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## Processing of information from the centromedian/parafascicular complex of the thalamus through the basal ganglia

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29 **ABSTRACT**

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

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

51 **SIGNIFICANCE STATEMENT**

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

54 connectivity of the CM/Pf with the other BG components and the integration of the information it  
55 provides to the network is poorly understood. By combining *in vivo* electrophysiological recordings  
56 with electrical or optogenetic stimulation, we show that neuronal activity in the BG output structure  
57 can be shaped by convergent information processed through trans-striatal, trans-subthalamic and  
58 direct thalamonigral subcircuits. Such high level of integration suggests an important functional weight  
59 of these different pathways on BG outflow, which needs to be considered to fully appreciate CM/Pf  
60 implication in BG physiological and pathological functioning.

61

## 62 **INTRODUCTION**

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

78 et al., 2009; Lanciego et al., 2009; Stefani et al., 2009; Fasano et al., 2012; Testini et al., 2016; Ilyas et  
79 al., 2019; Watson et al., 2021).

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

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

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

111

## 112 **MATERIAL AND METHODS**

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

118 *Mice.* We used CamKII $\alpha$ -IRES-Cre BAC mice (CamKII<sup>cre/+</sup> mice, purchased through European Mouse  
119 Mutant Archive, ID:EM01153) and Rosa26-LoxP-stop-ChR2-EYFP mice (ChR2-EYFP mice, purchased  
120 from Jackson Laboratories, stock number: 012569), both strains maintained on C57Bl/6J genetic  
121 background.

122 *Virally-mediated expression of opsins in CM/Pf neurons.* To drive channelrhodopsin (ChR2) expression  
123 in CM/Pf neurons, we used two different AAV vectors containing transgene to express either ChR2  
124 (AAV-Ef1 $\alpha$ -DIO-hChR2(H134R)-mCherry) or Cre recombinase (AAV-EF1 $\alpha$ -mCherry-IRES-WGA-Cre).  
125 These recombinant AAV vectors were serotyped with AAV5 coat proteins and packaged by the viral  
126 vector core at the University of North Carolina (UNC Vector Core, Chapel Hill, NC).

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

130 To achieve Cre-dependent expression of ChR2 in CM/Pf neurons independently on their projection  
131 site, we microinjected 1  $\mu$ l of AAV-Ef1a-DIO-hChR2(H134R)-mCherry into the CM/Pf of CamKII<sup>cre/+</sup> mice  
132 (-2.3 mm AP,  $\pm$ 0.7 mm ML, -3.1 mm DV). To achieve Cre-dependent expression of ChR2 in  
133 subpopulations of CM/Pf neurons based on their connectivity with distinct BG nuclei, we applied a  
134 strategy relying on the transcellular transport of Cre using a virus expressing Cre recombinase fused to  
135 the transcellular tracer protein wheat germ agglutinin (AAV5-EF1 $\alpha$ -mCherry-IRES-WGA-Cre; Gradinaru  
136 et al., 2010). Microinjections of this virus were performed in Rosa26-LoxP-stop-ChR2-EYFP mice into  
137 either the striatum (1  $\mu$ L at two dorso-ventral sites: +1.0 mm AP, +1.7 mm ML, -3.1 and -2.7 mm DV),  
138 the STN (0.2  $\mu$ L at one site at: -1.8 mm AP, +1.6 mm ML, -4.5 mm DV) or the SNr (0.3  $\mu$ L at one site: -  
139 2.8 mm AP, +1.35 mm ML, -4.4 mm DV), at coordinates defined from bregma with the incisor bar at -  
140 1.0 mm according to the stereotaxic mouse atlas of Paxinos and Franklin (2001). Cre recombinase fused  
141 to the transcellular tracer is then transported along with WGA to the cell-body of anatomically  
142 connected neurons where it activates opsin expression only in the cells defined by this connectivity.  
143 ChR2-expressing neurons were visualized by immunodetection of either mCherry in CamKII<sup>cre/+</sup> mice or  
144 of EYFP in Rosa26-LoxP-stop-ChR2-EYFP. Cortical afferent terminals were visualized in the CM/Pf by  
145 VGLUT1 immunolabelling. For primary antibody exposure, brain sections were incubated overnight at  
146 4°C in Rabbit anti-RFP (#600-401-379, Rockland), Chicken anti-GFP (#GFP-1020, Aves Labs, Davis, CA)  
147 and/or Guinea Pig anti-VGLUT1 (#AB5905, Merck) diluted to 1/1000. Secondary antibodies were Alexa  
148 Fluor 555 Donkey anti-Rabbit (#A-31572, Invitrogen), Alexa Fluor 488 Donkey anti-Chicken (#703-545-  
149 155, Jackson Immunosciences, Cambridgeshire, UK) and/or Alexa Fluor 633 Goat anti-Guinea Pig (#A-  
150 21050, Invitrogen) used at 1/500 for 1h30 at room temperature.



