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20

21 **ABSTRACT**

22 Accumulating evidence implicates the *parafascicular nucleus* of the thalamus (Pf) in basal ganglia
23 (BG)-related functions and pathologies. Despite Pf connectivity with all BG components, most
24 attention is focused on the thalamostriatal system and an integrated view of thalamic information
25 processing in this network is still lacking. Here, we addressed this question by recording the
26 responses elicited by Pf activation in single neurons of the *substantia nigra pars reticulata* (SNr), the
27 main BG output structure in rodents, in anaesthetized mice. We performed optogenetic activation of
28 Pf neurons innervating the striatum, the subthalamic nucleus (STN) or the SNr using virally-mediated
29 transcellular delivery of Cre from injection in either target in Rosa26-LoxP-stop-ChR2-EYFP mice to
30 drive channelrhodopsin expression. Photoactivation of Pf neurons connecting the striatum evoked an
31 inhibition often followed by an excitation, likely resulting from the activation of the trans-striatal
32 direct and indirect pathways, respectively. Photoactivation of Pf neurons connecting the SNr or the
33 STN triggered one or two early excitations, suggesting partial functional overlap of trans-subthalamic
34 and direct thalamonigral projections. Excitations were followed in about half of the cases by an
35 inhibition that might reflect recruitment of intranigral inhibitory loops. Finally, global Pf stimulation,
36 electrical or optogenetic, elicited similar complex responses comprising up to four components: one
37 or two short-latency excitations, an inhibition and a late excitation. These data provide evidence for
38 functional connections between the Pf and different BG components and for convergence of the
39 information processed through these pathways in single SNr neurons, stressing their importance in
40 regulating BG outflow.

41 **KEY WORDS:** basal ganglia, *substantia nigra pars reticulata*, optogenetics, *in vivo* electrophysiology,
42 intralaminar nuclei of the thalamus

43 **SIGNIFICANCE STATEMENT**

44 The thalamic centromedian /parafascicular (CM/Pf) complex is a major input and output station of
45 the basal ganglia (BG). While increasing knowledge is gained on the thalamostriatal circuitry, the

46 functional connectivity of the parafascicular nucleus (Pf) with the other BG components and the
47 integration of the information it provides to the network is poorly understood. By combining *in vivo*
48 electrophysiological recordings with electrical or optogenetic stimulation, we show that neuronal
49 activity in the BG output structure can be shaped by convergent information processed through
50 trans-striatal, trans-subthalamic and direct thalamonigral subcircuits. Such high level of integration
51 suggests an important functional weight of these different pathways on BG outflow, which needs to
52 be considered to fully appreciate Pf implication in BG physiological and pathological functioning.

53

54 **INTRODUCTION**

55 The parafascicular nucleus of the thalamus (Pf) is the primary subcortical source of excitatory
56 afferents to the basal ganglia (BG), where it mainly targets the striatum, the major input station of
57 the network. Over the last twenty years, significant knowledge has been gained on the complex
58 anatomy, physiology and functions of the microcircuits linking the Pf with the cerebral cortex and the
59 striatum (Smith et al., 2009; Mandelbaum et al., 2019), in particular its robust link with the striatal
60 cholinergic interneurons (Lapper and Bolam, 1992; Sidibé and Smith, 1999) that are now viewed as
61 central regulators of striatal functions (Abudukeyoumu et al., 2019). Hence, major progress has been
62 made in the dissection of the network mechanisms underlying the proposed key role of the Pf
63 projections to the striatum in processes as redirection of attention, associative learning, correct
64 action selection in an ongoing sensorimotor context and behavioral flexibility (Matsumoto et al.,
65 2001; Brown et al., 2010; Ding et al., 2010; Yamanaka et al., 2018; Mandelbaum et al., 2019).
66 Renewed interest for the Pf, long neglected in the models of BG functioning, also arose from clinical
67 and experimental data supporting its involvement in the pathophysiology of BG-related disorders,
68 including Parkinson's disease and Tourette's syndrome, and the potential of its deep brain
69 stimulation to alleviate symptoms of these diseases (Henderson et al., 2000; Smith et al., 2004;

70 Kerkerian-Le Goff et al., 2009; Lanciego et al., 2009; Stefani et al., 2009; Fasano et al., 2012; Testini et
71 al., 2016; Ilyas et al., 2019; Watson et al., 2021).

72 Amongst the thalamic nuclei, the Pf is characterized by its widespread connectivity with the entire
73 BG network, contrasting with its relatively sparse, although highly organized, connections with the
74 cerebral cortex. Besides its heavy input to the striatum, it provides weak to moderately dense inputs
75 to all the BG components (Sadikot et al., 1992; Bevan et al., 1995; Deschênes et al., 1996; Marini et
76 al., 1999; Lanciego et al., 2004; Yasukawa et al., 2004; Castle et al., 2005; Tandé et al., 2006) and, in
77 turn, receives a substantial innervation from the BG output structures (Sadikot and Rymar, 2009).
78 Single axon reconstruction showed that some Pf axons give rise to collaterals that arborize within the
79 striatum and extrastriatal BG components (Deschênes et al., 1996), including the subthalamic
80 nucleus (STN) that is also considered as an input station of the BG. However, dual retrograde tracing
81 study provided evidence that projections to the striatum and to the STN mostly arise from distinct
82 neuronal populations of the Pf (Féger et al., 1994), which has been recently confirmed using a
83 combination of Cre-dependent retrograde viruses (Watson et al., 2021). Whether or not Pf neurons
84 projecting to different extrastriatal targets are also mostly segregated has not been investigated in
85 depth. Furthermore, contrasting with the increasing knowledge on the function of the projections of
86 this thalamic complex to the striatum, little is known about the functional contributions of the
87 subcircuits it forms with the other BG components. The importance of these issues is pointed out by
88 a recent study demonstrating that the projections to STN while not to striatum contribute to
89 movement initiation (Watson et al., 2021).

