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Aversion encoded in the subthalamic nucleus

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Abstract

Activation of the subthalamic nucleus (STN) is associated with the stopping of ongoing behavior via the basal ganglia. However, we recently observed that optogenetic STN excitation induced a strong jumping/escaping behavior. We hypothesized that STN activation is aversive. To test this, place preference was assessed. Optogenetic excitation of the STN caused potent place aversion. Causality between STN activation and aversion has not been demonstrated previously. The lateral habenula (LHb) is a critical hub for aversion. Optogenetic stimulation of the STN indeed caused firing of LHb neurons, but with delay, suggesting the involvement of a polysynaptic circuit. To unravel a putative pathway, the ventral pallidum (VP) was investigated. VP receives projections from the STN and in turn projects to the LHb. Optogenetic excitation of STN-VP terminals caused firing of VP neurons and induced aversive behavior. This study identifies the STN as critical hub for aversion, potentially mediated via an STN-VP-LHb pathway.

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Introduction

The subthalamic nucleus (STN) is a small and bilaterally positioned nucleus which exerts excitatory influence over the indirect pathway of the basal ganglia (Kita, 1994; Kita and Kitai, 1987). The STN has long been associated with motor and cognitive processes (Bonnievie and Zaghoul, 2019; Schmidt and Berke, 2017; Weintraub and Zaghoul, 2013). Aberrant

33 STN activity is observed in patients with Parkinson's disease and obsessive-compulsive
34 disorder (OCD), but can be corrected by high-frequency electrical stimulation of the STN, so
35 called deep brain stimulation (DBS), which successfully alleviates both motor deficiency in
36 Parkinson's disease and compulsivity in OCD (Benabid et al., 2009; Blomstedt et al., 2015;
37 Mallet et al., 2008). However, while STN-DBS is highly beneficial to many severely afflicted
38 individuals, a number of adverse side-effects have been reported, including low emotional
39 state (Péron et al., 2013; Temel et al., 2006). This has not been easily understood, but high-
40 lights the need for revealing the full repertoire of regulatory roles executed by the STN.

41 Reward and punishment motivate learning through complex neuronal circuits (Jean-Richard-
42 Dit-Bressel et al., 2018). Studies of rewarding and aversive stimuli were long focused on
43 dopamine neurons in the ventral tegmental area (VTA) (Barker et al., 2016; Bromberg-Martin
44 et al., 2010; Lammel et al., 2014; Wise, 2004). However, with the seminal discovery that
45 glutamatergic neurons of the lateral habenula (LHb) respond to reward in an opposite
46 manner as dopamine neurons, LHb has gained considerable attention as an important brain
47 area in the regulation of aversive responses and negative reward-processing (Matsumoto
48 and Hikosaka, 2007). LHb neurons are activated both by aversive stimuli and the omission of
49 an expected reward (Gao et al., 1996; Matsumoto and Hikosaka, 2007). Further, LHb
50 hyperactivity has been associated with depression and negative symptoms of addiction (Hu,
51 2016).

52 The LHb receives regulatory input from several sources, many identified over the past few
53 years using optogenetic stimulations. For example, optogenetic excitation of glutamatergic
54 projections from the VTA and the lateral hypothalamus was shown to induce aversion
55 (Lazaridis et al., 2019; Lecca et al., 2017; Root et al., 2014; Stamatakis et al., 2016), as did
56 also stimulation of afferent fibers from the entopeduncular nucleus (EP) (Shabel et al.,
57 2012; Stephenson-Jones et al., 2016). In addition, optogenetic stimulation of glutamatergic
58 neurons in the ventral pallidum (VP) was recently shown to evoke post-synaptic activity in the
59 LHb and to drive place avoidance (Faget et al., 2018; Tooley et al., 2018). Thus, through
60 various sources of excitatory input, activation of the LHb mediates aversive behavior.

61

62 The STN projects directly to several of the structures recognized in aversive processing,
63 including the EP and VP, both directly connected to the LHb (Faget et al., 2018; Shabel et
64 al., 2012; Stephenson-Jones et al., 2016; Tooley et al., 2018). However, while ample studies
65 have addressed pallidal and habenular structures using optogenetics leading towards
66 increased understanding of their role in affective functions, only a limited set of optogenetic
67 studies have yet investigated the STN. Based on the strong interest in STN-DBS,

68 optogenetics in the STN has mainly been used to address motor functions in the context of
69 Parkinson's disease (Gradinaru et al., 2009; Sanders and Jaeger, 2016; Tian et al., 2018;
70 Yoon et al., 2016, 2014). Further, one recent study identified that optogenetic excitation of
71 the STN interrupted ongoing licking behavior in mice (Fife et al., 2017). STN neurons
72 express the *Pitx2* gene, which allows recombination of floxed alleles using Pitx2-Cre
73 transgenic mice (Martin et al., 2004). Confirming the selectivity of the Pitx2-Cre transgene in
74 directing opsin expression to the STN, we have previously shown that photostimulation of the
75 STN results in post-synaptic currents and glutamate release in basal ganglia target areas
76 (Schweizer et al., 2016, 2014; Viereckel et al., 2018). In a recent report, we show that
77 optogenetic excitation and inhibition result in opposite locomotor effects (Guillaumin et al.,
78 2020). Surprisingly, we also observed that STN excitation could induce an avoidance-like
79 behavior. When placed in an open and novel arena, mice displayed a strong escaping
80 behavior, characterized by attempts to jump out of the apparatus. This unexpected outcome
81 was evident immediately upon STN excitation (Guillaumin et al., 2020).

82 Based on these observations, we hypothesized that the STN is engaged in aversive
83 behavior. To test this, optogenetic stimulations followed through with electrophysiological
84 recordings and place preference analyses were implemented in Pitx2-Cre mice. Our results
85 identify place avoidance upon selective activation of either the STN itself or STN terminals in
86 the VP, and also demonstrate multi-synaptic STN-communication with the LHb. The findings
87 contribute to the current decoding of the neurocircuitry of aversive behavior.

88

89 **Results**

90 **STN neurons follow 20 Hz optogenetic stimulation.**

91 Optogenetics allows spatially and temporally precise control of neuronal activation which
92 under experimental conditions enables the correlation between distinct neuronal activation
93 and measurable behaviors in freely-moving animals (Deisseroth, 2011). To allow optogenetic
94 control of STN neurons, Pitx2-Cre mice were bilaterally injected with adeno-associated
95 (AAV) virus to express a construct encoding either Channelrhodopsin-2 (ChR2) fused with
96 eYFP (so called Pitx2/ChR2-eYFP mice) or, as controls, eYFP alone (Pitx2/eYFP; control
97 mice) (Figure 1A). Bilateral optic cannulas were placed above the STN (Figure 1A).
98 Histological analyses and *in vivo* extracellular recordings were performed to validate the
99 approach, and injections and cannulas placement were confirmed after completed behavioral
100 analyses.

101 Mice that displayed strong cellular eYFP labeling throughout the extent of the STN and in
102 which optic cannulas could be confirmed as positioned immediately above the STN, were

103 included in the statistical analyses of the electrophysiological and behavioral experiments
104 (Figure 1B). In addition to the STN, the para-subthalamic nucleus (pSTN), associated with
105 the medial STN, was eYFP-labeled to minor extent. Addressing eYFP-positive terminals in
106 target areas of the STN, these were detected in the GP (GP externa in humans), EP,
107 substantia nigra *pars reticulata* (SNr), substantia nigra *pars compacta* (SNc) and VP,
108 confirming that Pitx2-Cre-positive neurons of the STN reach these target areas (Figure 1B).

109 To ensure connectivity, *in vivo* single cell electrophysiological recordings upon optogenetic
110 stimulation of the STN were performed (Figure 1C,D). First, an optotagging protocol (Figure
111 1E) was used to stimulate and record within the STN. To observe the reaction of STN
112 neurons to photostimulation, peri-stimulus time histograms (PSTH protocol, 0.5 Hz, 5 ms bin
113 width, 5-8 mW) were created by applying a 0.5 Hz stimulation protocol for at least 100
114 seconds. Action potentials in ChR2-positive STN cells were successfully evoked by STN
115 photostimulation (Figure 1E).

116 Once neuronal activity of STN neurons returned to baseline, a photostimulation protocol
117 intended for behavioral experiments was validated (Behavior protocol, 20 Hz, 5 ms pulses, 5-
118 8 mW; 10 seconds) which increased the frequency and firing rate of STN neurons for the
119 whole duration of the stimulation, after which they returned to normal (Figure 1F).

120 The excitability of STN neurons was next confirmed by patch-clamp recordings in the STN of
121 Pitx2/ChR2-eYFP mice with the optic fiber placed above the recording site (Figure 1G).
122 ChR2-eYFP expression was strong in the STN (Figure 1H), and all of the STN neurons
123 tested responded to continuous (Figure 1I) or 20 Hz trains (Figure 1J) of light stimulation by
124 sustained ChR2-mediated currents. When light stimulation was applied in brain slices from
125 Pitx2/eYFP, no current were observed in STN neurons (n=3, data not shown).

126 Having confirmed optogenetics-driven activation of STN neurons, behavioral effects upon
127 optogenetic STN activation were next tested.

128 **Activation of STN neurons induces place avoidance**

129 To experimentally test the hypothesis that the STN drives affective behavior, we applied
130 optogenetic excitation in the real-time place preference paradigm (RT-PP) and the elevated
131 plus maze (EPM), representing validated tests for aversion and anxiety. First, Pitx2/ChR2-
132 eYFP and control mice were analyzed in the RT-PP. Here, the mouse moves around freely in
133 a three-compartment apparatus, in which entry into one of two main compartments (A or B)
134 is paired with photostimulation of the STN. A photostimulation-neutral, transparent, corridor
135 (neutral compartment) connects the main compartments, allowing free access to the whole
136 apparatus. In this setup, an aversive response to photostimulation would be displayed as
137 avoidance of entry into the light-paired compartment, and hence avoidance of activation of

138 the STN. Further, if the association is strong, this would be expected to give rise to similar
139 behavior even during days after the experience of STN activation due to conditioning effect
140 (Figure 2A). Pitx2/ChR2-eYFP mice showed an active avoidance of STN-photostimulation by
141 spending significantly less time in the light-paired compartment (A) than the unpaired
142 compartment (B) both during the two days of real-time exposure to photostimulation (Days 3,
143 4), and also during the conditioned test days (Days 5, 6) (Figure 2B,C). Control mice showed
144 an absence of stimulation-dependent response throughout the trials (Figure 2B).

145 Next, to test the ability for reversal of the avoidance behavior, a strategy was implemented in
146 which the previously light-unpaired compartment was paired with photostimulation, and the
147 stimulation protocol was repeated. On the first reversal day (Day 7), Pitx2/ChR2-eYFP mice
148 responded by spending time in all three compartments which resulted in lack of significant
149 preference for any compartment. On the second day (Day 8), mice again spent significantly
150 more time in the unpaired compartment (A). On the final conditioned test day (Day 9),
151 Pitx2/ChR2-eYFP mice spent a similar amount of time in the main compartments A and B
152 (Figure 2B,C).

153 Avoidance towards the light-paired compartment was corroborated by faster and shorter
154 distance moved compared to the non-light paired compartment across all conditioning days,
155 except Day 7 when no specific compartment was preferred or avoided (Figure 2D,E). Control
156 mice spent a comparable amount of time in A and B regardless of which compartment the
157 stimulation was applied in, and showed no significant effects in distance moved or speed
158 (Figure 2D,E). These findings identified a direct correlation between STN activation and
159 place avoidance both upon direct STN stimulation and upon exposure to aversion-paired
160 cues.

