to 3'): VGLUT2 first round upper caccgcccgaataaccacgg
641 and lower gcccaaagaccggttagc; GLS1 first round upper ttgtgtgacttctctaat and lower
642 atggtgtccaaagtgtag; GLS1 second round upper gtggcatgtatgacttct and lower
643 atggtgtccaaagtgtag. Products of the second round PCR were confirmed by sequencing,
644 and had the following sizes (in bp): TH 377, GAD67 702, VGLUT2 250 and GLS1 512.
645 Both first and second round amplifications was done with the following temperature cycle:
646 3 min at 94 °C, 35 cycles of 30 sec at 94 °C, 1 min at 58 °C, 3 min at 72 °C, followed by 7
647 min at 72 °C. 2 μl of the first round PCR product was used for the second round. PCR
648 products were separated on 1.5% agarose gels. Only clear bands were counted as
649 positive; runs with unclear bands or bands of incorrect size were discarded.

650 **RNA extraction and reverse transcription quantitative PCR (RT-qPCR)**

651 We used male and female juvenile (P30) DAT^{IRESc^{re}+/+} and littermate controls. Mice were
652 anesthetized with ketamine/xylazine. The ventral midbrain and dorsal striatum were
653 dissected and put in tubes with 300 μl Qiazol (Qiagen), a RNase-inhibitor buffer, and

654 rapidly frozen on dry ice. RNA extraction was done using the RNeasy Lipid Mini Kit
655 (Qiagen), according to the manufacturer's instructions, and stored in RNase-free water at -
656 80 °C until further processing. RNA concentrations were standardized to 1 µg per 10 µl
657 water using a NanoDrop 1000 Spectrophotometer (ThermoScientific). The 260:280 nm
658 absorbance ratio was measured to assess RNA quality; samples were excluded if the ratio
659 was outside the range 2.0 ± 0.2 , or if the RNA concentration was too low. Genomic DNA
660 elimination was performed using RNase-free DNase set (Qiagen). Reverse transcription
661 was carried with the RT² first-strand kit (Qiagen). Reverse transcription product (cDNA)
662 was diluted to a volume of 1 ml in water. The real time quantitative PCR (RT-qPCR) was
663 performed using an Opticon 2 DNA Engine (Bio-Rad) and microprofiler plates with primers
664 designed by SuperArray Biosciences (Qiagen). The primers were custom designed to
665 recognize cDNA for DAT, D1 and D2 receptors, TH, VMAT2. The cycle threshold (Ct)
666 values were normalized to GAPDH (Δ Ct). Relative copy number was obtained by
667 exponentiation of Δ Ct values (function $2^{-\Delta$ Ct}) multiplied by 1000.

668 **Quantitative GLS1 genotyping**

669 We used male and female adult (P90-150) Δ GLS1 HET mice and littermate controls, or
670 EMX1 GLS1 cHETs and littermate controls. Mice were anesthetized with
671 ketamine+xylazine, decapitated and brains quickly removed to ice-cold saline for
672 dissection. The hippocampus, prefrontal cortex, striatum, thalamus and ventral midbrain,
673 dissected from one hemisphere, were put in 96-well plates and sent to Transnetyx
674 (Cordova, TN) for quantitative genotyping using probe-based quantitative PCR (qPCR).
675 Allelic abundance was obtained from the mean of 4 qPCR determinations (2 runs done in
676 duplicate). The floxGLS1 and WT allele signals were normalized to the one-allele signal
677 from floxGLS1 heterozygous mice.

678 **Slice electrophysiology**

679 Recordings in the NAc shell were made from 300 µm coronal striatal slices, as described
680 previously (Chuhma et al., 2011). Animals were anesthetized with ketamine+xylazine.
681 Brains removed into ice-cold high-glucose aCSF saturated with carbogen (95% O₂ 5%
682 CO₂). The composition of the high-glucose aCSF was, in mM: 75 NaCl, 2.5 KCl, 26
683 NaHCO₃, 1.25 NaH₂PO₄, 0.7 CaCl₂, 2 MgCl₂ and 100 glucose, adjusted to pH 7.4. After 1
684 hour incubation in high-sucrose aCSF at room temperature to allow slices to recover,
685 slices were placed in a recording chamber with continuous perfusion of standard aCSF
686 equilibrated with carbogen, and maintained at 30-32 °C (TC 344B Temperature Controller,
687 Warner Instruments). Expression of ChR2 was confirmed by visualization of EYFP
688 fluorescence in DA neuron axons and varicosities. Whole-cell patch recording followed
689 standard techniques using glass pipettes (5–8 MΩ). For voltage clamp experiments, a
690 cocktail of antagonists was included in the perfusate to isolate AMPA-mediated responses:
691 SR95531 10 µM (GABA_A antagonist), CGP55345 3 µM (GABA_B antagonist), SCH23390
692 10 µM (D1 antagonist), (-)-sulpiride 10 µM (D2 antagonist), scopolamine 1 µM (muscarinic
693 antagonist) and dAP-5 50 µM (NMDA antagonist) (all from Tocris Bioscience). Patch
694 pipettes were filled with intracellular solution containing (in mM) 140 Cs⁺-gluconate
695 (voltage clamp recordings) or 140 K⁺-gluconate (current clamp recordings), 10 HEPES, 0.1
696 CaCl₂, 2 MgCl₂, 1 EGTA, 2 ATP-Na₂ and 0.1 GTP-Na₂ (pH 7.3). The Na⁺-channel blocker
697 lidocaine N-ethyl bromide (QX-314, 5 mM, Sigma-Aldrich) was added to the intracellular

698 solution in voltage clamp experiments to block active currents. For current clamp
699 experiments, no drugs were added to the perfusate; intracellular solution contained (in
700 mM): 140 K⁺-gluconate, 10 HEPES, 0.1 CaCl₂, 2 MgCl₂, 1 EGTA, 2 ATP-Na₂ and 0.1 GTP-
701 Na₂ (pH 7.3). Recordings were made with an Axopatch 200B (Molecular Devices); for
702 voltage clamp recordings (holding potential -70 mV), series resistance (6–35 MΩ) was
703 compensated online by 70%–80%. Liquid junction potentials (12–15 mV) were adjusted
704 online. ChR2 responses were evoked by field illumination with a high-power blue (470 nm)
705 LED (ThorLabs). GLU mediation was confirmed by blockade with 40 μM 6-cyano-7-
706 nitroquinoxaline-2,3-dione (CNQX, Tocris Bioscience). Data were filtered at 5 kHz with a 4-
707 pole Bessel filter, digitized (InstruTECH ITC-18 Interface, HEKA) at 5 kHz, and analyzed
708 using Axograph X (Axograph Scientific).

709
710 Recordings from putative DA neurons in adult (P59–P64) DAT GLS1 cHET mice and
711 CTRL littermates were made in 300 μm VTA/SN_c horizontal slices, blinded to genotype.
712 The medial optic tract defined the boundary between the SN_c and the VTA. SNc neurons
713 showing slow pacemaker firing and a prominent I_h were identified as DA neurons; in the
714 lateral VTA, large neurons with slow pacemaker firing and a prominent I_h were always
715 DAT-driven reporter positive (Chuhma, unpublished observation). VTA neurons in the
716 medial VTA with these properties are not always TH⁺ (Margolis et al., 2010), so VTA
717 recordings were restricted to the lateral VTA. Whole-cell patch recordings were made with
718 borosilicate glass pipettes (3–6 MΩ) with intracellular solution containing (in mM): 135 K⁺-
719 methanesulfonate, 5 KCl, 2 MgCl₂, 0.1 CaCl₂, 10 HEPES, 1 EGTA, 2 Na₂-ATP, 0.1 GTP
720 (pH 7.3), using an Axopatch 200B in fast current clamp mode. Since DA neurons were
721 spontaneously active, resting membrane potential was measured as the average of the
722 pacemaker fluctuation of the membrane potential after action potentials were truncated.
723 Input impedance was measured with -100 pA current pulses. Action potential threshold
724 was determined as the point where membrane potential change exceeded 10 mV/ms,
725 using AxographX automatic detection.

726 **Fast-scan cyclic voltammetry**

727 Recordings were done in adult (P71–P85) DAT GLS1 cHET::ChR2 and CTRL::ChR2 mice,
728 in 300 μm coronal slices through the striatum, as described previously for the
729 electrophysiology experiments. DA release was evoked by photostimulation (blue high-
730 power LED) and measured using carbon fiber electrodes, calibrated to 1 μM DA, post-
731 experiment. A triangle wave (-450 to +800 mV at 312.5 V/sec vs. Ag/AgCl) was applied to
732 the electrode at 10 Hz. Fibers were conditioned in the brain slice by cycling the fiber for 20-
733 30 minutes or until the current stabilized. Current was recorded using an Axopatch 200B
734 filtered at 10 kHz with a 4-pole Bessel filter, digitized at 25 kHz (ITC-18) using Igor Pro 6
735 (WaveMetrics) and analyzed with MATLAB R2014b (MathWorks).

736 **DA and DOPAC content**

737 To measure tissue DA and DOPAC content, mice underwent cervical dislocation; brains
738 were removed rapidly and flash frozen in isopentane. Tissue samples were obtained from
739 1 mm circular punches from 1 mm thick coronal sections, weighed, placed in 200 μl of
740 HeGA preservative solution (0.1 M Acetic Acid, 0.105% EDTA, 0.12% Glutathione, pH
741 3.7), homogenized (150 VT Ultrasonic homogenizer; Homogenizers.net), centrifuged and

742 supernatant frozen at -80°C pending analysis. Samples were separated by HPLC
743 coupled to an electrochemical detector. DA and DOPAC were separated with a reverse
744 phase C18 column (ChromSep SS 100 x 3.0 mm, Inertsil 3 ODS-3; Varian, Palo Alto, CA)
745 and a mobile phase containing: 75 mM NaH_2PO_4 , 25 mM citric acid, 25 μM EDTA, 100 μL /
746 tetraethylamine, 2.2 mM octanesulfonic acid sodium salt, 10% acetonitrile, 2% methanol,
747 pH 3.5. DA was oxidized with a coulometric electrode (Model 5014; ESA, Chelmsford,
748 MA), with conditioning cell set to a potential of -150 to -200 mV and the analytical cell set
749 to a potential of 350 mV. The concentration of DA and its metabolites was quantified using
750 an external standard curve from standards prepared in the same aCSF/preservative
751 mixture as the brain dialysates.

752 **PAG protein determination**

753 Protein analysis was performed using the Simon Simple Western assay (ProteinSimple).
754 Hippocampal tissue samples were dissected and homogenized in 100 μL lysis solution.
755 Lysis solution was prepared by mixing 1 mL of 1x lysis buffer (Cell Signalling Technology,
756 9803) containing 1 μL calyculin A and 0.5 μL okadaic acid (protein phosphatase inhibitors
757 from Sigma-Aldrich, C5552 and 08010 respectively) and 5 μL of protease inhibitor cocktail
758 (Sigma-Aldrich, P8340). After homogenization, the lysate was centrifuged at 12,000 rpm
759 for 30 min at 4°C . The supernatant was transferred to new tubes and frozen at -80°C
760 pending subsequent analysis. Tissue samples were diluted to a concentration of 0.2
761 mg/mL in ProteinSimple sample buffer. A master mix containing 10x sample buffer, 1M
762 DTT, and 10x fluorescent standard was added to the samples, which were then loaded in
763 the first row of a ProteinSimple cassette. A mixture of two rabbit polyclonal antibodies was
764 loaded in the second row: PAG antiserum (Norman Curthoys, Colorado State University,
765 Curthoys et al., 1976) diluted 1:200 and GAPDH (14C10) (Cell Signalling Technology,
766 2118S; AB Registry ID: AB_2107301) diluted 1:25. The luminol-S/peroxide
767 chemiluminescent detection mixture was loaded in the third row. Size-based separation,
768 immunoprobings, washing, and detection were done automatically by the Simon, which in
769 an automated sequence drew up the sample mixture, the antibodies, and then the
770 detection reagent into a capillary array. Chemiluminescence was measured along the
771 length of the capillary over time, and analyzed using ProteinSimple Compass software.

772 **Behavior**

773 **Motor performance.** A rotarod apparatus (accelerating model; Ugo Basile, Varese, Italy)
774 was used to measure motor learning and coordination. Mice were trained in the
775 accelerating speed mode at 0–20 (Day 1), 0–30 (Day 2), and 0–40 (Day 3) rpm, received
776 three trials per day, and performance was expressed as the time to the first fall.
777

778 **Novelty-induced locomotion, amphetamine-induced hyperlocomotion and**
779 **sensitization.** Novelty-induced exploration and reactivity to amphetamine were assessed
780 in the open field (Plexiglas activity chambers, 40.6 cm long \times 40.6 cm wide \times 38.1 cm high;
781 SmartFrame Open Field System, Kinder Scientific, Poway, CA) equipped with infrared
782 detectors to track animal movement. Testing took place under bright ambient light
783 conditions. Novelty-induced activity was recorded for 60 min, after which mice received an
784 i.p. injection of d-amphetamine hemisulfate (Sigma-Aldrich, A5880) or vehicle (saline) and
785 were returned to the open field for 90 min. This protocol was repeated for the

786 amphetamine sensitization studies.

787 **Anxiety.** Anxiety was measured in an elevated plus maze with two open arms and two
788 closed arms linked by a central platform. Two different size mazes were used, a smaller
789 one with shorter arms (28 cm) and 31 cm above the floor, and a larger one with longer
790 arms (45 cm) and 50 cm above the floor. Mice were put in the center of the maze and
791 allowed to explore for 5 min. Behavior was recorded with a video camera located above
792 the maze. In the smaller maze, the time spent in the open arms was scored using
793 TopScan (CleverSys, Reston, VA). In the larger maze, the time spent in the proximal and
794 distal open arms, and the number of entries into the open arms was scored using
795 AnyMaze (Stoelting, Wood Dale, IL).

796 **Fear conditioning.** Fear conditioning was assessed in rodent test chambers (20 cm
797 length x 16 cm width x 20.5 cm height; Med Associates, Fairfax, VT), equipped with a
798 ceiling and wall light, a speaker and a grid floor through which mild electrical shocks were
799 delivered. FreezeFrame video tracker (Coulbourn Instruments, Holliston, MA) was used to
800 measure freezing during the 3 phases of the procedure: conditioning (Day 1), tone test
801 (Day 2), and context test (Day 3). The same context was used for Day 1 and 3 (lemon
802 scent, grid floor and metal hall exposed, ceiling light on and wall light off), while a different
803 context was use on Day 2 (cinnamon scent, colored plastic sheets covered the floor and
804 halls, ceiling light off and wall light on). On Day 1, mice received 3 pairings of a tone (CS;
805 20 sec, 80 dB) and shock (US; 1 sec, 0.5 mA). On Day 2, the tone CS was delivered twice
806 (for 20 sec at 120 and 200 sec after the start of the session) during a 4 min session in a
807 different context, without the contextual cues associated with the shock US. On Day 3,
808 mice were tested for conditioned fear to the training context during a 4 min session,
809 without the tone CS or shock US. Sessions (4 min) were scored for freezing behavior.

810 **Latent inhibition** was assessed in the same test chambers used for fear conditioning.
811 Freezing was monitored during the four phases of the paradigm: pre-exposure and
812 conditioning (Day 1), context test (Day 2) and tone test (Day 3). The preexposure
813 stimulus/conditioned stimulus was an 80 dB tone and the unconditioned stimulus was a 1
814 sec, 0.70 mA shock. Mice were randomly assigned to a non-pre-exposed group (NPE;
815 received 3 CS/US pairings on Day 1) and a pre-exposed group (PE; received 20 CS
816 followed by 3 CS/US pairings on Day 1). On Day 1, 30 min before the behavioral test, mice
817 received clozapine (Sigma-Aldrich C6305, 1.5 mg/kg i.p., dissolved in a mixture of 1.5%
818 DMSO and saline) or vehicle. Clozapine is used as a positive control to demonstrate that
819 the limited number of pre-exposures does not elicit LI and yet are sufficient to reveal
820 potentiation of LI, thereby maximizing the dynamic range of the potentiation. On Day 3,
821 mice were put in a different context to measured freezing to the tone, which was presented
822 for 8 min. The same context was used for Days 1 and 2 (lemon scent, grid floor and metal
823 hall exposed, ceiling light on and wall light off), while a different context was use on Day 3
824 (anise scent, colored plastic sheets covered the floor and halls, ceiling light off and wall
825 light on). The scent was delivered to the chambers by placing a paper towel dabbed with
826 the scent solution under the chamber floor.