90 The present study was undertaken to provide an integrated view of thalamic information processing
91 through the BG network. To do so, we combined *in vivo* electrophysiological recordings of neurons in
92 the *substantia nigra pars reticulata* (SNr), the main basal ganglia output structure in rodents, with
93 stimulation of Pf, either electrical or by optogenetics, in anaesthetized mice. We recorded the
94 responses evoked by stimulating neuronal Pf subpopulations distinguished on the basis of their

95 connectivity with the striatum, the STN or the SNr, respectively, and of Pf neurons taken as a whole,
96 to decipher the contribution of the different circuits to the global responses. The results show that
97 trans-striatal, trans-subthalamic and direct thalamonigral circuits are functional and can convey
98 converging information onto single SNr neurons. They provide evidence for segregation of the
99 information transferred through the striatum and through extrastriatal pathways, but for partial
100 functional overlap between the trans-subthalamic and thalamonigral pathways, as also confirmed by
101 retrograde tracing experiments. Finally, they highlight the Pf-evoked responses recorded in the SNr
102 as a readout of the functionality of the different pathways by which the Pf impacts BG outflow.

103

104 **MATERIAL AND METHODS**

105 Animal experimental procedures were approved by the Comité National de Réflexion Ethique sur
106 l'Expérimentation Animale (Comité d'éthique de Marseille # 14) and were in agreement with the
107 European Communities Council Directive (2010/63/EU). All surgical procedures and
108 electrophysiological recordings were conducted in mice anaesthetized with intraperitoneal (i.p.)
109 injections of ketamine and xylazine (100 and 10 mg/kg, respectively)

110 *Mice.* We used CamKII α -IRES-Cre BAC mice (CamKII^{cre/+} mice, purchased through European Mouse
111 Mutant Archive, ID:EM01153, RRID:IMSR_EM:01153) and Rosa26-LoxP-stop-ChR2-EYFP mice (ChR2-
112 EYFP mice, purchased from Jackson Laboratories, stock number: 012569, RRID:IMSR_JAX:012569).
113 Both strains were maintained on C57Bl/6J genetic background and both sex were used for ethical
114 considerations.

115 *Virally-mediated expression of opsins in Pf neurons.* To drive channelrhodopsin (ChR2) expression in
116 Pf neurons, we used two different AAV vectors containing transgene to express either ChR2 (AAV-
117 Ef1 α -DIO-hChR2(H134R)-mCherry; titer: $\geq 10^{13}$ vg/ml) or Cre recombinase (AAV-EF1 α -mCherry-IRES-
118 WGA-Cre; titer: 1.6×10^{12} vg/ml). These recombinant AAV vectors were serotyped with AAV5 coat

119 proteins and packaged by the viral vector core at the University of North Carolina (UNC Vector Core,
120 Chapel Hill, NC).

121 Anaesthetized mice were mounted on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA).
122 Injections were made with a 10- μ l syringe, connected to the injector (30 G) by a polyethylene tubing,
123 and controlled by an injection pump at 0.3 μ l/min.

124 To achieve Cre-dependent expression of ChR2 in Pf neurons independently on their projection site,
125 we microinjected 1 μ l of AAV-Ef1a-DIO-hChR2(H134R)-mCherry into the Pf of CamKII^{cre/+} mice (-2.3
126 mm AP, \pm 0.7 mm ML, -3.1 mm DV). To achieve Cre-dependent expression of ChR2 in subpopulations
127 of Pf neurons based on their connectivity with distinct BG nuclei, we applied a strategy relying on the
128 transcellular transport of Cre using a virus expressing Cre recombinase fused to the tracer protein
129 wheat germ agglutinin (AAV5-EF1 α -mCherry-IRES-WGA-Cre; Gradinaru et al., 2010). Microinjections
130 of this virus were performed in Rosa26-LoxP-stop-ChR2-EYFP mice into either the striatum (1 μ L at
131 two dorso-ventral sites: +1.0 mm AP, +1.7 mm ML, -3.1 and -2.7 mm DV), the STN (0.2 μ L at one site
132 at: -1.8 mm AP, +1.6 mm ML, -4.5 mm DV) or the SNr (0.3 μ L at one site: -2.8 mm AP, +1.35 mm ML, -
133 4.4 mm DV), at coordinates defined from bregma with the incisor bar at -1.0 mm according to the
134 stereotaxic mouse atlas of Paxinos and Franklin (2001). At the injection site, transduced cells can be
135 visualized by mCherry immunodetection. These transduced cells express the Cre recombinase fused
136 to the transcellular tracer WGA which is secreted locally, taken up by the axon terminals of
137 synaptically connected neurons and transported to their cell-body, which allows opsin expression
138 only in the cells defined by this connectivity. ChR2-expressing neurons were visualized by
139 immunodetection of either mCherry in CamKII^{cre/+} mice or of EYFP in Rosa26-LoxP-stop-ChR2-EYFP.
140 Cortical afferent terminals were visualized in the Pf by VGLUT1 immunolabelling. For primary
141 antibody exposure, brain sections were incubated overnight at 4°C in Rabbit anti-RFP (#600-401-379,
142 RRID:AB_2209751, Rockland), Chicken anti-GFP (#GFP-1020, RRID:AB_10000240, Aves Labs, Davis,
143 CA) and/or Guinea Pig anti-VGLUT1 (#AB5905, RRID:AB_2301751, Merck) diluted to 1/1000.

144 Secondary antibodies were Alexa Fluor 555 Donkey anti-Rabbit (#A-31572, RRID:AB_162543,
145 Invitrogen), Alexa Fluor 488 Donkey anti-Chicken (#703-545-155, RRID:AB_2340375, Jackson
146 Immunotech, Cambridgeshire, UK) and/or Alexa Fluor 633 Goat anti-Guinea Pig (#A-21050,
147 RRID:AB_2535718, Invitrogen) used at 1/500 for 1h30 at room temperature. See table 2 for more
148 details.