161 **Aversion induced by optogenetic STN activation stronger than inherent aversion for** 162 **unsheltered exposure**

163 To assess a putative anxiogenic component and to further pinpoint the nature of the aversive
164 response observed in the RT-PP, mice were next analyzed in two versions of the EPM, the
165 regular EPM and a modified EPM setup established to compare any aversion to STN-
166 stimulation with a naturally occurring aversive situation (Figure 3A,D).

167 The regular EPM is commonly used to assess anxiety. Here, the innate response of mice is
168 their aversion towards the exposed open arms and a center zone, resulting in an avoidance
169 of these areas, and consequently, a preference for the sheltered space provided by the
170 closed arms. In the current experiment, STN-photostimulation was given throughout the trial
171 (Figure 3A). Similar as controls, Pitx2/ChR2-eYFP mice spent most of the time in the

172 sheltered space provided by the closed arms where duration was significantly higher
173 compared to exposed open arms and the center (Figure 3B). Number of entries into either
174 the open or closed arms was not significantly different between Pitx2/ChR2-eYFP and
175 control mice, but for both groups, the number of entries into the closed arms was statistically
176 higher than entry into open arms (Figure 3C). Since Pitx2/ChR2-eYFP mice showed the
177 same behavior as control mice, the results suggest that activation of the STN is not
178 associated with altered response towards the naturally aversive area, which they actively
179 avoid both in presence and absence of STN stimulation. These results suggest that the
180 aversive behavior upon STN-activation is likely not related to an anxiogenic effect.

181 Next, we re-designed the EPM test to directly compare the optogenetically induced aversive
182 behavior with the naturally aversive context offered by the exposed open arm and center
183 zone. In this EPM-avoidance setup, STN-photostimulation was activated directly upon entry
184 into one of the two closed arms and disabled by leaving them, while entry into open arms or
185 the center zone had no effect on STN-photostimulation (Figure 3D). The effect was striking;
186 Pitx2/ChR2-eYFP mice actively tried to avoid the previously preferred closed arms, showed
187 increased number of entries in all arms, and spent significantly more time in the open arms
188 and in the center (Figure 3E,F). These findings demonstrated that the aversive effect induced
189 by STN activation was stronger than the naturally aversive effect provided by unsheltered
190 exposure. Thus, while not confirming STN activation as anxiogenic, analysis in both the RT-
191 PP and EPM-avoidance paradigms demonstrated a causal relationship between STN
192 activation and aversive behavior.

193 **STN photoactivation induces excitatory post-synaptic responses in the LHb neurons** 194 **revealing poly-synaptic connectivity**

195 The LHb has recently emerged as an important brain area for aversive behavior, firmly
196 demonstrated by optogenetic analyses of the LHb itself as well as projections to the LHb
197 (Faget et al., 2018; Lazaridis et al., 2019; Lecca et al., 2017; Shabel et al., 2012;
198 Stephenson-Jones et al., 2016; Tooley et al., 2018). While STN activation has not previously
199 been associated with aversion, it has been shown that high frequency stimulation of the STN
200 in rats induces c-Fos expression in LHb (Tan et al., 2011) and modulation of LHb neuron
201 activity (Hartung et al., 2016).

202 The potential impact of STN-photostimulation on LHb activity was assessed using *in vivo*
203 extracellular recordings in Pitx2/ChR2-eYFP (Figure 4A). An optic fiber was positioned above
204 the STN (Figure 4C), and a stimulation protocol (PSTH 0.5 Hz, 5 ms pulses, 5-8 mW; at least
205 100 seconds) was implemented which evoked an excitatory response in 50% of the recorded
206 LHb neurons (onset latency =10.08 ms +/- 0.81 ms) (Figure 4B). Pontamine staining and

207 neurobiotin-marked neurons confirmed the positioning of the recording electrodes within the
208 LHb and enabled the reconstruction of neuronal positions (Figure 4D,E). No eYFP-positive
209 fibers were detected in the habenular area of Pitx2/ChR2-eYFP (Figure 4E), confirming the
210 absence of a direct connection between the STN and the LHb. These findings indicate a
211 multi-synaptic glutamatergic pathway between the STN and the LHb.

212 **Selected optogenetic activation STN-VP projections results in VP firing**

213 Several projections to the LHb are involved in aversion. With the lack of direct projection from
214 the STN to LHb, we decided to assess the putative role of the VP as the bridge between the
215 STN and LHb in the observed STN-driven aversion. While the STN projects to several basal
216 ganglia structures, the ventral pallidal target area, VP, was of particular interest as it sends
217 projections to both the VTA and LHb (Root et al., 2015; Tripathi et al., 2013). Further, the VP
218 was recently proposed to play a role in integrating rewarding and aversive information (Creed
219 et al., 2016; Itoga et al., 2016). Of mixed neurotransmitter phenotype, specifically the
220 glutamatergic neurons of the VP were recently identified as sending direct projections to the
221 LHb (Faget et al., 2018; Tooley et al., 2018). In addition, it has been shown that these VP
222 neurons receive input from the STN, among several regions (Tooley et al., 2018).

223 Focusing on the VP, to address the effect of STN optogenetic activation on the excitability of
224 VP neurons, sagittal slices from Pitx2/ChR2-eYFP and control mice were prepared for whole-
225 cell patch-clamp recordings. STN inputs were stimulated with 473 nm laser pulses through
226 an optic fiber placed above the VP (Figure 5A). VP neurons were all recorded in the area just
227 below the anterior commissure, a region rich in ChR2-eYFP-expressing STN axon terminals
228 (Figure 5B-C). The impact of the activation of the STN-VP pathway was first assessed in cell-
229 attached configuration. Trains of stimulation at 20 Hz induced a strong increase in the firing
230 activity of all recorded VP compared to basal firing activity (Figure 5D; $p < 0.05$), suggesting a
231 powerful excitatory drive from the STN on VP neurons. Next, STN-VP synaptic transmission
232 was recorded in whole-cell voltage-clamp configuration which confirmed the glutamatergic
233 nature of this pathway (Figure 5E). Altogether, these data support the strong impact of the
234 STN-VP pathway on the excitability of VP neurons.

235 **Activation of STN-VP terminals induces place avoidance**

236 Finally, to investigate if selective activation of the STN-VP pathway can drive aversive
237 behavior, optic cannulas were bilaterally implanted above the VP in a new batch of Pitx2-Cre
238 mice virally injected into the STN (following the terminology used above, these mice are
239 referred to as Pitx2/ChR2-eYFP/VP and Pitx2/eYFP/VP, respectively) (Figure 6A). This
240 allowed behavioral testing in the same RT-PP and EPM paradigms as used for the STN-
241 photostimulation, but now upon photostimulation directed to the ChR2-eYFP-positive STN-

242 terminals in the VP. Pitx2/ChR2-eYFP/VP mice showed an active avoidance behavior to VP-
243 photostimulation by spending significantly less time in the light-paired compartment (A) than
244 the unpaired compartment (B) both during the two days of real-time exposure to
245 photostimulation (Days 3, 4), and also during the first conditioned test days (Days 5) (Figure
246 6B). Control mice showed an absence of stimulation-dependent response throughout the
247 trials (Figure 6B,C).

248 Upon reversal of light-pairing (Days 7, 8), Pitx2/ChR2-eYFP/VP mice reversed their
249 preference by again spending significantly more time in the light-unpaired compartment (A).
250 The preference for the unpaired compartment was maintained on the final conditioned test
251 day (Day 9) when no light was applied (Figure 6B,C). Avoidance towards the light-paired
252 compartment was confirmed by shorter distance moved compared to the non-light paired
253 compartment across all test days similarly to what observed upon STN optogenetic
254 stimulation while, in the other hand, speed was not affected (Figure 6D,E).

255 These results suggest that the STN-VP pathway is involved in the aversive response
256 observed upon optogenetic STN activation.

257 **Activation of STN-VP terminals reduces the natural preference for a sheltered** 258 **environment**

259 Also the EPM protocols were used to analyse the Pitx2/ChR2-eYFP/VP mice (Figure 7A). A
260 similar response was observed upon VP-stimulation as was seen using STN-stimulation.
261 That is, in the regular EPM, photoactivation of the terminals in the VP did not induce any
262 visible difference between Pitx2/ChR2-eYFP/VP and control mice. Pitx2/ChR2-eYFP/VP
263 mice spent most of the time in the closed arms compared to open arms and center (Figure
264 7B). The number of entries into either the open or closed arms were not significantly different
265 between Pitx2/ChR2-eYFP/VP and control mice (Figure 7C). These results were in
266 accordance with those observed with STN activation.

267 Next, we tested the Pitx2/ChR2-eYFP/VP mice in the re-designed EPM-avoidance paradigm
268 (Figure 7D), where VP-photostimulation was activated directly upon entry into one of the two
269 closed arms and disabled by leaving them. As observed upon STN-photostimulation,
270 activation of STN-VP terminals in the VP induced a visible reduction of the time spent in the
271 closed arms with an increase of the time spent in the open arms and center. Pitx2/eYFP/VP
272 control mice spent significant more time in the closed arms compared to open arms and
273 center, while Pitx2/ChR2-eYFP/VP mice spent a similar amount of time in the three
274 compartments, annulling the natural preference for the closed arms (Figure 7E). Despite an
275 increased tendency in the number of entries comparing Pitx2/ChR2-eYFP/VP with
276 Pitx2/eYFP/VP control mice, no significant difference was detected (Figure 7F). In summary,

277 while mice did not show an anxiogenic phenotype, optogenetic stimulation of STN terminals
278 in the VP was sufficient to drive avoidance behavior.

279

280 **Discussion**

281 Three distinct sets of optogenetics-based experiments in mice expressing ChR2 selectively
282 in the STN enabled the identification of a previously unknown role of this clinically relevant
283 brain area in aversion: First, we identify that photostimulation of the STN drives aversive
284 behavior. Second, STN-photostimulation drives an excitatory response in LHb neurons.
285 Third, photostimulation of STN-terminals in the VP results in significant firing, and induces a
286 similar aversive response as when the STN itself is stimulated. Further, as no direct
287 projection is observed between the STN and LHb, and with a delay in the observed evoked-
288 firing of the LHb, the current results support an indirect connection between the STN and
289 LHb via the VP. The identification of a causal role of the STN in driving aversion opens up for
290 new possibilities in the current understanding and on-going decoding of aversive behavior.

291 The role of selective STN excitation in mediating emotional valence and motivated behaviors
292 has so far been poorly investigated. Aversive stimuli, such as the threat of a predator or the
293 perception of an imminent dangerous situation induce the activation of a “survival state”
294 designed to avoid or reduce the possible harmful outcome. In this context, aversive learning
295 allows the animals to detect the aversive stimulus and learn to actively avoid it, while
296 aversive Pavlovian conditioning serves to associate neutral stimuli to the hostile situation and
297 environment reducing the probability of a related behavior being expressed (Jean-Richard-
298 Dit-Bressel et al., 2018). Obtaining a reward or avoiding punishment shapes decision-making
299 and motivates learning with several brain regions playing a part in such vital responses
300 (Jean-Richard-Dit-Bressel et al., 2018). Using a recently validated optogenetic approach in
301 *Pitx2*-Cre mice in which STN neurons can be selectively excited and thus activated, our
302 findings now identify aversive responses upon STN activation, possibly by acting as an
303 upstream component of the LHb. Indeed, STN excitation caused an evoked excitation of LHb
304 neurons, demonstrating a common circuitry, but also found the association indirect. With the
305 observed STN-mediated excitation of VP neurons followed through with behavioral aversion,
306 the VP is here suggested as a brain area connecting the STN and LHb. However, given the
307 multitude of structures that communicate with the STN, additional experiments will be
308 required to fully pinpoint the STN circuitry of aversion. *Pitx2* gene expression is characteristic
309 of the STN, with expression starting early in STN development and persisting throughout life
310 (Dumas and Wallén-Mackenzie, 2019; Martin et al., 2004; Schweizer et al., 2016; Skidmore
311 et al., 2008). In previous work, we could verify the selectivity of the *Pitx2*-Cre transgene in

312 driving floxed opsin constructs to the STN, with photostimulation-induced post-synaptic
313 currents observed in pallidal (globus pallidus) and nigral (SNr) target areas (Viereckel, 2018,
314 Schweizer 2014). Thus, in addition to the post-synaptic response of VP neurons identified
315 here, also other STN target areas might be involved in mediating the observed behavioral
316 avoidance. While further investigations will be needed to fully outline the neurocircuitry of
317 STN-mediated aversion, the current findings clearly show that STN excitation caused both
318 immediate place avoidance and cue-induced avoidance behavior, thus directly linking STN
319 activation to aversion-processing. In this context, the STN, which in turn receives inputs from
320 cortical regions supporting hedonic processing (Degos et al., 2008; Frankle et al., 2006;
321 Haynes and Haber, 2013; Isaacs et al., 2018), might play a modulatory role in decision-
322 making in response to aversive conditions.

