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1 **Nicotinic acetylcholine receptors expressed by striatal interneurons inhibit striatal activity**
2 **and control striatal-dependent behaviors**

3

4 **Running title:** Nicotinic receptors in striatal interneurons

5

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29

30 **Conflict of interest**

31 The authors declare no competing financial interests.

32

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53 **Abstract**

54 Acetylcholine is an important modulator of striatal activity and it is vital to controlling striatal-
55 dependent behaviors including motor and cognitive functions. Despite this significance, the
56 mechanisms determining how acetylcholine impacts striatal signaling are still not fully understood.
57 In particular, little is known about the role of nicotinic acetylcholine receptors (nAChRs) expressed
58 by striatal interneurons. In the present study, we used fluorescent in situ hybridization (FISH) to
59 determine which neuronal types express the most prevalent beta2 nicotinic subunit in the mouse
60 striatum. Our data support a common view that nAChR expression is mostly restricted to striatal
61 interneurons. Surprisingly though, cholinergic interneurons (CINs) were identified as a population
62 with the highest expression of beta2 nicotinic subunit. To investigate the functional significance
63 of beta2-containing nAChRs in striatal interneurons, we deleted them by injecting the AAV-Cre
64 vector into the striatum of beta2-flox/flox male mice. The deletion led to alterations in several
65 behavioral domains, namely to an increased anxiety-like behavior, decrease in sociability ratio,
66 deficit in discrimination learning and increased amphetamine-induced hyperlocomotion and c-Fos
67 expression in mice with beta2 deletion. Further colocalization analysis showed that the increased
68 c-Fos expression was present in both medium spiny neurons and presumed striatal interneurons.
69 The present study concludes, that despite being relatively rare, beta2-containing nAChRs are
70 primarily expressed in striatal neurons by CINs and play a significant role in behavior.

71 **Significance statement:** A large variety of nicotinic acetylcholine receptors are expressed in the
72 striatum, a brain region that is crucial in the control of behavior. The complexity of receptors with
73 different functions is hindering our understanding of mechanisms through which striatal
74 acetylcholine modulates behavior. We focused on the role of a small population of beta2-
75 containing nicotinic acetylcholine receptors. We identified neuronal types expressing these

76 receptors and determined their impact in the control of explorative behavior, anxiety-like behavior,
77 learning and sensitivity to stimulants. Additional experiments showed that these alterations were
78 associated with an overall increased activity of striatal neurons. Thus, the small population of
79 nicotinic receptors represents an interesting target for a modulation of response to stimulant drugs
80 and other striatal-based behavior.

81

82 **Introduction**

83 A growing body of evidence suggests that interneurons (INs) play a key role in controlling striatal
84 functions despite the fact that they represent only a minority of striatal neurons (less than 5 % in
85 rodents) (Lee et al., 2017b; Rapanelli et al., 2017; Holly et al., 2019; Muñoz-Manchado et al.,
86 2016). Striatal interneurons are commonly divided into two main groups, cholinergic and
87 GABAergic (CINs and GABAINs, respectively). In the past decade, electrophysiological studies
88 indicated that unlike the principal striatal neurons (commonly referred to as medium spiny neurons,
89 MSNs), certain types of INs express functional nAChRs. For instance, somatostatin-expressing,
90 5HT₃A-expressing and neuropeptide Y-expressing (NPY+) GABAINs are activated by nicotine
91 and this activation leads to inhibition of MSNs activity (Faust et al., 2016; Assous et al., 2017;
92 English et al., 2011). These studies suggested that even though nAChRs are expressed by a
93 relatively small number of striatal neurons, these receptors have the power to modulate striatal
94 signaling and functions. Therefore, it has been hypothesized that nAChRs expressed by striatal
95 INs play a significant role in the control of striatal based behavior, e.g. supporting cognitive
96 flexibility (Faust et al., 2016; Assous, 2021), but a direct evidence was missing. Recently, several
97 novel types of GABAINs, each with distinct immunohistochemical and electrophysiological
98 properties, have been described in the striatum (Muñoz-Manchado et al., 2016; Assous et al., 2018;
99 Muñoz-Manchado et al., 2018). Including the classic groups of parvalbumin-, somatostatin- and
100 calretinin-expressing neurons, striatal GABAINs now seem to be composed of at least seven
101 distinct neuronal populations and this number may grow. Although individual types of striatal INs
102 express nAChRs in overall low levels and with varying subunit composition, beta2-containing
103 nAChRs are relatively the most common type (Quik and Wonnacott, 2011; Quik et al., 2009).
104 Until the emergence of single cell RNA sequencing (scRNA-seq), detailed information on the

105 expression of specific nicotinic subunits by individual types of striatal INs was lacking. A recent
106 scRNA-seq study distinguishing between seven different types of striatal GABAergic neurons suggested
107 that each GABAergic population may express different types of nAChRs (Munoz-Manchado et al.,
108 2018). Specifically, tyrosine hydroxylase-expressing GABAergic neurons were shown to be distinguished
109 by their expression of the alpha3 nicotinic subunit. Unlike the expression of the alpha3 subunit,
110 the beta2 subunit was not specifically examined in the study by (Munoz-Manchado et al., 2018).
111 Thus, we used this previously published scRNA-seq data and re-analyzed it in order to assess and
112 compare the expression of the beta2 subunit in different populations of striatal neurons (Fig. 2 a-
113 b). Surprisingly, the re-analysis showed no difference in beta2 expression not only between the
114 individual groups of GABAergic neurons but also between GABAergic neurons and MSNs. Therefore, expression of
115 beta2-containing nAChRs by striatal neurons requires further investigation.

116 In the present study, we used fluorescent in situ hybridization (FISH) with a probe targeting beta2
117 nicotinic subunit to evaluate its expression in combination with markers for all major neuronal
118 types present in the striatum. Unexpectedly, we found that the majority of beta2-containing
119 nAChRs are expressed by striatal CINs and to lesser extent by other types of INs. Then, to evaluate
120 a behavioral role of these receptors, we deleted beta2 nicotinic subunit specifically in striatal INs
121 while keeping intact receptors expressed on dopaminergic and glutamatergic striatal terminals. A
122 comprehensive behavioral analysis revealed that beta2 deletion in striatal INs leads to changes in
123 several behavioral domains including anxiety-like behavior, social and explorative behavior,
124 discrimination learning and sensitivity to amphetamine. Finally, our analysis of c-Fos expression
125 documented that these behavioral alterations are accompanied by increased activation of striatal
126 neurons, both MSNs and INs.

127

128 **Methods**

129 **Animals**

130 All experimental procedures complied with the directive of the European Community Council on
131 the use of laboratory animals (2010/63/EU) and were approved by the respective local animal
132 research committees. Mice were housed in a temperature and humidity-controlled room with a 12
133 hour light/dark cycle (lights ON at 6 AM local time). Standard rodent chow and water were
134 provided *ad libitum*. For the food-motivated T-maze task and instrumental task in the operant box
135 mice were mildly food-restricted and their weight was kept at 85-90 % of their free-food weight.
136 For assessment of behavioral responses to temporal restriction of access to food, the pellets were
137 removed from the cages 3 h after the lights on and they were returned back 6 h later. The regime
138 continued for 10 days. The FISH experiments were performed in three C57BL/6J wild type male
139 mice. In the rest of the study, a total number of 80 beta2-flox/flox mice were used. The original
140 breeding pairs of beta2-flox/flox mice were kindly provided by Prof. Michael Crair from Yale
141 University and their generation and genotyping protocol is described in details elsewhere
142 (Burbridge et al., 2014). The mice originally provided were homozygous on a mixed background
143 and we maintained them as such throughout the study (for ten generations). After detecting
144 behavioral differences between sexes in our pilot experiments, we decided to exclusively use male
145 mice in our behavioral and biochemical experiments. Mice between the ages of 2 to 8 months were
146 used throughout the study and the maximum age difference within a single cohort was 40 days.
147 After the stereotaxic surgery, some mice were separated and housed individually. To diminish any
148 effect of the cage/litter, whenever possible AAV-Cre injected and control animals were always
149 included in one cage.

150 **Experimental design and statistical analysis**

151 Four independent cohorts of mice were injected into the dorsal striatum (DS) and used for
152 behavioral experiments and three of these cohorts were used for c-Fos analysis. Cohorts 1&2 and
153 cohorts 3&4 always underwent the same behavioral tests. Selected tests were performed in all 4
154 cohorts and data pooled together. This resulted in some age differences between cohorts 1&2 vs.
155 3&4 when tested in specific tasks. Data for split performance of mice in the different cohorts are
156 shown in Figure 8 f-q. Animals were pseudorandomly assigned to an experimental group (mice
157 with the beta2 deletion) and a control group so that each litter/cage included both groups if possible.
158 Behavioral, immunofluorescence and RT-qPCR data were analyzed using GraphPad Prism
159 versions 7 and 8 (GraphPad Software, San Diego, CA). We used two-tailed Student's t-test for
160 comparison of two experimental groups with the following exceptions where the data were not
161 normally distributed: the number of dippings in the hole-board test was analyzed with a negative-
162 binomial regression using the glm.nb function of the package MASS (Venables, Ripley, and
163 Venables, 1994); the nest-building test was analyzed using a Bayesian zero-one-inflated beta
164 regression on the proportion of material used, via the brms package (Bürkner, 2017); the forced
165 swim test, the examination time of the non-social object and the sociability ratio in the social
166 preference test were assessed with a gamma regression using the `glm` function in R. The
167 percentage of c-Fos-positive CINs was analyzed by generalized linear mixed model. Two-way
168 ANOVA or repeated measures (RM) two-way ANOVA followed by Sidak's or Tukey's post-hoc
169 tests were used to evaluate the effects of two variables. In cases where some values were missing
170 (in tests of reversal learning where some mice were analyzed in the acquisition only since they
171 failed to reach the acquisition criteria and could not be moved to the reversal phase), we used a
172 mixed-effects analysis followed by post-tests. Significance was set at $p < 0.05$. Throughout the

173 manuscript, we report data as means \pm SEM and statistical analysis including p values and 95 %
174 confidence intervals (CI) for the differences in means.

175 **Re-analysis of *Chrb2* scRNA-seq data**

176 To assess between-population differences in *Chrb2* expression, we used a Bayesian hierarchical
177 generalized linear model with negative binomial response and varying intercepts for both cell
178 population group (glia, interneurons, MSNs) and the individual populations, using the brms R
179 package (Bürkner, 2017). To account for differences in sequencing depth, we used the
180 estimateSizeFactors function from the DESeq2 R package (Love et al., 2014). Plots were created
181 with the ggplot2 package (Wickham, 2016).

182 **Code accessibility**

183 The complete code to reproduce the analysis can be accessed at [https://github.com/cas-](https://github.com/cas-bioinf/chrb2_striatum/blob/main/Chrb2_scRNA_reanalysis.Rmd)
184 [bioinf/chrb2_striatum/blob/main/Chrb2_scRNA_reanalysis.Rmd](https://github.com/cas-bioinf/chrb2_striatum/blob/main/Chrb2_scRNA_reanalysis.Rmd).

185 **Double-probe fluorescent in situ hybridization (FISH)**

186 Adult mice (12.5 weeks) were euthanized by cervical dislocation. Brains were removed, rapidly
187 frozen in cold isopentane (-30°/-35°C) and serially sectioned in 10 series on cryostat at 16 μ m
188 thickness. Sections for each brain have been processed by in situ hybridization to allow systematic
189 quantitative analysis throughout the whole striatum. Double-probe FISH were performed as
190 previously reported (Dumas and Wallén-Mackenzie, 2019).

191 **Probes**

192 Double-probe FISH was performed using antisense riboprobes for the detection of the following
193 mRNAs: *Chrb2*: NM_009602 sequence 597-1517; *Drd1*: NM_010076 sequence 1756-2707 and
194 *Drd2* NM_010077 sequence 268-1187; *Chat*: NM_009891 sequence 526-1065; *Pvalb*:
195 NM_013645 sequence 74-591; *Npy*: NM_023456 sequence 13-453; *Sst*: NM_009215 sequence

196 143-401; Htr3a: NM_013561 sequence 641-1552. Synthesis of digoxigenin (DIG) and
197 fluorescein-labeled RNA probes were made by a transcriptional reaction with incorporation of
198 digoxigenin or fluorescein labelled nucleotides (Sigma-Aldrich; Reference 11277073910 and
199 11685619910). Specificity of probes was verified using NCBI blast.

200 *Procedure*

201 Cryosections were air-dried, fixed in 4 % paraformaldehyde (PFA) and acetylated in 0.25 % acetic
202 anhydride/100 mM triethanolamine (pH 8) followed by washes in PBS. Sections were hybridized
203 for 18 h at 65 °C in 100 µl of formamide-buffer containing 1 µg/ml DIG-labeled riboprobe and 1
204 µg/ml fluorescein-labeled riboprobe. Sections were washed at 65 °C with SSC buffers of
205 decreasing strength, and blocked with 20 % fetal bovine serum (FBS) and 1 % blocking solution.
206 For revelation steps, DIG epitopes were detected with HRP anti-DIG fab fragments at 1:2500
207 (Sigma-Aldrich; Reference 11207733910) and revealed using Cy3-tyramide at 1:100. Fluorescein
208 epitopes were detected with HRP anti-fluorescein fab fragments at 1:5000 (Sigma-Aldrich;
209 Reference 11426346910) and revealed using Cy2-tyramide at 1:250. Nuclear staining was
210 performed with DAPI. All slides were scanned at 20x resolution using the NanoZoomer 2.0-HT
211 (Hamamatsu, Japan). Laser intensity and time of acquisition were set separately for each riboprobe.
212 Images were analyzed using the NDP.view2 software (Hamamatsu Photonics). Regions of interest
213 were identified according to the Paxinos mouse brain atlas (Franklin and Paxinos, 2008). Positive
214 cells refer to a staining in a cell body clearly above background and surrounding a DAPI-stained
215 nucleus. Colocalization was determined by the presence of the signals for both probes in the soma
216 of the same cell. A manual counting was performed. The entire region of interest was evaluated.
217 A mean of 700, 1200, 520, 830 Chat, Npy, Pvalb and Sst neurons, respectively, were analyzed per
218 brain, on adjacent sections at 6 levels of rostral-caudal axis. For illustration purposes, the

219 NanoZoomer images were exported in TIFF format using NDP viewer. Images were corrected for
220 contrast, cropped on Photoshop 2021 (Adobe Systems, San Jose, California, United States), and
221 assembled on Illustrator 2021 (Adobe Systems).