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

164 *CM/Pf stimulation and analysis of evoked responses.* The CM/Pf (at: -2.3 mm AP, +0.7 mm ML, -3.2 mm  
165 DV) ipsilateral to the recorded SNr was stimulated through either bipolar coaxial stainless steel  
166 electrode (SNE-100, Rhodes Medical Instruments, Woodlands Hill, CA) or optical fiber (NA, 200  $\mu$ m  
167 diameter, Thorlabs, Canada). Electrical stimulation consisted of pulses of 600  $\mu$ s width and 10-20 V,  
168 delivered at 1 Hz. Optical stimulation consisted of pulses of blue light (1 ms, Laser 473 nm, 180-200  
169 mW) delivered at 1 Hz. Peristimulus time histograms (PSTHs) were generated from 140 stimulation  
170 trials. Spikes were discriminated from noise and stimulation artifact based on their amplitude, using  
171 the gate function of a 121 window discriminator (World Precision Instruments) and sampled on-line.  
172 Excitatory responses were defined as  $>$  50% increase vs. pre-stimulus in the number of spikes for at  
173 least three consecutive bins and were characterized by their latency, duration and amplitude.  
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 high spontaneous firing rate of SNr neurons in  
176 anaesthetized animals, inhibitory responses can be evidenced from 60 stimulations as previously

177 documented following cortical stimulations (Maurice et al., 1999). Numerical values are given as means  
178  $\pm$  SEM. Statistical analysis was performed using Sigma Plot 12.5 (Systat software Inc., San Jose, CA) and  
179 significance was assessed by performing appropriate statistical tests (Student's t test or, for multiple  
180 comparisons, one-way repeated measure ANOVA followed by Holm-Sidak post-hoc test).

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

189 *Retrograde labelling with cholera toxin subunit B (CTB).* Three adult (8-10 weeks) mice under  
190 anesthesia were placed in a David Kopf stereotaxic apparatus. Small craniotomies were made on the  
191 skull. Solutions of 150 nl of cholera toxin subunit B (CTB) conjugated to Alexa Fluor 594 (#C34777,  
192 Molecular probes, Eugene, OR) and Alexa Fluor 488 (#C34775, Molecular probes) at 1 $\mu$ g/ $\mu$ l in PBS  
193 were pressure-injected through a Hamilton syringe at a flow rate of 30 nl/min in the SNr and in the  
194 STN, respectively. The stereotaxic coordinates of the injection sites were as follows: SNr: -2.7 mm AP,  
195 +1.3 mm ML, -4.4 mm DV; STN: -1.7 mm AP; +1.6 mm ML; -4.35 mm DV. After surgery, mice were  
196 injected with 5 mg/kg carprofen (Rimadyl<sup>®</sup>, Pfizer) to reduce postoperative inflammation and pain.  
197 Ten days after CTB injection, mice were anaesthetized and perfused intracardially with 5 ml NaCl  
198 (0.9%) followed by 30 ml paraformaldehyde (4% in 0.12M PBS pH 7.2). Dissected brains were post-  
199 fixed in paraformaldehyde (4% in PBS) overnight at 4 °C and kept in a cryoprotective solution  
200 containing 30% of sucrose at 4 °C for 2 days and then frozen in dry ice and stored at -80°C. Frozen  
201 coronal (40  $\mu$ m thick) tissue sections were cut at -20°C with a cryostat (CM3050, Leica, Nussloch,