323 In a recent study, we could experimentally confirm the long-assumed role of the STN in
324 locomotion by demonstrating directly opposite motor effects by optogenetic excitation and
325 inhibition of the STN (Guillaumin et al., 2020). We found that STN excitation was generally
326 correlated with significant reduction in locomotor activity, while STN inhibition enhanced
327 locomotion, just as classical models of the regulatory role of the STN in basal ganglia motor
328 loops propose. However, we also found that these correlations were not true for all types of
329 behavior. Upon STN-photostimulation in the open field test, jumping and self-grooming
330 behaviors were induced, not reduced. Curiously, this was only observed in a non-challenging
331 environment, not when mice were engaged in complex motor tasks. While jumping and
332 grooming are distinctly measurable motor output, they might represent an emotional
333 manifestation (Smolinsky et al., 2009; Wahl et al., 2008). In fact, the mice attempted to jump
334 out of the experimental test arena as if to escape the stimulation. In a follow-up pilot study,
335 we observed the mice in their natural home-cage environment. STN-photostimulation was
336 turned on by remote control, and only in short periods. Immediately upon STN-stimulation,
337 *Pitx2/ChR2-eYFP* mice responded with self-grooming and attempts to escape the home-
338 cage. Self-grooming was mostly observed when the lid was on, preventing escape, while in
339 an open cage, most of the mice escaped by jumping over the walls (unpublished
340 observations).

341 These behavioral manifestations suggested to us that the mice experience STN activation as
342 strongly unpleasant. Identifying a causal role for the STN in neurocircuitry of aversion could
343 help the understanding of aversive conditions, including some of the adverse side-effects
344 observed upon STN-DBS. In the present study, we decided to fully explore these striking
345 observations. By implementing the RT-PP paradigm along with two different setups of the
346 EPM, the current results now allow both the pin-pointing of place aversion upon STN
347 activation, and also the segregation between aversion and anxiety. Further, in these

348 experimental arenas, mice move freely between compartments in which they receive, or do
349 not receive, optogenetic stimulation. As the mouse chooses where it will position itself
350 throughout the test session, there is no driving force for escape. However, our current
351 analyses clearly show that when allowed this choice, mice actively avoid areas in which they
352 receive stimulation of either the STN itself or STN-terminals within the VP. While mice do not
353 show measurable anxiety, significant place avoidance was evident in both the RT-PP and the
354 EPM-avoidance tests, and this avoidance behavior was strong enough to compete out the
355 natural aversion for an open unsheltered space.

356 STN activation has not been experimentally associated with aversion before. Instead, a main
357 brain area for aversion is the LHb. During the past years, several different glutamatergic
358 afferents to the LHb have proven responsible for mediating aversive behavior. For instance,
359 optogenetic stimulation of glutamatergic neurons of the EP (Shabel et al., 2012; Stephenson-
360 Jones et al., 2016), VP (Faget et al., 2018; Tooley et al., 2018) and lateral hypothalamus
361 (Lazaridis et al., 2019; Lecca et al., 2017) projecting to the LHb all induce place avoidance.
362 Now, our findings identifying optogenetically induced aversion in the RT-PP and EPM
363 avoidance tests upon stimulation of either the STN itself or its terminals in the VP, place the
364 STN as a distinct brain nucleus responsible for the control of aversion by the recruitment of
365 VP neurons. Using *in vivo* extracellular recordings, we confirmed the activational response of
366 the STN neurons themselves upon photostimulation, and also demonstrated that stimulation
367 of STN glutamatergic neurons drive neuronal activity in the LHb, while patch-clamp results
368 identify excitatory post-synaptic response in the VP. Together, these electrophysiological and
369 behavioral findings provide strong support for an STN-VP-LHb pathway for aversion. Our
370 findings are in accordance with recent observations identifying that activation of
371 glutamatergic projection from several regions to the LHb triggers place avoidance as well as
372 activation of LHb activity. Further, our results allow the identification of one more component
373 to the aversion circuitry, and establish the STN as putative upstream regulator of the VP-LHb
374 pathway.

375 Curiously, the VP is mostly a GABAergic structure, but recent studies demonstrated that it
376 also contains glutamatergic neurons projecting to the LHb (Faget et al., 2018; Tooley et al.,
377 2018). The glutamatergic population of the VP was recently shown to receive projections
378 from the STN, among several areas, and selective stimulation of glutamatergic VP neurons
379 was demonstrated to evoke EPSCs in the LHb and induce place avoidance (Tooley et al.,
380 2018). The glutamatergic population of the VP receives input from different cortical and
381 midbrain regions (Knowland et al., 2017; Tooley et al., 2018), and it remains to establish
382 which of these upstream structures, if any, is responsible for their activation in avoidance
383 behavior. However, our present results pin-point the STN as one distinct subcortical regulator

384 of VP activity. Further, the findings identify that the multisynaptic STN-VP-LHb circuit is
385 composed mainly of glutamatergic connections, which provides new insight into the role of
386 excitatory pathways in aversive behavior.

387 The clinical importance of the STN highlights the need for decoding the role of this small
388 nucleus in behavioral regulation. STN-DBS treatment for motor dysfunction in advanced
389 stage Parkinson's disease is a long established and highly successful clinical intervention
390 (Benabid et al., 2009). More recently, STN-DBS has also been approved as treatment for
391 highly refractory and treatment-resistant OCD, another disorder in which the STN shows
392 aberrant activity (Klavir et al., 2009; Mallet et al., 2008; Winter et al., 2008). However,
393 considering that numerous adverse side-effects of STN-DBS have been reported, including
394 low mood state, depression, personality changes and even suicide (Péron et al., 2013;
395 Temel et al., 2006), manipulating the STN might come with a certain risk. While this issue
396 has been challenging to resolve, any direct causality between STN excitation and behavioral
397 aversion in mice is clearly crucial to take into consideration as putatively important not only
398 experimentally, but also clinically.

399 The present results underscore the importance of decoding the full repertoire of behavioral
400 regulation executed by the STN not least to improve treatment prediction and outcome in any
401 intervention aiming to manipulate the STN and its pathways. Beyond Parkinson and OCD,
402 STN-DBS has recently gained attention as treatment for additional neuropsychiatric
403 disorders, including addiction (Pelloux and Baunez, 2013). This interest is strongly based on
404 findings that STN-DBS reduces symptoms of the Dopamine dysregulation syndrome in
405 Parkinson's disease (Bandini et al., 2007; Broen et al., 2011; Knobel et al., 2008; Smeding et
406 al., 2006). Also studies in rodents showing that experimental STN-DBS induces different
407 motivational responses depending on the nature of the reinforcers (Baunez et al., 2002;
408 Darbaky et al., 2005; Rouaud et al., 2010), and can reduce addictive behaviors (Lardeux and
409 Baunez, 2008; Rouaud et al., 2010; Wade et al., 2017), have high-lighted the STN in
410 addiction. However, it has also been shown that STN lesioning in rats led to altered
411 emotional state in response to various rewarding and aversive stimuli (Pelloux et al., 2014).
412 Taken together, both experimental and clinical data high-light the STN structure as important
413 in affective processing.

414 Here, we describe the identification of the STN as a non-canonical source of negative
415 emotional value. Well embedded within the brain circuitry of negative reinforcing properties,
416 the results point towards a pivotal role of the clinically relevant STN in aversive learning.
417 Evidently, aberrant activity of an STN-VP-LHb circuitry, as well as its manipulation, could
418 result in both beneficial and detrimental effects. Further studies will be needed to pinpoint the

419 nature of all pathways emerging from the STN that have an impact on rewarding vs aversive
420 responses. In a recent transcriptomics-histological effort, we identified gene expression
421 patterns that define distinct spatio-molecular domains within the STN structure (Wallén-
422 Mackenzie et al., 2020). Based on the current finding that STN encodes aversive behavior,
423 future experimental approaches should make an effort to correlate specific domains within
424 the STN with behavioral output. Such anatomical-functional mapping within the STN
425 structure would be important not least to aid towards improving spatial selectivity in STN-
426 based interventions.

427

428 **METHODS SECTION**

429 **Animal housing and ethical permits**

430 Pitx2-Cre_C57BL/6NTac transgenic mice were bred in-house and housed at the animal
431 facility of Uppsala University before and during behavioral experiments or at University of
432 Bordeaux after virus injections for *in vivo* electrophysiological experiments. Mice had access
433 to food and water *ad libitum* in standard humidity and temperature conditions and with a 12
434 hour dark/light cycle. PCR analyses were run to confirm the genotype of Pitx2-Cre positive
435 mice from ear biopsies. All animal experimental procedures followed Swedish (Animal
436 Welfare Act SFS 1998:56) and European Union Legislation (Convention ETS 123 and
437 Directive 2010/63/EU) and were approved by local Uppsala or Bordeaux (N°50120205-A)
438 Ethical Committee.

439 **Stereotaxic virus injection and optic cannula implantation**

440 *Virus injection:* Stereotaxic injections were performed on anesthetized Pitx2-Cre mice
441 maintained at 1.4-1.8 % (0.5-2 L/min, isoflurane-air mix v/v). Before starting the surgery, and
442 24h post-surgery, mice received subcutaneous injection of Carprofen (5 mg/Kg, Norocarp). A
443 topical analgesic, Marcain (1.5 mg/kg; AstraZeneca), was locally injected on the site of the
444 incision. After exposing the skull, holes were drilled in the skull for virus injections and skull
445 screws implantations for behavioral experiments. Mice were injected in the STN bilaterally
446 with a virus containing either a Cre-dependent Channelrhodopsin (rAAV2/EF1a-DIO-
447 hChR2(H134R)-eYFP) named Pitx2/ChR2-eYFP, or an empty virus carrying only a
448 fluorescent protein (rAAV2/EF1a-DIO-eYFP) named Pitx2/eYFP, respectively 3.8×10^{12} virus
449 molecules/mL, 2.7×10^{12} virus molecules/mL; 4.6×10^{12} virus molecules/mL (viruses purchased
450 from UNC Vector Core, Chapel Hill, NC, USA) at the following mouse brain coordinates (from
451 Paxinos and Franklin, 2013): anteroposterior (AP) = -1.90 mm, mediolateral (ML) = +/- 1.70
452 mm from the midline and 250 nL of virus was injected with a NanoFil syringe (World
453 Precision Instruments, Sarasota, FL, USA) at two dorsoventral levels (DV) = -4.65 mm and -
454 4.25 mm from the dura matter at 100 nL.min⁻¹.

455 *Optic cannula implantation:* Optic cannulas (Doric Lenses) were implanted directly after
456 completion of virus injections. Two skull screws were implanted in the skull to hold the optic
457 cannula-cement-skull complex. Primers were then applied and harden with UV light
458 (Optibond). Optic cannulas were implanted bilaterally above the STN (coordinates: AP = -
459 1.90 mm, ML = +/-1.70 mm from the midline DV = -4.30 mm) or the VP (coordinates: AP =
460 +0.45 mm, ML = +/-1.55 mm from the midline, DV = -4.00 mm), and fixed with dental cement.
461 1 mL of saline was injected subcutaneously at the end of the surgery.