222 **Stereotaxic surgeries**

223 Beta2-flox/flox mice were injected with the AAV5-eGFP or AAV5-Cre-GFP viral vectors (viral
224 titre 4.5×10^{12} vg/ml; UNC Vector Core) at 2 months of age. After mice were anesthetized by a
225 mixture of ketamine (87 mg/kg) and xylazine (13 mg/kg; both Vetoquinol), they were placed in
226 the stereotaxic frame (Stoelting Co.). Animals were injected with a total volume of 1 μ l per
227 hemisphere using four injections in total with the following coordinates: AP +1.5; ML \pm 1.4; DV -
228 3.0 and AP +1.3; ML \pm 1.6; DV -3.3. A microinjection pump (MICRO2T-UMP3-NL2010, World
229 Precision Instruments) was used for the infusions with a speed rate of 50 nl/min. For the behavioral
230 experiments, we injected 36 and 29 AAV5-Cre and AAV5-GFP mice, respectively. Viral
231 expression was verified in all animals by immunofluorescence (IF) after completion of behavioral
232 tests and 4 AAV5-Cre-injected animals were excluded from the study based on either insufficient
233 or excessive expression of the virus. We considered expression insufficient when viral expression
234 was completely absent from one or both hemispheres and excessive when it was significantly
235 leaking into the hippocampus, thalamus and/or cortex. IF analysis showed that the virus was
236 leaking into striatal neighbouring structures. However, the leaking was mostly unilateral, varied
237 between mice and did not systematically occur in a specific structure. The only exception was the
238 lateral septum (LS) which showed bilateral viral expression in two thirds of the AAV5-Cre-
239 injected animals (23 out of 32). Except for the LS, any other structure with bilateral viral
240 expression always occurred in less than half of the injected animals (Fig. 5d). To understand how
241 the variability in AAV injections could affect the investigated behavior, we also used a subset of

242 animals to analyze selected behavioral data in correlation to the extent of the AAV expression in
243 the DS. However, none of the analyzed behavioral parameters reached a significant correlation
244 with the AAV expression (Fig. 8 a-e).

245 **PCR and reverse transcription-quantitative PCR (RT-qPCR)**

246 To obtain samples for the PCR, mice were sacrificed by decapitation, brains were quickly removed,
247 and individual brain regions were dissected on ice, frozen on dry ice and stored at -80 °C until use.
248 DNA for the endpoint PCR and RNA for the RT-qPCR from non-striatal regions were isolated
249 using TriPure Isolation Reagent (Roche, Indiana, USA) and treated with DNase (Sigma). RNA for
250 the RT-qPCR from the striatum expressing AAV virus was prepared from punches taken
251 specifically in the AAV-expressing area and isolated by RNeasy Micro Kit (QIAGEN, Maryland,
252 USA). The endpoint PCR was used to confirm the deletion of exon 5 of the *Chrn2* gene induced
253 by the AAV-Cre injection into the DS of beta2-flox/flox mice. The primers used were 198#, 199#
254 and Bot2-09 as described in (Burbridge et al., 2014). The end point PCR was performed with Taq
255 DNA Polymerase (GeneDireX, Taiwan) according manufacturer's instructions and DNA
256 fragments were visualized on an agarose gel. For the RT-qPCR, the reverse transcription of the
257 RNA samples was performed with LunaScript RT SuperMix (New England Biolabs,
258 Massachusetts, USA) and the qPCR with LCC 480 SYBR Green Master at LCC 480 instrument
259 (Roche) according to manufacturers' instructions. Primers used in RT-qPCR were targeted against
260 exon 5 of the *Chrn2* gene: FW 5'-TGGCCATCCTGGTCTTCTAC-3' and RW 5'-
261 CGCCAGCAGCACAGAAATAC-3'. Beta-actin gene expression was used for normalization of
262 the data using primers reported by (Frahm et al., 2011). The relative quantification of gene
263 expression was done using the $2^{-\Delta\Delta CT}$ method.

264 **RNAscope analysis of the *Chrn2* expression**

265 Mice were sacrificed by decapitation. The brain was removed from the skull and immediately
266 frozen in isopentane pre-cooled in dry ice ($T < -35\text{ }^{\circ}\text{C}$), embedded in OCT medium and stored at -
267 $80\text{ }^{\circ}\text{C}$ for up to 3 months. On the day of slicing, the OCT block was placed in the pre-cooled
268 cryostat (Leica CM 3000) at $-20\text{ }^{\circ}\text{C}$ for 1h before being sectioned sagittally at $16\text{ }\mu\text{m}$. Sections that
269 contained both the DS and substantia nigra pars compacta (SNc) were selected for the *Chrn2*
270 RNA visualization. For visualization, we employed the RNAScope kit (ACDBio, California, USA)
271 and a custom-made probe for exon 5 of the *Chrn2* RNA following the manufacturer's instructions
272 for fresh-frozen tissue. Briefly, brain sections were fixed in 4 % PFA for 15 minutes and
273 dehydrated in increasing concentrations of ethanol (50 %, 70 %, 2x 100 %) for 5 minutes each.
274 After 30 minutes of incubation with Protease IV (ACDBio), the slices were hybridized with the
275 target probes (*Chrn2*, positive and negative controls) for 2 hours in the humidified incubator at
276 $40\text{ }^{\circ}\text{C}$. Several washes and incubations at $40\text{ }^{\circ}\text{C}$ with the amplification reagents (Amp-1, Amp-2,
277 Amp-3, and Amp-4) followed, as per manufacturer's instructions. Finally, the samples were stained
278 with DAPI (ACDBio) for 30 s and mounted with Prolong Gold antifade reagent (ThermoFisher,
279 Massachusetts, USA). The *Chrn2* RNA was detected with a Leica SP8 WLL MP confocal
280 microscope with HC PL APO 63x/1.40 NA objective. For each animal, three confocal images of
281 the DS and three of the SNc were analyzed by Fiji's manual multi-counting function throughout
282 the z-stacks (3 μm steps, 7 pictures). 5 cells per picture were randomly selected and the total
283 number of puncta in 5 cells was scored and reported.

284 **ELISA**

285 Concentration of striatal DA was measured using DA ELISA kit (ImmuSmol, France) according
286 to the manufacturer's instructions. Dissected striata from both hemispheres were pooled and
287 homogenized in 400 μl of Tris 40 mM, EDTA 1 mM and HCl 10 mM. For the ELISA procedure,

288 10 ul of 100x diluted original homogenate was used. Absorbance was measured by
289 spectrophotometer VICTOR Plate Reader (Perkin Elmer) at a wavelength of 450 nm. The
290 measurement was done in two separate batches. To control for differences between the two
291 measurements, we expressed the results as a percentage of controls.

292 **Behavioral testing**

293 All behavioral testing except the light/dark transition test was performed during the light phase (8
294 AM to 6 PM). Mice between the ages of 3 and 8 months were behaviorally tested with testing
295 being initiated 1 month after stereotaxic surgery. The order of behavioral tests performed in
296 individual cohorts were as follows (Figure 1): Cohorts 1 and 2: locomotor activity in the open field
297 → hole-board test → grooming test → marble burying test → forced swimming test (FST) →
298 novel object recognition (NOR) → nest building → cued Morris water maze (cued MWM) →
299 social preference task → T-maze task → elevated plus maze (EPM) → amphetamine-induced
300 hyperlocomotion. Cohorts 3 and 4: open field → FST → 1st evaluation of food-anticipatory
301 activity → social preference task → EPM → tail suspension test (TST) → nest building →
302 light/dark transition → instrumental learning with reversal in the operant box → 2nd evaluation of
303 food-anticipatory activity → amphetamine-induced hyperlocomotion. Before each behavioral
304 testing, mice were habituated to the testing room for at least 30 minutes.

305 ***Nest building***

306 Nest building behavior was evaluated as described in (Deacon, 2006). Mice were first individually
307 housed in new clean home cages. After 5 hours of habituation in the home cage and at least 3 hours
308 before the onset of the dark phase, 2.8-3 g of cellulose nestlet material was distributed in each cage.
309 The nest building activity was then evaluated using a scoring scale from 0 (untouched material) to

310 4 (complete, round, 3D nest), at times 10, 30, 60 and 180 minutes post material introduction. After
311 24 hours a last evaluation was recorded and the total amount of untorn material was weighed.

312 *Locomotion in the open field*

313 Mice were placed in the middle of a square plexiglass arena 40 x 40 cm and their locomotor activity
314 was recorded using a camera for 30 minutes in two consecutive days. The total distance travelled,
315 together with the time spent in the center and edges of the box, was analyzed by ToxTrac software
316 (Rodriguez et al., 2018). To test the acute effect of amphetamine, mice were habituated to the arena
317 for 30 minutes, then injected i.p. with either saline or amphetamine (Sigma) dissolved in saline (2
318 mg/kg) (Yates et al., 2007). After the injection, mice were returned to the arena and recorded for
319 an additional 90 minutes. Immediately after the test, mice were anesthetized and transcardially
320 perfused for c-Fos analysis.

321 *Elevated plus maze (EPM)*

322 We followed the protocol by (Walf and Frye, 2007). Mice were placed in the center of a cross
323 formed by two open and two closed arms. Using a camera placed above the maze, any movements
324 were recorded for 5 min. Based on the recordings, the time spent in each arm and number of entries
325 were manually evaluated.

326 *Light/dark transition*

327 The light/dark transition task was used for assessing anxiety-like behavior (Crawley and Goodwin,
328 1980). Half of a plexiglass 40 x 40 cm open field arena was enclosed in a black cardboard envelope
329 and divided into two halves, light and dark, using a black partition. The black insert had a small
330 opening in the front to allow the mouse to freely move between the light and the dark half of the
331 arena. The test was done during the dark phase of the light cycle (active phase) in a bright room.
332 Mice were placed in the center of the open part of the arena and allowed freely explore both parts.

333 The session was recorded and time spent in each part of the arena during the 10 min session was
334 manually scored.

335 ***Forced swimming test (FST)***

336 The FST for assessment of depressive-like behavior was performed as described in (Martyn et al.,
337 2012). A 2 L beaker was filled with 1.8 L of tap water whose temperature was maintained at 25-
338 27 °C throughout the experiment. The water was changed every 3 or 4 animals. For testing, mice
339 were gently placed into the beaker and recorded for 6 minutes. The time spent struggling for escape
340 vs. the time spent immobile was scored by an experimenter. Only 5 minutes of the test were scored
341 as the first minute was not evaluated.

342 ***Evaluation of food anticipation***

343 To determine the effect of the deletion on the food anticipation, the locomotor activity was
344 monitored for 20 days. During the monitoring, the mice were maintained individually in cages
345 equipped with infrared movement detectors attached above the cage in the center which allowed
346 for the detection of locomotor activity across the entire cage. The monitoring started under the
347 standard *ad libitum* conditions for 10 days, and were followed by monitoring under the regime
348 during which the food access was restricted to only 6 h centered to the middle of the light phase
349 of the light/dark cycle (Polidarová et al., 2013). A circadian activity monitoring system (Dr. H.M.
350 Cooper, INSERM, France) was used to measure activity at 1 min intervals. The resulting data were
351 analyzed using the ClockLab toolbox (Actimetry, Illinois, USA). Double-plotted actograms were
352 generated for visualization of data.

353 ***Social preference task***

354 We assessed the sociability of our mice using a three-chamber plexiglass apparatus (L x W x H,
355 90 x 23 x 23 cm) (Nadler et al., 2004). The two lateral compartments of the apparatus contained a

356 wire mesh pen cup and the middle compartment was empty. After 5 min of habituation in the
357 apparatus, a novel juvenile (4 to 7 weeks old) male mouse was placed into one of the pen cups
358 while the other remained empty. The tested animal was free to explore the compartments for an
359 additional 10 min and its behavior was recorded by a camera. The total time spent in each of the 3
360 compartments and the time spent interacting with the occupied and the unoccupied pen cup were
361 evaluated manually by a blinded experimenter.

362 *Novel object recognition (NOR)*

363 The procedure for testing memory was adapted from (Zhang et al., 2012). On day 1, mice were
364 habituated for 20 minutes in a clean empty home cage. The next day, mice were placed in the same
365 cage for 10 minutes and they were presented with two identical objects (small plastic black and
366 white striped cups). After 1 hour spent in their home cage, mice were placed back into the testing
367 cage for 5 minutes where one of the cups was replaced with a similar but novel object (a small
368 round plastic toy with a black stripe). Both the training and test sessions were recorded, and the
369 time spent exploring each individual object was manually scored by a blinded observer. Based on
370 the scores, the recognition index was expressed in percentage as $\text{Recognition index} = \frac{\text{time exploring novel object}}{\text{time exploring novel object} + \text{time exploring familiar object}} \times 100$. A lack
371 of innate bias for one of the two different objects was assessed by a pilot study.

373 *Cued Morris water maze (MWM)*

374 The cued version of the MWM was performed as described in (Rossato et al., 2006), with the
375 platform at water level, visible and with a black and white striped flag as a cue. Mice were tested
376 over two days with a novel platform and starting location each time. On day 1, the training
377 consisted of 8 consecutive trials with a maximum duration of 60 s interleaved with a 60 s inter-
378 trial interval. After 24 h, mice were probed for their retention in 2 trials, starting from positions

379 that were not used during the training. Latency to reach the platform and the distance travelled by
380 the animal was recorded with a Tracker software (Biosignal Group) and analyzed by CM Manager
381 version 0.4.0 (open source by Stepan Bahnik, available at:
382 https://github.com/bahniks/CM_Manager_0_4_0).

383 ***Grooming test***

384 Spontaneous and induced grooming was assessed by the grooming test. The test was performed as
385 described in (Wang et al., 2016). Before testing, mice were habituated to a novel empty cage for 5
386 minutes. After habituation, mice were recorded for 5 minutes (pre-spray phase), then removed
387 from the cage and slightly misted with water from a spray bottle before being returned to the test
388 cage. Recording continued for an additional 10 minutes (post-spray phase). Grooming events
389 (number of events and their duration) were scored by a blinded experimenter.

390 ***Marble burying test***

391 A compulsive-like behavior was tested using the marble burying test (Angoa-Pérez et al., 2013).
392 Twenty glass marbles were positioned evenly on the surface of a 5 cm high layer of clean bedding
393 in a standard holding cage. Mice were recorded for 15 minutes and the number of marbles buried
394 was evaluated by a blinded experimenter. The marble was considered buried if at least two thirds
395 were covered with bedding.

396 ***Hole-board test***

397 Exploratory behavior was assessed by the hole-board test that was performed according to (Wang
398 et al., 2016) and (Martos et al., 2017). Mice were placed in the 40 cm x 40 cm plexiglass arena
399 containing a plexiglass insertion with 16 equidistant 2 cm wide holes. Mouse were recorded for 30
400 minutes. The numbers and positions of head-dippings were scored by a blinded experimenter.

401 ***Response-based T-maze task***

402 To test the ability to discriminate between a correct and incorrect body turn (left or right), we used
403 a response-based T-maze task. We habituated the animals extensively before the training (Deacon
404 and Rawlins, 2006) and we adapted the procedure as described in (Okada et al., 2018). To perform
405 in the task mice were mildly food-restricted, and they were motivated by a sweetened condensed
406 milk, diluted in half of the original concentration with tap water. The volume of a single reward
407 was 40 ul. For the task, we used a cross-maze apparatus where one of the arms was always blocked
408 so the apparatus formed a T-shape with one starting and two target arms. The blocked and the
409 starting arm were opposite to each other and their positions was alternated so the starting positions
410 were pseudo-randomly changing for each animal. During the habituation period, reward was
411 placed in both target arms and mice were taught to leave the starting arm and reach and consume
412 the reward within 90 seconds. After the habituation, mice were moved to the acquisition phase
413 where the reward was only placed in either left or right arm. The correct side was based on the
414 actual starting position of the animal (Fig. 7h) so it alternated between the arms but the mouse was
415 always required to take the same body turn. Once the mice reached the learning criterion in the
416 acquisition phase (above 50 % in cohort 1, 70 % in cohort 2), they were moved to the reversal
417 phase where the correct body turn was switched. The mice that failed to reach the learning criterion
418 during the acquisition were not moved to the reversal phase but their acquisition data were included
419 in the analysis. Due to control mice learning the task faster than expected in the cohort 1, we
420 adjusted the learning criteria in the cohort 2 and the pooled data were expressed and analyzed as
421 the percentage of control performance.