827
828 The LI procedure was conducted over three days:

829 *Day 1: Preexposure/Conditioning* - Preexposed (PE) mice received 20 presentations of a
830 30 s tone CS at a variable interstimulus interval of 30 s; while the non-preexposed (NPE)
831 mice were confined to the chamber for an identical period of time without receiving the CS.
832 Conditioning began immediately upon completion of the PE in the same chamber, and
833 comprised 3 tone-shock CS-US pairings, given 3 min apart. Each trial began with the 30 s
834 tone CS; a foot shock immediately followed tone termination. Mice were observed for
835 freezing. After the last pairing, mice remained in the chamber for an additional 5 min.

836 *Day 2: Context Test* - Mice were tested for conditioned fear of the training context. Mice
837 were placed in the experiment chamber for 8 min and presented with neither tone nor
838 shock and observed for freezing.

839 *Day 3: Tone Test* - Mice were tested for conditioned fear induced by the tone presentation
840 in absence of the contextual cues associated with shock. Each mouse was placed in the
841 chamber for 12 min. After an acclimatization period of 3 min, the tone CS was delivered for
842 8 min (no shocks were administered), and mice observed for freezing.

843 **Sample size estimation**

844 Sample size estimates were made using G*Power (Faul et al., 2007). Sample sizes were
845 calculated using a power of 0.80 and an α of 0.05, as we assumed that a 4:1 ratio between
846 type 1 and type 2 errors was appropriate for all our experiments (Keppel, 1991 p. 75). The
847 predicted effects sizes were different for the behavioral, electrophysiology/voltammetry,
848 and stereology experiments. Since we were assessing the effects of a conditional
849 heterozygous manipulation, for the behavioral studies we predicted a medium effect size of
850 0.06 (partial η^2), which resulted in an estimated sample size range between 17 to 51 mice
851 per group (rotarod = 17; elevated plus maze short arms = 51; novelty-induced locomotion
852 = 22; amphetamine sensitization = 21). After running these first experiments in sequence
853 using samples sizes within the estimated range, we obtained significant F values with
854 effects sizes ranging from 0.06 to 0.15 and a better than predicted power of 0.9, which led
855 us to use smaller samples size in subsequent experiments (elevated plus maze longer
856 arms, acute amphetamine, fear conditioning, latent inhibition). For the electrophysiology
857 and voltammetry studies, which measured the direct effects of the conditional
858 heterozygous manipulation on synaptic release, we predicted an effect size of 0.1, which
859 resulted in an estimated sample size of 12 per group. For the stereology experiments, we
860 estimated a larger effect size of 0.2 based on previous experiments and pilot studies, for a
861 sample size of 4 per group.

862 **Statistical analysis**

863 In Figure 1, the stereological estimate of the number of TH⁺ only, PAG⁺ only and TH⁺ /
864 PAG⁺ cells in the VTA and SNc of juvenile wild-type mice was analyzed using a 3 (cell
865 type) X 2 (brain region) ANOVA. For the comparison between the relative number of TH⁺ /
866 PAG⁺ cells in juvenile (P25) and adult (P60) mice, a 2 (age) X 2 (brain region) ANOVA was
867 used. For the single cell RT-PCR data, the Chi-Square test was used to determine whether
868 TH⁺ / VGLUT2⁺ neurons preferentially expressed PAG.

869 In Figure 3, comparison of response amplitudes to single photostimulation was analyzed

870 using a 2 (genotype) X 2 (cell type) ANOVA. Comparison between genotypes of first
871 response amplitude to burst photostimulation was done for each cell type separately using
872 the nonparametric Mann-Whitney test, since samples were not normally distributed. For
873 the analysis of the amplitude of EPSCs induced by repeated burst photostimulation, data
874 was converted to percent of the first response amplitude and analyzed for each cell type
875 separately using a 2 (genotype) X 4 (pulses, repeated measures factor) ANOVA. Only the
876 results obtained from ChIs revealed a significant genotype X pulses interaction, which led
877 us to conduct further analysis of simple effects involving the non-repeated measures factor
878 (genotype) to detect the source of the interaction. To control for increased family-wise
879 type 1 errors due to multiple comparisons, we applied the Bonferroni correction for simple
880 effects and using $\alpha = 0.0125$. Finally, the analysis of the ratio of firing during burst (0 – 0.5
881 s from onset of train) and just after burst photostimulation (0.5 – 1 s from onset) was done
882 using a one-way ANOVA.

883 In Figure 4, genotypic differences in numbers of TH⁺ neurons, DA content and DOPAC/DA
884 ratio values were evaluated with one-way ANOVAs. For voltammetry data, the peak
885 amplitude of DA release evoked by consecutive bursts of photostimulation followed by a
886 single, or consecutive single pulses followed by burst, was analyzed using a 2 (genotype)
887 x 4 (pulses, repeated measures factor) ANOVA.

888 In Figure 5, the latency to fall from the rotarod was analyzed using a 2 (genotype) x 9
889 (trials, repeated measures factor) ANOVA. Locomotor counts in the open field were
890 analyzed using a 2 (genotype) x 6 (time, bins of 10 mins, repeated measures factor)
891 ANOVA. Behavior in the elevated plus maze and fear conditioning chambers was analyzed
892 using a one-way ANOVA to evaluate genotypic effects. Dose effects in amphetamine-
893 induced locomotion were analyzed using a 2 (genotype) x 3 (dose) ANOVA.

894 In Figure 6, for the sensitization experiment, locomotor activity during the first 2 habituation
895 days (vehicle injections) was analyzed separately using a 2 (genotype) x 2 (drug
896 treatment) x 2 (days, repeated measure factor) ANOVA. Locomotor activity during the
897 subsequent 5 test days (vehicle or amphetamine injections) was analyzed using a 2
898 (genotype) x 2 (drug treatment) x 5 (days, repeated measure factor) ANOVA. A significant
899 three-way interaction was further analyzed for simple effects. Within each drug treatment,
900 a 2 (genotype) x 5 (days, repeated measure factor) ANOVA was used. Only within the
901 amphetamine-treated groups was there a significant genotype X day interaction, which
902 allowed us to conduct a further analysis of simple effects involving the non-repeated
903 measures factor (genotype). Comparisons during the last 3 days of injections were
904 corrected by a Bonferroni adjustment ($\alpha = 0.016$). The data from the challenge day were
905 analyzed separately using a 2 (genotype) X 2 (drug treatment) ANOVA. In addition, data
906 obtained during the 90 min following injections was analyzed separately for each
907 amphetamine- and vehicle-treated group using a 2 (genotype) x 9 (time, bins of 10 min,
908 repeated measure factor) ANOVA.

909 For the latent inhibition experiment (in Figure 6), freezing before CS presentation was
910 analyzed separately for each genotype using a 2 (preexposure treatment) X 2 (time, bins
911 of 1 min, repeated measure factor) ANOVA. After CS presentation data were analyzed
912 using a 2 (preexposure treatment) X 8 (time, bins of 1 min, repeated measure factor)
913 ANOVA. A significant preexposure X time interaction was found for DAT GLS1 cHET mice,

914 allowing us to examine simple effects. The multiple comparisons for each 1 min time bin
915 after CS presentation were corrected by a Bonferroni adjustment ($\alpha = 0.006$). The data for
916 total amount of freezing during the CS were analyzed using a 2 (genotype) X 2
917 (preexposure treatment) ANOVA. We found a significant genotype X preexposure
918 interaction, allowing us to explore further the source of the interaction within each
919 genotype using one-way ANOVAs.

920 A few mice were removed from experiments because of procedural errors (mice were put
921 in the wrong treatment group, or tested in the wrong operant box).

922 **Acknowledgements**

923 We thank Shannon Wolfman, Celia Gellman, Benjamin Inbar, Lauren Rosko, Karin
924 Krueger, Leora Boussi and Sophia Tepler for technical assistance, Eugene Mosharov,
925 Hadassah Tamir, Benjamin Klein and David Hirschberg for advice, Norman Curthoys for
926 glutaminase antisera, and Theresa Swayne in The Confocal and Specialized Microscopy
927 Shared Resource of the Herbert Irving Comprehensive Cancer Center at Columbia
928 University, supported by NIH grant P30 CA013696. This work was supported by a
929 NARSAD Young Investigator award (SM), DA017978 and MH087758 (SR) and MH086404
930 (SR, HM).

931 **Author Contributions**

932 Conceptualization, SM, NC, IGS, SR; Methodology, SM, DS, HM, IGS, SR; Validation, SM,
933 SR; Formal Analysis, SM, SR; Investigation, SM, NC, AK, ACS, YW, AM, CS, IZS, GMT;
934 Resources, MGW, JLO, DS; Data Curation, SM, NC, AK, SR; Writing – Original Draft, SM;
935 Writing – Review & Editing, SM, NC, SR; Supervision, DS, HM, IGS, SR; Project
936 Administration, SR; Funding Acquisition, SM, HM, SR.

937 **Competing Interests**

938 None.

939

940 **REFERENCES**

- 941
- 942 Alabi AA, Tsien RW (2012) Synaptic vesicle pools and dynamics. Cold Spring Harb
943 Perspect Biol 4:a013680.
- 944 Alsio J, Nordenankar K, Arvidsson E, Birgner C, Mahmoudi S, Halbout B, Smith C, Fortin
945 GM, Olson L, Descarries L, Trudeau LE, Kullander K, Levesque D, Wallen-
946 Mackenzie A (2011) Enhanced sucrose and cocaine self-administration and cue-
947 induced drug seeking after loss of VGLUT2 in midbrain dopamine neurons in mice.
948 J Neurosci 31:12593-12603.
- 949 Bäckman CM, Malik N, Zhang Y, Shan L, Grinberg A, Hoffer BJ, Westphal H, Tomac AC
950 (2006) Characterization of a mouse strain expressing Cre recombinase from the 3'
951 untranslated region of the dopamine transporter locus. Genesis 44:383-390.
- 952 Bae N, Wang Y, Li L, Rayport S, Lubec G (2013) Network of brain protein level changes in
953 glutaminase deficient fetal mice. J Proteomics 80:236-249.
- 954 Beeler JA, Cao ZFH, Kheirbek MA, Ding Y, Koranda J, Murakami M, Kang UJ, Zhuang X
955 (2010) Dopamine-dependent motor learning: insight into levodopa's long-duration
956 response. Ann Neurol 67:639-647.
- 957 Billups D, Marx M-C, Mela I, Billups B (2013) Inducible presynaptic glutamine transport
958 supports glutamatergic transmission at the calyx of Held synapse. J Neurosci
959 33:17429-17434.
- 960 Birgner C, Nordenankar K, Lundblad M, Mendez JA, Smith C, le Greves M, Galter D,
961 Olson L, Fredriksson A, Trudeau LE, Kullander K, Wallen-Mackenzie A (2010)
962 VGLUT2 in dopamine neurons is required for psychostimulant-induced behavioral
963 activation. Proc Natl Acad Sci U S A 107:389-394.
- 964 Bocklisch C, Pascoli V, Wong JC, House DR, Yvon C, de Roo M, Tan KR, Luscher C
965 (2013) Cocaine disinhibits dopamine neurons by potentiation of GABA transmission
966 in the ventral tegmental area. Science 341:1521-1525.
- 967 Bromberg-Martin ES, Matsumoto M, Hikosaka O (2010) Dopamine in motivational control:
968 rewarding, aversive, and alerting. Neuron 68:815-834.
- 969 Chuhma N, Tanaka KF, Hen R, Rayport S (2011) Functional connectome of the striatal
970 medium spiny neuron. J Neurosci 31:1183-1192.
- 971 Chuhma N, Mingote S, Moore H, Rayport S (2014) Dopamine neurons control striatal
972 cholinergic neurons via regionally heterogeneous dopamine and glutamate
973 signaling. Neuron 81:901-912.
- 974 Chuhma N, Mingote S, Kalmbach A, Yetnikoff L, Rayport S (2017) Heterogeneity in
975 dopamine neuron synaptic actions across the striatum and its relevance for
976 schizophrenia. Biol Psychiatry 81:43-51.
- 977 Covey DP, Roitman MF, Garris PA (2014) Illicit dopamine transients: Reconciling actions
978 of abused drugs. Trends Neurosci:200-210.
- 979 Covey DP, Bunner KD, Schuweiler DR, Cheer JF, Garris PA (2016) Amphetamine

- 980 elevates nucleus accumbens dopamine via an action potential-dependent
981 mechanism that is modulated by endocannabinoids. *Eur J Neurosci* 43:1661-1673.
- 982 Curthoys NP, Kuhlenschmidt T, Godfrey SS, Weiss RF (1976) Phosphate-dependent
983 glutaminase from rat kidney. Cause of increased activity in response to acidosis and
984 identity with glutaminase from other tissues. *Arch Biochem Biophys* 172:162-167.
- 985 Danbolt NC (2001) Glutamate uptake. *Prog Neurobiol* 65:1-105.
- 986 Drui G, Carnicella S, Carcenac C, Favier M, Bertrand A, Boulet S, Savasta M (2013) Loss
987 of dopaminergic nigrostriatal neurons accounts for the motivational and affective
988 deficits in Parkinson's disease. *Mol Psychiatry* 19:358-367.
- 989 El Hage M, Masson J, Conjard-Duplany A, Ferrier B, Baverel G, Martin G (2012) Brain
990 slices from glutaminase-deficient mice metabolize less glutamine: a cellular
991 metabolomic study with carbon 13 NMR. *J Cereb Blood Flow Metab* 32:816-824.
- 992 El Mestikawy S, Wallen-Mackenzie A, Fortin GM, Descarries L, Trudeau LE (2011) From
993 glutamate co-release to vesicular synergy: vesicular glutamate transporters. *Nat*
994 *Rev Neurosci* 12:204-216.
- 995 Faul F, Erdfelder E, Lang AG, Buchner A (2007) G*Power 3: a flexible statistical power
996 analysis program for the social, behavioral, and biomedical sciences. *Behav Res*
997 *Methods* 39:175-191.
- 998 Fernandez Espejo E (2003) Prefrontocortical dopamine loss in rats delays long-term
999 extinction of contextual conditioned fear, and reduces social interaction without
1000 affecting short-term social interaction memory. *Neuropsychopharmacology* 28:490-
1001 498.
- 1002 Flagel SB, Clark JJ, Robinson TE, Mayo L, Czuj A, Willuhn I, Akers CA, Clinton SM,
1003 Phillips PEM, Akil H (2011) A selective role for dopamine in stimulus-reward
1004 learning. *Nature* 469:53-57.
- 1005 Ford CP, Phillips PEM, Williams JT (2009) The time course of dopamine transmission in
1006 the ventral tegmental area. *J Neurosci* 29:13344-13352.
- 1007 Fortin GM, Bourque MJ, Mendez JA, Leo D, Nordenankar K, Birgner C, Arvidsson E,
1008 Rymar VV, Berube-Carriere N, Claveau AM, Descarries L, Sadikot AF, Wallen-
1009 Mackenzie A, Trudeau LE (2012) Glutamate corelease promotes growth and
1010 survival of midbrain dopamine neurons. *J Neurosci* 32:17477-17491.
- 1011 Gaisler-Salomon I, Schobel SA, Small SA, Rayport S (2009a) How high-resolution basal-
1012 state functional imaging can guide the development of new pharmacotherapies for
1013 schizophrenia. *Schizophr Bull* 35:1037-1044.
- 1014 Gaisler-Salomon I, Wang Y, Chuhma N, Zhang H, Golumbic YN, Mihali A, Arancio O,
1015 Sibille E, Rayport S (2012) Synaptic underpinnings of altered hippocampal function
1016 in glutaminase-deficient mice during maturation. *Hippocampus* 22:1027-1039.
- 1017 Gaisler-Salomon I, Miller GM, Chuhma N, Lee S, Zhang H, Ghoddoussi F, Lewandowski
1018 N, Fairhurst S, Wang Y, Conjard-Duplany A, Masson J, Balsam P, Hen R, Arancio
1019 O, Galloway MP, Moore HM, Small SA, Rayport S (2009b) Glutaminase-deficient
1020 mice display hippocampal hypoactivity, insensitivity to pro-psychotic drugs and