462 **Single-cell extracellular recordings**

463 *Surgery:* *In vivo* single cell extracellular recordings started at least 4 weeks after virus
464 injections. Mice were anesthetized with a mix isoflurane-air (0.8-1.8 % v/v) and placed in a
465 stereotaxic apparatus. Optic fiber, optrode and glass micropipettes coordinates were AP = -
466 1.90 mm, ML = +/- 1.70 mm and DV = -4.30 mm for the STN and AP = -1.60 mm, ML = +/-
467 0.50 mm, DV = -2.00 to -3.30 mm for the LHb.

468 *STN Optotagging:* A custom-made optrode was made with an optic fiber (100 μ m diameter,
469 Thorlabs) connected to a laser (MBL-III-473 nm-100 mW laser, CNI Lasers, Changchun,
470 China) mounted and glued on the recording glass micropipette which was filled with 2%
471 pontamine sky blue in 0.5 μ M sodium acetate (tip diameter 1-2 μ m, resistance 10-15 M Ω).
472 The distance between the end of the optic fiber and the tip of the recording pipette varied
473 between 650 nm and 950 nm. Extracellular action potentials were recorded and amplified
474 with an Axoclamp-2B and filtered (300 Hz/0.5 kHz). Single extracellular spikes were collected
475 online (CED 1401, SPIKE2; Cambridge Electronic Design). The laser power was measured
476 before starting each experiment using a power meter (Thorlabs). The baseline was recorded
477 for 100 seconds for each neuron before starting any light stimulation protocols which were
478 set and triggered with Spike2 software. Light protocol consisted in a peristimulus time
479 histogram (PSTH, 0.5 Hz, 5 ms pulse duration, 5-8 mW) for at least 100 seconds followed,
480 after returned to baseline, by a "behavioral" protocol corresponding to the parameters used
481 for behavioral experiments (20 Hz, 5 ms pulse duration, 5-8 mW).

482 *LHb recordings:* An optic fiber and a recording pipette filled with either 2% pontamine sky
483 blue or 2% neurobiotin were respectively positioned in the STN and LHb. For each LHb
484 neurons, a PSTH was recorded upon STN optogenetic stimulation (PSTH 0.5 Hz, 5 ms
485 pulses, 5-8 mW) for at least 100 seconds. Neurons are considered as excited during the
486 PSTH protocol when, following the light pulses centered on 0, the number of spikes/5ms bin
487 is higher than the baseline (-500 ms to 0 ms) plus two times the standard deviation. Injection
488 of neurobiotin (2% neurobiotin in 0.5 M acetate sodium) by juxtacellular iontophoresis was
489 performed in some cases for precise spatial identification of excited neurons.

490 **Ex vivo electrophysiology**

491 STN and VP neurons were recorded in acute brain slices, prepared as previously described
492 (Dupuis et al., 2013; Froux et al., 2018). Briefly, Pitx2/eYFP and Pitx2/ChR2-eYFP mice (>
493 12 months) were deeply anesthetized with an i.p. injection of ketamine/xylazine (75/10
494 mg/kg) mixture and then perfused transcardially with cold (0-4°C) oxygenated (95% O₂ - 5%
495 CO₂) artificial cerebrospinal fluid (ACSF), containing the following (in mM): 230 sucrose, 26
496 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 0.5 CaCl₂, 10 MgSO₄ and 10 glucose (pH~7,35). The brain
497 was quickly removed, glued to the stage of a vibratome (VT1200S; Leica Microsystems,
498 Germany), immersed in the ice-cold ACSF and sectioned into 300 µm thick parasagittal
499 slices. Slices were transferred for 1 hour to a standard oxygenated ACSF solution, warmed
500 (~35°C) containing (in mM unless otherwise stated): 126 NaCl, 26 NaHCO₃, 2.5 KCl, 1.25
501 NaH₂PO₄, 2 CaCl₂, 2 MgSO₄, 10 glucose, 1 sodium pyruvate and 4.9 µM L-gluthathione
502 reduced. Single slices were then transferred to a recording chamber, perfused continuously
503 with oxygenated ACSF (without sodium pyruvate and L-gluthathione) heated at 32-34°C, and
504 visualized using infrared gradient contrast video microscopy (Ni-e workstation, Nikon) and a
505 60X water-immersion objective (Fluor APO 60X/1.00 W, Nikon).

506 Recordings from individual STN and VP neurons were performed with patch electrodes
507 (impedance, 3–8 MΩ) obtained from borosilicate glass capillaries (GC150F10; Warner
508 Instruments, Hamden, CT, USA) pulled with a horizontal puller (P-97; Sutter Instruments,
509 Novato, CA, USA). For cell-attached and whole-cell voltage-clamp recordings, pipettes were
510 filled with K-Gluconate-based internal solution containing (in mM): 135 K-gluconate, 3.8
511 NaCl, 1 MgCl₂.6H₂O, 10 HEPES, 0.1 EGTA, 0.4 Na₂GTP, 2 Mg_{1.5}ATP, 5 QX-314 and 5.4
512 biocytin (pH=7.2, ~292 mOsm). Recordings were obtained using a Multiclamp 700B amplifier
513 and Digidata 1440A digitizer controlled by Clampex 10.2 (Molecular Devices LLC). Signals
514 were sampled at 20 kHz and low-pass filtered at 4 kHz. Series resistance was monitored
515 throughout the experiment by voltage steps of -5 mV. Data were discarded when the series
516 resistance changed by >20%. Biocytin-filled neurons were identified. For *ex vivo* optogenetic
517 stimulation, a LED laser source (Prizmatix, Israel) connected to optic fiber (Ø: 500 µm) was
518 placed above the brain slice. Light intensities ranged from 4mW to 90mW at the tip of the
519 optic fiber depending of the type experiments. For cell body stimulation, continuous 100ms
520 long duration light stimulation was applied at low (4mW) and high (90 mW) intensities. To
521 evoke synaptic transmission, single pulses or train of stimulation (800 pulses at 20Hz) of 1ms
522 duration at full power (90mW) were used in order to maximize axon terminal depolarization
523 and efficient release of neurotransmitter. After electrophysiological recordings, slices were
524 fixed overnight in 4% paraformaldehyde, and maintained in PBS-azide at 0.2% at 4°C until
525 immunohistochemical processing.

526 **Histological analysis**

527 Following behavioral analyses, mice were deeply anesthetized and perfused transcardially
528 with phosphate-buffer-saline (PBS) followed by ice-cold 4% formaldehyde. Brains were
529 extracted and 60 μm sections were cut with a vibratome.

530 Fluorescent immunohistochemistry was performed to enhance the eYFP signal. All mice
531 were analysed. After rinsing in PBS, sections were incubated for 90 min in PBS 0.3% X-100
532 Triton containing 10% blocking solution (normal donkey serum) followed by an incubation
533 with primary antibody (chicken anti-GFP 1:1000, cat. no. ab13970, Abcam), diluted in 1%
534 normal donkey serum in PBS, overnight at 4°C. Next day, sections were rinsed in PBS plus
535 0.1% Tween-20 solution and incubated for 90 min with secondary antibody diluted in PBS
536 (A488 donkey anti-chicken 1:1000, cat. no. 703-545-155, Jackson Immunoresearch). After
537 rinsing in PBS containing 0.1% Tween-20, sections were incubated for 30 min with DAPI
538 diluted in distilled water (1:5000). Sections were mounted with a Fluoromount aqueous
539 mounting medium (Sigma, USA) and cover-slipped. Sections were scanned with
540 NanoZoomer 2-0-HT.0 (Hamamatsu) scanner and visualized with NDPView2 software
541 (Hamamatsu).

542 For *in vivo* electrophysiological experiments, a deposit was made at the last recording
543 coordinates by pontamine sky blue iontophoresis ($-20\ \mu\text{A}$, 25 min). Brains were then
544 collected after 0.9% NaCl and 4% PFA transcardial perfusion. 60 μm brain sections were cut
545 with a vibratome at the levels of the STN and the LHb, mounted with Vectashield medium
546 (Vector Laboratories), coverslipped and visualized with an epifluorescent microscope to
547 confirm eYFP expression, recording pipette and optic fiber positions. Neurons injected with
548 neurobiotin were revealed by first incubating the brain slices in a solution of
549 saturation/permeabilization (1X PBS-Triton X-100 0.3% and 1% BSA) for one hour at room
550 temperature followed by an incubation overnight at room temperature in a solution containing
551 1X PBS-Triton X-100 0.3% (v/v; Sigma-Aldrich) and Streptavidine-557 (1:500; R&D System
552 #NL 999).

553 To reveal biocytin-filled neurons after *ex vivo* recordings, brain slices were incubated
554 overnight in a solution of PBS 0.01 mM/Triton™ X100 0.3% (v/v; Sigma-Aldrich) containing
555 streptavidine-557 (1/1000; Cat# NL999; R&D system). Fluorescent immunohistochemistry was
556 used to enhance eYFP signal as described above but with a mouse anti-GFP (1/600;
557 Cat#11814460001, Roche or Cat#A11122; Life technologies) primary antibody and an Alexa-
558 Fluor Donkey anti-Mouse 488 (1/500; Cat#A21202, Life technologies) as secondary
559 antibody. The slices were then washed in PBS 0.01 mM before mounting onto slides in
560 Vectashield medium (CAT#H-1000, Vector laboratories). Images were acquired with a

561 fluorescence microscope (Axio Imager 2, Zeiss) or a confocal microscope (Leica, SP8) and
562 were analysed with Fiji.

563 **Behavioral testing**

564 After approximately four weeks of recovering from surgery, mice were tested in validated
565 behavioral tests by an experimenter blind to experimental groups, during which the same
566 stimulation protocol was used for Pitx2/ChR2-eYFP, Pitx2/ChR2-eYFP/VP and respective
567 controls: 473 nm light, 5 mW, 20 Hz, 5 ms pulse delivered by a MBL-III-473 nm-100 mW
568 laser (CNI Lasers, Changchun, China). Duration and condition of stimulation are specified for
569 each test. After completed behavioral tests, mice were sacrificed and brains analysed
570 histologically and for assessment of optic cannula position. Mice in which the optic cannulas
571 were not in correct position were excluded from the analysis. For each test, results were
572 obtained from groups of mice that followed the same battery of experiments. The initial
573 sample size was determined based by previous published studies with similar experimental
574 design.

575 **Habituation**

576 Three weeks after surgery and before the first behavioral test, all mice were handled and
577 habituated to the experimental room and to the optic cables to reduce the stress during the
578 day of the experiment. Before each behavioral test, mice were acclimatized for 30 minutes in
579 the experimental room.

580 **Real-time place preference (RT-PP)**

581 A three-compartment apparatus (Spatial Place Preference Box, Panlab, Harvard Apparatus)
582 was used for the RT-PP test. The apparatus is composed of two compartments with different
583 visual and tactile cues for both floors and walls and a connecting corridor (neutral
584 compartment) with transparent walls and floor. The study was carried out throughout 8
585 consecutive days preceded by one "*Habituation*" day. On each day of the experiment,
586 subjects were placed in a neutral cage for at least 3 minutes to recover after connecting the
587 optic cables. Mice were subsequently moved into the transparent corridor of the apparatus
588 where, after 30-50 s, the doors were removed and animals allowed to freely explore the
589 apparatus. The position of the mouse was detected by a camera positioned above the
590 apparatus. On "*Habituation*", "*Pre Test*" and "*Conditioning Place Test*" days (15 min), mice
591 were connected to the optic fiber but no light was paired to any chamber. On the "*Real Time*
592 *Conditioning*" days (30 min), one chamber was randomly chosen as a light-paired chamber
593 (counterbalanced across animals) while the other one was subsequently assigned as light-
594 paired chamber on the "*Reverse Real Time Conditioning*" days (30 min). Every time the
595 subject entered the light-paired chamber, the laser was activated according to the stimulation

596 protocol. Cumulative duration of the time spent in each compartment was recorded by using
597 the Ethovision XT13.0/14.0 tracking software (Noldus Information Technology, The
598 Netherlands). Mice that showed strong initial preference during the “Pre Test” for either one
599 of the two compartments (<25% or >75% of time spent) were excluded from the statistical
600 analysis.