422 ***Instrumental and reversal learning in the operant box***

423 Mice were tested using a standard operant chamber equipped with two fixed levers and a pellet
424 dispenser (Med Associates, Vermont, USA). Mice were rewarded with chocolate flavored cereal

425 pellets (20 mg each, Bio-Serv, New Jersey, USA). Throughout testing, mice received a maximum
426 of 1 session per day and they were usually tested 5 days per week. On day 1 of the pretraining,
427 mice were habituated to the box for 10 minutes. No reward was administered during the session.
428 After the session, four reward pellets per mouse were placed into the home cage for habituation to
429 the reward. On day 2, a 10-minute habituation to the box was performed again while four reward
430 pellets were placed into the feeder. Only mice that consumed the pellets were moved to the next
431 stage. On the 3rd day, mice were placed in the box for 30 minutes while the dispenser automatically
432 delivered 1 pellet every 2 minutes. Only mice that consumed all pellets were moved onto the next
433 stage. On the last day of pretraining, mice were required to press any lever (left or right) in order
434 to obtain a pellet reward. The criterion at this stage was to earn at least 30 rewards during a 30-
435 minute session while mice were allowed to earn maximum 40 rewards. During the acquisition
436 stage, mice were required to press only the correct lever (either left or right; the correct side was
437 chosen based on the last day of pretraining - it was the side that was less preferred) in order to
438 obtain the reward. Pressing the incorrect lever was recorded but it had no consequences. Mice were
439 allowed to earn a maximum of 40 rewards during a 30-minute session. The learning criterion
440 required to move to the next stage was to earn all 40 rewards while maintaining at least 80 %
441 correct presses. After completing the acquisition phase, mice were subjected to two more identical
442 sessions to ensure stable performance. In the following reversal phase, lever contingencies were
443 switched so the previously correct lever became incorrect and vice versa. Again, mice were
444 required to earn 40 rewards with at least 80 % accuracy. After reaching this criterion, they were
445 subjected to two more identical sessions.

446 **Immunofluorescence (IF)**

447 After the transcardial perfusion with 4 % PFA in PBS, brains were extracted and post-fixed
448 overnight in PFA 4 % at 4 °C. Overnight incubations with sucrose 10 % and 30 % followed. Brains
449 were cut with vibratome (Leica VT1000S) in 40 µm sections for either on-slide IF or stored in the
450 cryoprotective solution as free floating. All procedures except for the primary antibody incubation
451 were performed at room temperature. After the permeabilization step (1.2 % Triton X-100 in PBS
452 for 20 min), sections were washed in PBS for 10 min. For DARPP-32 staining, the
453 permeabilization step has been replaced by 3 consecutive washes of 5 min in ethanol 50 %, 70 %, 50 %.
454 For blocking of the non-specific binding, sections were incubated with 5 % normal goat
455 serum (NGS) in PBS for 1h. Primary antibodies used were chicken or rabbit anti-GFP (Abcam,
456 RRID: AB_300798, 1:1000, and ThermoFisher, RRID: AB_2536526, 1:200, respectively), rabbit
457 anti-c-Fos (Abcam, RRID: AB_2737414, 1:500) and goat anti-DARPP-32 (R&D Systems, RRID:
458 AF6259, 1:500) and guinea pig anti-VACHT (1:5000) (Gras et al., 2008). They were diluted in
459 PBS with 0.2 % Triton X-100 and 2 % NGS and incubated overnight at 4 °C. The next day, sections
460 were incubated with secondary antibodies (anti-chicken Alexa Fluor 488, anti-rabbit Alexa Fluor
461 594 and 680, anti-goat Alexa Fluor 594 and anti-guinea pig Alexa Fluor 594; Jackson
462 ImmunoResearch) in PBS with 0.2 % Triton X-100 and 2 % NGS, mounted on slides and
463 coverslipped with Fluoroshield medium (Sigma). Images were acquired through Leica SP8 WLL
464 MP confocal microscope, using HC PL FLUOTAR 5x and 10x and HC PL APO 40x objectives,
465 and Andor Dragonfly 503 - spinning disk confocal microscope (HCX PL APO 40x objective).
466 Quantification of c-Fos positive cells was performed with Fiji and CellProfiler software.

467 **IF Analysis of c-Fos expression**

468 Quantification of c-Fos positive cells was performed in beta2-del mice, in cohorts 2, 3 and 4 for a
469 total of 40 animals divided into 4 experimental groups: 8 and 9 control mice obtained saline and

470 amphetamine, respectively, 13 and 10 beta2-del mice received the same treatment. Two mice from
471 the saline/beta2-del group and 1 mouse from the amphetamine/beta2-del group were excluded as
472 outliers based on their locomotion or c-Fos expression data. For each animal, three confocal (40x
473 objective) maximal projection pictures per hemisphere, spanning throughout the DS – AP 1.5 and
474 AP 1.2 and AP 0.9, ML 1.5, DV 2.4 , were quantified using Fiji (Schindelin et al., 2012) and
475 CellProfiler software (Kamentsky et al., 2011). A pipeline was setup in CellProfiler, defining the
476 global thresholding strategy with otsu method for the nuclei (Hoechst) and MoG for GFP and c-
477 Fos. Objects with a typical diameter between 10- and 20-pixel units were counted. Clumped
478 objects were distinguished by Laplacian of Gaussian method. Blue-thresholding module with
479 intensity-calibrated resolution was used as mask for counting GFP and c-Fos objects. These values
480 were controlled by manual multi-counting in Fiji. Positive cells for each marker were then
481 expressed as percentage of the nuclei and averaged for each animal. Three animals from each
482 experimental group were used for analyzing the colocalization of c-Fos expressing cells with
483 DARPP-32. The analysis was done by using custom macros in Python and ImageJ Macro
484 Language with plugins (StarDist; JACoP; MorphoLibJ; 3D ImageJ Suite) (Bolte and Cordelieres,
485 2006; Ollion et al., 2013; Legland et al., 2016; Schmidt et al., 2018) available for the Fiji software
486 package to obtain suitable evaluation of the signal intensity throughout the z-stacks. 7 control mice
487 and 8 beta2-del mice were used for further evaluation of c-Fos expression in combination with
488 VACHT and GFP expression. For each animal, two confocal z-stack pictures (63x objective) per
489 hemisphere were quantified manually by using Fiji.

490 **Data availability**

491 Data generated in the study will be provided upon request.

492

493 **Results**

494 **mRNA coding for beta2 nicotinic subunit is expressed by specific groups of striatal INs, with**
495 **CINs showing the highest level of expression**

496 Although different subtypes of nAChRs are expressed by striatal neurons, the most common
497 nicotinic subunit is likely the beta2 subunit coded by the *Chrn2* gene (M. Picciotto et al., 2000;
498 Jaunarajs et al., 2015; Quik and Wonnacott, 2011; Quik et al., 2009). Therefore, to investigate the
499 function of nAChRs in the mouse striatum, we decided to focus on this subunit. First, we used a
500 previously published scRNA-seq study performed in the mouse striatum (Munoz-Manchado et al.,
501 2018) and re-analyzed the available data to assess the expression of the *Chrn2* gene, examining
502 the dataset GSE97478 from (Munoz-Manchado et al., 2018). We found that the *Chrn2* mRNA is
503 poorly expressed by all types of striatal neurons with little differences between individual groups
504 (Fig. 2 a-b). In all examined neuronal groups (MSNs and INs), a relatively large proportion of cells
505 showed no *Chrn2* transcripts. However, in all these groups there was a small proportion of cells
506 expressing *Chrn2* to some extent (Fig. 2a). When comparing the mean expression of *Chrn2*, we
507 could rule out substantial differences (fold change > 2) between groups of striatal neurons (Fig.
508 2b). Of note, non-neuronal cells showed minimal *Chrn2* expression and the proportion of cells
509 with zero transcripts was significantly higher and the mean expression lower than those in the
510 neuronal populations (Figure 2 a-b). The finding based on the scRNA-seq data was unexpected
511 since most evidence in literature suggest that MSNs express considerably less (if any) nAChRs
512 comparing to striatal INs. Therefore, we performed our own experiments using a different
513 approach, allowing us to examine *Chrn2* expression simultaneously in multiple neuronal types *in*
514 *situ*.

515 The phenotypes of the neurons expressing mRNA encoding the beta2 subunit of the nAChR
516 (Chrb2) gene were analyzed by coFISH in the mouse striatum (caudatum-putamen, CPU) (Fig.
517 3). Chrb2 mRNA was detected in scattered neurons all over the CPU (Fig. 3a). Several markers
518 including choline acetyltransferase (Chat, Fig.3 b-c), parvalbumin (Pvalb, Fig. 3d), neuropeptide
519 Y (Npy, Fig. 3e) and somatostatin (Sst, Fig. 3f) for INs and D1 or D2 dopamine receptors (Drd1
520 or Drd2, Fig. 3 g-h) for MSNs were co-analyzed with Chrb2. As expected, all these markers were
521 detected in the striatum with their specific expression profiles. Interestingly, Chrb2 was detected
522 in Chat+ neurons but also in Chat- neurons (Fig. 3 b-c) and the same observations were made for
523 Pvalb, Npy, Drd1 and Drd2 markers (Fig. 3 b-e, g-h). Chrb2 was virtually absent in Sst+ neurons
524 (Fig. 3f). A quantitative analysis at 6 different bregma levels (rostral to caudal part of the CPU
525 (0,98; 0,74; 0,5; 0,26; 0,02; -0,22) revealed that Chrb2 mRNA was expressed in the majority of
526 Chat+ neurons (96 %), but only in 24 % of Pvalb+ and 5.5 % of Npy+ neurons (Fig. 3i). Chat+
527 neurons expressing Chrb2 gene were distributed over the whole striatum all along the rostro
528 caudal axis, whereas Pvalb+ and Npy+ neurons expressing Chrb2 were located only in the
529 dorsolateral part of the striatum. Expression of serotonin receptor 5HT_{3A} (Htr3a gene) was also
530 analyzed as a marker for a presumably distinct population of INs (Faust et al., 2016). However,
531 no or only very rare Htr3a+ cells were detected in the CPU and no colocalization was detected
532 with Chrb2 mRNA in the cells (Fig. 3 k-m). Interestingly, independent analyses of Chrb2
533 expressing cells in sections probed for different markers showed that 80 %, 13 % and 7 % of
534 Chrb2 expressing neurons are also Chat+, Pvalb+ and Npy+, respectively, thus adding up to 100 %
535 of all Chrb2 expressing cells (Fig. 3j). Only 0.5 % of Drd1+ neurons displayed Chrb2 mRNA.
536 Finally, the Drd2+ neurons that also displayed Chrb2 gene were mostly large-sized, putatively
537 representing CINs (Le Moine et al., 1990). In addition, the proportion of Chrb2+ neurons that

538 were *Drd2*⁻ (20%) corresponded to the sum of the *Chrn2*⁺ neurons that were *Drd1*⁺
539 (*Chrn2*⁺/*Pvalb*⁺ and *Chrn2*⁺/*Npy*⁺).

540

541 **AAV-Cre injection into the DS of beta2-flox/flox mice induces a selective deletion of the beta2**
542 **nicotinic subunit in striatal neurons**

543 To investigate a functional role of beta2-containing nAChRs expressed by striatal INs, we deleted
544 the beta2 subunit by injecting AAV-Cre virus into the DS of beta2-flox/flox mice carrying floxed
545 exon 5 of the *Chrn2* gene (Fig. 4a). The successful deletion of exon 5 was confirmed as an
546 additional 400 bp fragment on agarose gel (Fig. 4a). Importantly, the deletion fragment was only
547 present in striatal and not in midbrain samples which indicates that the virus did not travel
548 retrogradely from the striatum into the SNc. We also measured beta2 mRNA by RT-qPCR,
549 specifically targeting exon 5. We found a significant decrease in the level of beta2 mRNA isolated
550 from striatal punches taken inside the AAV-expressing area of AAV-Cre-injected mice (95 % CI
551 for the difference between means [-103.0; -79.35], $t(11)=16.95$, $p<0.0001$, two-tailed t-test). The
552 expression of beta2 mRNA remained unaffected in the cortex and midbrain and the expression of
553 the complementary nicotinic subunit alpha4 measured in striatal punches also did not change after
554 beta2 deletion (Fig. 4 e-h). Finally, we visualized the decrease of *Chrn2* mRNA expression using
555 RNAscope. The analysis showed a marked decrease of mRNA puncta in the striatum of AAV-
556 Cre-injected mice compared to control animals (95 % CI for the difference between means [-90.17;
557 -42.94], $t(4)=7.824$, $p=0.0014$, two-tailed t test) while there was no difference in the SNc (Fig. 4
558 b-d).

559 Midbrain DA neurons express their nAChRs on axonal terminals in the striatum where they control
560 the release of striatal DA (Cachope et al., 2012; Threlfell et al., 2012). We used ELISA to measure

561 DA concentrations in striatal homogenates from control and AAV-Cre-injected mice and we did
562 not find any difference between the groups (Fig. 4i). However, our experimental approach cannot
563 exclude more subtle changes in the release of striatal DA or changes limited to individual striatal
564 subregions. Taken together, these experiments confirmed that the injection of the AAV-Cre virus
565 caused a deletion of the beta2 subunit only at the site of injection. This was also confirmed by
566 direct examination of the spread of the virus in both coronal and sagittal sections from brains of
567 AAV5-Cre-injected animals. While we detected a strong viral expression in the DS (Fig. 5 b-c),
568 we detected negligible expression in the midbrain region. Specifically, in one sagittal section, we
569 found 6 fluorescent nuclei in the SNc, suggesting that while the retrograde transport of this virus
570 is possible, it is rare, and likely does not contribute to a behavioral phenotype. Results of IF
571 analysis of the viral expression and viral leakage into structures beyond the DS are schematically
572 shown in Figure 5 d.

573

574 **Mice with a deletion of the beta2 subunit in striatal neurons show increased anxiety-like**
575 **behavior and changes in social preference task**

576 In the next part of the study, we wanted to examine if beta2-containing nAChRs expressed by INs
577 in the striatum play a role in controlling striatal-dependent behaviors. The striatum is a large
578 structure with different subregions controlling different functions. In our study, we primarily
579 focused on the DS including both medial and lateral part. The beta2-containing nAChRs were
580 previously shown to play a role in fundamental behaviors in mice including feeding, motivation
581 and sleep/wake behavior (Dezfuli et al., 2020; Konsolaki et al., 2016; Léna et al., 2004). We
582 subjected a group of mice with the beta2 deletion in the DS (further referred to as beta2-del mice)
583 and control mice to a battery of behavioral tasks that are known to depend on striatal functions or,

584 in some cases, on nAChRs. However, investigating these domains did not reveal any significant
585 difference between control and beta2-del mice (Fig. 6 a-b, i-j, Fig. 8 f, g).

586 We also measured spontaneous locomotion in the open field in two consecutive days, thus probing
587 activity in novel and familiar environment. Control and beta2-del mice were not significantly
588 different, although there was a trend to hyperactivity on day 1 (novel environment) and the groups
589 were very similar on day 2 (familiar environment) (day vs. group interaction 95 % CI [-0.6509;
590 16.20], $F(1, 59)=3.409$, $p=0.0699$; two-way ANOVA) (Fig. 6 c-d, Fig. 8h). When we analyzed the
591 time mice spent in the centre versus in the edges of the open field arena, we found that beta2-del
592 mice spent less time in the centre compared to controls (main effect of group: 95 % CI for the
593 difference between means [-8.475; -1.676], $F(1, 59)=8.925$, $p=0.0041$; two-way ANOVA), namely
594 during the first day of the test (difference between means on day 1: 95 % CI [-12.0; -3.122],
595 $p=0.0004$; day 2: 95 % CI [-7.028; 1.845], $p=0.3412$; Sidak's post-test) (Fig. 6e, Fig. 8 a, i).