- 1021 potentiated latent inhibition: relevance to schizophrenia. *Neuropsychopharmacology*
1022 34:2305-2322.
- 1023 Hazan L, Gaisler-Salomon I (2014) Glutaminase1 heterozygous mice show enhanced
1024 trace fear conditioning and Arc/Arg3.1 expression in hippocampus and cingulate
1025 cortex. *Eur Neuropsychopharmacol* 24:1916-1924.
- 1026 Hnasko TS, Edwards RH (2012) Neurotransmitter corelease: mechanism and
1027 physiological role. *Annu Rev Physiol* 74:225-243.
- 1028 Hnasko TS, Hjelmstad GO, Fields HL, Edwards RH (2012) Ventral tegmental area
1029 glutamate neurons: electrophysiological properties and projections. *J Neurosci*
1030 32:15076-15085.
- 1031 Hnasko TS, Chuhma N, Zhang H, Goh GY, Sulzer D, Palmiter RD, Rayport S, Edwards
1032 RH (2010) Vesicular glutamate transport promotes dopamine storage and
1033 glutamate corelease in vivo. *Neuron* 65:643-656.
- 1034 Ikemoto S (2007) Dopamine reward circuitry: two projection systems from the ventral
1035 midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain Res Rev*
1036 56:27-78.
- 1037 Ishikawa M, Otaka M, Neumann PA, Wang Z, Cook JM, Schluter OM, Dong Y, Huang YH
1038 (2013) Exposure to cocaine regulates inhibitory synaptic transmission from the
1039 ventral tegmental area to the nucleus accumbens. *J Physiol* 591:4827-4841.
- 1040 Ishikawa T, Sahara Y, Takahashi T (2002) A single packet of transmitter does not saturate
1041 postsynaptic glutamate receptors. *Neuron* 34:613-621.
- 1042 Iwasaki S, Takahashi T (2001) Developmental regulation of transmitter release at the calyx
1043 of Held in rat auditory brainstem. *J Physiol* 534:861-871.
- 1044 Joseph MH, Peters SL, Moran PM, Grigoryan GA, Young AM, Gray JA (2000) Modulation
1045 of latent inhibition in the rat by altered dopamine transmission in the nucleus
1046 accumbens at the time of conditioning. *Neuroscience* 101:921-930.
- 1047 Kaneko T (2000) Enzymes responsible for glutamate synthesis and degradation. In:
1048 Handbook of Chemical Neuroanatomy (Storm-Mathisen J, Ottersen OP, eds), pp
1049 203-230: Elsevier.
- 1050 Kaneko T, Akiyama H, Nagatsu I, Mizuno N (1990) Immunohistochemical demonstration of
1051 glutaminase in catecholaminergic and serotonergic neurons of rat brain. *Brain Res*
1052 507:151-154.
- 1053 Kapur S (2003) Psychosis as a state of aberrant salience: a framework linking biology,
1054 phenomenology, and pharmacology in schizophrenia. *Am J Psychiatry* 160:13-23.
- 1055 Keppel G (1991) Design and analysis: a researcher's handbook, 3rd Edition. Englewood
1056 Cliffs, N.J.: Prentice Hall.
- 1057 Kim JI, Ganesan S, Luo SX, Wu YW, Park E, Huang EJ, Chen L, Ding JB (2015) Aldehyde
1058 dehydrogenase 1a1 mediates a GABA synthesis pathway in midbrain dopaminergic
1059 neurons. *Science* 350:102-106.
- 1060 Lee J, Finkelstein J, Choi JY, Witten IB (2016) Linking cholinergic interneurons, synaptic

- 1061 plasticity, and behavior during the extinction of a cocaine-context association.
1062 Neuron 90:1071-1085.
- 1063 Liss B, Neu A, Roeper J (1999) The weaver mouse gain-of-function phenotype of
1064 dopaminergic midbrain neurons is determined by coactivation of *wvGirk2* and K-
1065 ATP channels. *J Neurosci* 19:8839-8848.
- 1066 Lodge DJ, Grace AA (2012) Divergent activation of ventromedial and ventrolateral
1067 dopamine systems in animal models of amphetamine sensitization and
1068 schizophrenia. *Int J Neuropsychopharmacol* 15:69-76.
- 1069 Lüscher C, Malenka RC (2011) Drug-evoked synaptic plasticity in addiction: from
1070 molecular changes to circuit remodeling. *Neuron* 69:650-663.
- 1071 Madisen L et al. (2012) A toolbox of Cre-dependent optogenetic transgenic mice for light-
1072 induced activation and silencing. *Nat Neurosci* 15:793-802.
- 1073 Margolis EB, Coker AR, Driscoll JR, Lemaître A-I, Fields HL (2010) Reliability in the
1074 identification of midbrain dopamine neurons. *PLoS ONE* 5:e15222.
- 1075 Marx M-C, Billups D, Billups B (2015) Maintaining the presynaptic glutamate supply for
1076 excitatory neurotransmission. *J Neurosci Res* 93:1031-1044.
- 1077 Masson J, Darmon M, Conjard A, Chuhma N, Ropert N, Thoby-Brisson M, Foutz A, Parrot
1078 S, Miller GM, Jorisch R, Polan J, Hamon M, Hen R, Rayport S (2006) Mice lacking
1079 brain/kidney phosphate-activated glutaminase (GLS1) have impaired glutamatergic
1080 synaptic transmission, altered breathing, disorganized goal-directed behavior and
1081 die shortly after birth. *J Neurosci* 26:4660-4671.
- 1082 Mendez JA, Bourque MJ, Dal Bo G, Bourdeau ML, Danik M, Williams S, Lacaille JC,
1083 Trudeau LE (2008) Developmental and target-dependent regulation of vesicular
1084 glutamate transporter expression by dopamine neurons. *J Neurosci* 28:6309-6318.
- 1085 Mihali A, Subramani S, Kaunitz G, Rayport S, Gaisler-Salomon I (2012) Modeling
1086 resilience to schizophrenia in genetically modified mice: a novel approach to drug
1087 discovery. *Expert Rev Neurother* 12:785-799.
- 1088 Mingote S, Chuhma N, Kusnoor SV, Field B, Deutch AY, Rayport S (2015a) Functional
1089 connectome analysis of dopamine neuron glutamatergic connections in forebrain
1090 regions. *J Neurosci* 35:16259-16271.
- 1091 Mingote S, Masson J, Gellman C, Thomsen GM, Lin CS, Merker RJ, Gaisler-Salomon I,
1092 Wang Y, Ernst R, Hen R, Rayport S (2015b) Genetic pharmacotherapy as an early
1093 cns drug development strategy: testing glutaminase inhibition for schizophrenia
1094 treatment in adult mice. *Front Syst Neurosci* 9:165.
- 1095 Morales M, Margolis EB (2017) Ventral tegmental area: cellular heterogeneity, connectivity
1096 and behaviour. *Nat Rev Neurosci* 18:73-85.
- 1097 Nelson AJ, Thur KE, Horsley RR, Spicer C, Marsden CA, Cassaday HJ (2011) Reduced
1098 dopamine function within the medial shell of the nucleus accumbens enhances
1099 latent inhibition. *Pharmacol Biochem Behav* 98:1-7.
- 1100 Paladini CA, Roeper J (2014) Generating bursts (and pauses) in the dopamine midbrain

- 1101 neurons. *Neuroscience* 282:109-121.
- 1102 Peleg-Raibstein D, Knuesel I, Feldon J (2008) Amphetamine sensitization in rats as an
1103 animal model of schizophrenia. *Behav Brain Res* 191:190-201.
- 1104 Robinson MJ, Fischer AM, Ahuja A, Lesser EN, Maniates H (2016) Roles of "wanting" and
1105 "liking" in motivating behavior: gambling, food, and drug addictions. *Curr Top Behav*
1106 *Neurosci* 27:105-136.
- 1107 Robinson TE, Berridge KC (2008) Review. The incentive sensitization theory of addiction:
1108 some current issues. *Philos Trans R Soc Lond B Biol Sci* 363:3137-3146.
- 1109 Roiser JP, Howes OD, Chaddock CA, Joyce EM, McGuire P (2013) Neural and behavioral
1110 correlates of aberrant salience in individuals at risk for psychosis. *Schizophr Bull*
1111 39:1328-1336.
- 1112 Romaniuk L, Honey GD, King JR, Whalley HC, McIntosh AM, Levita L, Hughes M,
1113 Johnstone EC, Day M, Lawrie SM, Hall J (2010) Midbrain activation during
1114 Pavlovian conditioning and delusional symptoms in schizophrenia. *Arch Gen*
1115 *Psychiatry* 67:1246-1254.
- 1116 Root DH, Wang HL, Liu B, Barker DJ, Mod L, Szocsics P, Silva AC, Magloczky Z, Morales
1117 M (2016) Glutamate neurons are intermixed with midbrain dopamine neurons in
1118 nonhuman primates and humans. *Sci Rep* 6:30615.
- 1119 Rozas G, Lopez-Martin E, Guerra MJ, Labandeira-Garcia JL (1998) The overall rod
1120 performance test in the MPTP-treated-mouse model of Parkinsonism. *J Neurosci*
1121 *Methods* 83:165-175.
- 1122 Salamone JD, Correa M (2012) The mysterious motivational functions of mesolimbic
1123 dopamine. *Neuron* 76:470-485.
- 1124 Schobel SA, Lewandowski NM, Corcoran CM, Moore H, Brown T, Malaspina D, Small SA
1125 (2009) Differential targeting of the CA1 subfield of the hippocampal formation by
1126 schizophrenia and related psychotic disorders. *Arch Gen Psychiatry* 66:938-946.
- 1127 Schultz W (2013) Updating dopamine reward signals. *Curr Opin Neurobiol* 23:229-238.
- 1128 Stuber GD, Hnasko TS, Britt JP, Edwards RH, Bonci A (2010) Dopaminergic terminals in
1129 the nucleus accumbens but not the dorsal striatum corelease glutamate. *J Neurosci*
1130 30:8229-8233.
- 1131 Tani H, Dulla CG, Farzampour Z, Taylor-Weiner A, Huguenard JR, Reimer RJ (2014) A
1132 local glutamate-glutamine cycle sustains synaptic excitatory transmitter release.
1133 *Neuron* 81:888-900.
- 1134 Tritsch NX, Sabatini BL (2012) Dopaminergic modulation of synaptic transmission in cortex
1135 and striatum. *Neuron* 76:33-50.
- 1136 Tritsch NX, Granger AJ, Sabatini BL (2016) Mechanisms and functions of GABA co-
1137 release. *Nat Rev Neurosci* 17:139-145.
- 1138 Trudeau LE, Hnasko TS, Wallen-Mackenzie A, Morales M, Rayport S, Sulzer D (2014) The
1139 multilingual nature of dopamine neurons. *Prog Brain Res* 211:141-164.
- 1140 Vezina P (2004) Sensitization of midbrain dopamine neuron reactivity and the self-

- 1141 administration of psychomotor stimulant drugs. *Neurosci Biobehav Rev* 27:827-839.
- 1142 Wang DV, Viereckel T, Zell V, Konradsson-Geuken A, Broker CJ, Talishinsky A, Yoo JH,
1143 Galinato MH, Arvidsson E, Kesner AJ, Hnasko TS, Wallen-Mackenzie A, Ikemoto S
1144 (2017) Disrupting glutamate co-transmission does not affect acquisition of
1145 conditioned behavior reinforced by dopamine neuron activation. *Cell Rep* 18:2584-
1146 2591.
- 1147 Weiner I (2003) The "two-headed" latent inhibition model of schizophrenia: modeling
1148 positive and negative symptoms and their treatment. *Psychopharmacology (Berl)*
1149 169:257-297.
- 1150 Wen J-L, Xue L, Wang R-H, Chen Z-X, Shi Y-W, Zhao H (2015) Involvement of the
1151 dopaminergic system in the consolidation of fear conditioning in hippocampal CA3
1152 subregion. *Behav Brain Res* 278:527-534.
- 1153 Wilson NR, Kang J, Hueske EV, Leung T, Varoqui H, Murnick JG, Erickson JD, Liu G
1154 (2005) Presynaptic regulation of quantal size by the vesicular glutamate transporter
1155 VGLUT1. *J Neurosci* 25:6221-6234.
- 1156 Winton-Brown TT, Fusar-Poli P, Ungless MA, Howes OD (2014) Dopaminergic basis of
1157 salience dysregulation in psychosis. *Trends Neurosci* 37:85-94.
- 1158 Xenias HS, Ibáñez-Sandoval O, Koos T, Tepper JM (2015) Are striatal tyrosine
1159 hydroxylase interneurons dopaminergic? *J Neurosci* 35:6584-6599.
- 1160 Yamaguchi T, Qi J, Wang HL, Zhang S, Morales M (2015) Glutamatergic and
1161 dopaminergic neurons in the mouse ventral tegmental area. *Eur J Neurosci* 41:760-
1162 772.
- 1163 Young AMJ, Moran PM, Joseph MH (2005) The role of dopamine in conditioning and latent
1164 inhibition: what, when, where and how? *Neurosci Biobehav Rev* 29:963-976.
- 1165 Zhang S, Qi J, Li X, Wang H-L, Britt JP, Hoffman AF, Bonci A, Lupica CR, Morales M
1166 (2015) Dopaminergic and glutamatergic microdomains in a subset of rodent
1167 mesoaccumbens axons. *Nat Neurosci* 18:386-392.
- 1168 Zweifel LS, Argilli E, Bonci A, Palmiter RD (2008) Role of NMDA receptors in dopamine
1169 neurons for plasticity and addictive behaviors. *Neuron* 59:486-496.
- 1170 Zweifel LS, Parker JG, Lobb CJ, Rainwater A, Wall VZ, Fadok JP, Darvas M, Kim MJ,
1171 Mizumori SJY, Paladini CA, Phillips PEM, Palmiter RD (2009) Disruption of
1172 NMDAR-dependent burst firing by dopamine neurons provides selective
1173 assessment of phasic dopamine-dependent behavior. *Proc Natl Acad Sci U S A*
1174 106:7281-7288.
- 1175
- 1176

1177 **Table 1 - Behaviors affected in DAT GLS1 cHET mice.**

Psychological domain	Behavioral test	Affected in DAT GLS1 cHETs
Motor skills and exploration	Rotarod	–
	Novelty-induced locomotion	–
Anxiety	Open field – center time	–
	Elevated plus maze	–
Associative learning	Fear conditioning	–
Psychostimulant response	Acute Amph-induced hyperlocomotion	–
	Amph sensitization	Attenuated
Attention	Latent inhibition	Potentiated

1178

1179 **FIGURE LEGENDS**

1180 **Figure 1 - Expression of phosphate-activated glutaminase (PAG) in mouse ventral** 1181 **midbrain DA neurons.**

1182 (A) Confocal mosaic z-projected image of the ventral midbrain showing TH (green, left)
1183 and PAG (magenta, right) immunoreactivity. Merged image (center) shows that some TH⁺
1184 DA neurons co-express PAG (white). The specificity of the PAG antibody was verified in
1185 GLS1 KO mice; see [Figure 1—figure supplement 1A](#).

1186 (B) Magnified confocal images in the VTA (left) and SNc (right) showing TH⁺ only (thin blue
1187 arrow), PAG⁺ only (blue arrow head) and TH⁺/PAG⁺ cells (thick blue arrow).

1188 (C) Stereological counts of TH⁺ only (green), PAG⁺ only (magenta) and TH⁺ / PAG⁺ (white)
1189 cells in the VTA and SNc of juvenile (P25) wild type mice (n = 4). Cell numbers in the VTA
1190 (TH⁺ only = 4681 , PAG⁺ only = 3411, TH⁺ / PAG⁺ = 3673) were greater than in the SNc
1191 (TH⁺ only = 2564, PAG⁺ only = 2909, TH⁺ / PAG⁺ = 2595) (two-way ANOVA: main effect of
1192 brain region, $F_{(1,18)} = 18.36$; $p < 0.001$; effect size (ES) partial $\eta^2 = 0.51$), but the relative
1193 proportions of cell types did not differ between regions (main effect of cell type, $F_{(2,18)} =$
1194 1.22 ; $p = 0.318$; cell type X brain region interaction, $F_{(2,18)} = 2.70$; $p = 0.094$).

1195 (D) Single-cell RT-PCR analysis of cells expressing TH mRNA, in the VTA and SNc of
1196 juvenile mice (P25-37), showing the percentage of cells that co-expressed PAG and
1197 VGLUT2 mRNA. In the VTA, most cells were either TH⁺ only (7/22) or
1198 TH⁺/PAG⁺/VGLUT2⁺(8/22); there were also TH⁺/PAG⁺ cells (5/22) and rarely
1199 TH⁺/VGLUT2⁺ (2/22). In the SNc, most cells were either TH⁺ only (5/12) or TH⁺/PAG⁺ cells
1200 (6/12); and rarely TH⁺/PAG⁺/VGLUT2⁺ (1/12). No TH⁺ cells expressed GAD mRNA. For
1201 the full coexpression analysis, including GAD mRNA, see [Figure 1—figure supplement](#)
1202 [1B, 1C](#).