601 **Elevated plus maze (EPM)**

602 EPM analysis was carried out in an apparatus with the shape of a plus, made of two open
603 arms (35 cm length) and two closed arms (35 cm length) with walls (15 cm high) and crossed
604 in the middle to create a center platform. The EPM apparatus adjusted for mice is elevated
605 50 cm from the floor. Mice were placed in a neutral cage for 5 minutes to recover from optic
606 cable connection. Mice were next placed individually in the center of the maze facing one of
607 the open arms and allowed to freely explore the apparatus for 10 minutes with light delivered
608 for the total duration of the test according to the stimulation protocol. The results of the test
609 were recorded with a camera placed above the EMP arena. Time spent in arms and number
610 of entries in arms were manually scored by experimenter blind to experimental groups
611 through subsequent video analysis.

612 **Modified version of the EPM (EPM avoidance)**

613 The re-designed version of the EPM test aims to assess the aversive effect induced by the
614 photostimulation in comparison to the natural aversion experienced in the open arms of the
615 apparatus. In the EPM avoidance test, photostimulation was activated upon entry into any of
616 the closed arms, and disabled by leaving it. In contrast, visiting the open arms or occupying
617 the center zone had no effect on photostimulation. The animal was placed in the center zone
618 of the arena and allowed to freely explore the apparatus for 15 min. Time spent in each
619 compartment and number of entries were recorded by the Ethovision XT13.0/14.0 tracking
620 software (Noldus Information Technology, The Netherlands).

621 **Statistics**

622 Data are expressed on the plots as means \pm SEM. For behavioral analysis, repeated
623 measures two-way ANOVA were performed followed by Bonferroni's or Tukey's multiple
624 comparisons where appropriate. For single cell extracellular recordings, Friedman test was
625 performed followed by Dunn's multiple comparisons. For *ex vivo* electrophysiology, paired t-
626 test was used (GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla
627 California USA). See Supplementary file 1.

628

629

630 **Figure legends**

631 **Figure 1. Photostimulation in the STN in Pitx2-ChR2-eYFP mice excites STN neurons**

632 (A) Graphical representation of stereotaxic bilateral injection into the STN of an rAAV
633 carrying a Cre-dependent Channelrhodopsin 2 (ChR2) fused with eYFP (ChR2-eYFP), or
634 eYFP alone, into Pitx2-Cre mice (Pitx2/ChR2-eYFP mice and Pitx2/eYFP control mice,
635 respectively) followed by canula implantation above the STN. (B) Coronal sections showing
636 eYFP fluorescence (green) in STN neurons and its target areas projections in a
637 representative Pitx2/ChR2-eYFP mouse; from left to right: STN cell bodies, STN target areas
638 projections in the VP, GP, EP, SNr and SNc; nuclear marker (DAPI, blue); scale, 250 μ m. (C)
639 Procedure for STN optotagging experiments. (D) eYFP-positive neurons (green) in the STN;
640 pontamine blue sky deposit (red dot) at the last recorded coordinate; nuclear marker (DAPI,
641 blue) (scale, 200 μ m). (E) Averaged PSTH of STN-excited neurons upon light stimulation
642 (0.5 Hz, 5 ms pulse duration, 5-8 mW). (F) Example of an excited STN neuron during a
643 behavioral protocol (20 Hz, 5 ms pulse duration, 5-8 mW) with the frequency and the
644 recording trace. (G) Frequency of STN neurons before, during and after 10 s light stimulation
645 following the behavioral protocol (20 Hz, 5 ms pulse duration, 5-8 mW); data expressed as
646 mean \pm SEM, n=7, both *p=0.15. (H) Graphical representation of a para-sagittal slice
647 obtained from a Pitx2/ChR2-eYFP mouse with a patch-clamp electrode and an optic fiber
648 placed above the STN. (I) Confocal images depicting a STN neuron filled with biocytin (BC)
649 and expressing the ChR2-eYFP construct (visualized by eYFP). Scale bar: 10 μ m. (J)
650 Representative example of typical ChR2-mediated currents induced by a 1 s blue light (λ =
651 470 nm) pulses at intensities of 4mW and 90 mW, respectively. (K) ChR2-mediated currents
652 induced by a train of blue light of 40 s duration at 20 Hz (individual light pulses of 1 ms).
653 Abbreviations: STN, subthalamic nucleus; VP, ventral pallidum; GP, globus pallidus, EP;
654 entopeduncular nucleus; SNr, substantia nigra *pars reticulata*; SNc, substantia nigra *pars*
655 *compacta*.

656 **Figure 2. Optogenetic activation of STN neurons induces place aversion**

657 (A) Graphical representation of the real-time place preference (RT-PP) setup in which
658 Pitx2/ChR2-eYFP and control mice were tested. (B) Percentage of time that Pitx2/ChR2-
659 eYFP and control mice spent in each compartment expressed as mean \pm SEM. Dark blue filled
660 circles (compartment A) and dark blue filled squares (compartment B) indicate
661 photostimulation in that compartment. (Left panel) n=12 Pitx2/ChR2-eYFP, compartment A
662 vs. compartment B, ***p<0.001; (Right panel) n=10 Pitx2/eYFP controls. (C) Representative
663 occupancy heat-maps for Pitx2/ChR2-eYFP mice show less time spent in the
664 photostimulation-paired chamber than in the unpaired chamber. (D) Distance moved in each
665 compartment expressed as mean \pm SEM throughout the course of the experiment. Light blue

666 filled histograms indicate photostimulation in that compartment. (Left panel), *** $p < 0.001$.
667 (Right panel) $n = 10$ Pitx2/eYFP control mice. (E) Speed in each compartment expressed as
668 mean \pm SEM. (Left panel) $n = 12$ Pitx2/ChR2-eYFP mice, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
669 (Right panel) $n = 10$ Pitx2/eYFP control mice.

670 **Figure 3. Aversion induced by optogenetic STN activation stronger than inherent**
671 **aversion for unsheltered exposure**

672 (A) Schematic representation of the elevated plus maze (EPM) in which Pitx2/ChR2-eYFP
673 and control mice were tested; STN-photostimulation delivered for 10 minutes throughout the
674 entire test arena. (B) Percentage of time spent in arms expressed as mean \pm SEM, $n = 17$
675 Pitx2/ChR2/eYFP, open arms vs. closed arms *** $p < 0.001$; center vs. closed arms,
676 *** $p < 0.001$; $n = 11$ Pitx2/eYFP mice, open arms vs. closed arms, *** $p < 0.001$; center vs. closed
677 arms, *** $p < 0.001$. (C) Number of entries in arms expressed as mean \pm SEM, $n = 17$
678 Pitx2/ChR2/eYFP mice, open arms vs. closed arms * $p < 0.05$; $n = 11$ Pitx2/eYFP, open arms
679 vs. closed arms, ** $p < 0.01$. (D) Graphical representation of a modified version of the EPM
680 test and representative heat map showing occupancy. (E) Time spent in arms expressed as
681 mean \pm SEM, $n = 14$ Pitx2/ChR2/eYFP, open arms vs. closed arms, * $p < 0.05$; $n = 11$
682 Pitx2/eYFP, open arms vs. closed arms, *** $p < 0.001$; center vs. closed arms, *** $p < 0.001$;
683 Pitx2/ChR2/eYFP vs. Pitx2/eYFP, open arms * $p < 0.05$, closed arms *** $p < 0.001$, center
684 *** $p < 0.001$. (F) Number of entries in arms expressed as mean \pm SEM, Pitx2/ChR2/eYFP vs.
685 Pitx2/eYFP, open arms *** $p < 0.001$; Closed arms ** $p < 0.01$.

686 **Figure 4. STN-photoactivation in Pitx2-ChR2-eYFP mice induces excitatory post-**
687 **synaptic responses in neurons of the lateral habenula (LHb)**

688 (A) Procedure of LHb recordings experiments with STN-photostimulation in Pitx2/ChR2-
689 eYFP mice. (B) Close-up of the averaged PSTH of LHb-excited neurons upon STN
690 photostimulation and overlap of 10 traces of a LHb neuron centred on the 5 ms light pulse.
691 (C) Representative image of coronal section showing eYFP fluorescence (green) in STN cell
692 bodies of Pitx2/ChR2/eYFP mice with optic cannula track above the STN; nuclear marker
693 (DAPI, blue); scale, 250 μ m. (D) Reconstructed mapping of LHb recorded neurons ($N = 44$
694 neurons recorded in 3 mice). Dark blue dots indicate LHb neurons excited during the
695 stimulation protocol; light blue dots correspond to excited neurons outside of the LHb; grey
696 dots correspond to neurons which did not respond to the light. (E) Excited LHb neuron
697 injected with neurobiotin. No eYFP-positive fibers in the LHb.

698 **Figure 5. Selective optogenetic activation of projections from the STN to the ventral**
699 **pallidum (VP) results in firing of VP neurons**

700 (A) Graphical representation of a para-sagittal slice obtained from a Pitx2/ChR2-eYFP/VP
701 mouse with a patch-clamp electrode and optic fiber placed above the ventral pallidum (VP).
702 (B) Epifluorescence image depicting the globus pallidus (GP) and VP. VP neurons were
703 recorded below the anterior commissure (ac). (C) Confocal images showing a VP neuron in a
704 Pitx2/ChR2-eYFP/VP mouse filled with biocytin (BC); surrounded by STN axon terminals
705 expressing ChR2-eYFP (eYFP). Scale bar: 10 μ m. (D) Representative firing rate of a VP
706 neuron during ON/OFF periods of light stimulation (20Hz, 1ms, for 40 s) and corresponding
707 population data (n=5, *p<0.05, paired t-test). Note the strong increase of firing produced by
708 light stimulation of STN axon terminals. (E) Typical light-evoked excitatory synaptic current
709 (EPSC) which is abolished by AMPA/NMDA receptors antagonists (DNXQ, 20 μ m; D-APV,
710 50 μ m). (F) Population graph of light-evoked EPSCs (n=6). (F) Graph showing the
711 pharmacological blockade of light-evoked EPSC (n=2) by DNQX and D-APV.

712 **Figure 6. Selective photoactivation within the ventral pallidum (VP) of STN-VP**
713 **projections is sufficient to drive place aversion**

714 (A) Graphical representation of the real time place preference (RT-PP) experiment in which
715 Pitx2/ChR2-eYFP/VP and corresponding control mice were tested. (B) Percentage of time
716 spent in each compartment express as mean \pm SEM. Dark blue filled circles (compartment A)
717 and dark blue filled squares (compartment B) indicate photostimulation in that compartment.
718 (Left panel) n=4 Pitx2/ChR2/eYFP/VP, compartment A vs. compartment B, ***p<0.001;
719 (Right panel) n=3 Pitx2/eYFP/VP controls. (C) Representative occupancy heat-maps for
720 Pitx2/ChR2-eYFP mice less time spent in the photostimulation-paired chamber than in the
721 unpaired chamber. (D) Distance moved in each compartment expressed as mean \pm SEM
722 throughout the course of the experiment. Light blue filled histograms indicate
723 photostimulation in that compartment. (Left panel) n=4 Pitx2/ChR2/eYFP/VP mice,
724 compartment A vs. compartment B,***p<0.001. (Right panel) n=3 Pitx2/eYFP/VP control
725 mice. (E) Speed in each compartment expressed as mean \pm SEM. (Left panel) n=4
726 Pitx2/ChR2/eYFP/VP mice. (Right panel) n=3 Pitx2/eYFP/VP control mice.