149 *Electrophysiological recordings.* Mice under anesthesia were fixed in a stereotaxic frame (Horsley-
150 Clarke apparatus, Unimécanique, Epinay-sur-Seine, France) and body temperature was maintained at
151 36.5°C. All Recordings were performed 8-10 weeks after injection of the viruses. Single-unit activity
152 of neurons in the SNr (at: -3.3/-2.9 mm AP, +1.3/+1.5 mm ML, -3.7/-4.6 mm DV) was recorded
153 extracellularly using glass micropipettes (25-35 M Ω) filled with a 0.5M sodium chloride solution
154 containing 1.5% neurobiotin (Vector Laboratories, Burlingame, CA). Action potentials were recorded
155 using a Axoclamp-2B amplifier (Molecular Devices, Union City, CA), amplified, filtered with an AC/DC
156 amplifier (DAM 50, Tektronix, Courtabœuf, France). Data were sampled on-line at 10 kHz rate on a
157 computer connected to a CED 1401 interface and off-line analyzed using Spike2 software (Cambridge
158 Electronic Design, Cambridge, UK). Nigral neurons were identified as non-dopaminergic by their
159 classically defined electrophysiological characteristics: narrow spikes (width \leq 2 ms) and ability to
160 present high-frequency discharge ($>$ 10 Hz) without decrease in spike amplitude (Bunney et al., 1973;
161 Deniau et al., 1978).

162 *Pf stimulation and analysis of evoked responses.* The Pf (at: -2.3 mm AP, +0.7 mm ML, -3.2 mm DV)
163 ipsilateral to the recorded SNr was stimulated through either bipolar coaxial stainless steel electrode
164 (SNE-100, Rhodes Medical Instruments, Woodlands Hill, CA) or optical fiber (NA 0.22, 200 μ m
165 diameter, Thorlabs, Canada). Electrical stimulation consisted of pulses of 600 μ s width and 10-20 V,
166 delivered at 1 Hz. Optical stimulation consisted of pulses of blue light (1 ms, Laser 473 nm, 180-200
167 mW) delivered at 1 Hz. Peristimulus time histograms (PSTHs) were generated from 140 stimulation
168 trials with a bin size of 1 ms. Spikes were discriminated from noise and stimulation artifact based on

169 their amplitude, using the gate function of a 121 window discriminator (World Precision Instruments)
170 and sampled on-line. Excitatory responses were defined as > 50% increase vs. pre-stimulus in the
171 number of spikes for at least three consecutive bins and were characterized by their latency,
172 duration and amplitude (number of spikes within the excitatory response period minus the mean
173 number of spontaneous spikes emitted in basal condition within the same duration of time).
174 Inhibitory responses corresponded to the time interval during which no spike was observed and were
175 characterized by their latency and duration. Given the maintenance of a sustained tonic activity of
176 SNr neurons despite the decrease in their firing rate in our anaesthetized animals, inhibitory
177 responses can be evidenced from 60 stimulations as previously documented following cortical
178 stimulations (Maurice et al., 1999). Only responses obtained from animals with both a correct
179 targeting of injection sites and a correct positioning of the optical fiber and the recording electrodes
180 were analysed. Numerical values are given as means \pm SEM. Statistical analysis was performed using
181 Prism 9 (GrapPad Software, San Diego, CA). Statistically significant differences were tested using
182 Student's t-test or Mann-Whitney Rank Sum test as indicated for each situation. Significance level
183 was set at $p < 0.05$. Numerical p-values and corresponding n-values are given in table 1.

184 *Juxtacellular labeling using neurobiotin.* Some Pf neurons were juxtacellularly-labeled with
185 neurobiotin according to Pinault (1996). Briefly, positive current pulses (2-6 nA, 200 ms, 50% duty
186 cycle) were applied until single-unit activity became robustly entrained by the pulses for at last 5
187 minutes. Two to four hours after labeling, mice were euthanized by a lethal dose of anesthetic and
188 transcardially perfused with 5 ml sodium chloride solution followed by 20 ml of 4%
189 paraformaldehyde in phosphate buffer (PBS). Brains were removed and cryoprotected in sucrose
190 30% in PB and frozen until cutting. Serial frozen section (40 μ m) were incubated overnight at room
191 temperature in Triton (0.3%) phosphate buffer saline (PBS) containing Cy3-streptavidin (1:1 000,
192 RRID:AB_2535718, Thermo Fisher Scientific).

193 *Retrograde labelling with cholera toxin subunit B (CTB)*. Three adult (8-10 weeks) mice under
194 anesthesia were placed in a David Kopf stereotaxic apparatus. Small craniotomies were made on the
195 skull. Solutions of 150 nl of cholera toxin subunit B (CTB) conjugated to Alexa Fluor 594 (#C34777,
196 Molecular probes, Eugene, OR) and Alexa Fluor 488 (#C34775, Molecular probes) at 1 μ g/ μ l in PBS
197 were pressure-injected through a Hamilton syringe at a flow rate of 30 nl/min in the SNr and in the
198 STN, respectively. The stereotaxic coordinates of the injection sites were as follows: SNr: -2.7 mm AP,
199 +1.3 mm ML, -4.4 mm DV; STN: -1.7 mm AP; +1.6 mm ML; -4.35 mm DV. A similar experiment was
200 performed consisting of injections of CTB conjugated to Alexa Fluor 488 (150 nl) in the STN combined
201 with injections of CTB conjugated Alexa Fluor 594 (450 nl) in the striatum (+1.0 mm AP, +1.7 mm ML,
202 -2.9 mm DV) in three additional mice. After surgery, mice were injected with 5 mg/kg carprofen
203 (Rimadyl[®], Pfizer) to reduce postoperative inflammation and pain. Ten days after CTB injection, mice
204 were anaesthetized and perfused intracardially with 5 ml NaCl (0.9%) followed by 30 ml
205 paraformaldehyde (4% in 0.12M PBS pH 7.2). Dissected brains were post-fixed in paraformaldehyde
206 (4% in PBS) overnight at 4 °C and kept in a cryoprotective solution containing 30% of sucrose at 4 °C
207 for 2 days and then frozen in dry ice and stored at -80°C. Frozen coronal (40 μ m thick) tissue sections
208 were cut at -20°C with a cryostat (CM3050, Leica, Nussloch, Germany) at Pf level. The sections were
209 then mounted on SuperFrost Plus glass slides (Fisher Scientific, Elancourt, France). The sections were
210 inspected under a Zeiss confocal microscope (510 Meta, Zeiss, Oberkochen, Germany) at low
211 magnification. Colocalization analysis into the Pf was performed by counting the number of cells
212 labelled both with CTB conjugated to Alexa Fluor 594 and CTB conjugated to Alexa Fluor 488 in 13
213 coronal sections for the combined injections in the SNr and STN or in 9 coronal sections for the
214 combined injections in the STN and the striatum. Photomicrographs were obtained at the highest
215 magnification available (63 \times oil-immersion lens, NA 1.4; 1024 \times 1024 pixels) and with the appropriate
216 band-pass and long-pass filter setting to avoid cross-talk.