1203 (E) Comparison of the relative number of TH⁺ / PAG⁺ cells in juvenile (P25) and adult
1204 (P60) mice. In both the VTA and SNc, there was a significant increase in the number of
1205 TH⁺ / PAG⁺ cells. # indicates a significant main effect of age (two-way ANOVA, $F_{(1,10)} =$
1206 8.26 ; $p = 0.017$, ES partial $\eta^2 = 0.45$); there was no significant region effect ($F_{(1,10)} = 2.154$;
1207 $p = 0.173$), nor interaction, ($F_{(1,10)} = 0.846$; $p = 0.379$).

1208 **Figure 1—figure supplement 1. Expression of PAG in dopamine neurons**

1209 (A) Validation of the phosphate-activated glutaminase (PAG) antibody in GLS1 KO mice
1210 (neonates (P0) were used since KOs survive only for a few hours). Immunoreactivity was
1211 absent in GLS1 KO brain. Sagittal sections are shown. Abbreviations: Ctx, cortex; Hipp,
1212 hippocampus.

1213 (B) Sample gel images of single-cell reverse transcription (RT) PCR from the VTA (top)
1214 and the SN (bottom). For each region, the upper gel shows the multiplex result for
1215 glutamate decarboxylase (GAD67, 702 bp), tyrosine hydroxylase (TH, 377 bp), vesicular
1216 glutamate transporter (VGLUT2, 250 bp); the lower gel shows PAG (512 bp). Numbers on
1217 the top of each image are cell numbers; each lane in the multiplex gel (top) and PAG gel
1218 (bottom) was from the same cell.

1219 (C) Euler diagrams showing RT-PCR results in the VTA (top) and the SN (bottom).
1220 Numbers inside each square indicate the number of cells expressing the gene or
1221 combination of genes. TH⁺ cells are grouped (green square) in the diagram on the left;
1222 TH⁻ cells are divided into those expressing GAD67 (gray squares) and VGLUT2 (blue
1223 squares), on the right. PAG expressing cells are indicated by magenta-filled magenta
1224 squares. Cells expressing TH, VGLUT2 and PAG are indicated by yellow filled squares.
1225 There was no overlap of TH and GAD.

1226 **Figure 2 - DA neuron selective PAG deletion**

1227 (A) PCR screens for the floxed GLS1 allele (left) and Δ GLS1 allele (right) in brain regions
1228 from both GLS1^{lox/lox} and DAT GLS1 cKO mice. The Δ GLS1 allele was present solely in
1229 DAT GLS1 cKO ventral midbrain. dStr, dorsal striatum; HIPPP, hippocampus; VMB, ventral
1230 midbrain; CTX, cortex. Gel is representative of 3 replications.

1231 (B) Single-cell rtPCR analysis of TH expressing cells in the VTA in DAT^{IRESc^{re}/+} and DAT
1232 GLS1 cKO mice. In the VTA of DAT^{IRESc^{re}/+} mice, 11/30 TH cells expressed PAG mRNA,
1233 while in DAT GLS1 cKO none did (0/38 cells).

1234 (C) Confocal photomicrographs of the VTA from DAT^{IRESc^{re}/+} and DAT GLS1 cKO mice
1235 showing TH⁺ only (thin blue arrow) and PAG⁺ only (blue arrow head) and TH⁺/PAG⁺ cells
1236 (thick blue arrow). There were no TH⁺/PAG⁺ cells in the DAT GLS1 cKO ventral midbrain.

1237 Expression of dopaminergic markers and amphetamine-induced hyperlocomotion were not
1238 affected in DAT^{IRESc^{re}} mice; see **Figure 2—figure supplement 1**. These mice were control
1239 (CTRL) mice in subsequent experiments.

1240 **Figure 2—figure supplement 1. Expression of dopaminergic markers and** 1241 **amphetamine-induced hyperlocomotion were not affected in DAT^{IRESc^{re}} mice.**

1242 (A) The relative mRNA expression of dopamine transporter (DAT), tyrosine hydroxylase
1243 (TH), vesicular monoamine transporter 2 (VMAT2) and dopamine D2 receptor (D2R) in the
1244 ventral midbrain (left), and D1R and D2R in dorsal striatum (dStr, right) of DAT^{IRESc^{re}/+} and
1245 wild-type littermates (CTRL). A multivariate ANOVA showed no genotypic effect for any of
1246 the dopaminergic markers (ventral midbrain, DAT, $F_{(1,8)} = 0.061$, $p = 0.811$; TH, $F_{(1,8)} =$
1247 0.320 , $p = 0.587$; VMAT2, $F_{(1,8)} = 1.742$, $p = 0.223$; D2, $F_{(1,8)} = 3.903$, $p = 0.084$; dStr, D1 =
1248 $F_{(1,10)} = 0.384$, $p = 0.549$; $F_{(1,10)} = 0.851$, $p = 0.004$).

1249 (B) Amphetamine (Amph) stimulated locomotion. Total locomotor counts (*i.e.*, beam
1250 breaks) in the open field made over 2.5 hours following Vehicle (0 mg/kg) or Amph, 3 or 5
1251 mg/kg (i.p.). A two-way ANOVA showed a main effect of drug ($F_{(2,28)} = 83.1$; $p < 0.001$, ES
1252 partial $\eta^2 = 0.86$), but no significant main effect of genotype ($F_{(1,28)} = 0.846$; $p = 0.366$) or
1253 significant interaction ($F_{(2,28)} = 28.2$; $p = 0.973$).

1254 (C) Time course of Amph-evoked locomotion. There were no genotypic differences for
1255 either the 3 mg/kg (top) or 5 mg/kg doses (bottom). The repeated measures (RM) ANOVA
1256 showed no significant main effect of genotype (3 mg/kg dose, $F_{(1,10)} = 0.003$, $p = 0.960$;
1257 5 mg/kg dose, $F_{(1,9)} = 1.322$, $p = 0.280$) or time X genotype interaction (3 mg/kg dose,
1258 $F_{(14,140)} = 0.784$, $p = 0.685$; 5 mg/kg dose, $F_{(14,126)} = 1.663$, $p = 0.071$); there was a main

- 1259 effect of time (3 mg/kg dose, $F_{(14,140)} = 20.5$, $p < 0.0001$, ES partial $\eta^2 = 0.67$; 5 mg/kg dose,
1260 $F_{(14,126)} = 20.5$, $p < 0.0001$, ES partial $\eta^2 = 0.69$).
- 1261 Numbers of cells are shown above each bar in the graphs.
- 1262 **Figure 3 – DA neuron GLU cotransmission is attenuated in DAT GLS1 cHETs at**
1263 **phasic firing frequencies.**
- 1264 (A) Schematic of a coronal slice (-1.34 mm from bregma) indicating the location of the
1265 patch-clamp recordings in the medial NAc shell. DA neuron excitatory responses evoked
1266 by photostimulation (blue circles) were measured from ChIs and SPNs (left). See also
1267 **Figure 3—figure supplement 1.**
- 1268 (B) Representative traces (left) of EPSCs generated by a single-pulse photostimulation
1269 (blue bar) at 0.1 Hz recorded from ChIs and SPNs (middle). Traces shown are averages of
1270 10 consecutive traces. Comparison is made between responses in CTRL (black traces)
1271 and DAT GLS1 cHET mice (gray traces); all responses were completely blocked by CNQX
1272 (40 μ M; red traces). Summary of average EPSC amplitude after single-pulse
1273 photostimulation (right). # indicates a significant main effect of cell type (two-way ANOVA,
1274 $F_{(1,36)} = 25.6$, $p < 0.001$, ES partial $\eta^2 = 0.42$); there was no significant genotype effect
1275 ($F_{(1,36)} = 1.084$, $p = 0.305$), nor interaction ($F_{(1,36)} = 0.628$, $p = 0.433$). See also **Figure 3—**
1276 **figure supplement 2.**
- 1277 (C) Representative traces of EPSCs generated by burst photostimulation (5 pulses at 20
1278 Hz) recorded from ChIs (top) and SPNs (bottom). Summary of the average EPSC
1279 amplitudes after burst photostimulation (right) are shown as percentage of the first
1280 response, which did not differ between genotypes (ChIs, CTRL 95 ± 29 pA vs. cHET $107 \pm$
1281 12 pA, Mann-Whitney, ChIs, $p = 0.14$; SPNs, CTRL 27 ± 4 pA vs. cHET 28 ± 5 pA, Mann-
1282 Whitney, $p = 0.88$). The shaded violet bar at the bottom of the graphs represents the
1283 average baseline noise (ChIs 3.8 ± 0.4 pA; SPNs 3.5 ± 0.3 pA). For ChIs, repeated
1284 measures (RM) ANOVA revealed a significant pulses X genotype interaction ($F_{(3,54)} = 28.2$,
1285 $p = 0.006$, ES partial $\eta^2 = 0.27$), main effect of pulses ($F_{(3,54)} = 20.9$, $p < 0.001$), and main
1286 effect of genotype ($F_{(1,18)} = 5.06$, $p = 0.037$). * indicates significant difference from CTRL
1287 ($p = 0.006$) after applying a Bonferroni correction for 4 comparisons ($\alpha = 0.0125$). For
1288 SPNs, \diamond # indicates a significant main effect of genotype ($F_{(1,18)} = 4.6$, $p = 0.047$, ES partial
1289 $\eta^2 = 0.20$) and main effect of pulses ($F_{(3,54)} = 7.7$, $p < 0.001$, ES partial $\eta^2 = 0.30$) by RM
1290 ANOVA; but no significant interaction ($F_{(3,54)} = 2.0$, $p = 0.101$).
- 1291 (D) Effect of photostimulation mimicking DA neuron bursting (5 pulses at 20 Hz) on ChI
1292 firing. Representative traces are shown above (left), with peristimulus histograms summing
1293 ten consecutive traces (0.1 s bin) below. Ratio of firing during burst photostimulation (0 –
1294 0.5 s from onset of train) and after (0.5 – 1 s from onset) to baseline firing (right). * indicates
1295 significant effect of genotype (one-way ANOVA, $F_{(1,33)} = 7.0$, $p = 0.013$, ES partial $\eta^2 =$
1296 0.17).
- 1297 (E) Colored-coded tables showing action potential counts in 50 ms intervals, prior to,
1298 during and after DA terminal photostimulation for CTRL (left) and DAT GLS1 cHET mice
1299 (right). The blue horizontal bar at the bottom of each table indicates the duration of burst

1300 photostimulation, with onset at time 0.

1301 The number of cells is shown in the graphs above the bars or next to the lines. In this and
1302 subsequent figures, error bars represent SEM.

1303 **Figure 3—figure supplement 1. Comparison between CTRL::ChR2 and DAT GLS1**
1304 **cHET::ChR2 mice showing selective ChR2 expression in DA neurons did not differ**
1305 **between genotypes.**

1306 (A) ChR2-EYFP expression (green) in the ventral midbrain was restricted to TH+ cells
1307 (magenta), with similar colocalization (white) in both CTRL::ChR2 mice (left) and DAT
1308 GLS1 cHET::ChR2 mice (right).

1309 (B) Stereological counts of ChR2-EYFP and TH+ cells in the VTA and SNc of CTRL::ChR2
1310 (n=3) and DAT GLS1 cHET::ChR2 mice (n=3). Values are presented as percent of the
1311 total number of cells counted in each region, for each genotype (VTA, CTRL::ChR2= 5097
1312 ± 817 cells and DAT GLS1 cHET::ChR2= 3891± 628 cells; SNc, CTRL::ChR2 = 4782 ±
1313 889 cells and DAT GLS1 cHET::ChR2= 3345 ± 453 cells; Kruskal-Wallis test in each
1314 region showed no genotype effect). The percentage of TH⁺/ChR2-EYFP⁺ cells did not
1315 differ genotypically, in the VTA or SNc (Kruskal-Wallis test).

1316 (C) TH⁺ interneurons (magenta) in the dorsal striatum (dStr) did not express ChR2-EYFP
1317 (green).

1318 **Figure 3—figure supplement 2. Comparison between CTRL::ChR2 and DAT GLS1**
1319 **cHET::ChR2 mice showing that intrinsic electrophysiological membrane properties**
1320 **and spontaneous EPSCs measured in NAc shell cells did not differ between**
1321 **genotypes.**

1322 (A) ChIs and SPNs are identifiable based on their electrophysiological signature under
1323 current clamp. The ChI (left) had a resting membrane potential around -70 mV, fired
1324 spontaneously (black trace), and showed a voltage sag with hyperpolarizing current
1325 injection (green trace). The SPN (right) had a deep resting membrane potential around -
1326 100 mV, did not fire spontaneously (black), showed no sag with hyperpolarizing current
1327 injection (green trace), and fired rapidly with depolarizing current injection, after a delay
1328 (blue trace).

1329 (B) The average baseline membrane potential (V_{rest}) was more negative in SPNs than in
1330 ChIs, but not genotypically different. A two-way ANOVA showed a main effect of cell type
1331 ($F_{(1,62)} = 128.3$, $p < 0.0001$, ES partial $\eta^2 = 0.67$), indicated by the #, but no main effect of
1332 genotype ($F_{(1,62)} = 1.67$, $p = 0.201$) or significant interaction ($F_{(1,62)} = 0.138$, $p = 0.711$).

1333 (C) Action potential (AP) threshold in ChIs and SPNs. A two-way ANOVA showed no main
1334 effect of genotype ($F_{(1,62)} = 0.53$, $p = 0.819$) or cell type ($F_{(1,62)} = 2.78$, $p = 0.100$), or
1335 significant interaction ($F_{(1,62)} = 0.480$, $p = 0.491$).

1336 (D) Input impedance was significantly higher in ChIs compared to SPNs, but not
1337 statistically different between genotypes. A two-way ANOVA showed a main effect of cell
1338 type ($F_{(1,62)} = 15.7$, $p < 0.001$, ES partial $\eta^2 = 0.20$), indicated by the #, but no main effect

- 1339 of genotype ($F_{(1,62)} = 0.233$, $p = 0.631$) or significant interaction ($F_{(1,62)} = 1.96$, $p = 0.167$).
- 1340 (E) The hyperpolarization-activated cation current (I_h) ratio was lower in the ChIs than
1341 SPNs, revealing the presence of an I_h in ChIs but not SPNs. A two-way ANOVA showed a
1342 main effect of cell type ($F_{(1,62)} = 15.0$, $p < 0.001$, ES partial $\eta^2 = 0.20$), indicated by the #,
1343 but no main effect of genotype ($F_{(1,62)} = 0.001$, $p = 0.976$) or significant interaction ($F_{(1,62)} =$
1344 0.856 , $p = 0.358$).
- 1345 (F) Characterization of photostimulated DA neuron evoked EPSCs in ChIs and SPNs
1346 under voltage clamp revealed that rise time (from 10% to 90% of peak amplitude) was
1347 faster in SPNs than ChIs, but not genotypically different. A two-way ANOVA showed a
1348 significant cell type effect ($F_{(1,46)} = 28.4$, $p < 0.0001$, ES partial $\eta^2 = 0.08$) indicated by the
1349 #, but no main effect of genotype ($F_{(1,46)} = 1.02$, $p = 0.305$) or significant interaction ($F_{(1,46)}$
1350 $= 1.02$, $p = 0.305$).
- 1351 (G) Decay times of evoked EPSCs under voltage clamp. A two-way ANOVA showed no
1352 main effect of genotype ($F_{(1,46)} = 2.135$; $p = 0.151$) or cell type ($F_{(1,46)} = 0.458$; $p = 0.502$),
1353 or significant interaction ($F_{(1,46)} = 1.331$; $p = 0.255$).
- 1354 (H) Amplitude of spontaneous EPSCs measured under voltage clamp (holding potential -
1355 70 mV) in ChIs and SPNs. A two-way ANOVA showed a main effect of cell type ($F_{(1,36)} =$
1356 5.85 , $p < 0.021$, ES partial $\eta^2 = 0.14$), indicated by the #, but no main effect of genotype
1357 ($F_{(1,36)} = 0.257$, $p = 0.615$) or significant interaction ($F_{(1,36)} = 1.68$, $p = 0.203$).
- 1358 (I) Frequency of spontaneous EPSCs measured under voltage clamp (holding potential -
1359 70 mV) in ChIs and SPNs. A two-way ANOVA showed no main effect of genotype ($F_{(1,36)} =$
1360 0.308 , $p = 0.582$) or cell type ($F_{(1,36)} = 0.764$, $p = 0.388$), or significant interaction ($F_{(1,36)} =$
1361 1.97 , $p = 0.169$).
- 1362 Numbers of cells are shown above each bar or circle in the graph.