202 Germany) at Pf level. The sections were then mounted on SuperFrost Plus glass slides (Fisher  
203 Scientific, Elancourt, France). The sections were inspected under a Zeiss confocal microscope (510  
204 Meta, Zeiss, Oberkochen, Germany) at low magnification. Photomicrographs were obtained at the  
205 highest magnification available (63× oil-immersion lens, NA 1.4; 1024×1024 pixels) and with the  
206 appropriate band-pass and long-pass filter setting to avoid cross-talk.

207

## 208 **RESULTS**

### 209 **SNr responses elicited by optogenetic activation of subpopulations of CM/Pf neurons distinguished** 210 **on the basis of their connectivity**

211 The CM/Pf is connected to the SNr through different BG pathways, including the trans-striatal, trans-  
212 subthalamic and the direct thalamonigral pathways. To assess the functionality of these different  
213 pathways, we recorded the responses elicited in SNr neurons by the optogenetic activation of  
214 subpopulations of CM/Pf neurons segregated based on their connections with the striatum, STN or  
215 SNr, respectively (Fig. 1). This connectivity-based strategy relies on the expression of the opsin in the  
216 projection neurons innervating a given target owing to the transcellular transport of Cre from this  
217 target, using virally-mediated expression of the WGA-Cre. For instance, 5 weeks after the unilateral  
218 injection of the virus into the striatum of Rosa26-LoxP-stop-ChR2-EYFP mice, robust ChR2-EYFP  
219 expression was observed in a subset of CM/Pf neurons strictly in the ipsilateral side (Fig. 2A), as well  
220 as in other neuronal populations known as innervating the striatum, such as cortical projection  
221 neurons and nigral dopaminergic cells (data not shown). Although the opsin will be expressed in all the  
222 systems innervating the virally-injected structure, restricted activation of the thalamic projection  
223 system will be achieved by light delivery into the CM/Pf. The limitation of this strategy might be the  
224 recruitment, besides CM/Pf neurons projecting to either targets, of axon collaterals within the CM/Pf  
225 of other neuronal systems projecting to the injected BG component. Previous work in the cat using  
226 dual retrograde tracing provided evidence for collateralized projections from the cerebral cortex to

227 the CM/Pf and the striatum (Royce, 1983; Paré and Smith, 1996), although the number of double  
228 labelled cortical neurons was few compared to the single labelled ones from either target (Royce,  
229 1983). To investigate the potential implication of CM/Pf collaterals of corticostriatal projections in our  
230 experimental paradigm, we labelled cortical terminals into the CM/Pf using VGLUT1 immunoreactivity  
231 as a specific marker in Rosa26-LoxP-stop-ChR2-EYFP mice after WGA-Cre viral injection into the  
232 striatum (Fig. 2B). VGLUT1 and EYFP immunostained profiles were spatially segregated, suggesting that  
233 cortico-CM/Pf fibers are not expressing ChR2 at detectable level following WGA-Cre viral delivery into  
234 the striatum and thus will not be recruited by CM/Pf light shining. The next step was to validate the  
235 functionality of the ChR2 expressed using the WGA system by performing single unit extracellular  
236 recordings of CM/Pf neurons. As illustrated in figure 2C, some of the recorded CM/Pf neurons were  
237 efficiently driven by 1 ms blue light pulses, and their juxtacellular labeling with neurobiotin showed  
238 that these light-driven neurons actually expressed ChR2-EYFP, confirming the efficiency of our  
239 optogenetic strategy.