596 Spending less time in the central part of a novel environment indicates an increase in the anxiety-
597 like behavior. Therefore, we used additional tasks to examine the anxiogenic effect of a beta2
598 deletion. The EPM did not reveal any effect of the beta2 deletion (Fig. 6f, Fig. 8k) while in the
599 light/dark transition task, we found that the beta2-del mice spend slightly but significantly less
600 time in the light part of the apparatus compared to controls (95 % CI for the difference between
601 means [-115.1; -3.595], $t(27)=2.184$, $p=0.0378$; two-tailed t-test) (Fig. 6g, Fig. 8b). Finally, we
602 saw no difference between the control and beta2-del mice in immobility time during the FST used
603 for evaluation of behavioral despair (Fig. 6h, Fig. 8j).

604 Previously, it has been shown that social behavior depends on different types of INs in the DS
605 (Rapanelli et al., 2017). For the assessment of social behavior in beta2-del mice, we used the social
606 preference task (Fig. 6 k-n, Fig. 8 c-d, l-o). In contrast to control animals who significantly

607 preferred the mouse chamber, the beta2-del mice did not show this preference and the time they
608 spent in with a mouse or an object did not differ (chamber vs. group interaction, 95 % CI [49.63;
609 166.4], $F(1, 116)=13.43$, $p=0.0004$; two-way ANOVA) (Fig. 6k, Fig. 8l). When we directly
610 compared the time that the two groups spent in the social chamber, we found a significant decrease
611 in beta2-del mice (95 % CI for the difference between means [-117.1; -22.64], $p=0.0022$, Sidak's
612 post-test) (Fig. 6k). To enhance our understanding of the behavior occurring during the task, we
613 analyzed time that the two groups of mice spent directly interacting (sniffing or pawing) with the
614 social and non-social stimulus. Surprisingly, the time spent interacting with the mouse did not
615 differ between controls and beta2-del mice (95 % CI for the difference between means [-20.95;
616 13.14], $t(58)=0.4590$, $p=0.6480$, two-tailed t-test) (Fig. 6l, Fig. 8m). In contrast, the beta2-del mice
617 showed notably longer time spent examining the non-social stimulus (95 % CI for beta2-
618 del/controls ratio [1.19; 2.1], $p=0.003$, generalized linear model) (Fig. 6m, Fig. 8 c, n). Thus, the
619 lower sociability ratio in the beta2-del mice was purely driven by an increased object exploration
620 (Fig. 6n, Fig. 8 d, o). In summary, the beta2-del mice showed no changes in weight, nest building,
621 food-anticipatory activity, locomotion and depressive-like behavior while they showed an
622 increased anxiety-like behavior in two out of three tests. In addition, the most marked behavioral
623 change in beta2-del mice was an impairment in social preference task driven by a relatively higher
624 interest in inanimate objects compared to social stimuli.

625

626 **Deletion of the beta2 nicotinic subunit in striatal neurons impairs response discrimination** 627 **learning but has no effect on cognitive flexibility**

628 In the next set of experiments, we focused on evaluating of cognitive functions in beta2-del mice.
629 Striatal ACh is indispensable for intact cognitive flexibility (Prado et al., 2016; Favier et al., 2020)

630 and nAChRs expressed by striatal INs have been also hypothesized to play a role in this cognitive
631 domain. Thus, we employed multiple tests to investigate cognitive flexibility and goal-directed
632 behavior. These tests included cued MWM for goal-directed behavior, grooming test, marble
633 burying test and hole board test for compulsive-like and repetitive behavior and lever-pressing task
634 in the operant box for reversal learning, but we did not find a significant alteration in any of them.
635 (Fig. 7a-h). In addition, episodic-like memory was not impaired in the beta2-del mice as
636 documented by NOR test (Fig. 7i). Finally, we trained beta2-del mice in a response-based T-maze
637 task to examine their egocentric navigation, discrimination and reversal learning (Fig. 7 j-l, Fig.
638 8e). In this task, mice were trained to navigate to a reward whose position was dependent on a
639 mouse's body turn. Two different starting arms of the maze were pseudo-randomly alternated so
640 that mice would not learn the position of the reward from the spatial cues (Fig. 7j). In the
641 acquisition phase of the task, mice were trained to make the correct body turn and in the following
642 reversal phase, the correct turn was changed to the opposite direction. We found that beta2-del
643 mice needed significantly more training sessions to reach the accuracy criteria during the
644 acquisition phase of the T-maze task (mean percentage of the control values \pm SEM in beta2-del:
645 178.8 ± 5.9 , 95 % CI for the difference between means [11.76; 145.9], $t(13.96)=2.521$, $p=0.0245$;
646 Welsh's t-test) (Fig. 7k, Fig. 8e). In contrast, the reversal phase did not show an impairment in
647 beta2-del mice. Instead, the data were more consistent with a better performance in beta2-del
648 animals (mean percentage of the control values \pm SEM in beta2-del: 78.4 ± 7.2 , 95 % CI for the
649 difference between means [-51.69; 8.489], $t(20)=1.497$, $p=0.1499$, two-tailed t-test) (Fig. 7l). In
650 summary, cognitive tests showed no impairment of cognitive flexibility in beta2-del mice but they
651 identified the impairment of the initial discrimination learning in the egocentric navigation-based
652 T-maze task.

653

654 **Deletion of the beta2 nicotinic subunit increases c-Fos expression in the DS and leads to a**
655 **higher sensitivity to amphetamine**

656 The FISH analysis showed that beta2-containing nAChRs are predominantly expressed by striatal
657 INs and their expression by MSNs is negligible. Based on this finding we asked if the beta2
658 deletion would lead to a decreased activity of striatal INs and what would be the ultimate effect on
659 the activity of MSNs. To answer this question, beta2-del and control mice received i.p. injection
660 of either saline or amphetamine (Fig. 9a). Following the injection, we measured locomotion as a
661 behavioral marker of striatal activity and we also evaluated c-Fos expression in the DS as a
662 biochemical correlate of neuronal activity. The comparison of controls and beta2-del mice during
663 habituation period (before injection) revealed a significantly higher locomotor activity in the
664 beta2-del (95 % CI for the difference between means [4.787; 27.29], $t(42)=2.877$, $p=0.0063$, two-
665 tailed t-test) (Fig. 9 b, d, Fig. 8p). When we analyzed the activity of mice after the injection, we
666 found a significant effect of amphetamine (main effect of treatment: 95 % CI [58.03; 154.9], $F(1,$
667 $40)=19.75$, $p<0.0001$, two-way ANOVA) and no effect of group or interaction (Fig. 9 c, d, Fig.
668 8q). When further examined with post-tests, we found a marked difference between the saline- and
669 amphetamine-injected beta2-del mice (95 % CI [52.83; 225.8], $p=0.0006$, Tukey's multiple
670 comparisons test) and no difference between the saline- and amphetamine-injected controls,
671 possibly due to older age of the experimental animals (8 months, see Fig. 1).

672 In addition to measuring locomotion as a behavioral marker of MSN activity, we also evaluated c-
673 Fos expression in the DS. Similar to the analysis of locomotor activity, we found a significant
674 effect of amphetamine (main effect of treatment: 95 % CI [0.5088; 2.307], $F(1, 33)=10.15$,
675 $p=0.0031$, two-way ANOVA). In addition, there was an effect of beta2 deletion (main effect of

676 group: 95 % CI [0.1795; 1.977], $F(1, 33)=5.958$, $p=0.0202$; two-way ANOVA) and post-tests
677 showed only a significant difference between the saline- and amphetamine-injected beta2-del mice
678 (95 % CI [0.2063; 3.452], $p=0.0222$, Tukey's multiple comparisons test) (Fig. 9 e, g). Given the
679 similarity of the locomotion and c-Fos expression data, we decided to test if locomotor activity is
680 correlated with the level of c-Fos expression. Indeed, the distance travelled after injection was
681 positively correlated with the percentage of c-Fos-expressing cells in the DS (Fig. 9f), suggesting
682 that both parameters can be used as indicators of striatal activity. In summary, beta2-del mice
683 showed an increase in both behavioral and biochemical marker of striatal activity. The striatum is
684 composed of two different types of MSNs with antagonistic effect on locomotor activity and
685 several types of INs which effect on locomotion is largely unknown. To obtain more information
686 on the identity of c-Fos expressing neurons associated with the increased locomotion, we stained
687 striatal sections in a subgroup of ctrl and beta2-del mice with the antibody against dopamine- and
688 cAMP-regulated neuronal phosphoprotein (DARPP-32), a marker of MSNs (Fig. 10a). As
689 expected, most of the c-Fos-expressing neurons were also stained for the DARPP-32, as MSNs
690 form about 95 % of all striatal neurons. However, between 5-10 % of c-Fos-expressing neurons in
691 all examined sections were DARPP-32 negative, presumably representing activated striatal INs
692 (Fig. 10 a-b). The proportion of DARPP-32 negative/c-Fos positive neurons was similar in all
693 experimental groups, indicating that both the beta2 deletion and the amphetamine treatment
694 increased the activity in both MSNs and INs (Fig. 10b). Finally, we hypothesized that among the
695 different types of striatal INs, those with significant beta2 deletion, namely CINs, should show a
696 decrease in c-Fos expression. We tested this hypothesis by triple staining against GFP, c-Fos and
697 vesicular acetylcholine transporter (VACHT), a marker for CINs. Analysis of c-Fos and VACHT
698 labeling revealed that percentage of c-Fos-positive neurons out of all VACHT-positive neurons

699 was lower in beta2-del mice compared to controls, although due to low number of CINs, and
700 between-mice variability, the evidence is not completely conclusive (95 % credible interval for the
701 odds ratio between ctrl and beta2-del [0.09; 1.02], generalized linear mixed model) (Fig. 10 c-d).

702

703 **Discussion**

704 Although ACh is widely accepted as a key modulator of striatal signaling and function, the
705 mechanisms underlying this modulation are still elusive. In particular, little is known about the
706 expression and function of nAChRs expressed by individual populations of striatal neurons. In the
707 present study, we used FISH simultaneously probing all major markers of striatal neurons to
708 evaluate expression of beta2-containing nAChRs in the striatum. We also deleted beta2 nicotinic
709 subunit in striatal INs to reveal a functional significance of the relatively scarce population of
710 receptors. Finally, our analysis of c-Fos expression indicated that the beta2-containing nAChRs
711 expressed by striatal INs have overall inhibitory effect on other striatal neurons, both MSNs and
712 INs.

713 **Expression of beta2 nAChR by striatal neurons**

714 Our FISH analysis showed that *Chrnb2* mRNA is not particularly abundant in the striatum and it
715 is present almost exclusively in INs, specifically in CINs, PV+ and NPY+ INs. The current
716 knowledge on the expression of nAChRs by individual types of striatal neurons primarily arises
717 from electrophysiological studies that detect changes in neuronal firing after the application of
718 nicotine or nAChRs antagonists coupled with optogenetic activation of striatal cholinergic
719 interneurons (English et al., 2011; Koós and Tepper, 2002; Luo et al., 2013; Sullivan et al., 2008;
720 Ibáñez-Sandoval et al., 2015; Faust et al., 2015; Faust et al., 2016; Assous et al., 2017). Most of
721 these studies found the expression of nAChRs in striatal GABAergic INs (Sullivan et al., 2008; Faust et

722 al., 2016; Faust et al., 2015; English et al., 2011). The FISH data presented here broaden this view:
723 first, they confirm that beta2-containing nAChRs are primarily expressed by striatal INs and not
724 by the MSNs; second, they show that majority of Chrb2 mRNA is expressed by CINs in adult
725 mouse striatum. While certain level of Chrb2 expression by CINs has been previously suggested
726 (Lim et al., 2014; Assous, 2021), CINs have been rarely viewed as the main site of Chrb2
727 expression among individual populations of striatal INs. It should be noted that the literature on
728 nicotinic expression by striatal neurons has been quite diverse and sometimes contradictory. In
729 particular, there is data that is both in favor (Koós and Tepper, 2002) and against (English et al.,
730 2011) the expression of nAChRs by fast-spiking interneurons. Some discrepancies can also be
731 found in the case of MSNs where at least one study confirms the presence of nAChRs on MSNs
732 (Liu et al., 2007) while others do not (Luo et al., 2013; Matsubayashi et al., 2001). Several factors
733 may contribute to these discrepancies. First, mRNA expression does not necessarily reflect
734 expression on the protein level. Second, in all neuronal populations presented in (Munoz-
735 Manchado et al., 2018), there was a large proportion of cells displaying no expression. This overall
736 low expression may contribute to the contradictory data in literature on nAChRs' expression by
737 striatal neurons. Lastly, it has been suggested that the expression of nAChRs in the striatum is
738 strongly age- and learning-dependent (Havekes et al., 2011). In our experiments, we used 3-4
739 months old mice while in electrophysiological studies the age of animals is usually lower. Thus,
740 we speculate that the nAChR expression in individual striatal neuronal types flexibly changes
741 during lifetime and is controlled by behavioral state and other mechanisms beyond just the neuron's
742 identity.

743 **Behavioral significance of beta2-containing nAChRs**

744 The DS is involved in the control of multiple behavioral domains. In order to investigate which of
745 them are influenced by beta2-containing nAChRs expressed by striatal INs, we performed
746 numerous behavioral tests. The behavioral phenotype observed in beta2-del mice is generally in
747 agreement with previous studies reporting that centrally expressed nAChRs control explorative
748 and social behavior, anxiety and higher cognitive functions (Koukouli and Changeux, 2020; Avale
749 et al., 2011; Picciotto et al., 2015). However, the global knockout (KO) of beta2 nicotinic subunit
750 has been reported to increase social approach in the social preference test (Avale et al., 2011;
751 Guillem et al., 2011) and higher activity of $\alpha 4\beta 2$ nAChRs lead to increased anxiety in mice
752 (Labarca et al., 2001). During the social preference task, our beta2-del mice spent less time in the
753 social compartment compared to controls but the time spent directly interacting with the social
754 stimulus was not decreased. Instead, the time spent interacting with the non-social object was
755 significantly higher, resulting in a lower sociability ratio. The apparent social deficit in beta2-del
756 mice may be therefore more related to impaired exploratory behavior and an altered response to
757 novelty that was also previously reported in beta2-KO mice (Granon et al., 2003; Maubourguet et
758 al., 2008). In line with this, beta2-del mice also showed hyperactivity in a novel environment.
759 However, we found a discrepancy between the open field test that did not reveal hyperactivity in
760 beta2-del mice and the amphetamine test where the beta2-del were clearly hyperactive during the
761 pre-injection habituation period. This can be caused by several factors: the two tasks were
762 performed at different ages as we tested our mice between 3 and 8 month of age and the open field
763 and amphetamine test were the first and the last task performed, respectively. In relation to this,
764 the beta2 deletion was induced 1 month before the open field testing while it was in place for 6
765 months already in case of the amphetamine test. During that period, additional compensatory
766 changes could develop to contribute to hyperactivity in the older beta2-del mice. It should be also

767 noted that the broad age span during which the behavioral testing took place could have influenced
768 other behavioral domains as well.