1363 **Figure 4 – PAG reduction in DA neurons does not alter the number of DA neurons or**
1364 **striatal DA function.**

- 1365 (A) Stereological-estimate of the total number of DA neurons (TH^+ neurons) in the VTA
1366 and SNc in one hemisphere showed no difference between genotypes (one-way ANOVA:
1367 VTA, $F_{(1,6)} = 0.149$, $p = 0.713$; SNc, $F_{(1,6)} = 0.085$, $p = 0.781$). There were no differences in
1368 DA neuron intrinsic electrophysiological properties; see **Figure 4—figure supplement 1**.
- 1369 (B) Tissue DA content in the NAc and dStr (left) and DA turnover measured by DOPAC/DA
1370 ratio (right) did not differ between genotypes by one-way ANOVA (NAc DA content, $F_{(1,10)}$
1371 $= 0.070$, $p = 0.794$; NAc DOPAC/DA, $F_{(1,10)} = 0.078$, $p = 0.783$; dStr DA content, $F_{(1,10)} =$
1372 0.078 , $p = 0.783$; dStr DOPAC/DA, $F_{(1,10)} = 1.68$, $p = 0.211$).
- 1373 (C) FSCV recordings in the medial NAc shell. A representative voltammogram is shown
1374 above a schematic of a coronal slice (-1.34 mm from bregma) indicating the recording
1375 configuration.
- 1376 (D) DA release evoked by three consecutive single photostimulation pulses followed by a

1377 burst (5 pulses at 20Hz) (above), or by three consecutive bursts followed by a single
1378 (below). Representative recordings of evoked DA release are shown with dashed boxes
1379 indicating initial traces that were enlarged and superimposed on the left, showing that DA
1380 release dynamics did not differ between genotypes for the single (above) or burst (below)
1381 responses. DA release dynamics did not differ between genotypes for consecutive singles
1382 followed by a burst (above) or repeated bursts followed by a single pulse (below). The
1383 average evoked DA release is shown on the graph (right). For consecutive single pulses
1384 followed by a burst, a RM ANOVA revealed a significant main effect of pulses ($F_{(3,69)} =$
1385 135.1 , $p < 0.001$, ES partial $\eta^2 = 0.85$); there was no effect of genotype ($F_{(1,23)} = 0.069$, $p =$
1386 0.795) nor interaction ($F_{(3,69)} = 0.247$, $p = 0.864$). For the consecutive bursts followed by a
1387 single, a RM ANOVA revealed a significant main effect of pulses ($F_{(3,66)} = 124.5$; $p <$
1388 0.001); there was no effect of genotype or interaction. Dopamine release in the NAc core
1389 and dStr was also not affected in DAT GLS1 cHETs; see [Figure 4—figure supplement 2](#).

1390 Numbers of mice or the number of slices (FSCV) are shown in each graph above the bars.

1391 **Figure 4—figure supplement 1. Electrophysiological properties of putative DA**
1392 **neurons in the ventral midbrain.**

1393 (A) DA neuron pacemaker firing recorded in the ventral tegmental area (VTA, left) or
1394 substantia nigra pars compacta (SNc, right), in CTRL and DAT GLS1 cHET slices.

1395 (B) Graph of average firing frequency. Numbers of cells recorded are shown above the
1396 bars.

1397 (C) Input impedance.

1398 (D) Baseline membrane potential (V_{rest}).

1399 (E) Action potential threshold.

1400 There was no genotypic difference in either the VTA or SNc for any of these measures by
1401 one-way ANOVA (firing frequency, VTA, $F_{(1,27)} = 0.238$, $p = 0.630$; SNc, $F_{(1,29)} = 2.59$, $p =$
1402 0.118 ; input impedance, VTA, $F_{(1,27)} = 0.005$, $p = 0.945$; SNc, $F_{(1,29)} = 1.48$, $p = 0.233$;
1403 baseline membrane potential, VTA, $F_{(1,27)} = 0.658$, $p = 0.424$; SNc, $F_{(1,29)} = 0.140$, $p =$
1404 0.711 ; action potential threshold, VTA, $F_{(1,27)} = 0.480$, $p = 0.494$; SNc, $F_{(1,29)} = 0.567$, $p =$
1405 0.458). These results indicate that basic DA neuron properties are not affected in DAT
1406 GLS1 cHET mice, nor was there evidence for cell deterioration.

1407 The number of cells is shown in the graphs above the bars or circles.

1408 **Figure 4—figure supplement 2. Dopamine release in nucleus accumbens core and**
1409 **dorsal striatum, measured by fast-scan cyclic voltammetry (FSCV), was not affected**
1410 **in DAT GLS1 cHET mice.**

1411 (A) Schematic of a coronal slice with recording site in the nucleus accumbens (NAc) core.

1412 (B) Representative FSCV traces, organized as in B.

1413 (C) Average evoked DA release in the NAc core. Graphs correspond to traces in E. In the

1414 upper graph, RM ANOVA showed a significant main effect of pulses ($F_{(3,36)} = 22.903$, $p <$
1415 0.0001 , ES partial $\eta^2 = 0.656$), but no main effect of genotype ($F_{(1,12)} = 0.32$, $p = 0.523$) or
1416 significant interaction ($F_{(3,36)} = 0.418$, $p = 0.741$). In the lower graph, RM ANOVA showed a
1417 significant main effect of pulses ($F_{(3,36)} = 60.79$, $p < 0.0001$, ES partial $\eta^2 = 0.835$), but no
1418 main effect of genotype ($F_{(1,12)} = 0.249$, $p = 0.627$) or significant interaction ($F_{(3,36)} = 0.210$,
1419 $p = 0.889$).

1420 (D) Schematic of a coronal slice with recording site in the medial dorsal striatum (dStr). DA
1421 release evoked by photostimulation was measured using FSCV. A representative cyclic
1422 voltammogram is shown in the upper left.

1423 (E) Representative FSCV traces of photostimulated DA release. The first two responses in
1424 each trace (dashed box) are enlarged and superimposed on the left. The upper pair of
1425 traces shows responses to 3 single photostimulations followed by a burst; the lower pair to
1426 3 burst photostimulations followed by a single.

1427 (F) Average evoked DA release in the dStr. Graphs correspond to traces in B. In the upper
1428 graph, RM ANOVA showed a significant main effect of photostimulation ($F_{(3,36)} = 48.52$, $p <$
1429 0.0001 , ES partial $\eta^2 = 0.802$), but no main effect of genotype ($F_{(1,12)} = 0.072$, $p = 0.793$) or
1430 significant interaction ($F_{(3,36)} = 0.26$, $p = 0.854$). In the lower graph, RM ANOVA showed a
1431 significant main effect of pulses ($F_{(3,36)} = 37.257$, $p < 0.0001$, ES partial $\eta^2 = 0.756$), but no
1432 main effect of genotype ($F_{(1,12)} = 0.084$, $p = 0.777$) or significant interaction ($F_{(3,36)} = 0.083$,
1433 $p = 0.969$).

1434 The numbers of slices recorded are shown above the first pair of bars in the graphs.

1435 **Figure 5 – Motor performance, anxiety and amphetamine-induced hyperlocomotion**
1436 **are unaffected in DAT GLS1 cHETs**

1437 (A) Motor performance on an accelerating rotarod over 3 days showed no difference
1438 between genotypes (RM ANOVA, significant effect of trials, $F_{(8,520)} = 22.9$, $p < 0.0001$, ES
1439 partial $\eta^2 = 0.26$); there was no effect of genotype ($F_{(1,65)} = 0.018$, $p = 0.894$; nor interaction
1440 $F_{(8,520)} = 0.562$, $p = 0.809$).

1441 (B) Locomotor activity in the open field for one hour revealed no genotypic difference in
1442 novelty-induced locomotion and habituation (RM ANOVA, main effect of time, $F_{(5,510)} =$
1443 193.0 , $p < 0.0001$, ES partial $\eta^2 = 0.65$); no effect of genotype ($F_{(1,102)} = 0.664$, $p = 0.417$;
1444 nor interaction, $F_{(5,510)} = 0.329$, $p = 0.895$).

1445 (C) Exploration in the elevated-plus maze (5 min) showed no genotypic difference in
1446 percentage of time spent in the open arms (left) (one way-ANOVA, $F_{(1,22)} = 0.004$, $p =$
1447 0.949) or time spent in the open arms per entry (right) (one way-ANOVA, $F_{(1,22)} = 0.547$,
1448 $p = 0.467$).

1449 (D) Fear conditioning to tone (left) measured as the average percentage of freezing during
1450 the CS (two tone presentations) or to a context previously paired with a shock (right)
1451 showed no genotypic differences (one-way ANOVA, tone fear conditioning, $F_{(1,16)} = 1.145$,
1452 $p = 0.300$; context fear conditioning, $F_{(1,16)} = 0.207$, $p = 0.655$).

1453 (E) Amphetamine-induced locomotor activity recorded over 90 min post injection showed
1454 no genotypic difference in the dose-dependent responses (two-way ANOVA, main effect of
1455 drug treatment, $F_{(2,66)} = 34.8$, $p < 0.0001$, ES partial $\eta^2 = 0.51$; no effect of genotype, $F_{(2,66)}$
1456 $= 0.068$, $p = 0.795$; nor interaction, $F_{(2,66)} = 0.18$, $p = 0.836$).

1457 The number of mice is shown in the graphs above the bars or next to the lines.

1458 **Figure 6 – DAT GLS1 cHET mice showed attenuated amphetamine sensitization and**
1459 **potentiated latent inhibition**

1460 (A) Schematic of amphetamine sensitization protocol.

1461 (B) Locomotor activity in the open field after vehicle (Veh) or Amphetamine (Amph)
1462 injection. There were no between group differences in activity on the habituation days
1463 (Days 1 and 2). Over the subsequent 5 treatment days, CTRL mice showed sensitization
1464 to Amph while DAT GLS1 cHET mice did not (RM ANOVA, significant genotype X
1465 treatment X day interaction, $F_{(4,296)} = 4.4$, $p = 0.002$, ES partial $\eta^2 = 0.06$; RM ANOVA
1466 within amphetamine-treated mice, significant genotype X day interaction, $F_{(4,160)} = 5.9$, $p <$
1467 0.001 , ES partial $\eta^2 = 0.112$). * $p < 0.016$ indicates significantly different from CTRL Amph-
1468 treated mice, after Bonferroni correction for 3 comparisons ($\alpha = 0.016$). On the Veh
1469 challenge day (day 18), Amph-treated mice showed a modest increase in locomotion
1470 relative to Veh-treated mice independent of genotype. # indicates significant treatment
1471 effect ($F_{(1,74)} = 4.03$, $p = 0.048$; partial $\eta^2 = 0.052$), but no main effect of genotype ($F_{(1,74)} <$
1472 0.001 , $p = 1$) or significant interaction ($F_{(1,74)} = 0.163$, $p = 0.688$). On the challenge day
1473 (Day 19), Amph-treated mice showed increased locomotion relative to Veh-treated mice
1474 independent of genotype. # indicates significant treatment effect (two-way ANOVA: $F_{(1,74)}$
1475 $= 13.7$, $p < 0.001$, ES partial $\eta^2 = 0.112$), with no significant genotype effect ($F_{(1,74)} = 2.76$,
1476 $p = 0.101$), but a trend for interaction ($F_{(1,74)} = 3.18$, $p = 0.078$).

1477 (C) On the Amph challenge day Veh-treated (left) and Amph-treated mice (right) received
1478 Amph and activity was monitored for 90 min. Veh-treated mice showed no genotypic
1479 difference in their response to Amph (RM ANOVA genotype effect, $F_{(1,74)} = 0.012$, $p = 0.91$;
1480 genotype X time interaction, $F_{(1,74)} = 0.53$, $p = 0.83$). Amph-treated CTRL mice showed a
1481 sensitized response to Amph while DAT GLS1 cHET did not. \diamond # indicate a significant
1482 genotype difference (RM ANOVA, $F_{(1,40)} = 89.3$, $p = 0.034$, ES partial $\eta^2 = 0.107$), and
1483 significant effect of time ($F_{(8,320)} = 12.8$, $p < 0.0001$, ES partial $\eta^2 = 0.243$), but no
1484 significant interaction ($F_{(8,320)} = 0.576$, $p = 0.798$).

1485 stopGLS1 mice, with a global GLS1 HET reduction, show attenuated amphetamine
1486 sensitization; see [Figure 6—figure supplement 1](#). Δ GLS1 HET mice, generated by
1487 breeding floxGLS1 mice with mice expressing cre under the control of the ubiquitous
1488 tamoxifen-inducible ROSA26 promoter ([Figure 6—figure supplement 2](#)), also show
1489 attenuated amphetamine sensitization ([Figure 6—figure supplement 3](#)).

1490 (D) Schematic of latent inhibition protocol.

1491 (E) On the tone test day (Day 3), the percent time freezing for the 3 min before and 8 min
1492 after CS (tone) presentation are shown for CTRL (left) and DAT GLS1 cHET mice (right).

1493 CTRL non-preexposure (NPE) and preexposure (PE) groups did not differ, evidencing no
1494 LI (RM ANOVA during CS, preexposure effect, $F_{(2,12)} = 0.127$, $p = 0.728$; preexposure X
1495 time interaction, $F_{(7,84)} = 1.66$, $p = 0.129$). DAT GLS1 cHET NPE and PE groups did not
1496 differ before CS presentation (PE effect, $F_{(1,20)} = 0.646$, $p = 0.431$; interaction, $F_{(2,40)} = 2.12$,
1497 $p = 0.132$); during CS presentation, PE mice showed less freezing than NPE mice,
1498 evidencing potentiated LI (RM ANOVA, significant time X PE treatment interaction, $F_{(7,140)} =$
1499 2.88 , $p = 0.008$, ES partial $\eta^2 = 0.126$). * $p < 0.006$ indicates significant different between
1500 PE and NPE groups, after Bonferroni correction for 8 comparisons ($\alpha = 0.006$).

1501 (F) Percent total time freezing during 8 min CS presentation on the tone test (Day 3). DAT
1502 GLS1 cHET PE mice, but not CTRL mice, showed less freezing during CS presentation,
1503 evidencing potentiated LI (two-way ANOVA, significant genotype X PE treatment
1504 interaction, $F_{(1,32)} = 5.3$, $p = 0.028$, ES partial $\eta^2 = 0.334$; no significant genotype effect,
1505 $F_{(1,32)} = 0.145$, $p = 0.71$, nor PE effect, $F_{(1,32)} = 1.52$, $p = 0.227$). Within the NPE group, there
1506 was no genotype effect, showing that learning was not affected in DAT GLS1 cHETs
1507 ($F_{(1,15)} = 1.56$, $p = 0.23$). * indicates significant pre-exposure effect within the DAT GLS1
1508 cHET group by ANOVA ($F_{(1,20)} = 10.03$, $p = 0.005$, ES partial $\eta^2 = 0.334$). stopGLS1 mice
1509 (Gaisler-Salomon et al., 2009b), as well as Δ GLS1 HET mice (**Figure 6—figure**
1510 **supplement 3**), both with a global GLS1 reduction, show potentiation of LI.

1511 (G) Schematic of the EMX1 GLS1 cHET mouse brain (sagittal view) illustrating the GLS1
1512 cHET genotype in forebrain. See **Figure 6—figure supplement 4**.

1513 (H) Novelty-induced locomotion and habituation to the open field did not differ between
1514 CTRL (white circles) and EMX1 GLS1 cHET mice (grey circles). RM ANOVA showed a
1515 significant time effect, $F_{(5,170)} = 138.1$, $p < 0.0001$, ES partial $\eta^2 = 0.802$; no significant
1516 genotype effect, $F_{(1,34)} = 0.599$, $p = 0.44$; and no significant interaction, $F_{(5,170)} = 0.820$, $p =$
1517 0.537 .

1518 (I) Both CTRL and EMX1 GLS1 cHET mice showed sensitization to Amph during the
1519 5 treatment days (RM ANOVA: days X drug treatment effect, $F_{(4,128)} = 11.33$, $p < 0.0001$,
1520 ES partial $\eta^2 = 0.259$; there was no significant day X drug treatment X genotype
1521 interaction, $F_{(4,128)} = 0.161$, $p = 0.96$). On the Veh challenge day, there were no significant
1522 differences between genotypes of drug-treatment groups. On the Amph challenge day,
1523 Amph-treated mice showed a sensitized response relative to Veh-injected mice,
1524 independent of genotype. # indicates a significant main effect of drug treatment ($F_{(1,32)} =$
1525 16.83 , $p < 0.0001$, ES partial $\eta^2 = 0.330$).

1526 (J) EMX1 GLS1 cHET mice did not show potentiation of LI. Percent time freezing during
1527 the 8 min CS presentation on the tone test day (Day 3) did not differ between NPE and PE
1528 groups, independent of genotype (two-way ANOVA: no significant main effect of genotype,
1529 $F_{(1,35)} = 0.281$, $p = 0.60$; PE, $F_{(1,35)} = 0.163$, $p = 0.69$; or interaction, $F_{(1,35)} = 0.586$, $p = 0.45$).
1530 EMX1 GLS1 cHET mice, as well as Δ GLS1 HET and DAT GLS1 cHET mice, showed
1531 clozapine-induced potentiation of LI (**Figure 6—figure supplement 5**).