217

218 **RESULTS**

219 **SNr responses elicited by optogenetic activation of subpopulations of Pf neurons distinguished on**
220 **the basis of their connectivity**

221 The Pf is connected to the SNr through different BG pathways, including the trans-striatal, trans-
222 subthalamic and the direct thalamonigral pathways. To assess the functionality of these different
223 pathways, we recorded the responses elicited in SNr neurons by the optogenetic activation of
224 subpopulations of Pf neurons segregated based on their connections with the striatum, STN or SNr,
225 respectively (Fig. 1). This connectivity-based strategy relies on the expression of the opsin in the
226 projection neurons innervating a given target owing to the transcellular transport of Cre from this
227 target, using virally-mediated expression of the WGA-Cre. For instance, 5 weeks after the unilateral
228 injection of the virus into the striatum of Rosa26-LoxP-stop-ChR2-EYFP mice, robust ChR2-EYFP
229 expression was observed in a large subset of Pf neurons strictly in the ipsilateral side (Fig. 2A), in few
230 other thalamic nuclei, notably the ventral posteromedial (VPM) nucleus, as well as in other neuronal
231 populations known as innervating the striatum, such as cortical projection neurons and nigral
232 dopaminergic cells (data not shown). Although the opsin was expressed in all the systems innervating
233 the virally-injected structure, restricted activation of the Pf thalamic projection system was achieved
234 by light delivery into the Pf. The limitation of this strategy might be the recruitment, besides Pf
235 neurons projecting to either targets, of axon collaterals within the Pf of other neuronal systems
236 projecting to the injected BG component. Previous work in the cat using dual retrograde tracing
237 provided evidence for collateralized projections from the cerebral cortex to the Pf and the striatum
238 (Royce, 1983; Paré and Smith, 1996), although the number of double labelled cortical neurons was
239 few compared to the single labelled ones from either target (Royce, 1983). To investigate the
240 potential implication of Pf collaterals of corticostriatal projections which could result in axonal reflex
241 in our experimental paradigm, we labelled cortical terminals into the Pf using VGLUT1
242 immunoreactivity as a specific marker in Rosa26-LoxP-stop-ChR2-EYFP mice after WGA-Cre viral

243 injection into the striatum (Fig. 2B). VGLUT1 and EYFP immunostained profiles were spatially
244 segregated, suggesting that cortico-Pf fibers are not expressing ChR2 at detectable level following
245 WGA-Cre viral delivery into the striatum and thus will not be recruited by Pf light shining. The next
246 step was to validate the functionality of the ChR2 by performing single unit extracellular recordings
247 of Pf neurons. As illustrated in figure 2C, some of the recorded Pf neurons were efficiently driven by 1
248 ms blue light pulses, and their juxtacellular labeling with neurobiotin showed that these light-driven
249 neurons actually expressed ChR2-EYFP, confirming the efficiency of our optogenetic strategy.

250 The impact of the specific activation of the Pf-striatal circuit was then investigated in SNr neurons
251 (Fig. 3). Figure 3A illustrates the WGA-Cre expression in the virally-injected striatum and the
252 expression of ChR2 in the synaptically-connected Pf neurons in a mice used for electrophysiological
253 recordings. A large number of both transduced cells in the striatum (Fig. 3A1) and of ChR2-expressing
254 cells in the PF (Fig. 3A2), as reported in Fig. 2A1, was consistently observed in the recorded animals.
255 Photo-activation of the Pf neurons projecting to the striatum evoked responses in 22 out of 68
256 recorded SNr cells. Among the responding cells, 10 presented an inhibition followed by an excitation
257 (Fig. 3B), 8 cells displayed only an inhibition (Fig. 3C) whereas the 4 remaining neurons showed only
258 late excitation (not shown). The population PSTH in figure 3D depicts two components in the average
259 response, namely an inhibition followed by an excitation, whose characteristics are detailed in figure
260 3E. It is to note that there was a competition between the inhibition and the following excitation.
261 This was evidenced by the fact that *i)* the inhibition was longer (21.5 ± 2.0 ms vs. 14.1 ± 2.1 ms; $t(16)$
262 = 2.525; $p = 0.023$; Student's *t*-test) when it was not followed by an excitation ($n = 8$) and *ii)* the
263 excitation, when alone ($n = 4$), had a shorter latency (18.1 ± 5.0 ms vs. 35.2 ± 4.2 ms; $t(12) = 2.334$; p
264 = 0.038; Student's *t*-test), a tendency for a longer duration (23.0 ± 4.8 ms vs. 14.3 ± 1.7 ms; $t(12) =$
265 2.155; n.s. $p = 0.052$; Student's *t*-test) and a stronger amplitude (71.1 ± 21.7 spikes vs. 19.7 ± 4.4
266 spikes; $U = 3$; $p = 0.014$: Mann-Whitney rank sum test).

267 Next, the impact of the specific activation of the Pf-subthalamic circuit was examined. Figure 4A
268 illustrates an injection site in the STN (Fig. 4A1) and the transcellularly-driven expression of ChR2 in
269 the connected Pf neurons (Fig. 4A2). Photo-activation of Pf neurons projecting to the STN evoked
270 responses in 8 out of 78 examined SNr cells. These responses consisted of one (6/8) or two early
271 excitations (2/8), followed, in most of the cases (n=5, 62.5 %), by an inhibition, as illustrated in figure
272 4B. The population PSTH (Fig. 4C) further shows that the excitations are distributed in two peaks with
273 latencies <5ms or ≥5ms. Characteristics of the population responses are detailed in figure 4D.