769 In addition to changes in explorative behavior and locomotion, there was an evidence of increased
770 anxiety-like behavior in both the open field and light/dark transition tasks. While changes in this
771 behavior have not been commonly reported in genetic mouse models with beta2-containing
772 nAChR alterations, nicotine and nicotinic agonists and antagonists have been implicated in the
773 control of anxiety (Salín-Pascual and Basañez-Villa, 2003; Mineur et al., 2007; Mineur et al., 2013;
774 Picciotto et al., 2015). Finally, in beta2-del mice we found an impairment of discrimination
775 learning in the acquisition phase of the response-based T-maze task. This was rather unexpected
776 as striatal ACh has not been previously shown to play a role in the acquisition of discrimination
777 learning (Okada et al., 2018) and instead, its role in cognitive flexibility is well recognized (Prado
778 et al., 2016). However, we were not able to detect any cognitive flexibility impairment in beta2-
779 del mice, even though we used several different tasks to reveal it. It is conceivable that the
780 impairment of discrimination learning detected in the present study reflects an impaired function
781 of striatal GABAergic neurons expressing nAChRs, as recent studies showed the involvement of striatal
782 SST+ and fast spiking (putative PV+) GABAergic neurons in instrumental learning and egocentric
783 navigation in the T-maze (Holly et al., 2019; Owen et al., 2018).

784 In summary, it is quite remarkable that the selective deletion of relatively scarce population of
785 receptors was able to induce a specific behavioral phenotype and that these receptors play a
786 significant role in the control of striatal signaling. It should be noted here that the presented data
787 apply only to male mice that were used exclusively throughout the study. However, a small cohort
788 of female mice that we used in some pilot experiments suggested that the behavioral phenotype

789 induced by beta2 deletion in females may be rather different and the topic requires further
790 investigation.

791 **Activating effect of beta2 deletion on striatal neurons**

792 Through the analysis of c-Fos expression in the striatum, we show that the deletion of beta2-
793 containing nAChRs has an overall activating effect on striatal neurons. This is seemingly in
794 accordance with electrophysiological data suggesting that nicotinic activation of striatal GABAergic
795 leads to inhibition of MSNs (Faust et al., 2016). However, the data indicate that the effect of beta2
796 deletion in our experiments is more complicated. First, majority of beta2-containing nAChRs is
797 expressed by CINs themselves and as CINs realize a very complex control over striatal signaling,
798 it is currently impossible to conclusively predict the effect of this deletion. Second, as shown by
799 our IF analysis, almost 10 % of the activated c-Fos expressing neurons are DARPP-32-negative
800 cells, presumably INs or possibly astrocytes (Groves et al., 2018). Because neither amphetamine
801 nor beta2-deletion had any effect on the proportion of DARPP-32-negative cells, the idea that it is
802 mostly MSNs that are dis-inhibited by the beta2 deletion seems unlikely. Rather, the activating
803 effect of the deletion on locomotion and sensitivity to stimulant agrees with the general notion that
804 CINs have overall inhibitory effect, opposing the striatal activation by dopamine. This view is
805 further supported by our data showing that in contrast to the overall increase in c-Fos induced by
806 beta2 deletion, CINs show rather a decrease of c-Fos expression.

807 In conclusion, our results show that nAChRs expressed by striatal CINs and other INs inhibit
808 striatal activity. Through this inhibition they modulate behaviors, including social and exploratory
809 behavior, anxiety-like behavior and learning. Future studies should determine a possible effect of
810 age and learning on nAChR striatal expression and function.

811

812 **Author contributions**

813 A.A., A.S., V.B., S.D. and H.J. participated in the research design; A.A., S.D., I.R.B., A.T., J.M.,
814 R.R., P.H., S.N. and H.J. conducted experiments; M.M. performed reanalysis of the scRNA-seq
815 data; M.C. performed analysis of DARPP-32 and Fos colocalization; A.A., S.D., A.S. and H.J.
816 performed data analysis; A.A., M.M., A.S., V.B., S.D. and H.J. wrote or contributed to the writing
817 of the manuscript; all authors read and approved the final version of the manuscript.

818

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1021

1022 **Figure legends**

1023 **Figure 1: Behavioral tests performed in individual cohorts of mice.**

1024 The order of tests in each cohort and the approximate age of tested animals are shown. We indicate
1025 each month of age with an arrow, each arrow can host 4 dots representing the 4 weeks of the month.
1026 On this time line, we are showing the order of the behavioral tasks in the two different cohorts.
1027 The maximum age gap between the youngest and oldest animals in the cohorts is 6 weeks.

1028

1029 **Figure 2: *Chrn2* expression in individual cell populations in the striatum.**

1030 (a) Expression of *Chrn2* across cell populations from (Munoz-Manchado et al., 2018). Each point
1031 is a cell. The color represents the proportion of cells with the given number of transcripts in each
1032 population. The cells below the horizontal red line have no *Chrn2* transcripts. The triangle
1033 represents an outlier cell that had 13 reads. (b) Differences between groups of cell populations as
1034 fitted by a Bayesian hierarchical generalized-linear model. Displayed are 50% (thick) and 95%
1035 (thin) posterior credible intervals for the difference of a given group (vertical axis) from a baseline
1036 group (subplot title). Fold change > 1 indicates the group on the vertical axis has higher mean
1037 expression than the baseline group. ASTRO: astrocytes; ENDO: endothelial cells; OLIGOS:
1038 oligodendrocytes; CHAT: cholinergic interneurons; NPY_NGC: NPY-expressing neurogliaform
1039 interneurons (INs); Pthlh: parathyroid hormone like hormone-expressing INs; Pvalb:
1040 Parvalbumine-expressing INs; Sst: somatostatin-expressing INs; Th: tyrosinhydroxylase-
1041 expressing INs; MSND1: D1-expressing MSNs; MSND2: D2-expressing MSNs.

1042

1043 **Figure 3: Double-labeling riboprobe fluorescent in situ hybridization (FISH) of serial**
1044 **sections identifying neuronal phenotypes expressing *Chrn2* gene in mouse striatum.**

1045 (a) Overview of *Chrn2* expression in the caudatum-putamen (CPU) at bregma 0.5. *Chrn2* (red),
1046 DAPI (Blue). (b) Schematic summary of findings mapping the extent of *Chrn2* and *Chat* overlap
1047 within CPU subnuclei. (c-h) Close ups of codetection of *Chrn2* (red) with (in green) (c) *Chat*, (d)
1048 *Pvalb*, (e) *Npy*, (f) *Sst*, (g) *Drd1*, (h) *Drd2*. Arrows point to cells positive for one mRNA only (red
1049 or green) or double-positive (yellow arrows). i: Quantitative analysis of the proportion of *Chat*,
1050 *Pvalb* or *Npy* gene expressing neurons in the mouse striatum, displaying *Chrn2* mRNA. j:
1051 Quantitative analysis of the proportion of *Chrn2* gene expressing neurons in mouse striatum
1052 displaying also *Chat*, *Pvalb* or *Npy* mRNA. In i and j: Percentage indicates the result of
1053 quantification per CPU from 3 male mice. A mean of 700, 1200, 520, 830 *Chat*, *Npy*, *Pvalb* and
1054 *Sst* neurons, respectively, were analyzed per brain. (k-m) *Htr3a*⁺ cells in mouse coronal brain
1055 section. (l) Only minimum to none of *Htr3a*⁺ cells were found in the CPU. (m) Several cells
1056 strongly positive for *Htr3a* are shown in the cortex. CPU: caudate putamen; Ms: medial septum;
1057 aca: anterior commissure; Ctx: cortex.

1058

1059 **Figure 4: Striatal injection of AAV-Cre leads to a specific decrease of beta2 mRNA in the**
1060 **striatum of beta2-flox/flox mice.**

1061 (a) Upper: A diagram showing exon 5 of the *Chrn2* gene flanked by loxP sites in beta2-flox/flox
1062 mice. Locations of the three primers used in the ELFO analysis below are also indicated. Lower:
1063 The ELFO analysis shows samples from two representative mice injected with AAV-GFP or
1064 AAV-Cre-GFP in the DS. The lower row on the gel shows 200 bp products of #198 and #199

1065 primers, the upper additional band shows an additional 400 bp product of #198 and Bot2-09
1066 primers indicating excision of exon 5. (b) RNAscope visualization of *Chrn2* mRNA in the DS
1067 and SNc in two representative mice injected with AAV-GFP or AAV-Cre-GFP. *Chrn2* mRNA
1068 puncta are missing in the DS injected with Cre while the SNc of the same animal shows no decrease
1069 in puncta density. (c) Quantification of *Chrn2* mRNA puncta in the DS. $t(4)=7.824$, $p=0.0014$,
1070 two-tailed t-test. (d) Quantification of *Chrn2* mRNA puncta in the SNc. $t(4)=0.5321$, $p=0.6228$,
1071 two-tailed t-test. (e) *Chrn2* mRNA expression analyzed by RT-qPCR in striatal punches from
1072 AAV-expressing area of mice injected with AAV-GFP or AAV-Cre-GFP. $t(11)=16.95$, $p<0.0001$,
1073 two-tailed t-test. (f) *Chrna4* mRNA expression analyzed by RT-qPCR in striatal punches from
1074 AAV-expressing area. $t(11)=0.5191$, $p=0.6140$, two-tailed t-test. (g-h) *Chrn2* mRNA expression
1075 analyzed by RT-qPCR in the cortex (g) and midbrain (h), cortex: $t(11)=0.1788$, $p=0.8613$;
1076 midbrain: $t(6)=0.7645$, $p=0.4736$; two-tailed t-test. (i) Concentration of dopamine measured by
1077 ELISA in homogenates prepared from mice injected with AAV-GFP or AAV-Cre-GFP.
1078 $t(14)=1.106$, $p=0.2874$, two-tailed t-test. Number of samples used for analysis is indicated in
1079 graphs. All graphs show individual values and means \pm SEM. str: striatum; ctx: cortex; mdr:
1080 midbrain.

1081

1082 **Figure 5: Expression of the AAV-Cre-GFP virus injected in the DS of beta2-flox/flox mice.**

1083 (a) Scheme of the viral injections, 2 sites per hemisphere (frontal section). (b) A representative
1084 image of the injection extent in the DS. An enlarged image shows transduction by the AAV5
1085 serotype used in the study. (c) A sagittal brain section shows an expression of the AAV-Cre-GFP
1086 in the DS and an absence of the viral expression in the midbrain. (d) Scheme of the degree of the
1087 AAV-Cre-GFP expression in mice used for behavioral testing. The shaded areas show the extent

1088 of expression and the numbers in individual regions represent the number of mice that showed
1089 viral expression in that region. A darker shade is proportional to a higher number of mice showing
1090 expression in the region. Note that two of the animals (out of 32) used in behavioral tests died
1091 prematurely and were not included in the histological analysis.

1092

1093 **Figure 6: Beta2-del mice show increased anxiety-like behavior and changes in the social task.**

1094 (a) Weight of beta2-del and control mice 1 month post-surgery. $n=30$, $n(\text{ctrl})=27$. $t(55)=0.4069$,
1095 $p=0.6857$, two-tailed t-test. (b) The amount of nestlet material left untorn in the nest building task.
1096 $n=32$, $n(\text{ctrl})=29$. 95 % credible interval for difference in untorn material proportion (%) [-0.6; 20],
1097 Bayesian generalized linear model. (c) Left: Distance travelled during 30 minutes in the open field
1098 task measured on two consecutive days. $n=32$, $n(\text{ctrl})=29$. Effect of group: $F(1, 59)=1.255$,
1099 $p=0.2672$; effect of day: $F(1, 59)=17.81$, $p<0.0001$; group vs. day interaction: $F(1, 59)=3.409$,
1100 $p=0.0699$; two-way ANOVA. Locomotor activity divided into 5-minute time bins is shown as
1101 extended data in Figure 6-1. Right: Representative track records of a control and beta2-del mouse
1102 during the first day in the open field. Note the lower preference for the central part of the arena by
1103 the beta2-del mouse. (d) Locomotor activity in open field divided into 5-minute time bins,
1104 measured on day 1 (left) and 2 (right). (e) Time spent in the center of the open field arena measured
1105 on two consecutive days. $n=32$, $n(\text{ctrl})=29$. Effect of group: $F(1, 59)=8.925$, $p=0.0041$; effect of
1106 day: $F(1, 59)=38.56$, $p<0.0001$; group vs. day Interaction: $F(1, 59)=6.494$, $p=0.0134$; two-way
1107 ANOVA. (f) Time spent in the open and closed arms of the EPM apparatus. $n=31$, $n(\text{ctrl})=26$.
1108 Effect of group: $F(1, 165)=0.000$, $p>0.999$; effect of arm: $F(2, 165)=138.6$, $p<0.0001$; genotype
1109 vs. arm interaction, $F(2, 165)=0.1530$, $p=0.8583$; two-way ANOVA. (g) Time spent in the light
1110 part of the arena during the light/dark task. $n=16$, $n(\text{ctrl})=13$. $t(27)=2.184$, $p=0.0378$; two-tailed t-

1111 test. (h) Time spent immobile during the FST task. n=32, n(ctrl)=29. 95% CI for beta2-del/controls
1112 ratio [0.88; 1.22], p = 0.658, generalized linear model. (i) Representative double-plotted locomotor
1113 activity records (actograms) of one control (left) and one beta2-del (right) mouse. The mice were
1114 maintained in the original light/dark cycle (grey area corresponds to darkness), and then they were
1115 exposed to a restricted feeding (RF) regime (arrow) for 10 days. The time of food availability
1116 during 6 h centered to the middle of the light phase is shown as the dashed rectangle. Time intervals
1117 during which the activity was compared between the controls and the beta2-del mice are shown at
1118 the bottom of the actograms. n=15, n(ctrl)=10. (j) Comparison of locomotor activity of the control
1119 (circles, open column) and the beta2-del (squares, grey column) mice under *ad libitum* (upper
1120 graph) and restricted feeding (lower graph) conditions as assessed in four intervals during the 24
1121 h cycle depicted in Fig. 6i. The activity is expressed as the ratio of total daily activity of each
1122 mouse. Data were analyzed by multiple t-test: upper graph, 0-3, p=0.7522; 3-9, p=0.7941; 9-12,
1123 p=0.8045; 12-24, p=0.8953; lower graph, 0-3, p=0.3174, 3-9, p=0.8551, 9-12, p=0.4827, 12-24,
1124 p=0.4526. (k) Time spent in the individual chambers of the apparatus during the social preference
1125 task. n=32. n(ctrl)=28. Effect of group: F(1, 116)=1.158, p=0.2842; effect of chamber: F(1,
1126 116)=1.041, p=0.3098; group vs. chamber interaction: F(1, 116)=13.43, p=0.0004; two-way
1127 ANOVA. (l) Time spent directly interacting with a juvenile mouse during the social preference
1128 task. t(58)=0.4590, p=0.6480, two-tailed t-test. (m) Time spent directly interacting with an
1129 inanimate object during the social preference task. p=0.003, generalized linear model. (n)
1130 Sociability in beta2-del and control mice expressed as a ratio of time spent interacting with a mouse
1131 versus time spent interacting with an object. p=0.009; generalized linear model. All graphs are
1132 showing means \pm SEM. In selected tasks, we also correlated main behavioral parameters to the

1133 extent of AAV expression in the DS (Fig. 8 a-e). In tasks that were performed in all cohorts 1-4,
1134 we analyzed performance of mice divided according to the age (cohort 1&2 vs. 3&4) (Fig. 8 f-q).