1532 The number of mice is shown in the graphs above the bars or next to the lines. See
1533 Source Data for the full statistical analysis.

1534 **Figure 6 – figure supplement 1. stopGLS1 HET with a global PAG reduction show**
1535 **attenuated amphetamine sensitization.**

1536 (A) While potentiation of LI is seen in stopGLS1 mice (Gaisler-Salomon et al., 2009b),
1537 amphetamine sensitization had not been tested. To test for amphetamine sensitization in
1538 stopGLS1 HET mice, Amph (4 mg/kg) or Veh was administered over 4 consecutive days.
1539 Amph-treated CTRL mice showed a sensitized response to Amph while Amph-treated
1540 stopGLS1 HET mice did not. A three-way RM ANOVA revealed a significant day X
1541 genotype interaction ($F_{(3,120)} = 3.4$, $p = 0.021$, ES partial $\eta^2 = 0.078$), a treatment X
1542 genotype interaction ($F_{(1,40)} = 5.85$, $p = 0.020$, ES partial $\eta^2 = 0.128$), and a trend for a day
1543 X treatment X genotype interaction ($F_{(3,120)} = 2.6$, $p = 0.058$, ES partial $\eta^2 = 0.060$).
1544 Analysis of genotype and treatment effects on each day revealed significant genotype X
1545 treatment interactions on Day 3 ($F_{(1,40)} = 7.68$, $p = 0.008$, ES partial $\eta^2 = 0.161$) and Day 4
1546 ($F_{(1,40)} = 7.00$, $p = 0.012$, ES partial $\eta^2 = 0.149$), but not on Days 1 or 2. Analysis of simple
1547 effects on Days 3 and 4 revealed a genotype effect within the Amph-treated groups
1548 indicated by * (Day 3, $F_{(1,20)} = 8.29$, $p = 0.009$, ES partial $\eta^2 = 0.313$; Day 4, $F_{(1,20)} = 9.13$, p
1549 $= 0.007$, ES partial $\eta^2 = 0.616$). One week later (Day 11), all mice received a lower
1550 challenge dose of Amph (2 mg/kg; gray shading). Amph-treated CTRL mice showed a
1551 significantly increased response to Amph compared to Veh-treated CTRL mice, revealing
1552 sensitization. In contrast, Amph-treated stopGLS1 HET mice showed a slightly increased
1553 response to Amph compared to Veh-treated stopGLS1 HET mice, showing reduced
1554 expression of sensitization. A two-way ANOVA revealed a significant treatment X genotype
1555 interaction ($F_{(1,40)} = 4.5$, $p = 0.039$, ES partial $\eta^2 = 0.103$). Analyses within each genotype,
1556 showed a significant effect of drug treatment for CTRL mice ($F_{(1,19)} = 24.8$, $p < 0.001$, ES
1557 partial $\eta^2 = 0.566$), and a trend for treatment in stopGLS1 HET mice ($F_{(1,21)} = 4.1$, $p =$
1558 0.055 , ES partial $\eta^2 = 0.164$). * indicates significantly different from Amph-treated CTRL
1559 mice, analyses of simple main effects.

1560 (B) Time course of Amph-induced locomotion for CTRL and stopGLS1 HET mice on the
1561 challenge day (Day 11). There were no genotypic differences in baseline activity, prior to
1562 Amph injection. After Amph injection, Veh-treated mice — receiving Amph for the first time
1563 — showed a modest locomotor response that did not differ genotypically (left graph).
1564 Amph-treated mice showed a robust locomotor response to Amph, greater in CTRL than
1565 stopGLS1 HET mice (right graph). These differences were supported by a RM ANOVA
1566 within each treatment, showing no time X genotype interaction for Veh-treated mice ($F_{(8,160)}$
1567 $= 0.782$, $p = 0.619$), but a significant time X genotype interaction for Amph-treated mice
1568 ($F_{(8,160)} = 3.9$, $p < 0.0001$, ES partial $\eta^2 = 0.165$). Taken together, these results indicate that
1569 stopGLS1 HET mice show attenuated amphetamine sensitization.

1570 Numbers of mice are shown above the bars. Abbreviations: Amph - amphetamine; Veh -
1571 vehicle. See [Source Data](#) for the full statistical analysis.

1572 **Figure 6 – figure supplement 2. Breeding Δ GLS1 HET mice (with a global GLS1**
1573 **reduction) from floxGLS1 mice.**

1574 (A) Inducible Rosa26^{creERT2} :: GLS1^{lox/+} mice (pink outline) were used to produce a global
1575 heterozygous GLS1 inactivation in adulthood by tamoxifen-induced recombination of the
1576 floxGLS1 allele. These Rosa26^{creERT2} :: GLS1 ^{Δ /+} mice (gray with pink outline) were bred

1577 with wild-type (WT) C57BL6 mice (white) to generate Δ GLS1 HET mice (gray).
1578 (B) Expression of PAG in the hippocampus (HIPP) of Rosa26^{creERT2} :: GLS1 ^{Δ /+} mice after
1579 tamoxifen revealed that the protein was reduced to 55.5% of control levels measured in
1580 Rosa26^{creERT2} mice. These mice were bred with WT mice to generate Δ GLS1 HET mice. *
1581 indicates significant difference from CTRL (Rosa26^{creERT2}) (ANOVA, $F_{(1,7)} = 20.15$, $p =$
1582 0.003 , ES partial $\eta^2 = 0.742$).

1583 (C) GLS1 allelic abundance for WT and floxGLS1 alleles in Δ GLS1 HET mice showed one
1584 WT allele and the absence of the floxGLS1 allele (blue bars) in the hippocampus (HIPP),
1585 prefrontal cortex (PFC), dorsal striatum (dStr), thalamus (Thal) and ventral midbrain
1586 (VMB), further validating the global heterozygous GLS1 deletion. Allelic abundance data
1587 were normalized to CTRL values in GLS1 lox/+ (gray line)..

1588 **Figure 6 – figure supplement 3. Δ GLS1 HET mice show reduced novelty-induced**
1589 **locomotion, attenuated amphetamine sensitization and potentiated latent inhibition.**

1590 (A) Novelty-induced locomotion, but not habituation, was reduced in Δ GLS1 HET mice.
1591 \diamond indicates a significant main effect of genotype (RM ANOVA: $F_{(1,33)} = 5.98$, $p < 0.020$, ES
1592 partial $\eta^2 = 0.153$). # indicates a significant main effect of time ($F_{(5,165)} = 91.92$, $p < 0.001$,
1593 ES partial $\eta^2 = 0.736$); there was no significant interaction ($F_{(5,165)} = 0.942$, $p = 0.455$).

1594 (B) Δ GLS1 HET mice were tested for amphetamine sensitization following a similar
1595 protocol and drug dose to that used for DAT GLS1 cHET mice (Figure 6A). After Veh
1596 injections (Days 1 and 2), Δ GLS1 HET mice were overall less active than CTRL mice. This
1597 was supported by a 2 (genotype) x 2 (treatment) x 2 (days) ANOVA that showed a
1598 significant main effect of genotype ($F_{(1,31)} = 17.03$, $p < 0.001$, ES partial $\eta^2 = 0.355$)
1599 indicated by \diamond , but no other significant main effects or interactions. During the 5
1600 consecutive treatment days, Amph-treated Δ GLS1 HET mice showed both a blunted
1601 response to acute amphetamine and no sensitization. This is reflected in the 2 (genotype)
1602 x 2 (treatment) x 5 (days) ANOVA by the absence of a significant genotype X drug
1603 treatment X days interaction ($F_{(4,124)} = 0.733$, $p = 0.585$), but a significant genotype X drug
1604 treatment interaction ($F_{(1,31)} = 13.2$, $p = 0.001$, ES partial $\eta^2 = 0.299$). Analysis of genotype
1605 and treatment effects on each day revealed significant genotype X treatment interactions
1606 on all days except Day 4 (Day 3, $F_{(1,31)} = 10.14$, $p = 0.03$; Day 4, $F_{(1,31)} = 3.73$, $p = 0.063$;
1607 Day 5, $F_{(1,31)} = 15.00$, $p = 0.001$; Day 6, $F_{(1,31)} = 11.64$, $p = 0.002$; Day 7, $F_{(1,31)} = 10.56$, $p =$
1608 0.003). Analysis of simple effects on Days 3, 5, 6 and 7 revealed a genotype effect within
1609 the Amph-treated groups indicated by * (Day 3, $F_{(1,19)} = 18.21$, $p < 0.001$; Day 5, $F_{(1,19)} =$
1610 21.57 , $p < 0.001$; Day 6, $F_{(1,19)} = 18.97$, $p < 0.001$; Day 7, $F_{(1,19)} = 17.82$, $p < 0.001$; ES
1611 partial $\eta^2 > 0.400$ for all). After a withdrawal period, on Day 19, all mice received Amph
1612 (2.5 mg/kg). Amph-treated CTRL mice showed a sensitized locomotor response compared
1613 to Δ GLS1 HET mice, yet due to a ceiling effect the responses of Amph-treated and Veh-
1614 treated CTRL mice did not differ. \diamond indicates significant main effect of genotype (two-way
1615 ANOVA: $F_{(1,31)} = 10.6$, $p = 0.003$, ES partial $\eta^2 = 0.254$). There was no significant drug
1616 treatment effect ($F_{(1,31)} = 3.64$, $p = 0.066$) or interaction ($F_{(1,31)} = 2.56$, $p = 0.120$). Four
1617 days later (Day 23), mice received a low-dose Amph challenge (1.25 mg/kg). Amph-
1618 treated CTRL mice showed a sensitized response compared to Veh-treated mice and
1619 Amph-treated Δ GLS1 HET mice (two-way ANOVA: significant genotype X drug treatment

1620 interaction, $F_{(1,31)} = 4.22$, $p = 0.048$, ES partial $\eta^2 = 0.120$). * indicates significant genotype
1621 effect for Amph-treated mice (ANOVA, $F_{(1,31)} = 5.20$, $p = 0.034$, ES partial $\eta^2 = 0.215$).

1622 (C) Δ GLS1 HET mice were tested for potentiation of LI, following the same protocol as
1623 used for the DAT GLS1 cHETs (Figure 7A). The graph shows percent time during the
1624 8 min CS presentation on the test day (Day 3). There was no difference between NPE and
1625 PE CTRL groups. Δ GLS1 HET PE mice froze less during the CS exposure revealing a
1626 potentiated LI response (two-way ANOVA: significant genotype X PE interaction, $F_{(1,24)} =$
1627 5.40 , $p = 0.029$, ES partial $\eta^2 = 0.183$). * indicates a significant PE effect for Δ GLS1 HETs
1628 (ANOVA: $F_{(1,10)} = 11.2$, $p = 0.007$, ES partial $\eta^2 = 0.530$).

1629 Numbers of mice are shown either next to the lines or above the bars. Abbreviations:
1630 Amph - amphetamine; Veh - vehicle; PE - preexposed group; NPE - non-preexposed
1631 group. See [Source Data](#) for the full statistical analysis.

1632 **Figure 6 – figure supplement 4. Conditional forebrain PAG reduction in EMX1 GLS1**
1633 **cHET mice.**

1634 (A) Validation of the forebrain-specific GLS1 deletion in EMX1 GLS1 cKO mice using PAG
1635 immunoreactivity. P18 mice were used, as EMX1 GLS1 cKO mice die by P21. PAG
1636 immunoreactivity in EMX1 GLS1 cKO mice was absent in HIPP and PFC, but not Thal.

1637 (B) GLS1 allelic abundance for WT and floxGLS1 alleles in EMX1 GLS1 cHET mice
1638 showed that the floxGLS1 allele was reduced to 38% in the HIPP and 44% in the PFC, but
1639 not affected in the dStr and Thal, further validating the regional specificity of the EMX1^{cre}-
1640 induced heterozygous GLS1 reduction. Allelic abundance data were normalized to CTRL
1641 values in GLS1 lox/+ (gray line).

1642 (C) PAG protein expression in the hippocampus of EMX1 GLS1 cHET mice. PAG protein
1643 was reduced to 52% of CTRL. * indicates significant difference from CTRL (EMX1^{cre}) (one-
1644 way ANOVA, $F_{(1,19)} = 51.38$, $p < 0.0001$, ES partial $\eta^2 = 0.730$).

1645 The number of mice is shown above the bars.

1646 **Figure 6 – figure supplement 5. Clozapine-induced potentiation of latent inhibition in**
1647 **EMX1 GLS1 cHET, Δ GLS1 HET and DAT GLS1 cHET mice.**

1648 (A) Schematic of the LI protocol to test the effect of clozapine using the same protocol
1649 used for the DAT GLS1 cHETs (Figure 6D). Both NPE and PE groups received a single
1650 injection of clozapine (1.5 mg/kg) on Day 1, 30 min before being put in the conditioning
1651 boxes.

1652 (B) EMX1 GLS1 cHET mice were tested for potentiation of LI following pretreatment with
1653 clozapine. Graph shows percent freezing on the tone test (Day 3) during the 8 min CS
1654 presentation. Clozapine decreased freezing in CTRL and EMX1 GLS1 cHET PE groups,
1655 revealing potentiation of LI. # indicates a significant main PE effect (two-way ANOVA,
1656 $F_{(1,23)} = 13.13$, $p = 0.001$, ES partial $\eta^2 = 0.363$); there was no significant main effect of
1657 genotype ($F_{(1,23)} = 0.452$, $p = 0.508$) or interaction ($F_{(1,23)} = 0.002$, $p = 0.967$). The percent
1658 time freezing of clozapine-treated NPE groups was similar to the freezing reported for

1659 vehicle-treated NPE groups (dashed grey line) indicating that clozapine did not affect
1660 aversive associative learning.

1661 (C) Δ GLS1 HET (left) and DAT GLS1 cHET (right) mice were tested for potentiation of LI
1662 following pretreatment with clozapine. Clozapine pretreatment selectively decreased
1663 freezing in the PE groups, revealing potentiation of LI, independent of genotype. #
1664 indicates a significant main PE effect (two-way ANOVA: for Δ GLS1 HETs, $F_{(1,23)} = 6.74$,
1665 $p = 0.016$, ES partial $\eta^2 = 0.227$; DAT GLS1 cHETs, $F_{(1,24)} = 6.06$, $p = 0.021$, partial $\eta^2 =$
1666 0.202); there was no significant main effect of genotype (Δ GLS1 HETs, $F_{(1,23)} = 0.120$, $p =$
1667 0.732 ; DAT GLS1 cHETs, $F_{(1,24)} = 0.069$, $p = 0.794$) or interaction (Δ GLS1 HETs, $F_{(1,23)} =$
1668 0.051 , $p = 0.824$; DAT GLS1 cHETs, $F_{(1,24)} = 1.978$; $p = 0.172$). The percent time freezing
1669 of clozapine-treated NPE groups was similar to the freezing reported for Veh-treated NPE
1670 groups (dashed gray line) indicating that clozapine did not affect aversive associative
1671 learning.