240 Photo-activation of the CM/Pf neurons projecting to the striatum evoked responses in 22 out of 68  
241 recorded SNr cells (Fig. 3). Among the responding cells, 10 presented an inhibition followed by an  
242 excitation (Fig. 3A), 8 cells displayed only an inhibition (Fig. 3B) whereas the 4 remaining neurons  
243 showed only late excitation (not shown). The population PSTH in figure 3C evidences two components  
244 in the average response, namely an inhibition followed by an excitation, whose characteristics are  
245 detailed in figure 3D. It is to note that there was a competition between the inhibition and the following  
246 excitation. This was evidenced by the fact that *i*) the inhibition was longer ( $21.5 \pm 2.0$  ms vs.  $14.1 \pm 2.1$   
247 ms;  $p = 0.023$ ; Student's *t*-test) when it was not followed by an excitation ( $n = 8$ ) and *ii*) the excitation,  
248 when alone ( $n = 4$ ), had a shorter latency ( $18.1 \pm 5.0$  ms vs.  $35.2 \pm 4.2$  ms;  $p = 0.038$ ; Student's *t*-test),  
249 a tendency for a longer duration ( $23.0 \pm 4.8$  ms vs.  $14.3 \pm 1.7$  ms; n.s.  $p = 0.052$ ; Student's *t*-test) and a  
250 stronger amplitude ( $71.1 \pm 21.7$  spikes vs.  $19.7 \pm 4.4$  spikes;  $p = 0.02$ ; Mann-Whitney rank sum test).

251 Photo-activation of CM/Pf neurons projecting to the STN evoked responses in 8 out of 78 examined  
252 SNr cells (Fig. 4). These responses consisted of one (6/8) or two early excitations (2/8), followed, in  
253 most of the cases (n=5, 62.5 %), by an inhibition, as illustrated in figure 4A. The population PSTH (Fig.  
254 4B) further shows that the excitations are distributed in two peaks with latencies <5ms or ≥5ms.  
255 Characteristics of the population responses are detailed in figure 4C.

256 Photo-activation of CM/Pf neurons projecting to SNr evoked responses in 14 out of 53 recorded SNr  
257 cells (Fig. 5). These responses consisted of a single (12/14, Fig. 5A and 5B) or a double excitation (2/14)  
258 followed, in half of the cells, by an inhibition (Fig. 5B). Population PSTH in figure 5C evidences these  
259 excitations followed by the inhibition in the average population response. The distribution displays a  
260 peak with a shoulder reminiscent of the second peak of the trans-STN responses (≥5ms, see also Fig  
261 4C). We then used the same latency thresholds (<5ms or ≥5ms) to distinguish the two excitations and  
262 provide the characteristics of the population responses (Fig. 5D).

263

#### 264 **SNr responses evoked by global CM/Pf stimulation**

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

267 Responses evoked by electrical stimulation of the CM/Pf were analyzed in 54 SNr responding cells  
268 recorded in 18 mice (Fig. 6). They presented up to 4 components: one or two early excitations, an  
269 inhibition and a late excitation. Inhibition was recorded in the majority of the cells (n = 37 of 54; 68.5  
270 %), the most often (30 of 37), preceded by one or two short latency excitations (Fig. 6A-C). The  
271 inhibition was in some cases followed by a late excitation (13 out of 37 cells) (Fig. 6C). In the 17  
272 remaining SNr cells out of the 54 (31.5 %), excitation without inhibition were recorded, consisting in  
273 one (3 cells, Fig. 6D) or two (7 cells; Fig. 6E) short latency excitations the more often followed (9 cells)  
274 by a late latency excitation. All the components were found together in 17% (n = 9) of the responding

275 cells. Figure 6F provides the pie-chart of the different types of responses expressed as percent of total  
276 responding cells. The characteristics of each of these components were determined by pooling all  
277 evoked responses together to measure the latency (L) and the duration (D) of each events as well as  
278 the amplitude (A) of the excitations. Results are summarized in table 1.