1135

1136 **Figure 7: Beta2-del mice show a deficit in the acquisition of the T-maze task but they have**
1137 **no impairment of cognitive flexibility.**

1138 (a) Latency to reach a visible platform in the cued-MWM task. n=15, n(ctrl)=16. Effect of group,
1139 $F(1, 29)=0.3785$, $p=0.5432$; effect of day: $F(1, 29)=48.16$, $p<0.0001$; group vs. day interaction:
1140 $F(1, 29)=0.6274$, $p=0.4347$; two-way ANOVA. (b) Time spent grooming during the grooming test
1141 before and after spraying the mice with water. n=15, n(ctrl)=16. Effect of group, $F(1, 58)=0.5290$,
1142 $p=0.4699$; effect of phase: $F(1, 58)=123.5$, $p<0.0001$; group vs. phase interaction: $F(1, 58)=0.2765$,
1143 $p=0.6010$; two-way ANOVA. (c) Number of buried marbles during the marble burying test. n=15,
1144 n(ctrl)=16. $t(29)=0.2103$, $p=0.8349$, two-tailed t-test. (d) The probability of returning to the same
1145 hole during the hole-board test. n=15, n(ctrl)=16. $t(29)=1.096$, $p=0.2823$, two-tailed t-test. (e) The
1146 percentage of visited holes (out of 16) during the hole-board test. $t(29)=1.611$, $p=0.1180$, two-
1147 tailed t-test. (f) The total number of head dippings during the hole-board test. $p=0.27$, generalized
1148 linear model. (g) Number of sessions needed to reach criteria during the acquisition and reversal
1149 phase of the instrumental task. n=11, n(ctrl)=8. $F(1, 33)=2.488$, $p=0.1243$, mixed-effects model.
1150 (h) Percentage of correct presses during different stages of a simple instrumental task in the operant
1151 box. Effect of group: $F(1, 17)=0.01091$, $p=0.9180$; effect of session: $F(2.537, 41.44)=57.05$,
1152 $p<0.0001$; group vs. session interaction: $F(6, 98)=0.3622$, $p=0.9011$; mixed-effects model. (i)
1153 Recognition index in the NOR test. n=15, n(ctrl)=16. $t(21.56)=1.708$, $p=0.1021$, Welch's t-test. (j)
1154 A diagram showing two alternating starting positions in the response-based T-maze task and the
1155 respective location of the reward. (k) Number of sessions needed to reach learning criteria during

1156 the acquisition phase of the T-maze task expressed as percentage of control performance. n=14,
1157 n(ctrl)=14. $t(13.96)=2.521$, $p=0.0245$; Welsh's t-test. (l) Number of sessions needed to reach
1158 learning criteria during the reversal phase of the T-maze task expressed as percentage of control
1159 performance. n=11, n(ctrl)=8. $t(20)=1.497$, $p=0.1499$, two-tailed t-test. All graphs are showing
1160 means \pm SEM. In selected tasks, we also correlated main behavioral parameters to the extent of
1161 AAV expression in the DS (Extended data, Fig. 6-2). In tasks that were performed in all cohorts
1162 1-4, we analyzed performance of mice divided according to the age (cohort 1&2 vs. 3&4)
1163 (Extended data, Fig. 6-3).

1164

1165 **Figure 8: Additional analysis of selected behavioral tasks.**

1166 (a-e) Main parameters of selected behavioral tasks correlated to AAV expression in the dorsal
1167 striatum. The individual parameters are (a) time spent in the center (%) during the open field test
1168 on day 1, (b) time spent in light (s) during the light/dark task, (c) time interacting with object (s)
1169 during the social preference test, (d) mouse/object interaction time (ratio) during the social
1170 preference test and (e) number of sessions needed to reach performance criteria in the acquisition
1171 phase of the T-maze task. Note that none of the parameters shows a significant correlation with
1172 the AAV expression although the correlation almost reaches significance in (c). (f-q) Graphs
1173 showing split performance for cohort 1&2 vs. 3&4. Individual performances corresponding to
1174 mice in cohort 1&2 (green circles and triangles for controls and beta2-del, respectively) and 3&4
1175 (red circles and triangles for controls and beta2-del, respectively) are shown. Only tests that were
1176 performed in both cohorts are shown: (f) weight after surgery, (g) nest building test, (h-i)
1177 locomotion in the open field, (j) forced swim test, (k) elevated plus maze, (l-o) social preference
1178 task, (p-q) locomotion in amphetamine experiment before (p) and after (q) injection. Note that

1179 when performance of ctrl and beta2-del mice was analyzed separately for cohort 1&2 vs. 3&4,
1180 only the difference in "Time interacting with object" (n) reached the significance in both cohorts
1181 before pooling the data.

1182

1183 **Figure 9: Beta2-del mice are hyperactive and show higher amphetamine-induced c-Fos**
1184 **expression compared to controls.**

1185 (a) A diagram showing the experimental design. (b) Distance travelled during the 30 minutes of
1186 habituation (pre-injection) in the open field. $n=24$, $n(\text{ctrl})=20$. $t(42)=2.877$, $p=0.0063$, two-tailed
1187 t-test. (c) Distance travelled in the open field for the 90 minutes following an acute saline or
1188 amphetamine injection. $n(\text{saline/amphetamine})=11/13$, $n(\text{ctrl; saline/amphetamine})=9/11$. Effect
1189 of group: $F(1, 40)=3.519$, $p=0.068$; effect of treatment: $F(1, 40)=19.75$, $p<0.0001$; group vs.
1190 treatment interaction: $F(1, 40)=1.886$, $p=0.1773$; two-way ANOVA. (d) Locomotion during the
1191 amphetamine experiment divided into 5-minute time bins. We also analyzed distance travelled
1192 before and after injection in individual cohorts of mice divided according to the age (cohort 1&2
1193 vs. 3&4) (Extended data, Fig. 6-3). (e) Analysis of c-Fos expression in the DS of beta2-del and
1194 control mice injected either with saline or amphetamine. $n(\text{saline/amphetamine})=11/9$, $n(\text{ctrl;}$
1195 $\text{saline/amphetamine})=8/9$. An average of 1000 nuclei per picture were analyzed in 6 brain sections
1196 per mouse. Effect of group: $F(1, 33)=5.958$, $p=0.0202$; effect of treatment: $F(1, 33)=10.15$,
1197 $p=0.0031$; group vs. treatment interaction: $F(1, 33)=0.9106$, $p=0.3469$; two-way ANOVA. (f)
1198 Distance traveled after injection correlated to c-Fos expression. (g) Right: A representative section
1199 counterstained with Hoechst showing GFP and c-Fos expression. A white square shows a typical
1200 area in the DS that was used for c-Fos analysis. Left: Representative images counterstained with

1201 Hoechst showing baseline and amphetamine-induced c-Fos expression in beta2-del and control
1202 animals. All graphs are showing means \pm SEM.

1203

1204 **Figure 10: c-Fos expressing neurons include both DARPP-32+ MSNs and DARPP-32- INs.**

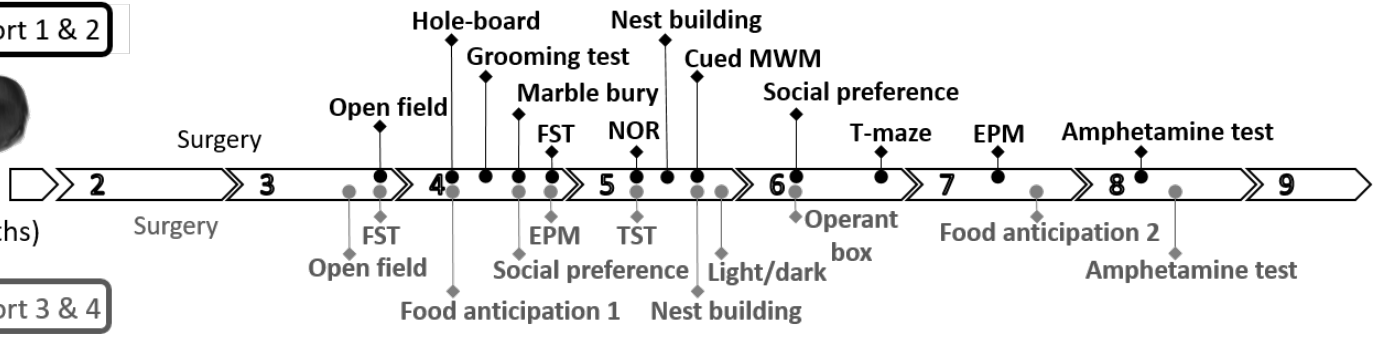
1205 (a) Representative images showing baseline and amphetamine-induced c-Fos expression in beta2-
1206 del and control animals in combination with DARPP-32 and GFP staining (indicating the AAV-
1207 expressing area with presumed beta2 deletion). From the left, merged images are showing: c-Fos
1208 double labelling with GFP; c-Fos double labelling with DARPP-32; c-Fos triple labelling with
1209 GFP and DARPP-32; the combination of the three markers over Hoechst counterstain for nuclei.
1210 The white arrowheads are highlighting c-Fos positive cells, not expressing DARPP-32. The white
1211 squares show different combinations of the used markers. (b) Number of c-Fos-positive and
1212 DARPP-32-negative cells expressed as percentage of all c-Fos-positive neurons in beta2-del and
1213 control mice injected with either saline or amphetamine. $n(\text{saline/amphetamine})=3/3$, $n(\text{ctrl};$
1214 $\text{saline/amphetamine})=3/3$. An average of 25 c-Fos positive, DARPP-32 negative neurons were
1215 analyzed in two brain sections per mouse. Effect of group: $F(1, 8)=0.6286$, $p=0.4507$; effect of
1216 treatment: $F(1, 8)=1.541$, $p=0.2497$; group vs. treatment interaction: $F(1, 8)=0.4934$, $p=0.5023$;
1217 two-way ANOVA. (c) Number of c-Fos-positive neurons out of all VACHT-positive neurons in
1218 control and beta2-del mice. Saline and amphetamine-injected mice were pooled together in each
1219 group. $n(\text{ctrl})=7$, $n(\text{beta2-del})=8$. An average of 23 VACHT positive neurons were analyzed in 4
1220 brain sections per mouse. 95 % credible interval for the odds ratio between ctrl and beta2-del [0.09;
1221 1.02], generalized linear mixed model. Graphs are showing means \pm SEM. (d) Representative
1222 images showing amphetamine-induced c-Fos expression in control and beta2-del animals in
1223 combination with VACHT and GFP staining (indicating the AAV-expressing area with presumed

1224 beta2 deletion). Left: two main panels show merged images for the three markers c-Fos (magenta),
1225 GFP (green) and VACHT (grey). Right: different combinations of two markers are shown, c-
1226 Fos/VACHT, GFP/VACHT and c-Fos/GFP.

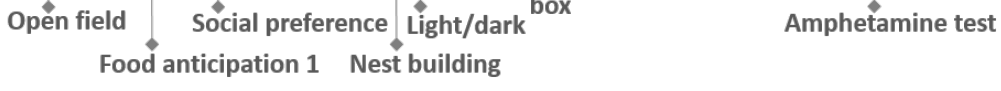
DS Cohort 1 & 2

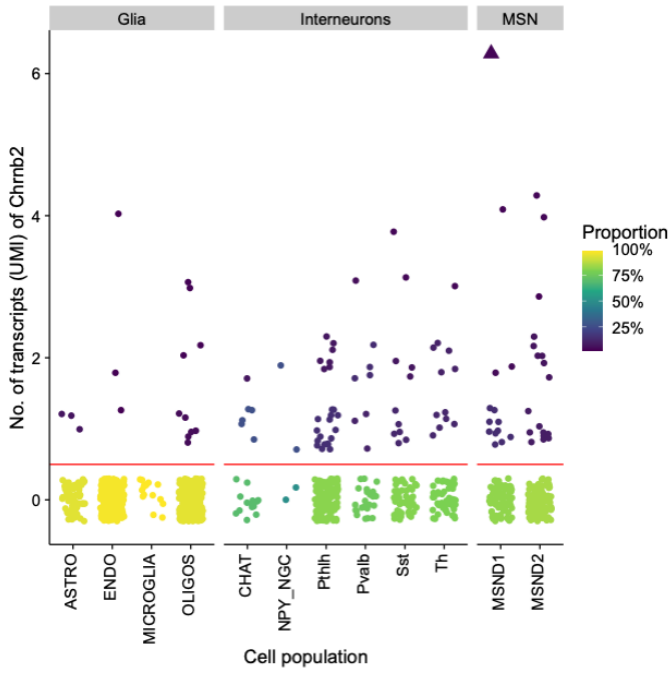
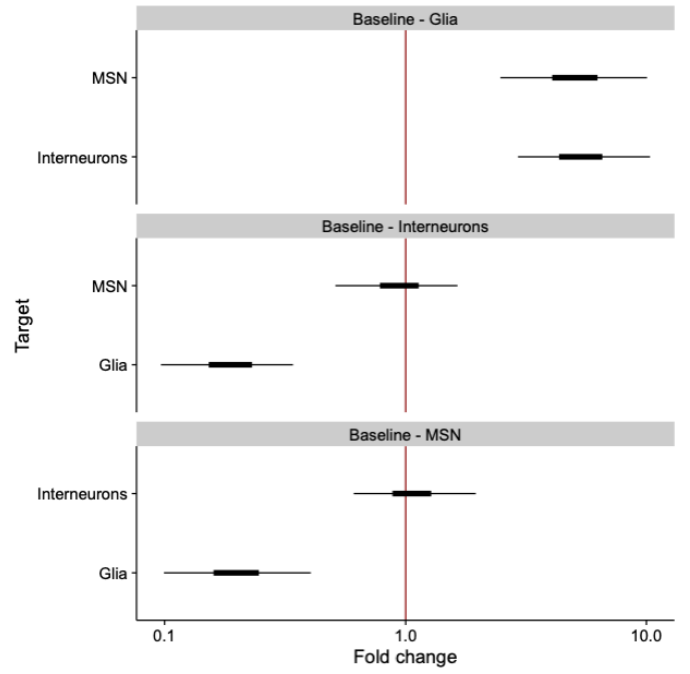