727 **Figure 7. Optogenetic activation within the ventral pallidum (VP) of STN-VP terminals**
728 **reduces the natural preference for a sheltered environment**

729 (A) Schematic representation of elevated plus maze (EPM) with photostimulation delivered
730 for 10 minutes in the entire arena. (B) Percentage of time spent in arms expressed as mean
731 \pm SEM, n=4 Pitx2/ChR2/eYFP/VP mice, open arms vs. closed arms, ***p<0.001; center vs.
732 closed arms, ***p<0.001; n=3 Pitx2/eYFP/VP control mice, open arms vs. closed arms,
733 ***p<0.001; center vs. closed arms, ***p<0.001. (C) Number of entries in EPM arms
734 expressed as mean \pm SEM. (D) Graphical representation of a modified version of the EPM
735 test and representative occupancy heat map. (E) Time spent in EPM arms expressed as

736 mean \pm SEM, n=3 Pitx2/eYFP/VP, open arms vs. closed arms, **p<0.01; center vs. closed
737 arms, **p<0.01. (F) Number of entries in arms expressed as mean \pm SEM.

738

739 **Supplementary file 1**

740 Detailed description of the statistics used for the different experiments.

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748

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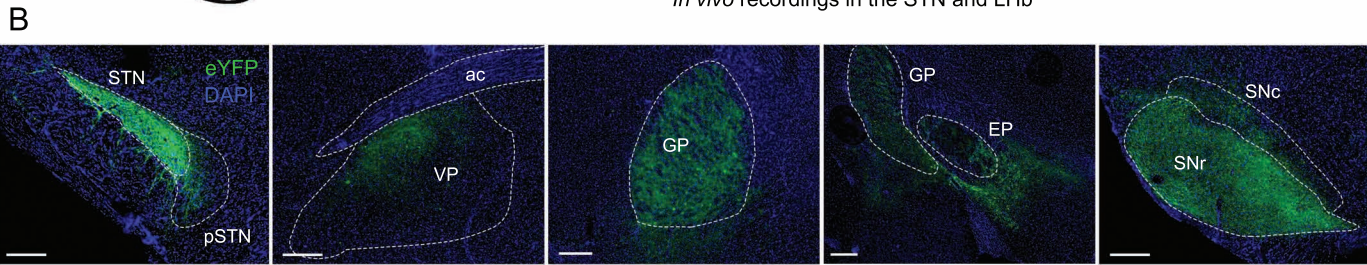
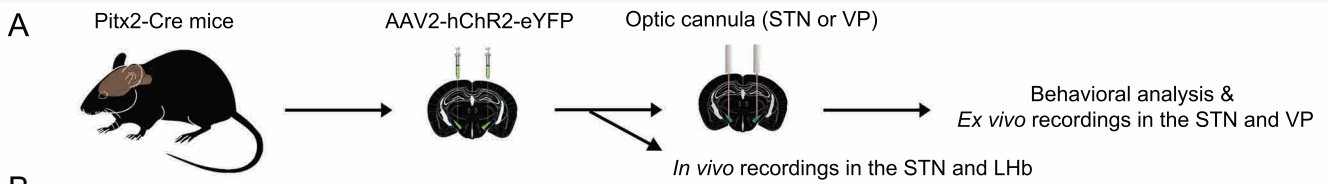
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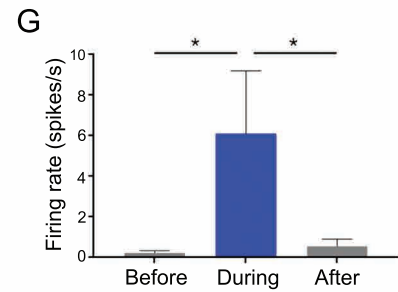
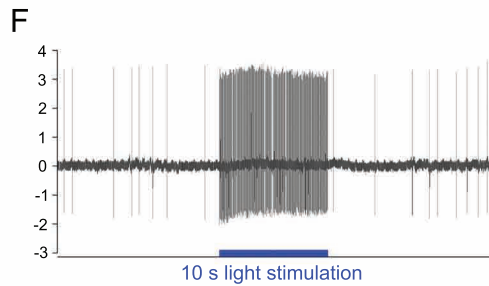
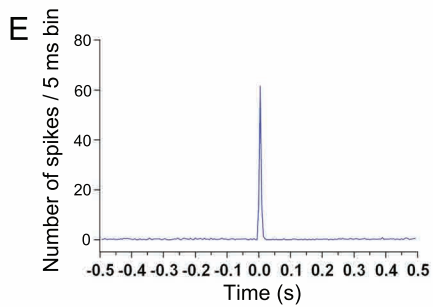
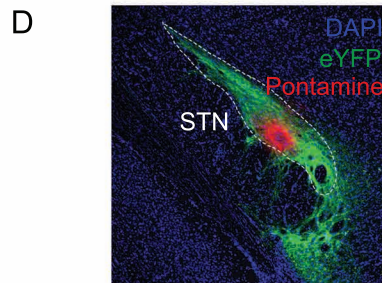
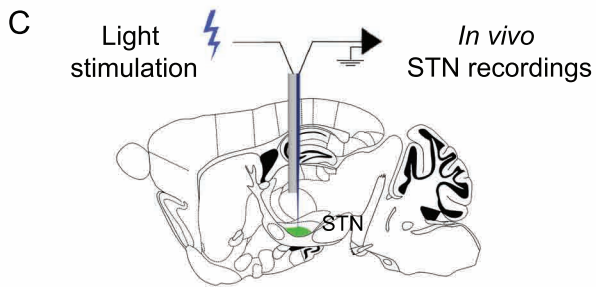
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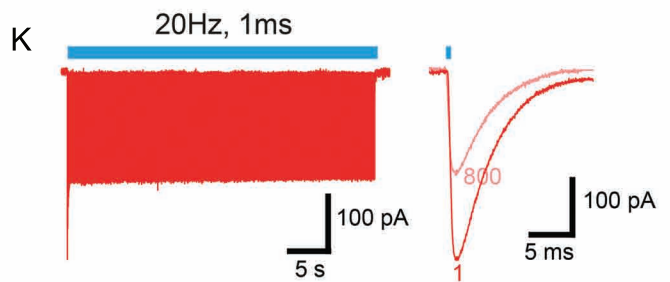
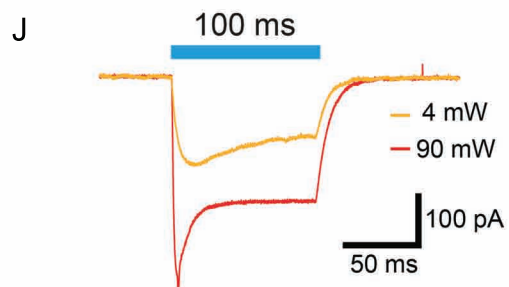
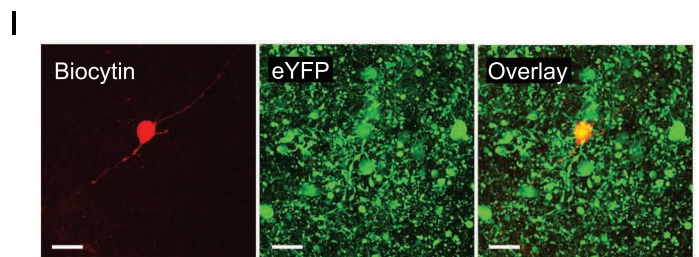
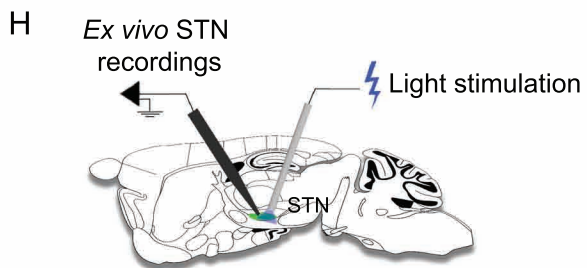
Experimental protocol and histological analysis



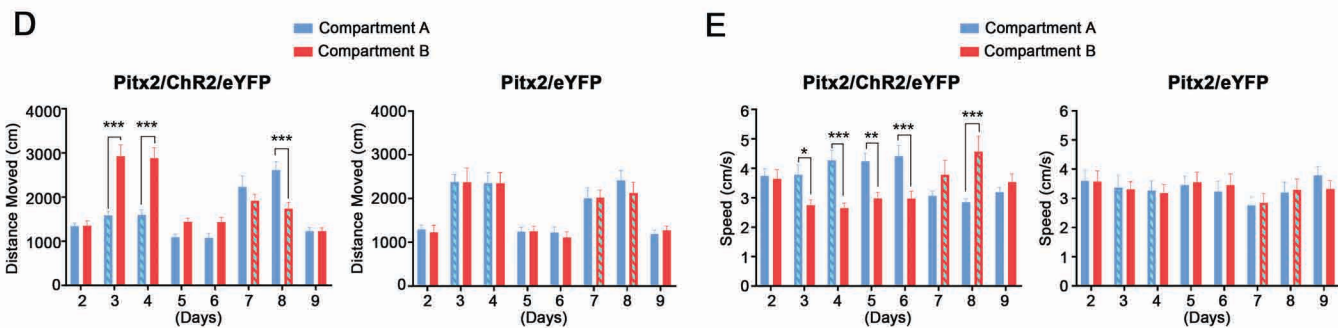
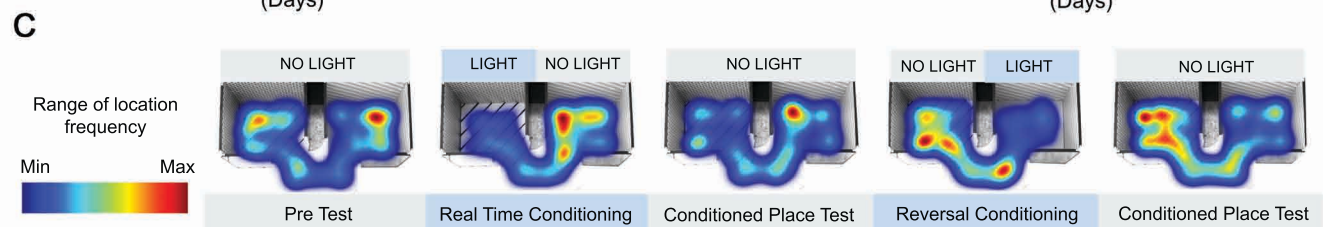
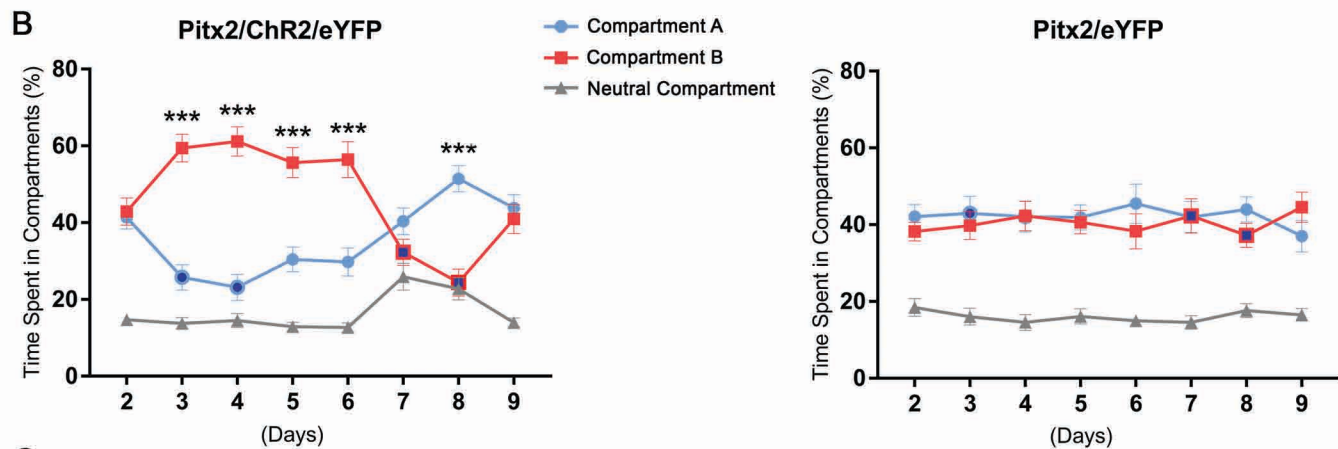
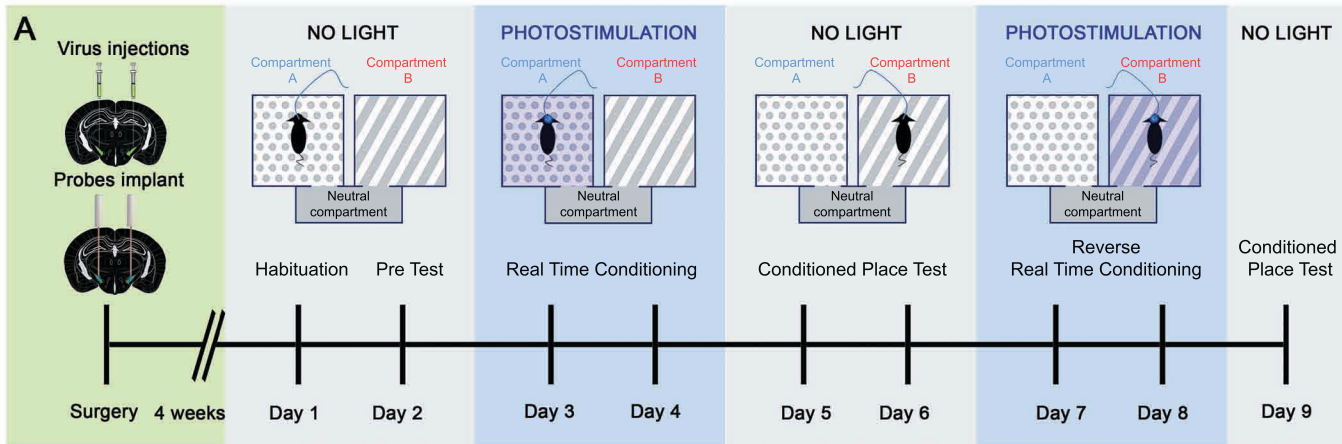
In vivo electrophysiological recordings in the STN