1672 The number of mice is shown above the bars.

Figure 1

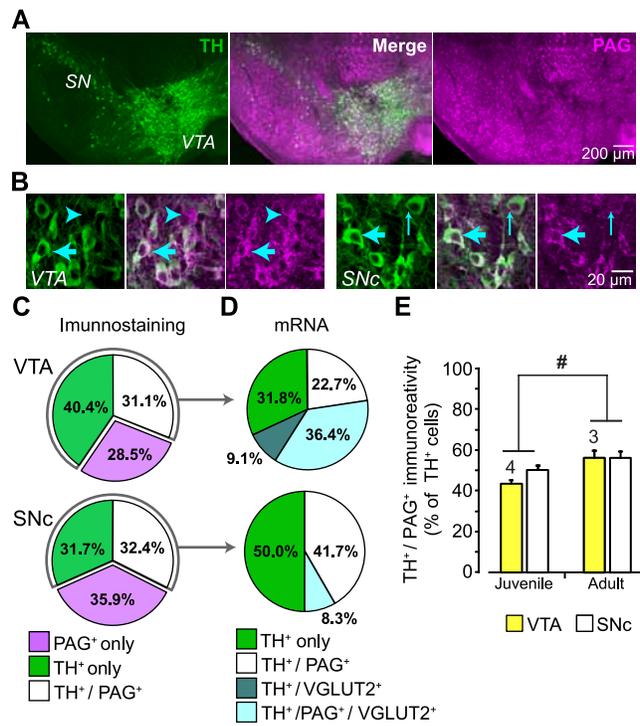


Figure 1 - Suppl 1

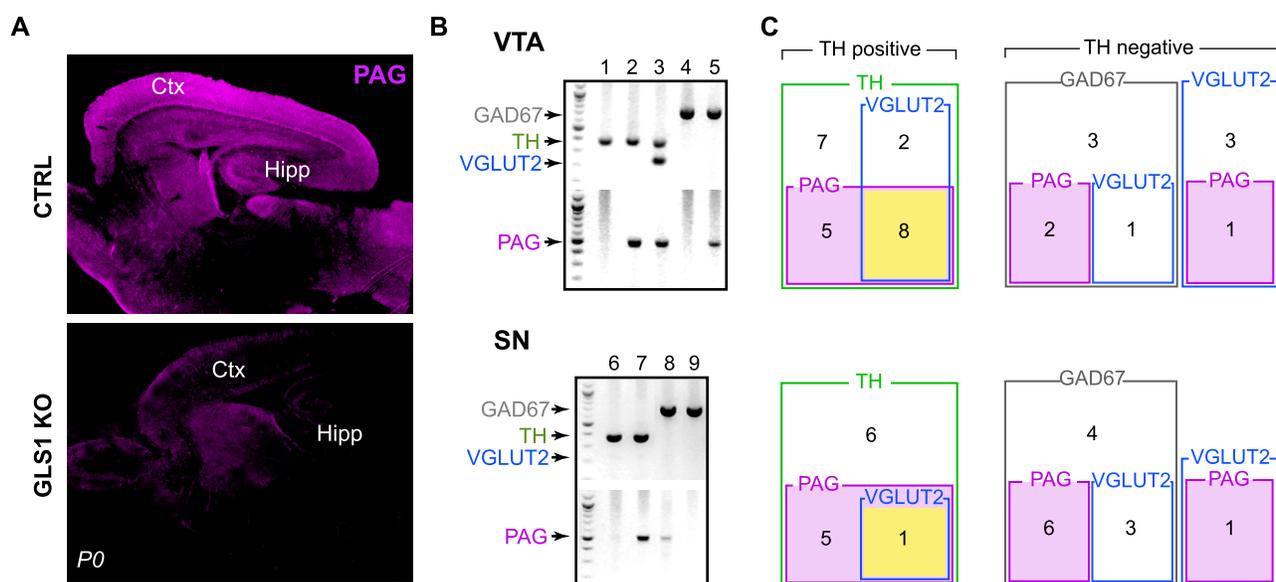


Figure 2

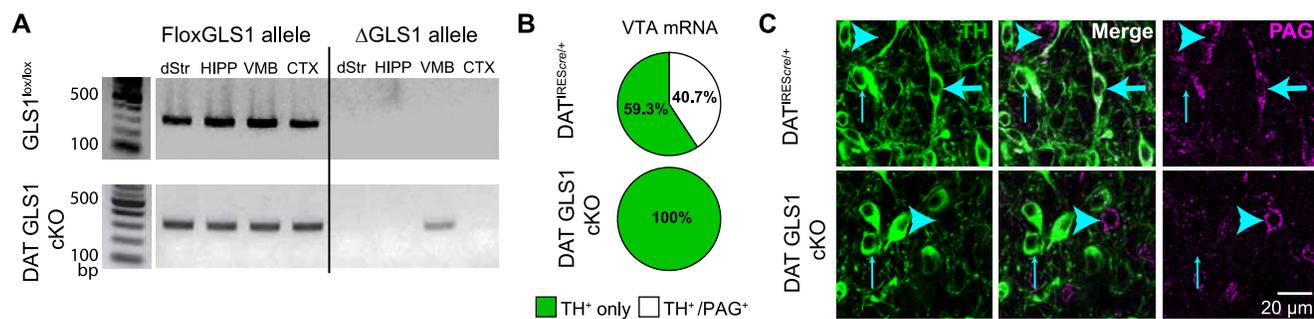


Figure 2 - Suppl 1

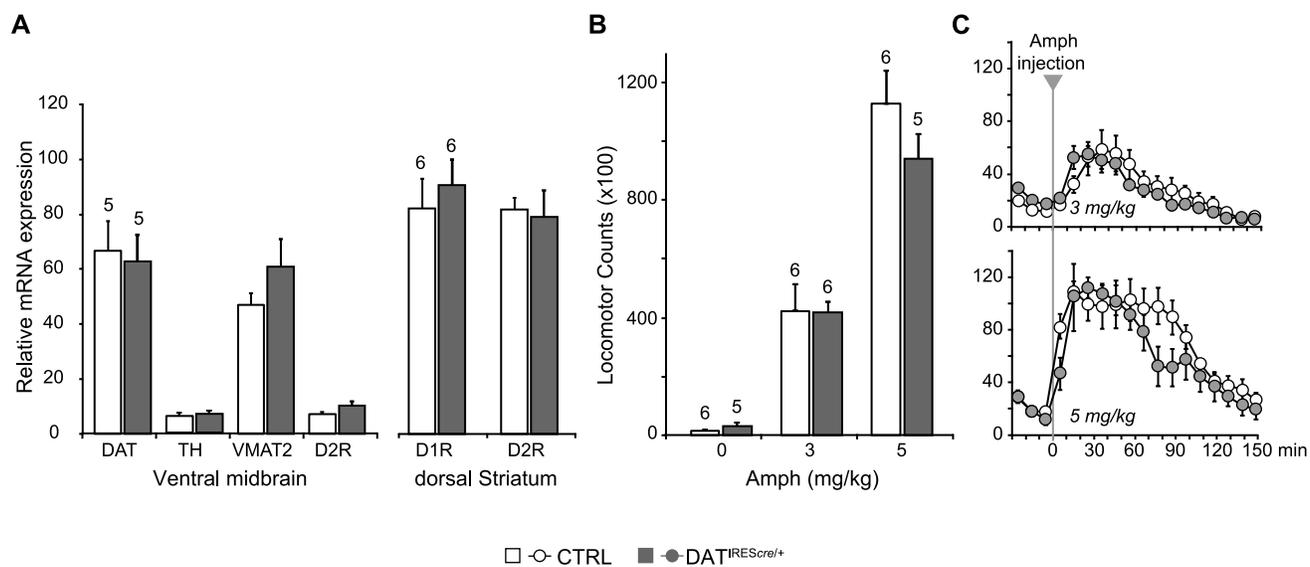


Figure 3

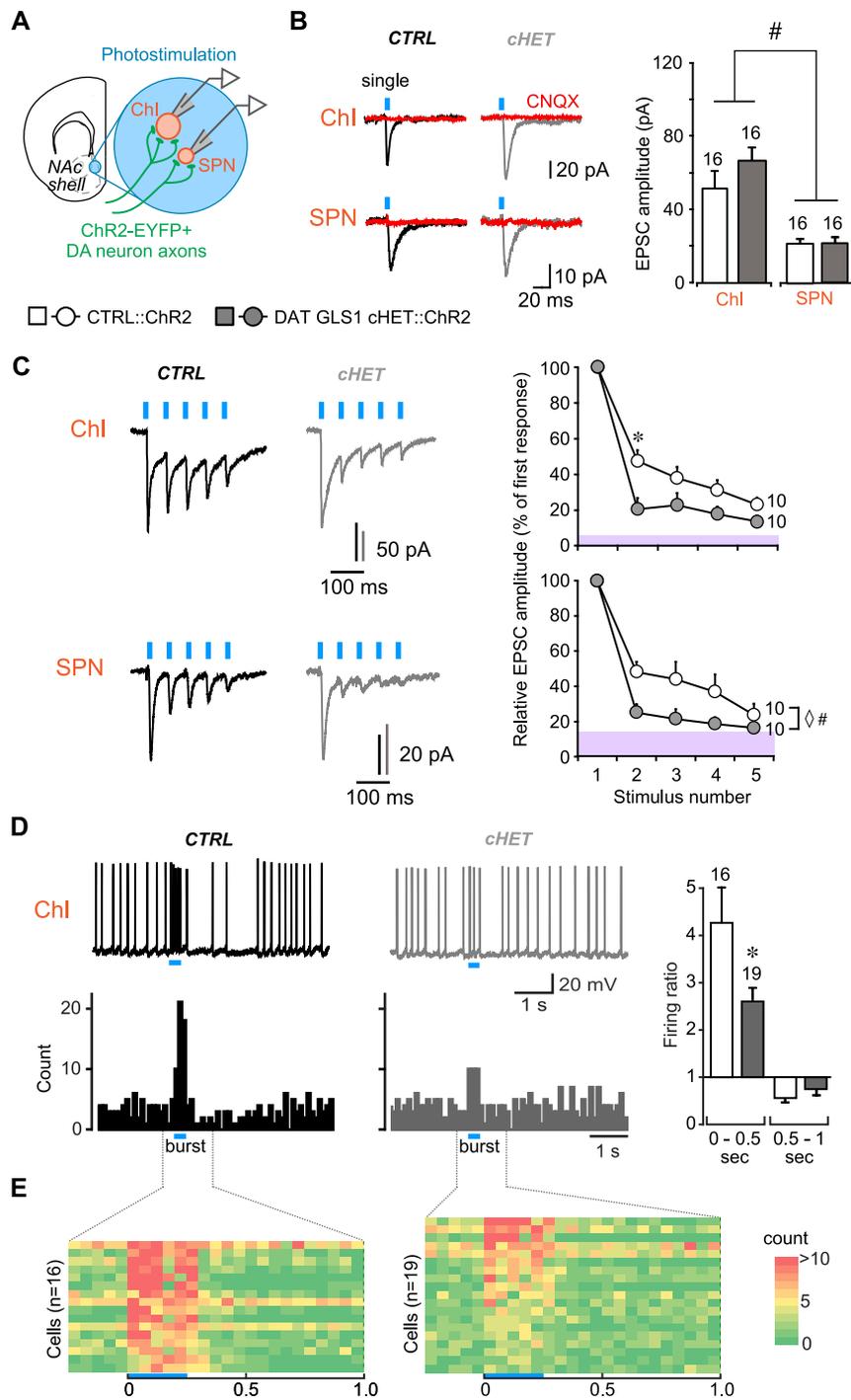


Figure 3- Suppl 1

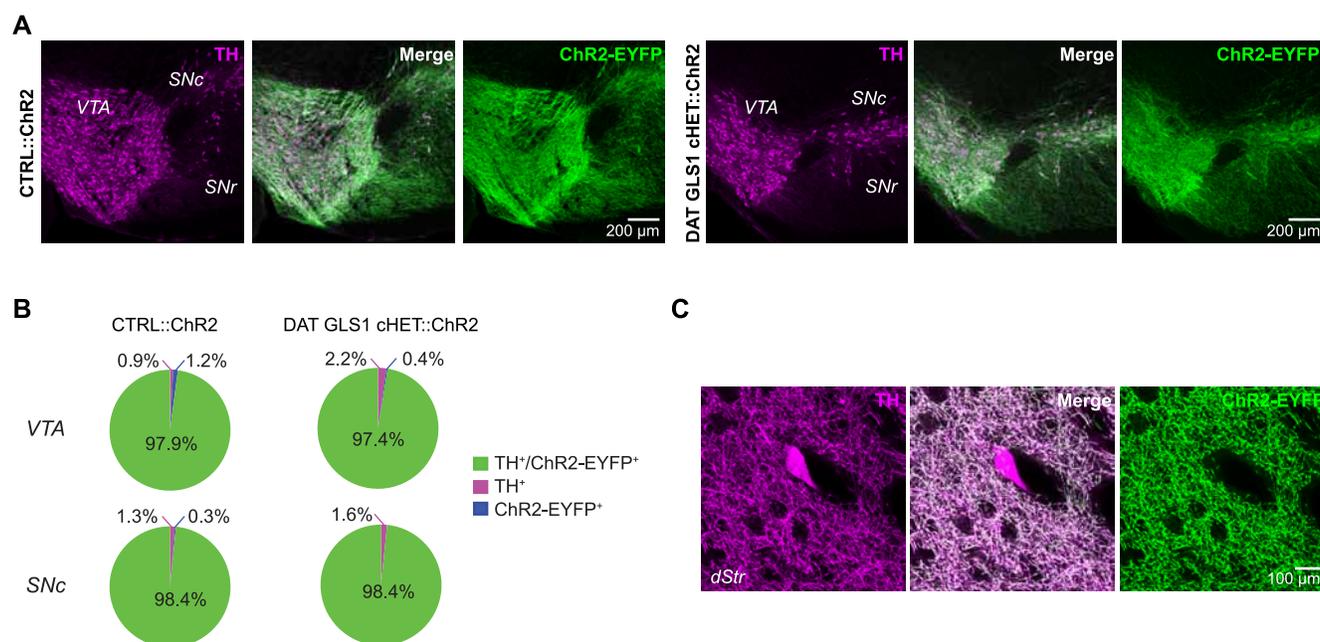


Figure 3 - Suppl 2

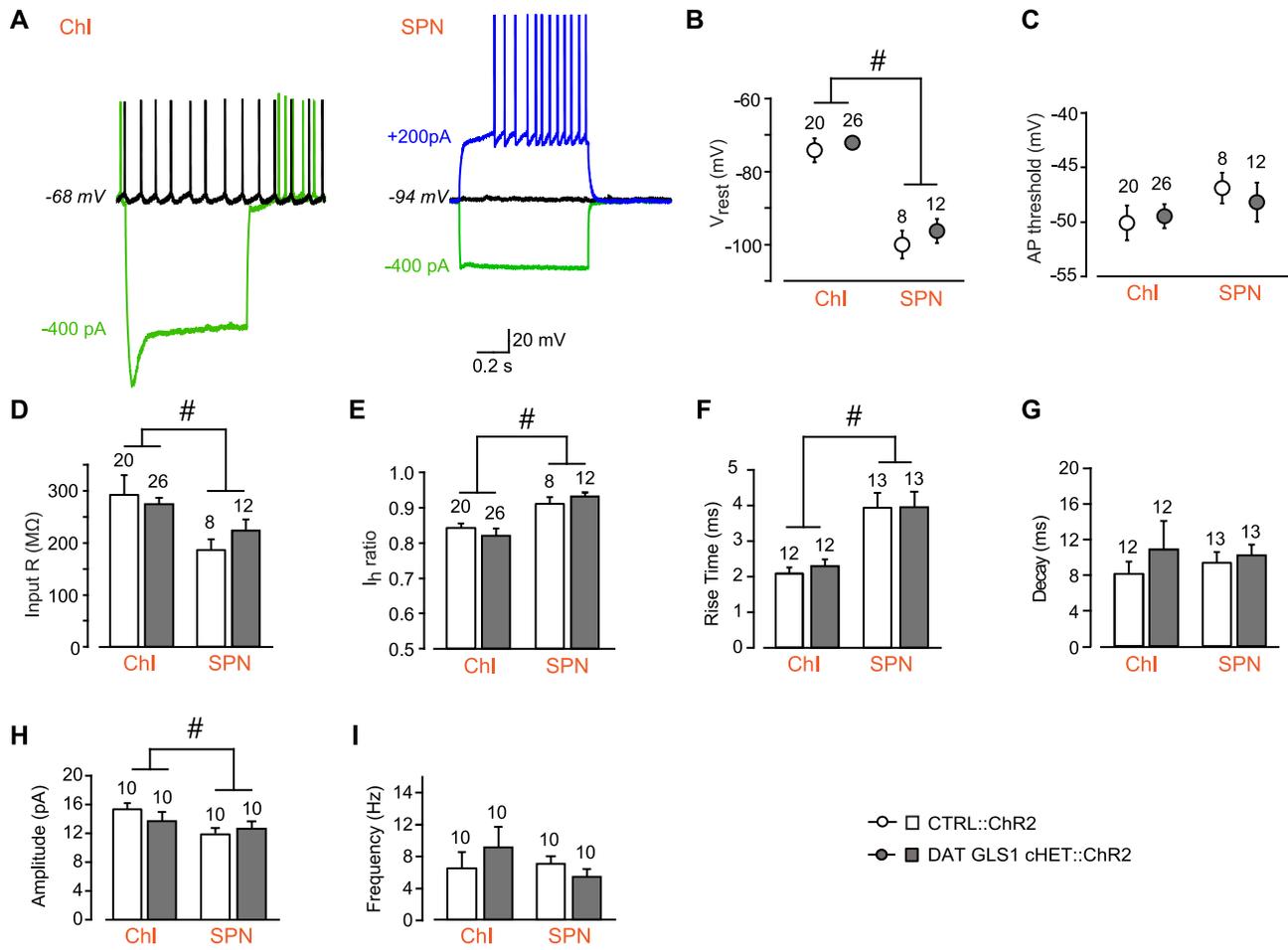


Figure 4