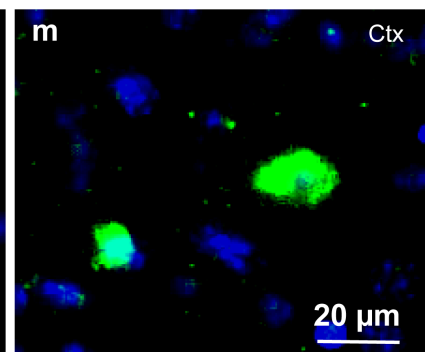
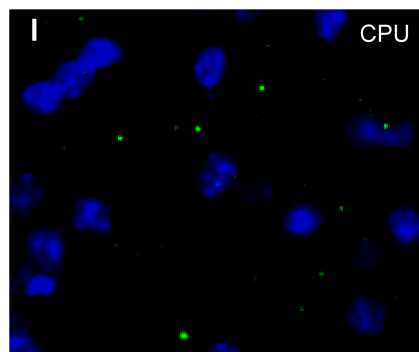
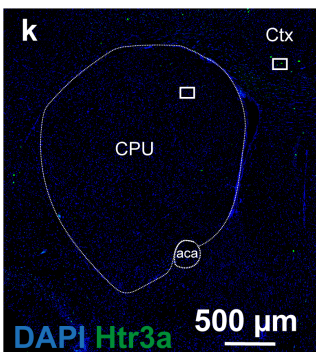
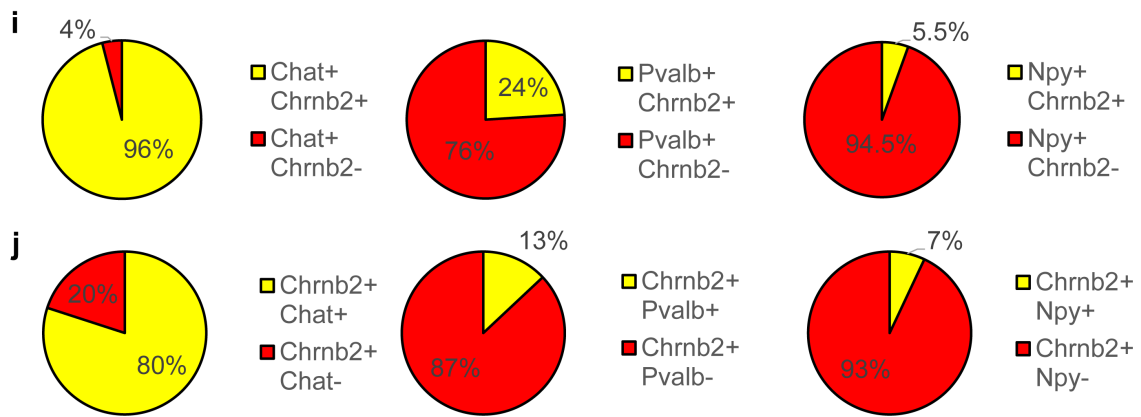
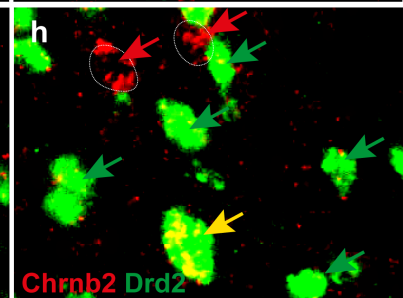
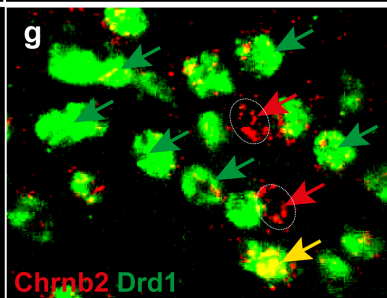
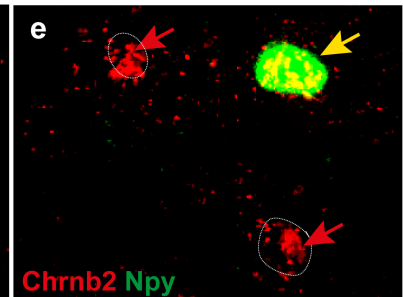
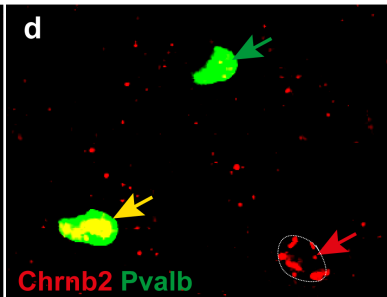
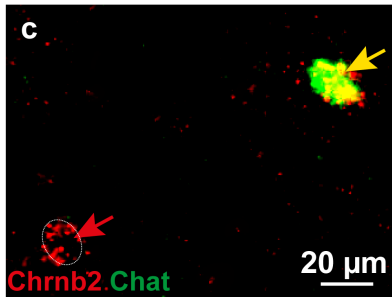
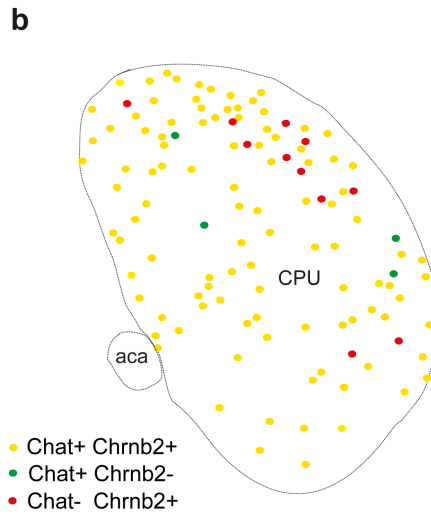
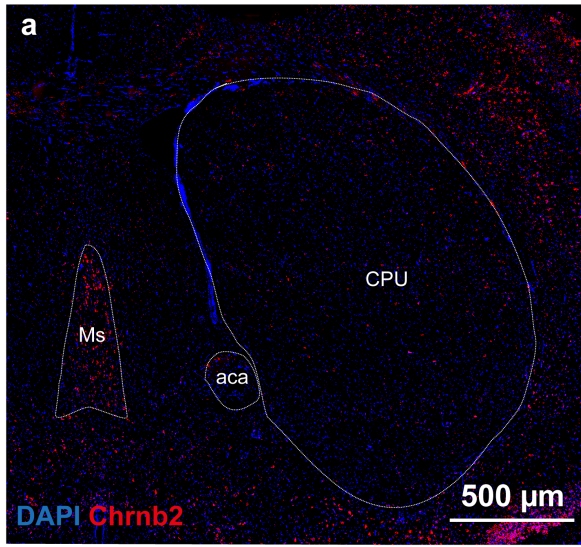
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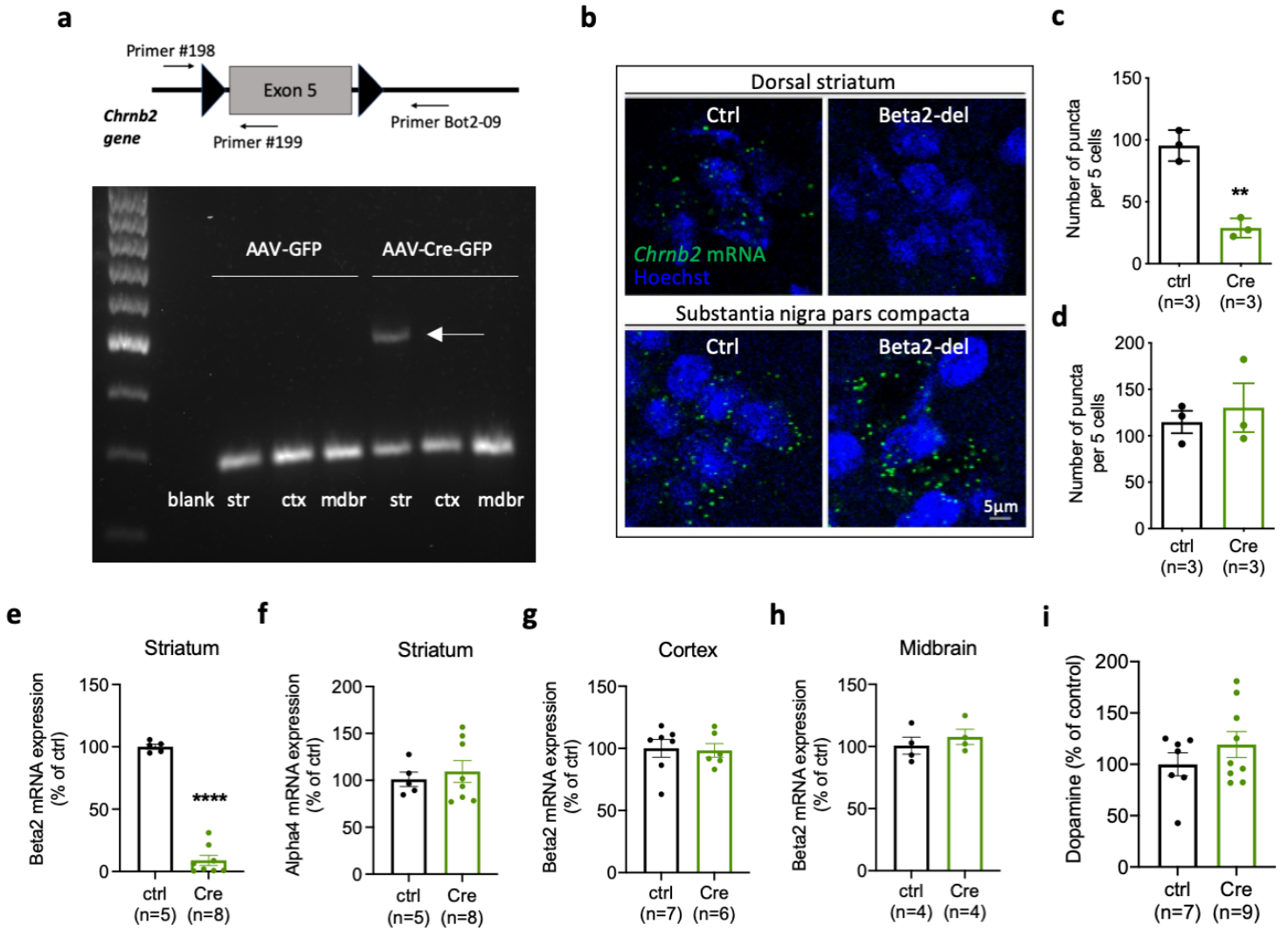


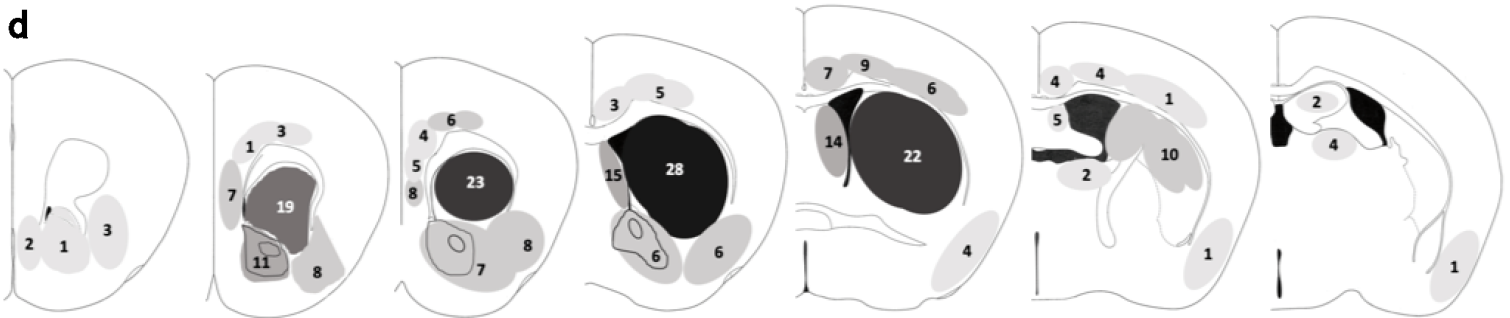
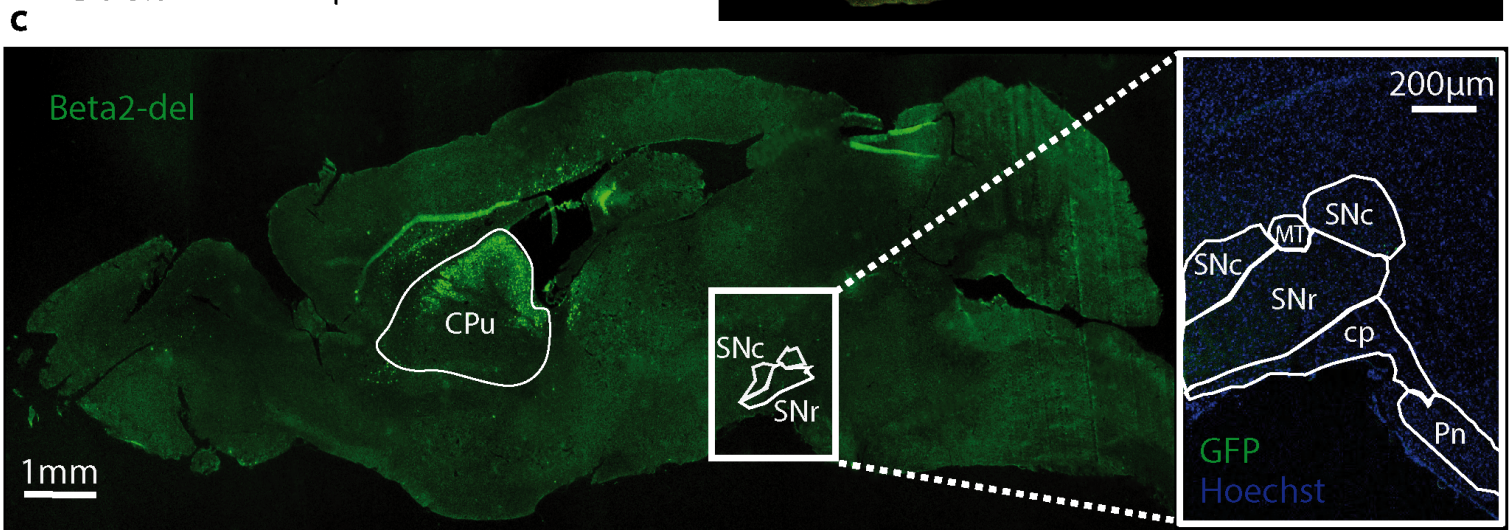
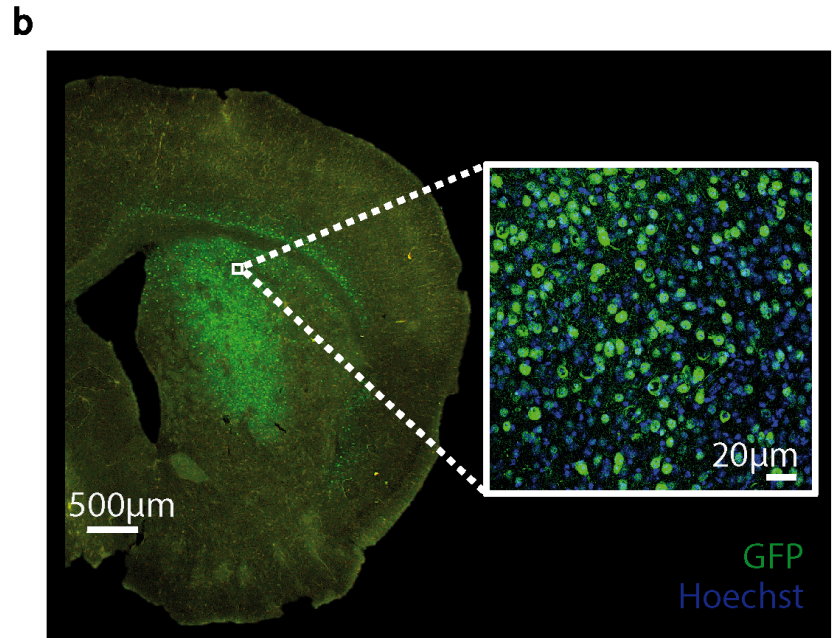
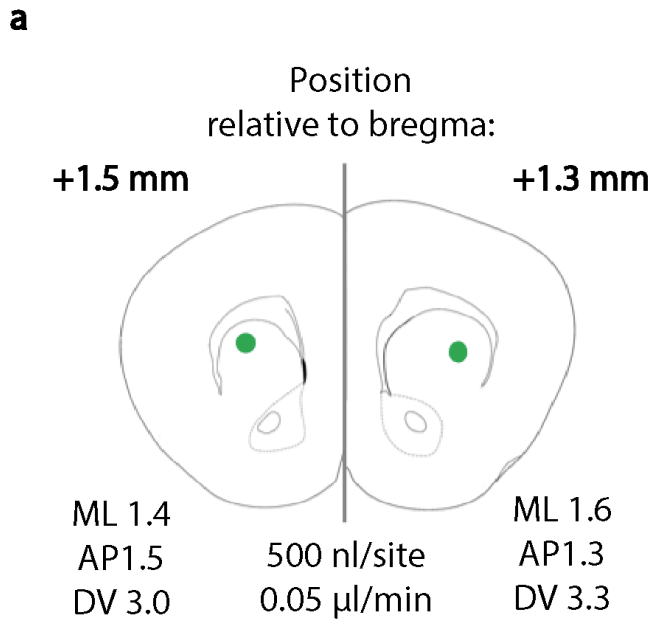
DS Cohort 3 & 4