274 Finally, the impact of the specific activation of the direct thalamonigral projection system was
275 assessed. Figure 5A depicts an injection site in the SNr (Fig. 5A1) and the retrogradely driven
276 expression of ChR2 in the connected Pf neurons (Fig. 5A2). Out of the virally-injected BG
277 components, SNr is the one from which the weaker ChR2 expression is found in the Pf. Photo-
278 activation of Pf neurons projecting to SNr evoked responses in 14 out of 53 recorded SNr cells. These
279 responses consisted of a single (12/14, Fig. 5C) or a double excitation (2/14, Fig. 5B) followed, in half
280 of the cells, by an inhibition. Population PSTH in figure 5D evidences these excitations followed by
281 the inhibition in the average population response. The distribution displays a peak with a shoulder
282 reminiscent of the second peak of the trans-STN responses (≥5ms, see also Fig 4C). We then used the
283 same latency thresholds (<5ms or ≥5ms) to distinguish the two excitations and provide the
284 characteristics of the population responses (Fig. 5E).

285

286 **SNr responses evoked by stimulating Pf neuronal populations as a whole**

287 We then investigated the global transfer of information from the Pf through the BG, by recording the
288 responses elicited in SNr neurons by Pf stimulation, either electrically or by optogenetics.

289 Responses evoked by electrical stimulation of the Pf were analyzed in 54 SNr responding cells
290 recorded in 18 mice (Fig. 6). They presented up to 4 components: one or two early excitations, an

291 inhibition and a late excitation. Inhibition was recorded in the majority of the cells ($n = 37$ of 54; 68.5
292 %), the most often (30 of 37), preceded by one or two short latency excitations (Fig. 6A-C). The
293 inhibition was in some cases followed by a late excitation (13 out of 37 cells) (Fig. 6C). In the 17
294 remaining SNr cells out of the 54 (31.5 %), excitation without inhibition were recorded, consisting in
295 one (3 cells, Fig. 6D) or two (7 cells; Fig. 6E) short latency excitations the more often followed (9 cells)
296 by a late latency excitation. All the components were found together in 17% ($n = 9$) of the responding
297 cells. Figure 6F provides the pie-chart of the different types of responses expressed as percent of
298 total responding cells. The characteristics of each of these components were determined by pooling
299 all evoked responses together to measure the latency (L) and the duration (D) of each events as well
300 as the amplitude (A) of the excitations. Results are summarized in table 1.

301 There was no obvious topographical distribution of the SNr cells presenting those different complex
302 responses to Pf stimulation and these responses were reproducible overtime in a given neuron for
303 identical stimulation parameters. By contrast, modifying the intensity or the current polarity of the
304 stimulation could alter the respective weight of each component of the evoked responses, or evoke
305 different patterns of responses in the same individual neuron (not shown), presumably by recruiting
306 populations of Pf neurons with different projections within the BG network.

307 The complex responses evoked by Pf electrical stimulation in SNr neurons were reminiscent of the
308 responses evoked by cortical stimulation (Maurice et al., 1999). Whether electrical stimulation of the
309 Pf recruits the cerebral cortex and remotely activates the corticostriatal pathway either
310 orthodromically via thalamocortical projections, or through axonal reflex via cortical axons branching
311 both in the thalamus and the striatum is an important matter of concern. The possibility of
312 orthodromic recruitment is limited by the sparse innervation of the cerebral cortex from the Pf. The
313 above reported lack of EYFP expression in VGluT1-labelled terminals within the Pf after virus injection
314 in the striatum suggest that collaterals of corticostriatal projections are weak, if present, in mice; as
315 previously reported in cat (Royce, 1983; Paré and Smith, 1996). To nevertheless exclude contribution

316 of axonal reflex, we developed an optogenetic strategy to achieve selective activation the Pf neurons
317 and compared the elicited responses with those evoked by electrical Pf stimulation. This was done by
318 expressing ChR2-mCherry specifically in Pf neurons by injecting Cre-dependent AAV5 carrying the
319 ChR2-mCherry into the Pf of mice expressing Cre under the promoter of the CaMKII, a marker of
320 projection neurons including those of the thalamus and the cerebral cortex. 5 weeks following AAV
321 injections into the Pf, numerous mCherry-expressing neurons were found in the Pf (Fig. 7A), whereas
322 no mCherry neuron was detected into the cerebral cortex (Fig. 7B), confirming that corticothalamic
323 neurons in our experimental setting do not express ChR2. Moreover, contrasting with the dense
324 network of fibers in the striatum, very sparse mCherry fibers were detected in the cerebral cortex
325 (Fig. 7C), in compliance with the literature. Together, these data support the view that the responses
326 driven by light delivery locally into the Pf will result most directly from the activation of Pf neurons.

327 Responses evoked by photostimulation of Pf neurons were then investigated in 41 SNr responding
328 cells recorded in 5 CaMKII^{cre/+} mice (Fig. 8), 8-10 weeks post-AAV injection into the Pf. Similar to the
329 electrical stimulation, in most tested cells (n = 37 of 41 responding neurons; 90%), photostimulation
330 of the Pf induced an inhibition (Fig. 8A, 8B and 8C), that was, in 27 out of 37 of the cells, preceded
331 and/or followed by excitation. In the 4 (10%) remaining cells, Pf stimulation evoked only excitation
332 (Fig. 8D and 8E). Figure 8F summarizes the different patterns of responses expressed as percent of
333 total responding cells. Note that the variety and the distribution of the components were similar to
334 those recorded under electrical stimulation (fig. 8F). As for the electrical stimulation, no obvious
335 topographical distribution of the cells with distinct types of responses could be observed. Here again,
336 the characteristics of each component were determined by pooling these complex responses
337 altogether to calculate the latency and the duration of each component as well as the amplitude for
338 the excitations. Table 1 summarizes the characteristics of each component of the responses evoked
339 by electrical and optogenetic activation of Pf. Differences between the two modes of stimulation
340 were not obvious.