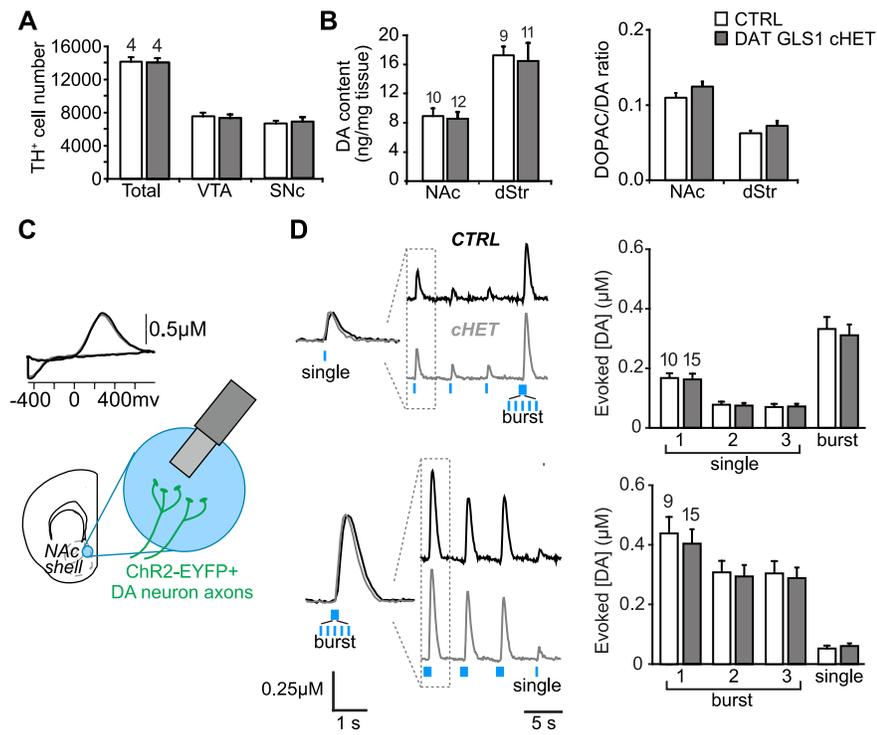


Figure 4 - Suppl 1

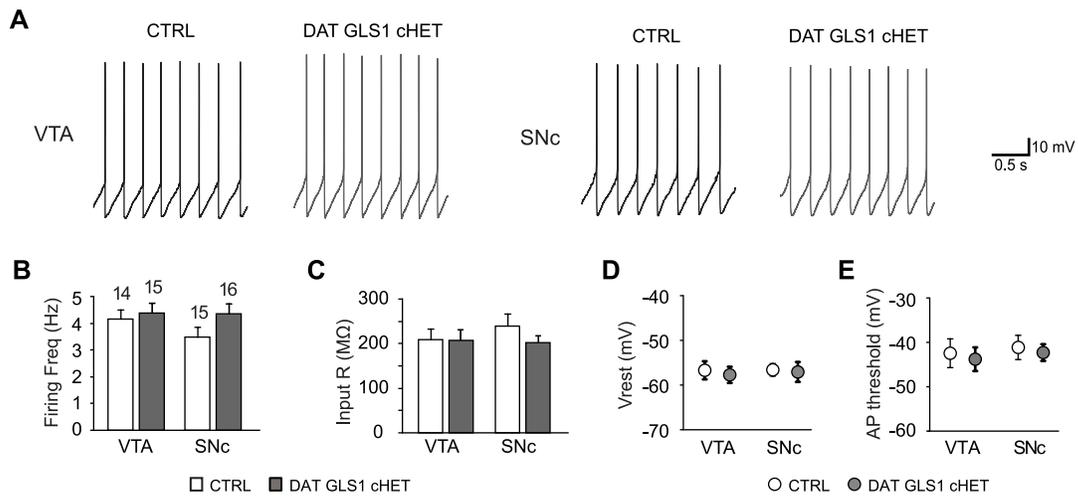


Figure 5

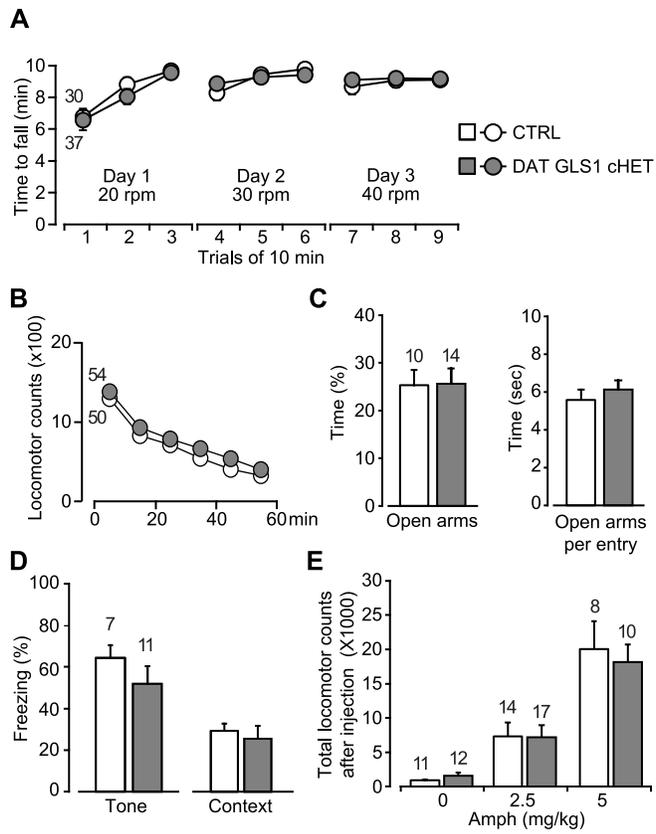


Figure 6

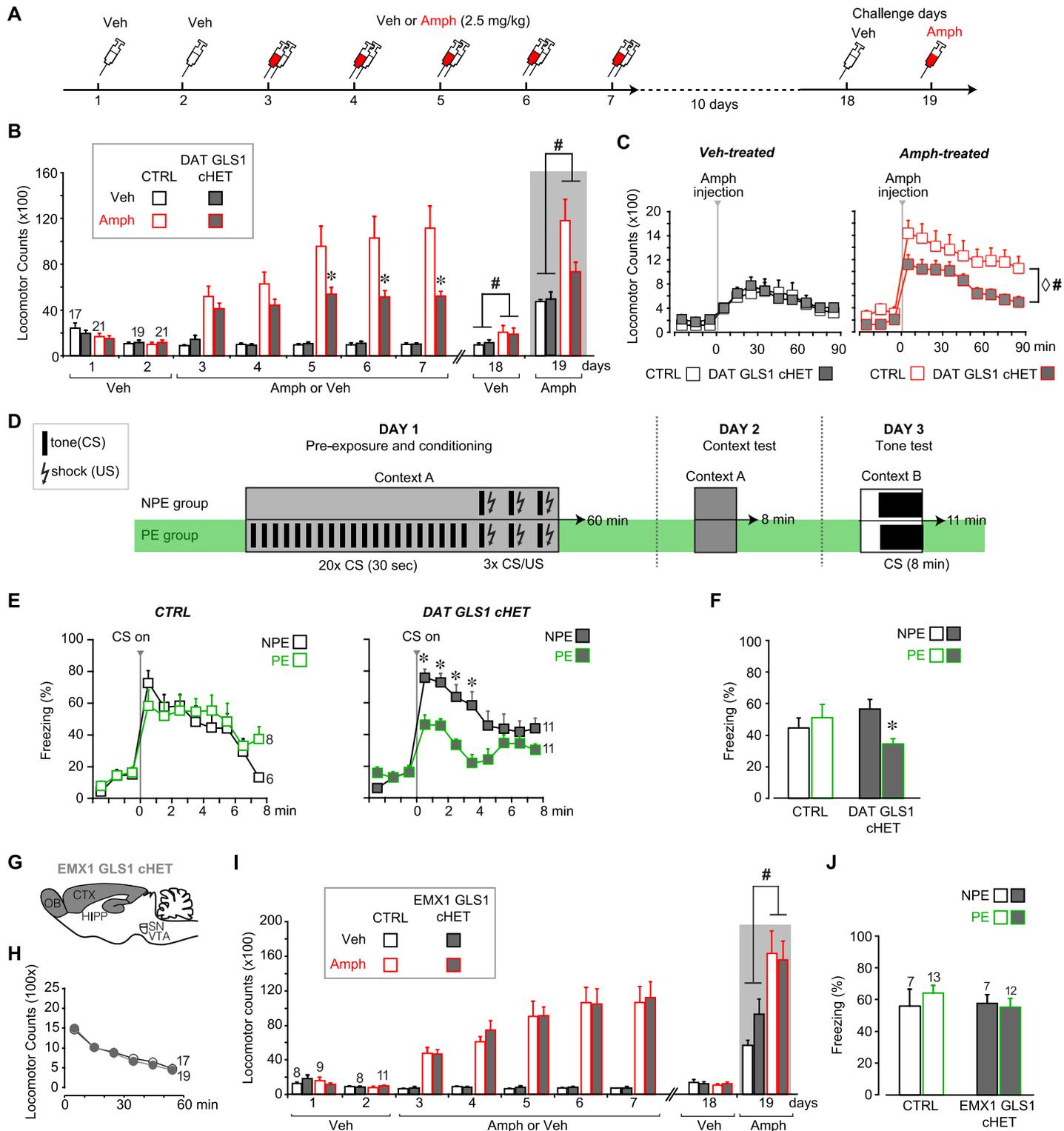


Figure 6 - Suppl 1

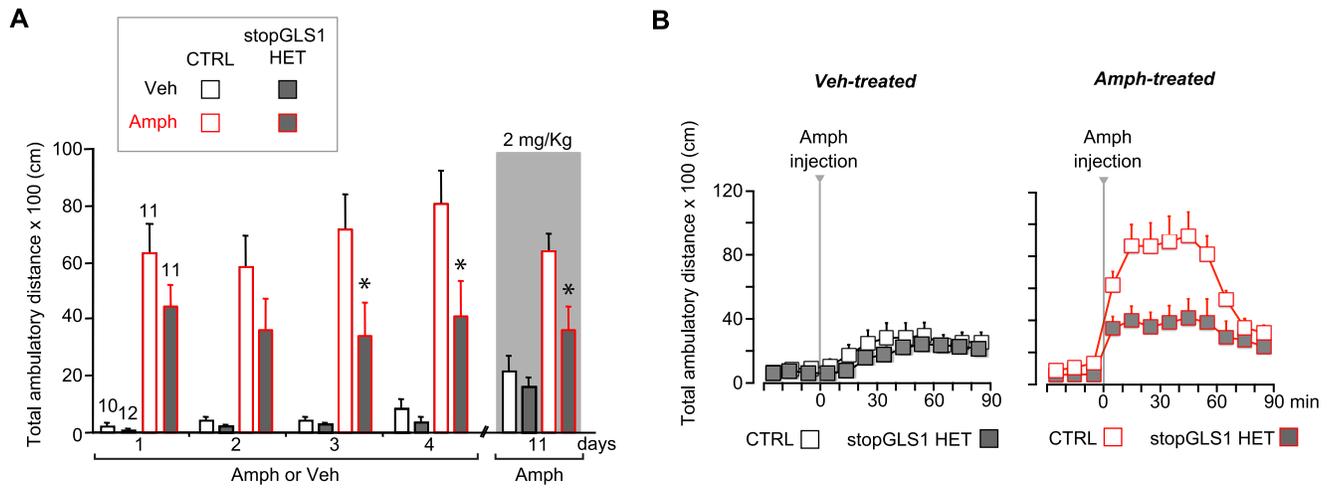


Figure 6 - Suppl 2

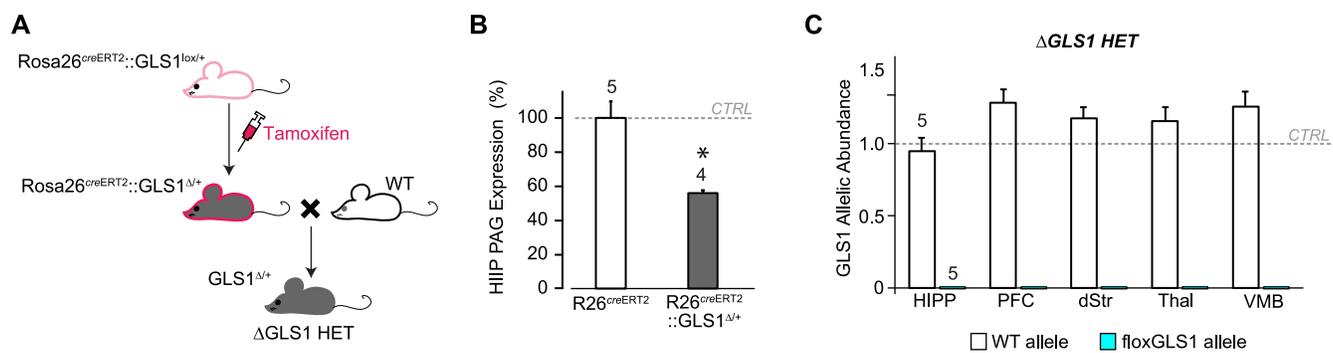


Figure 6 - Suppl 3

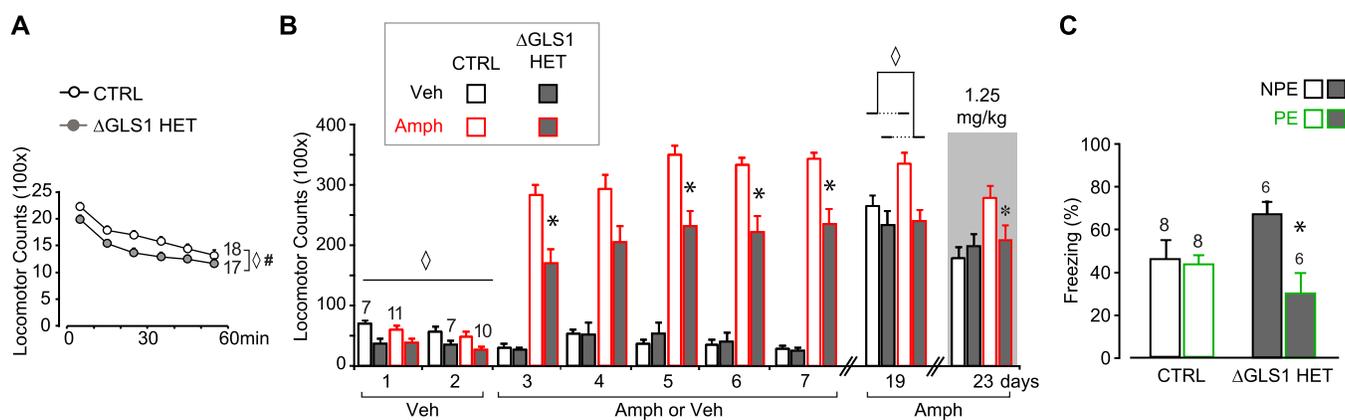


Figure 6 - Suppl 4

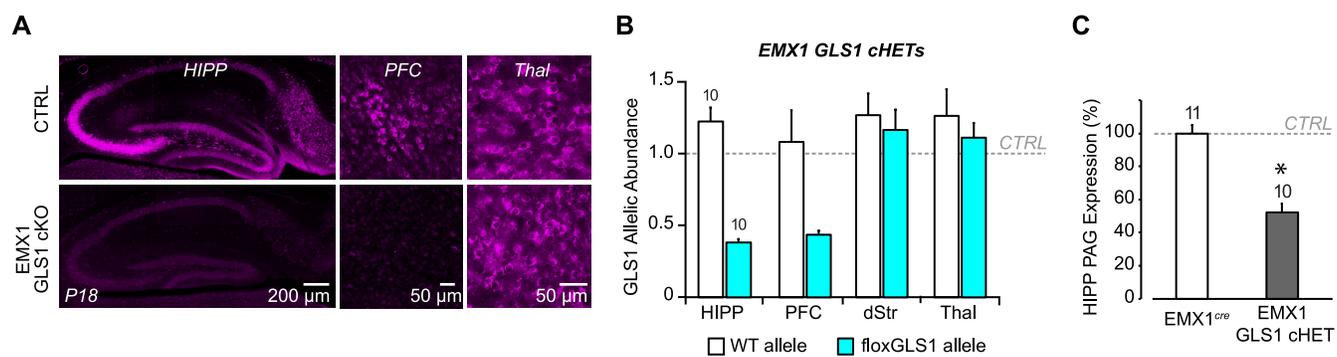


Figure 6 - Suppl 5