Ex vivo electrophysiological recordings in the STN

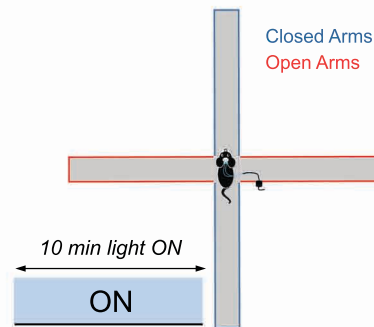


Real-Time Place Preference (RT-PP) : STN

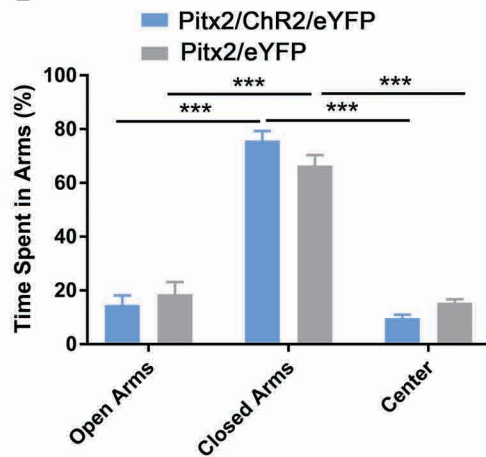


Elevated Plus Maze (EPM) : STN

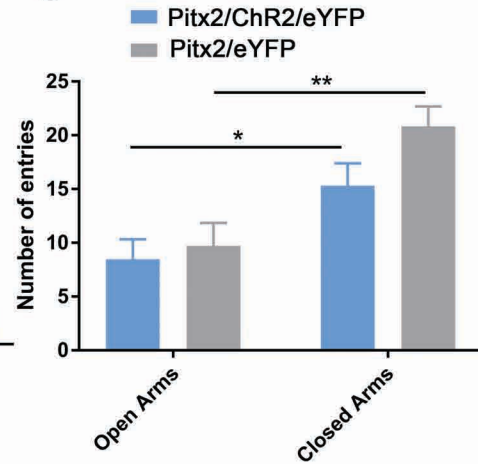
A Regular EPM



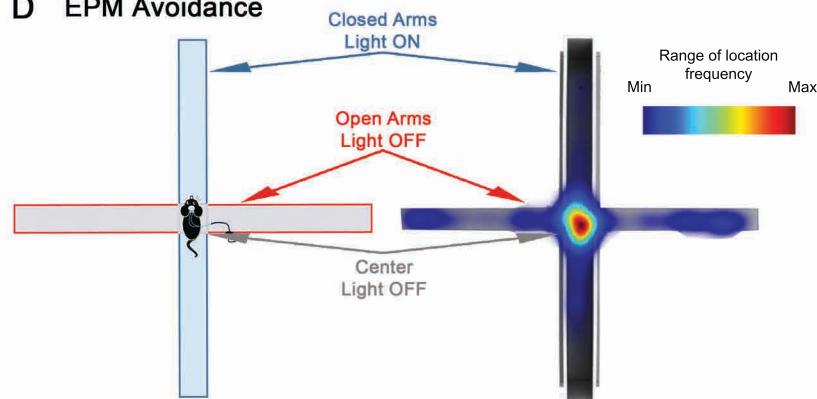
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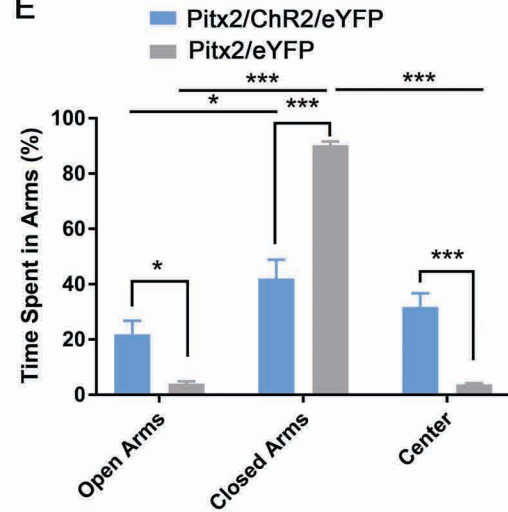
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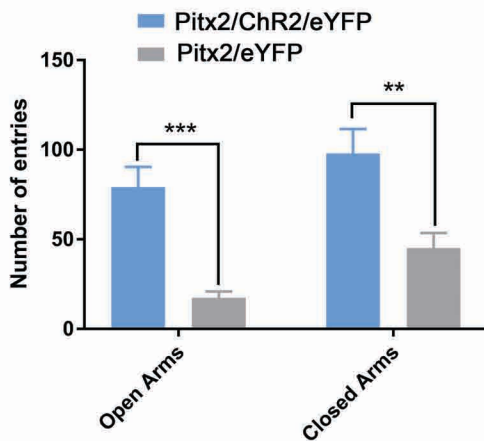
D EPM Avoidance

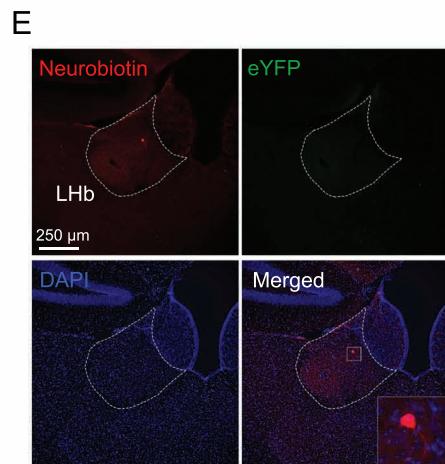
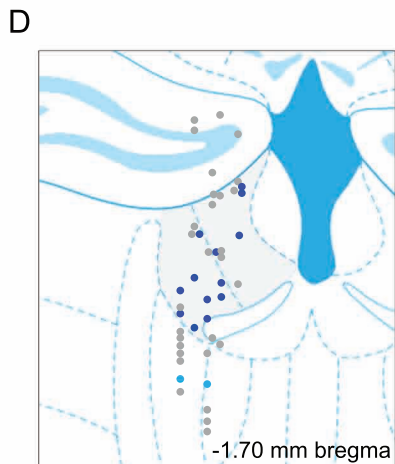
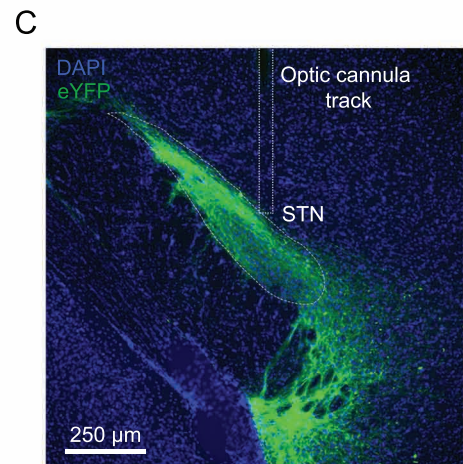
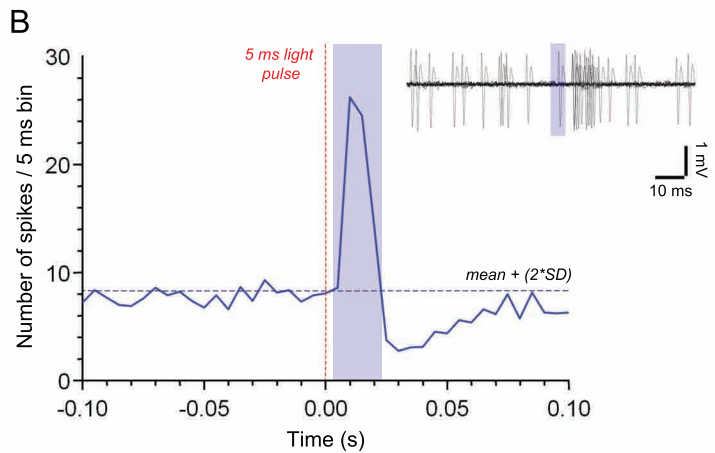
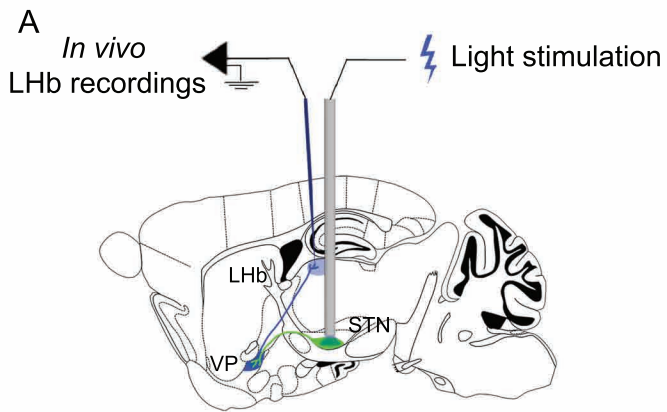