341

342 **Dual retrograde labeling of Pf neurons from the STN and the SNr**

343 To investigate whether Pf neurons projecting to the STN and to the SNr were branched, two
344 fluorophore-conjugated variants of CTB were injected into the STN and the SNr in 3 mice. The
345 labeling at the injection sites covered the anterior 2/3 and the lateral 2/3 extents of both structures.
346 231 Pf cells were retrogradely labelled with Alexa Fluor 488-CTB injected into the STN and 165 cells
347 with Alexa Fluor 594-CTB injected into the SNr. A large population of cells showed co-localization of
348 the two markers (Figure 9), representing 40.9 ± 12.1 % of the cells labelled in the Pf from both
349 targets. The observation of these double-labelled neurons indicates that thalamosubthalamic and
350 thalamonigral pathways arise in significant proportion from branched Pf neurons projecting to both
351 sites. Using a similar approach, and in agreement with the literature (Féger et al., 1994; Watson et
352 al., 2021), we confirmed that, on the contrary, Pf-STR and Pf-STN projections arise from largely
353 segregated populations, as shown by the fact that only 3.0 ± 2.0 % of the cells showed co-labelling
354 among 342 Pf cells retrogradely labeled from the STR and 246 labeled from the STN.

355

356 **DISCUSSION**

357 This study provides physiological evidence for functional connections between the Pf and the main
358 input (striatum and STN) and output (SNr) structures of the BG, and for possible convergence of the
359 information conveyed through these pathways on single SNr neuron. They further indicate that the
360 information processed through trans-striatal and extrastriatal projections arises from functionally
361 segregated populations of Pf neurons, whereas information conveyed through the STN and direct
362 nigral projections originates from partly overlapping populations, as confirmed by retrograde tracing.
363 While highly reproducible in a given cell for a given setting of stimulation parameter, the responses
364 were variable from cell to cell, as reported previously regarding the transfer of cortical information

365 through the BG (Maurice et al., 1999). This variability is likely due to the topographical relationship
366 between the stimulated Pf region and the recorded SNr neuron. This interpretation is consistent with
367 the observation that modifying the stimulation intensity or polarity can impact the response pattern,
368 presumably due to the recruitment of a less or more extended region of the Pf connected with the
369 recorded neuron. The composition of the evoked responses is unlikely to be affected by the
370 anesthesia. For instance, one can note that cortical stimulation evokes similar triphasic responses
371 (excitation-inhibition-excitation) in SNR neurons in awake animals and under different anesthesia
372 (Maurice et al., 1999; Nishimuta et al., 2002; Degos et al., 2005; Sano et al., 2013; Koketsu et al.,
373 2021). However, we cannot exclude an impact of the anesthesia on the characteristics (latency,
374 duration, amplitude) of the response components.

375 In the different stimulation paradigms tested, the responses were usually polymodal, suggesting the
376 involvement of different subcircuits, even when targeting neurons identified based on their
377 connectivity with a given component of the BG. This was expected for the neurons projecting to the
378 striatum, as the information can be processed through the direct and the indirect trans-striatal
379 pathways, but not for neurons projecting to the STN or SNr. For instance, optogenetic stimulation of
380 the Pf neurons projecting to the striatum elicited an inhibition, the most often followed by an
381 excitation. These responses can be attributed to the recruitment of striatal medium spiny neurons
382 (MSNs) of the direct and of the indirect pathway, respectively, which exert inhibitory versus
383 excitatory influence onto SNr neurons. Indeed, thalamostriatal terminals have been reported to
384 target both types of MSNs, with the direct pathway MSNs receiving a greater density of thalamic
385 synapses than those of the indirect pathway (Huerta-Ocampo et al., 2014), which could account for
386 the more consistent recording of the inhibitory versus the late excitatory component of the
387 responses. One cannot exclude a role of striatal interneurons, in particular cholinergic interneurons.
388 These interneurons receive particularly dense Pf innervation and can exert complex influence on
389 MSNs by acting via different subtypes of muscarinic cholinergic receptor, by co-releasing glutamate
390 (Higley et al., 2011) and indirectly via the control of GABA release by dopaminergic terminals (Nelson

391 et al., 2014) and interneurons via nicotinic receptors (English et al., 2012; Luo et al., 2013; Faust et
392 al., 2015, 2016). Activation of Pf neurons projecting to either the STN or the SNr unexpectedly
393 elicited complex responses, consisting of one or two early excitations followed in most cases by a
394 brief inhibition. The lack of such early excitation component in the responses elicited by the
395 activation of Pf neurons projecting to the striatum suggests that these neurons do not provide
396 significant functional input to the STN or the SNr. This complies with and extends previous
397 observations in the rat (Féger et al., 1994) and the mouse (Watson et al., 2021) showing that the Pf
398 projections to the STN and striatum arise mostly from separate neuronal populations. Indeed, they
399 emphasize a functional segregation of Pf information processing via the striatum and extrastriatal
400 sites. Such a segregation supports the view of differential implication in BG functions, as recently
401 exemplified by the demonstration of the contribution of Pf projections to STN but not to striatum in
402 movement initiation (Watson et al., 2021). The fact that the activation of Pf neurons projecting to
403 either the STN or the SNr triggers excitations with similar bimodal distribution, or even two
404 excitations in the same cell, suggests that the two structures are connected (at least in part) by the
405 same thalamic neuronal population. This is confirmed by our dual retrograde fluorescent labeling
406 using CTB, showing that a non-negligible proportion of Pf neurons targeting these structures
407 innervate both of them. Therefore, stimulation of Pf neurons projecting to either the STN or the SNr
408 would trigger both a monosynaptic and a di-synaptic response. These responses will involve
409 respectively collateralized and non-collateralized projections for STN and vice versa for SNr. In
410 principle, light delivery in the Pf following viral injection in the SNr might also trigger antidromic
411 activation of SNr neurons that reciprocally project to the Pf (Tsumori et al., 2000). However
412 antidromic SNr activation is unlikely to account for the early excitations because they do not show a
413 fixed latency and do not collide with spontaneous orthodromic spikes emitted by SNr neurons.
414 Regarding the brief inhibition that in most cases follows the early excitatory responses, it could result
415 from the recruitment of the potent inhibitory microcircuit formed by the dense network of
416 intranigral recurrent collaterals (Deniau et al., 1982; Brown et al., 2014). One cannot exclude,

417 however, the contribution of the thalamic input to the globus pallidus, which could result in SNr
418 inhibition either directly or via STN inhibition in our experimental paradigm if Pf projections to the
419 STN and the SNr are collaterals of projections to the globus pallidus. Finally, the recently evidenced
420 efferent projections of the STN neurons innervated by the Pf onto the globus pallidus in addition to
421 the SNr (Watson et al., 2021) might also be involved.