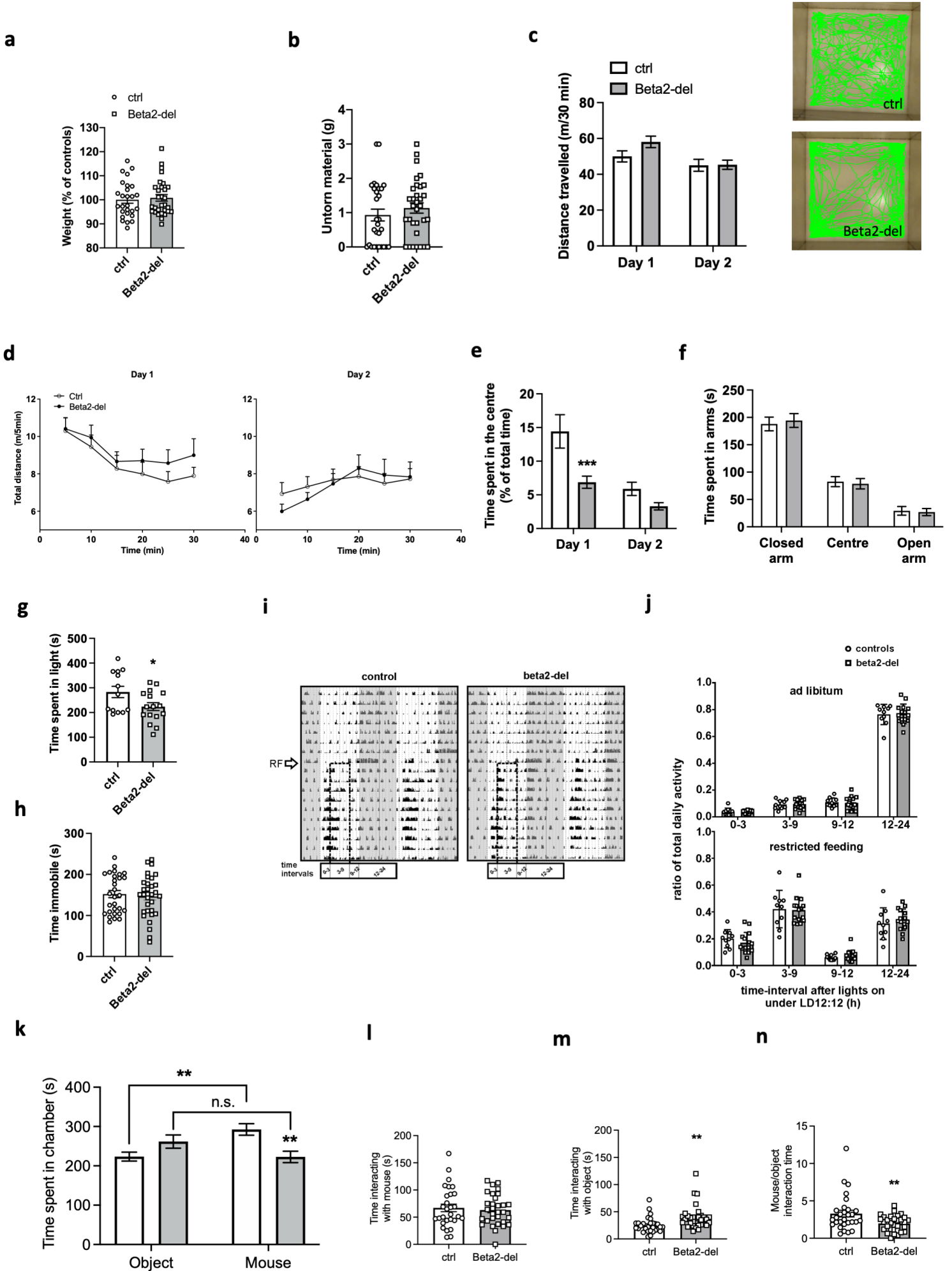


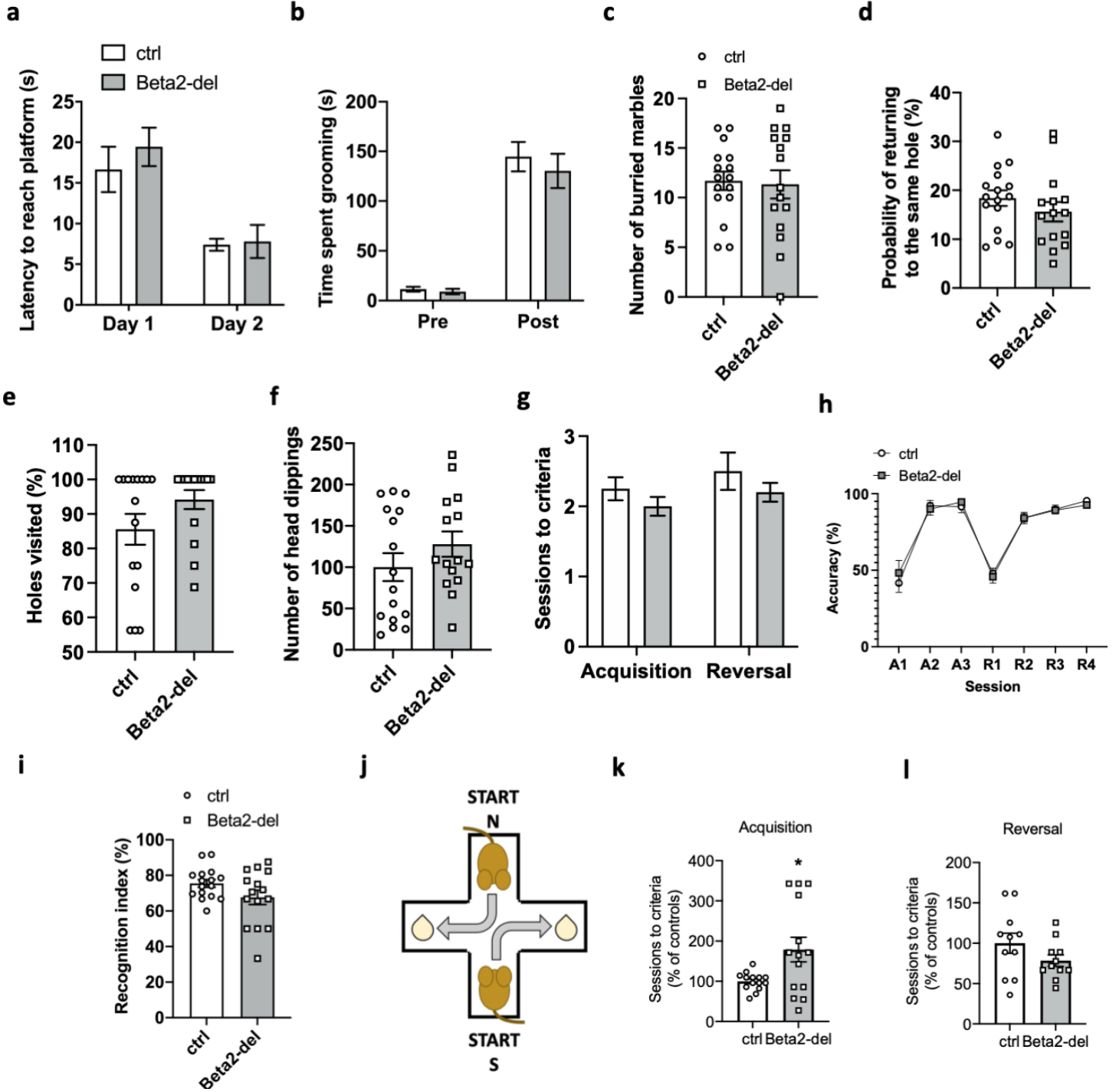
a**b**

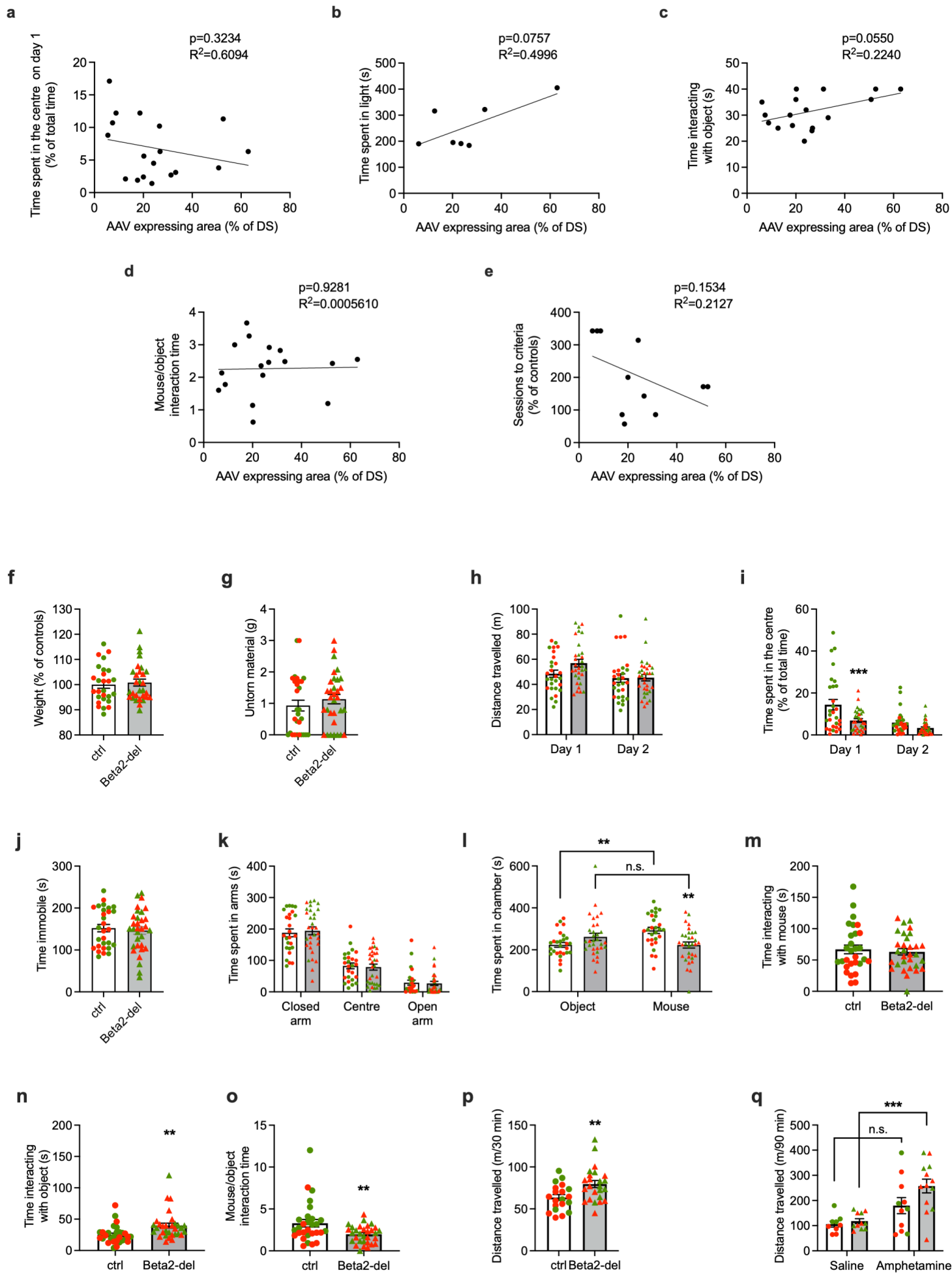


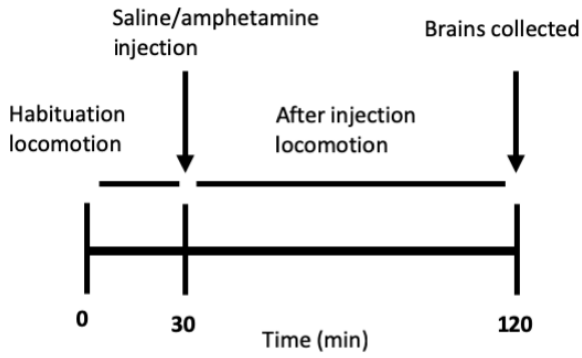
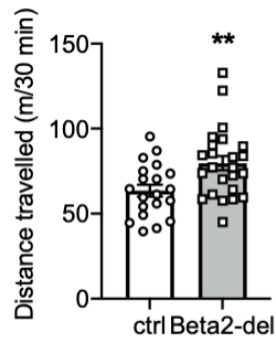
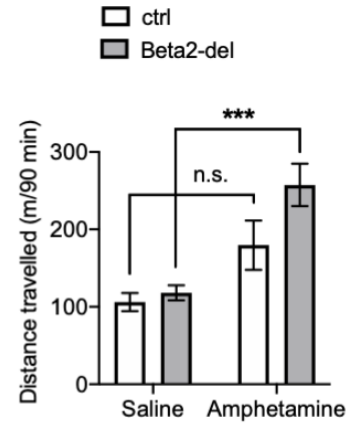
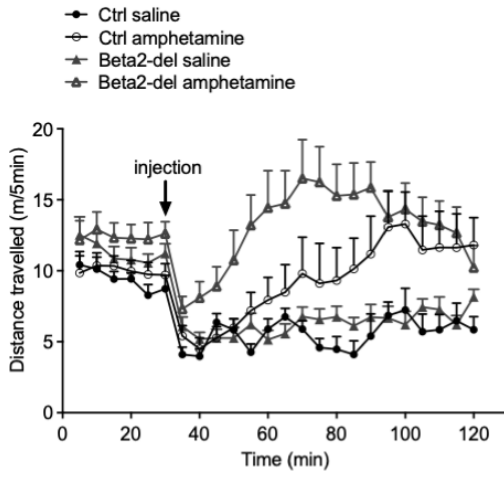
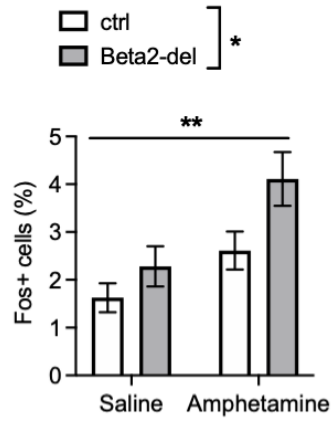
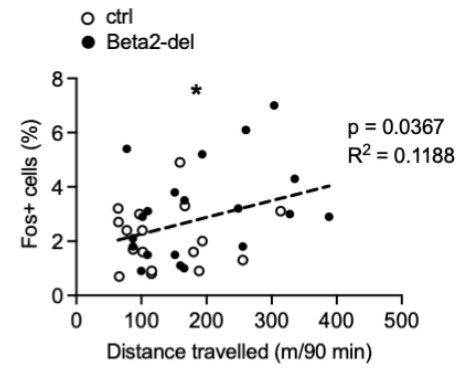










a**b****c****d****e****f****g**