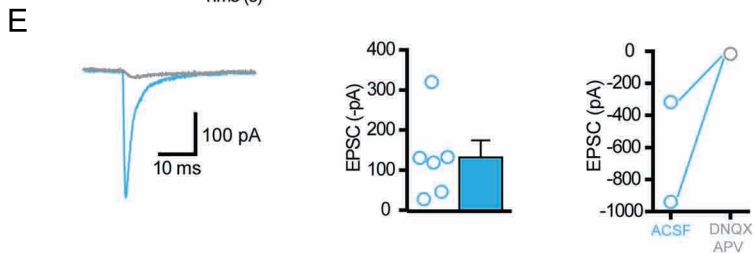
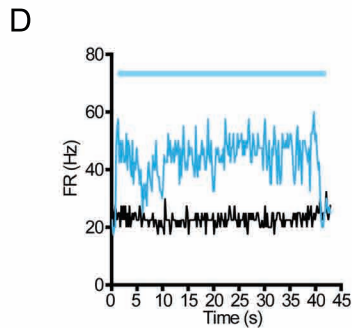
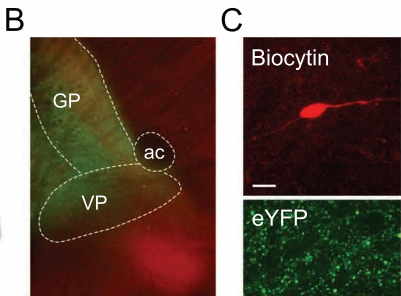
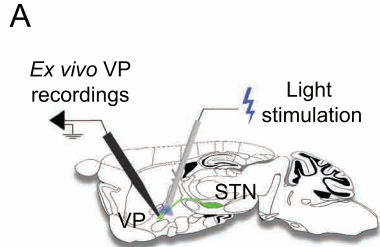
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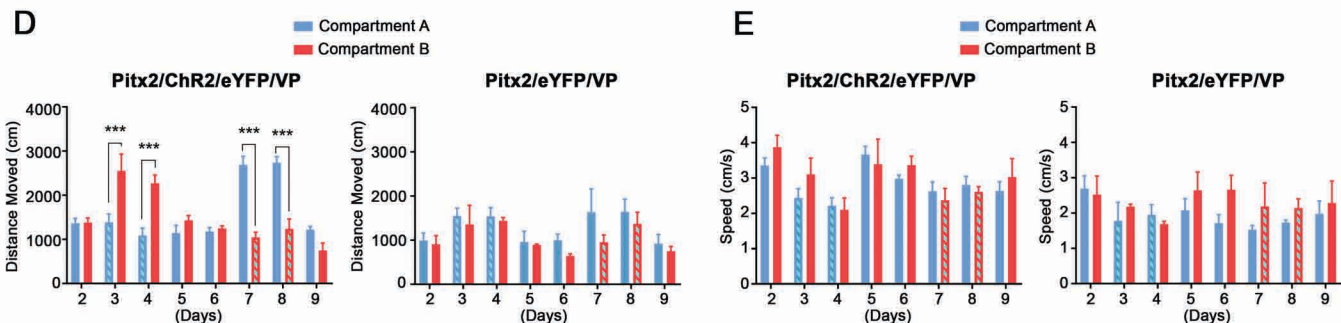
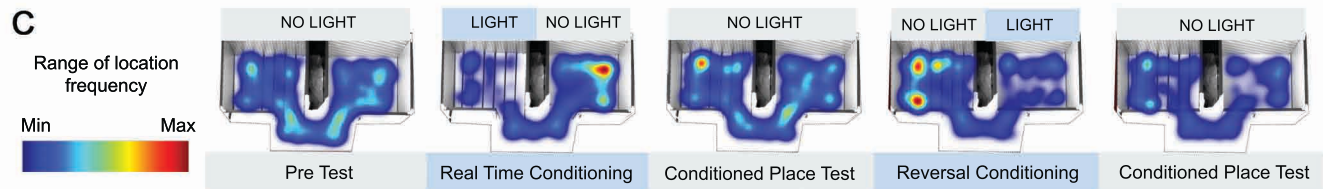
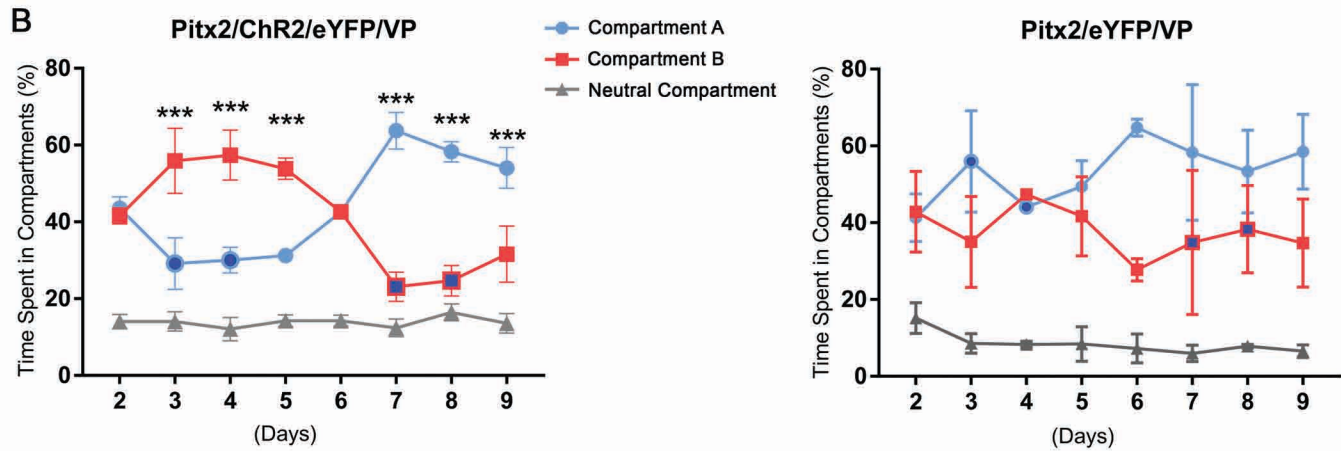
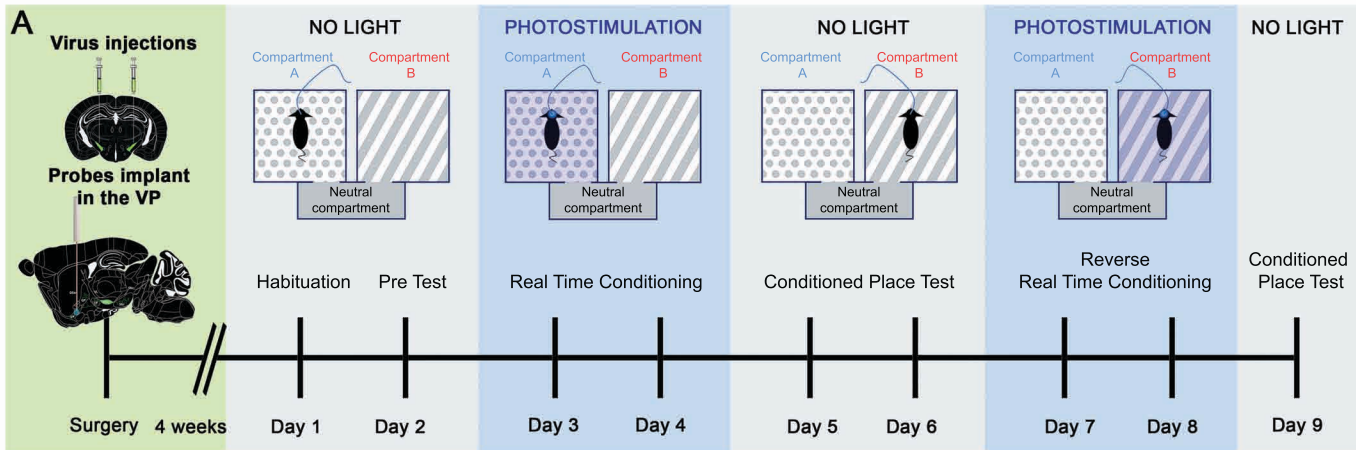
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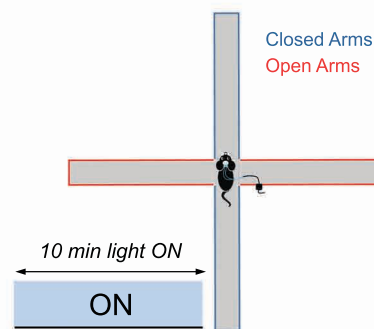


Real-Time Place Preference (RT-PP) : STN-VP

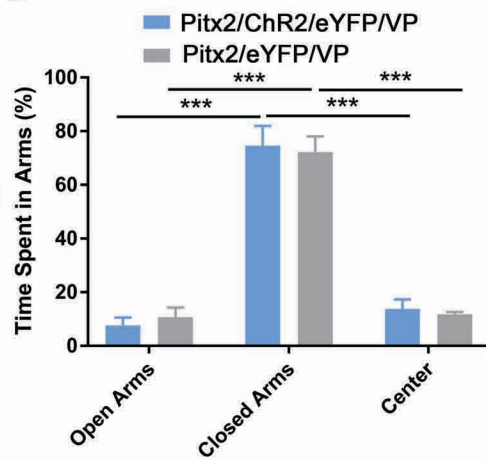


Elevated Plus Maze (EPM) : STN-VP

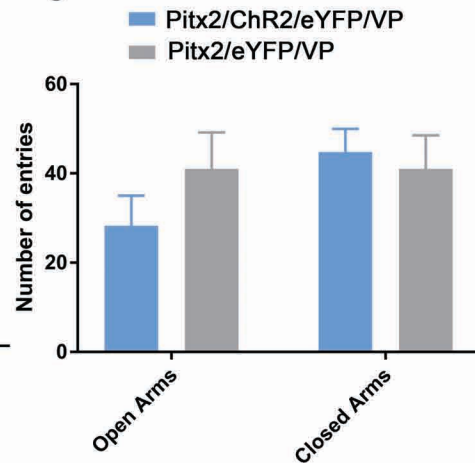
A Regular EPM



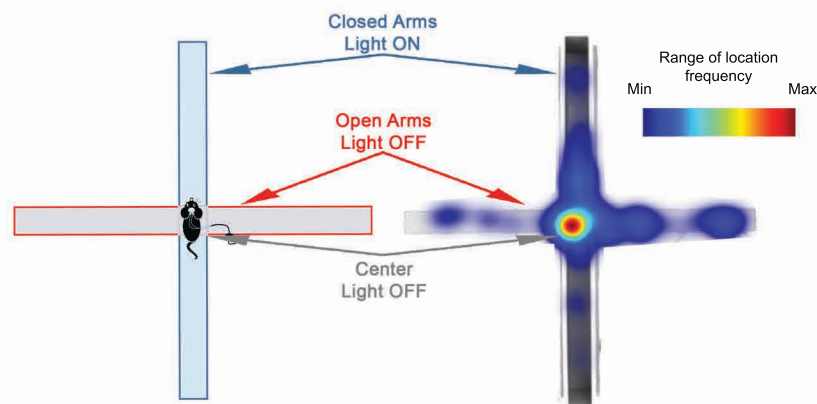
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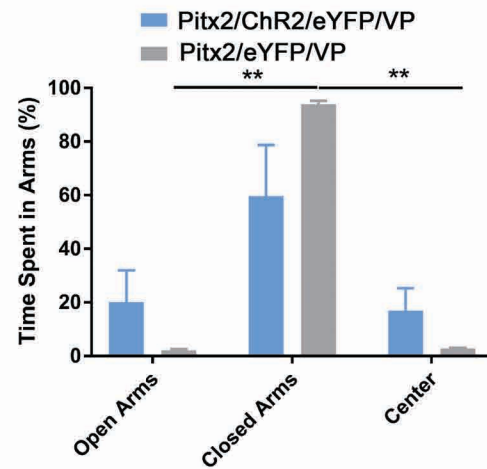
C



D EPM Avoidance



E



F