422 After having characterized the responses elicited in the SNr by optogenetic activation of neuronal
423 subpopulations of Pf distinguished on the basis of their connectivity with the striatum, the STN and
424 the SNr, we aimed at examining whether and how the information processed through these
425 pathways are integrated in the BG output structure. To do so, we examined the responses evoked by
426 activation of Pf neuronal populations as a whole using either electrical stimulation or optogenetics.
427 The response patterns were highly similar in the two paradigms, excluding major contribution, in the
428 responses to electrical Pf stimulation, of axonal reflex involving pathways collateralizing onto both Pf
429 and a BG component, as the projections from the cerebral cortex that branch in the Pf and striatum
430 (Paré and Smith, 1996). These responses were in most cases multiphasic, the more complex including
431 4 components, namely two early excitations followed by an inhibition and a late excitation. Given the
432 connectivity-based responses reported above, the early excitations are likely to result from the
433 recruitment of the trans-subthalamic and direct nigral pathways, the inhibition from recruitment of
434 the trans-striatal direct pathway (presumably reinforced by local inhibitory microcircuits following
435 SNr activation or recruitment of globus pallidus), and the late excitation from the activation of the
436 indirect trans-striatal pathway. Taken as a whole, these data suggest that, although Pf projections to
437 extrastriatal structures are weak in terms of density of innervation, as compared to the massive
438 thalamostriatal pathway, they should not be considered as functionally less important, since they
439 have the potential to shape the activity of SNr neurons. Previous work in monkey called for a
440 reappraisal of the functional weight of extrastriatal Pf projections based on the observation that their
441 long arborization, although poorly branched, covered a relatively large portion of each BG nucleus
442 (Tandé et al., 2006). Moreover, in the rat, axons from the Pf have been shown to contact more

443 proximal dendrites of STN neurons (Bevan et al., 1995), which places them in position to exert potent
444 action onto the contacted neurons. If this is a general feature of extrastriatal projections, thalamic
445 axons could then exert powerful action on the different components of the BG network.

446 In conclusion, our study shows that the trans-striatal, trans-subthalamic and direct nigral pathways
447 formed from Pf neurons are functional and although more or less segregated can provide converging
448 information onto SNr neurons. The responses Pf activation as a whole in the SNr thus provide a read-
449 out of the functionality of the different subcircuits by which information from the Pf can be
450 processed through the BG to regulate the network outflow, which might be useful to detect changes
451 in these circuits occurring in different treatment or pathological conditions, as previously done for
452 the cortically-driven BG circuits. Here, Pf neuronal populations were activated artificially either
453 together or separately based on their connectivity, and what remains to be determined is whether
454 and when these different components of the thalamo-BG circuits are recruited under physiological
455 conditions in connection with behavior. Recent evidence has been provided for converging thalamic
456 and cortical information onto direct and indirect MSNs in the striatal matrix, with a predominance of
457 the cortical input (Huerta-Ocampo et al., 2014). Moreover, distinct cortical-thalamic-striatal circuits
458 through the Pf have been revealed, placing this thalamic complex as a critical node that organizes
459 limbic, associative and somatosensory information in the BG (Mandelbaum et al., 2019). Whether
460 cortical and thalamic projections to STN neurons are also organized in such sophisticated circuits
461 would need to be investigated. In addition, Pf is in position to also provide direct information to the
462 output structures of the network. Characterizing the information that flows within the BG through
463 striatal and extrastriatal thalamic pathways is then a major issue that needs to be addressed to fully
464 appreciate Pf implication in BG functioning in physiological and pathological conditions.

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484

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614

615

616 **FIGURE LEGENDS:**

617 **Figure 1.** Schematic of the strategy used to assess the functionality of the thalamostriatal,
618 thalamosubthalamic and thalamonigral pathways. In three different sets of experiments, expression
619 of WGA-Cre was achieved by viral delivery of mCherry-IRES-WGA-Cre into the striatum (1), the
620 subthalamic nucleus (STN; 2) or the *substantia nigra pars reticulata* (SNr, 3) in Rosa26-LoxP-stop-
621 Chr2-EYFP mice. Chr2 is expressed in the locally transduced neurons, and, after transneuronal
622 retrograde transport of WGA-Cre, in the connecting ones, including the Pf projection neurons. Light
623 was then delivered in the Pf to activate the neuronal subpopulation connecting the virally-injected
624 basal ganglia target, and the evoked responses were recorded in the SNr. Pf: *parafascicular nucleus*
625 of the thalamus; GPe: external segment of the *globus pallidus*; SNr; *substantia nigra pars reticulata*;
626 STN: *subthalamic nucleus*.

627 **Figure 2.** Optogenetic strategy to activate subpopulations of Pf neurons distinguished on the basis of
628 their connectivity with BG components: Example of the thalamostriatal neurons. **A**,
629 Channelrhodopsin expression in the Pf as revealed by EYFP immunodetection in coronal brain slices
630 following injection of AAV carrying the EF1 α ::mCherry-IRES-WGA-Cre cassette in the striatum
631 (maximum intensity projection, 40 μ m depth). A strong labelling was observed in the ipsilateral Pf
632 (**A1** and **A2** at higher magnification) but not in the contralateral side. **B1**, Relationship between EYFP
633 expressing neurites and VGLUT1 expressing axon terminals (single confocal section). **B2** and **B3**,
634 higher magnification of the boxed area in **B1** showing the absence of EYFP immunolabelling in
635 VGLUT1 axon terminals. **B4**, Colocalization analysis of EYFP and VGLUT1 fluorescence confirms that
636 the two markers are spatially segregated. **C**, Efficiency of the optogenetic strategy. Raw trace
637 recording of a Pf neuron (**C1**) whose firing was driven by 1 ms-blue light pulses (10 Hz). The cell body
638 of this recorded neuron juxtacellularly labeled with neurobiotin (NBT, red) also shows EYFP
639 immunolabelling (green) indicating that it actually expressed Chr2 (**C2**). fr: *fasciculus retroflexus*; Pf:
640 *parafascicular nucleus of the thalamus*; EYFP: *enhanced yellow fluorescent protein*; NBT: *neurobiotin*;

641 *VGLUT1*: vesicular glutamate transporter 1; A.U.: arbitrary unit; DAPI: 4',6-diamidino-2-phenylindole.
642 Scale bars: 200 μm in **A1** and 20 μm in **A2**.