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

285 The complex responses evoked by CM/Pf electrical stimulation in SNr neurons were reminiscent of the  
286 responses evoked by cortical stimulation (Maurice et al., 1999). Whether electrical stimulation of the  
287 CM/Pf recruits the cerebral cortex and remotely activates the corticostriatal pathway either  
288 orthodromically via thalamocortical projections, or through axonal reflex via cortical axons branching  
289 both in the thalamus and the striatum is an important matter of concern. The possibility of orthodromic  
290 recruitment is limited by the sparse innervation of the cerebral cortex from the CM/Pf. The above  
291 reported lack of EYFP expression in VGluT1-labelled terminals within the CM/Pf after virus injection in  
292 the striatum suggest that collaterals of corticostriatal projections are weak, if present, in mice; as  
293 previously reported in cat (Royce, 1983; Paré and Smith, 1996). To nevertheless exclude contribution  
294 of axonal reflex, we developed an optogenetic strategy to achieve selective activation the CM/Pf  
295 neurons and compared the elicited responses with those evoked by electrical CM/Pf stimulation. This  
296 was done by expressing ChR2-mCherry specifically in CM/Pf neurons by injecting Cre-dependent AAV5  
297 carrying the ChR2-mCherry into the CM/Pf of mice expressing Cre under the promoter of the CaMKII,  
298 a marker of projection neurons including those of the thalamus and the cerebral cortex. 5 weeks  
299 following AAV injections into the CM/PF, numerous mCherry-expressing neurons were found in the

300 CM/Pf (Fig. 7A), whereas no mCherry neuron was detected into the cerebral cortex (Fig. 7B),  
301 confirming that corticothalamic neurons in our experimental setting do not express ChR2. Moreover,  
302 contrasting with the dense network of fibers in the striatum, very sparse mCherry fibers were detected  
303 in the cerebral cortex (Fig. 7C), in compliance with the literature. Together, these data support the  
304 view that the responses driven by light delivery locally into the CM/Pf will result most directly from the  
305 activation of CM/Pf neurons.

306 Responses evoked by photostimulation of CM/Pf neurons were then investigated in 41 SNr responding  
307 cells recorded in 5 CaMKII<sup>cre/+</sup> mice (Fig. 8), 8-10 weeks post-AAV injection into the CM/Pf. Similar to  
308 the electrical stimulation, in most tested cells (n = 37 of 41 responding neurons; 90%),  
309 photostimulation of the CM/Pf induced an inhibition (Fig. 8A, 8B and 8C), that was, in 27 out of 37 of  
310 the cells, preceded and/or followed by excitation. In the 4 (10%) remaining cells, CM/Pf stimulation  
311 evoked only excitation (Fig. 8D and 8E). Figure 8F summarizes the different patterns of responses  
312 expressed as percent of total responding cells. Note that the variety and the distribution of the  
313 components were similar to those recorded under electrical stimulation (fig. 8F). As for the electrical  
314 stimulation, no obvious topographical distribution of the cells with distinct types of responses could  
315 be observed. Here again, the characteristics of each component were determined by pooling these  
316 complex responses altogether to calculate the latency and the duration of each component as well as  
317 the amplitude for the excitations. Table 1 summarizes the characteristics of each component of the  
318 responses evoked by electrical and optogenetic activation of CM/Pf. Differences between the two  
319 modes of stimulation were not statistically significant (n.s.; Mann-Whitney Rank Sum test).

320

### 321 **CM/Pf neurons retrogradely labelled from the STN and the SNr**

322 To investigate whether CM/Pf neurons projecting to the STN and to the SNr were branched, two  
323 fluorophore-conjugated variants of CTB were injected into the STN and the SNr in 3 mice. The  
324 populations labelled in the CM/Pf from either target show a weak overlap (Fig. 9). The observation of

325 such double-labelled neurons indicates that thalamosubthalamic and thalamonigral pathways arise, at  
326 least in part, from branched CM/Pf neurons projecting to both sites.