643 **Figure 3.** Responses evoked in the SNr by the photoactivation of Pf neurons projecting to the
644 striatum. **A**, Representative images illustrating the virus injection site in the striatum, as shown by
645 mCherry immunodetection (A1), and the resulting retrograde transduction of ChR2 in the Pf as
646 visualized by YFP immunodetection (A2); the asterisk marks the tip of the optical fiber. **B** and **C**,
647 Raster plots and PSTH (1 ms bins) illustrating examples of evoked responses that consisted in either
648 an inhibition followed by an excitation (**B**) or a single inhibition (**C**). **D**, Population PSTH of the overall
649 responses evoked in SNr neurons ($n = 18$ cells from $N = 5$ animals, 2 males and 3 females). PSTHs,
650 generated from 140 stimulation trials, are aligned on the light pulse delivery (vertical blue line) and
651 the light shaded color represents SEM. **E**, Characteristics of the responses evoked. Nb of Sp.: number
652 of spikes; cc: corpus callosum; Cx: cerebral cortex; D3V: dorsal third ventricle; fr: fasciculus
653 retroflexus. Scale bars: 200 μm in **A1** and 100 μm in **A2**.

654 **Figure 4.** Responses evoked in the SNr by the photoactivation of Pf neurons projecting to the STN. **A**,
655 Representative images illustrating the virus injection site in the STN, as shown by mCherry
656 immunodetection (**A1**), and the resulting retrograde transduction of ChR2 in the Pf as visualized by
657 YFP immunodetection (**A2**). **B**, Raster plot and PSTH (1 ms bins) of an evoked response consisting in
658 an excitation followed by an inhibition. **C**, Population PSTH of the responses evoked in SNr neurons (n
659 = 8 cells from $N = 4$ animals, 1 male and 3 females) expressed as in Figure 3. **D**, Characteristics of the
660 responses evoked. Excit.: excitation; fr: fasciculus retroflexus, STN: subthalamic nucleus. Scale bars:
661 100 μm in **A1** and **A2**

662 **Figure 5.** Responses evoked in SNr by the photoactivation of Pf neurons projecting to the SNr.
663 Representative images illustrating the virus injection site in the SNr, as shown by mCherry
664 immunodetection (**A1**), and the resulting retrograde transduction of ChR2 in the Pf as visualized by
665 YFP immunodetection (**A2** and magnification in **A3**); Arrowhead indicates the canula track and

666 asterisk marks the tip of the optical fiber. The evoked responses consisted in early excitations (**B and**
667 **C**) that were often followed by an inhibition. **D**, Population PSTH of the responses evoked in SNr
668 neurons (n = 14 cells from N = 6 animals, 3 males and 3 females) expressed as in Figure 3. **E**,
669 Characteristics of the responses evoked. Excit.: excitation. fr: fasciculus retroflexus; Pf: parafascicular
670 nucleus ; SNr: substantia nigra pars reticulata. Scale bars: 200 μm in **A1** and **A2**; 50 μm in **A3**.

671 **Figure 6.** Patterns of responses evoked in SNr cells by Pf electrical stimulation. **A-E**, Representative
672 PSTHs (1ms bins) illustrating the diversity of the responses recorded in individual SNr neurons; red
673 lines indicate the time of electrical shock delivery. **F**, Pie-chart illustrating the percentage of each
674 type of responses exhibited by the overall responding cells (n = 54 cells from N = 18 animals, 10
675 males and 8 females). Excit: excitation; Inhib: inhibition.

676 **Figure 7.** Representative photomicrographs of the typical mCherry expression patterns in coronal
677 sections at the level of the Pf (**A**), the cerebral cortex (**B**) and the striatum (**C**) of a transgenic
678 CaMKII^{cre/+} mice following AAV-ChR2-mCherry virus injection into the Pf. mCherry expression is
679 revealed by immunostaining with an antibody against RFP (magenta). The right panels (scale 10 μm)
680 are magnification of the boxed areas in the corresponding left panels (scale 100 μm). Both labelled
681 cell bodies and neurites were observed in the Pf (**A1, A2**) whereas the cerebral cortex (**B1, B2**) and
682 the striatum (**C1, C2**) contained only axonal fibers. Note the very sparse cortical staining compared
683 with the dense striatal labelling. cc: *corpus callosum*; D3V: dorsal third ventricle; fr: *fasciculus*
684 *retroflexus*; Pf: parafascicular nucleus; STR: striatum. Scale bars: 200 μm in A1, B1, C1 and 10 μm in
685 A2, B2 and C2.

686 **Figure 8.** Patterns of responses evoked in SNr cells by Pf photostimulation. **A-E**, Representative PSTHs
687 (1 ms bins) illustrating the diversity of the responses evoked in distinct SNr cells and their similarity
688 with those induced by Pf electrical stimulation (see Fig. 6); Blue lines indicate the time of light flash (1
689 ms) delivery. **F**, Pie-chart of the percentage of each type of response in the overall responding cells (n
690 = 41 cells from N = 4 animals, 1 male and 3 females). Excit: excitation; Inhib: inhibition.

691 **Figure 9.** Confocal image of retrogradely labelled cell bodies in the Pf after Alexa Fluor 488-CTB
692 injection into the STN (green) combined with Alexa Fluor 594-CTB injection into the SNr (magenta).
693 **A**, merged image; **B**, **C**, single channels. The dually labelled cells are indicated by arrows. Scale bar: 20
694 μm .