327

## 328 **DISCUSSION**

329 This study provides physiological evidence for functional connections between the CM/Pf and the main  
330 input (striatum and STN) and output (SNr) structures of the BG, and for possible convergence of the  
331 information conveyed through these pathways on single SNr neuron. They further indicate that the  
332 information processed through trans-striatal and extrastriatal projections arises from functionally  
333 segregated populations of CM/Pf neurons, whereas information conveyed through the STN and direct  
334 nigral projections originates from partly overlapping populations, as confirmed by retrograde tracing.

335 While highly reproducible in a given cell for a given setting of stimulation parameter, the responses  
336 were variable from cell to cell, as reported previously regarding the transfer of cortical information  
337 through the BG (Maurice et al., 1999). This variability is likely due to the topographical relationship  
338 between the stimulated CM/Pf region and the recorded SNr neuron. This interpretation is consistent  
339 with the observation that modifying the stimulation intensity or polarity can impact the response  
340 pattern, presumably due to the recruitment of a less or more extended region of the CM/Pf connected  
341 with the recorded neuron.

342 In the different stimulation paradigms tested, the responses were usually polymodal, suggesting the  
343 involvement of different subcircuits, even when targeting neurons identified based on their  
344 connectivity with a given component of the BG. This was expected for the neurons projecting to the  
345 striatum, as the information can be processed through the direct and the indirect trans-striatal  
346 pathways, but not for neurons projecting to the STN or SNr. For instance, optogenetic stimulation of  
347 the CM/Pf neurons projecting to the striatum elicited an inhibition, the most often followed by an  
348 excitation. These responses can be attributed to the recruitment of striatal medium spiny neurons



349 (MSNs) of the direct and of the indirect pathway, respectively, which exert inhibitory versus excitatory  
350 influence onto SNr neurons. Indeed, thalamostriatal terminals have been reported to target both types  
351 of MSNs, with the direct pathway MSNs receiving a greater density of thalamic synapses than those of  
352 the indirect pathway (Huerta-Ocampo et al., 2014), which could account for the more consistent  
353 recording of the inhibitory versus the late excitatory component of the responses. Activation of CM/Pf  
354 neurons projecting to either the STN or the SNr unexpectedly elicited complex responses, consisting  
355 of one or two early excitations followed in most cases by a brief inhibition. The lack of such early  
356 excitation component in the responses elicited by the activation of CM/Pf neurons projecting to the  
357 striatum suggests that these neurons do not provide significant functional input to the STN or the SNr.  
358 This complies with and extends previous observations in the rat (Féger et al., 1994) and the mouse  
359 (Watson et al., 2021) showing that the CM/Pf projections to the STN and striatum arise mostly from  
360 separate neuronal populations. Indeed, they emphasize a functional segregation of CM/Pf information  
361 processing via the striatum and extrastriatal sites. Such a segregation supports the view of differential  
362 implication in BG functions, as recently exemplified by the demonstration of the contribution of CM/Pf  
363 projections to STN but not to striatum in movement initiation (Watson et al., 2021). The fact that the  
364 activation of CM/Pf neurons projecting to either the STN or the SNr triggers excitations with similar  
365 bimodal distribution, or even two excitations in the same cell, suggests that the two structures are  
366 connected (at least in part) by the same thalamic neuronal population. This is confirmed by our dual  
367 retrograde fluorescent labeling using CTB, showing that some CM/Pf neurons project both to the STN  
368 and the SNr. Therefore, stimulation of CM/Pf neurons projecting to either the STN or the SNr would  
369 trigger both a monosynaptic and a di-synaptic response. These responses will involve respectively  
370 collateralized and non-collateralized projections for STN and vice versa for SNr. Regarding the brief  
371 inhibition that in most cases follows the early excitatory responses, it could result from the recruitment  
372 of the potent inhibitory microcircuit formed by the dense network of intranigral recurrent collaterals  
373 (Deniau et al., 1982; Brown et al., 2014). One cannot exclude, however, the contribution of the  
374 thalamic input to the globus pallidus, which could result in SNr inhibition either directly or via STN

375 inhibition in our experimental paradigm if CM/Pf projections to the STN and the SNr are collaterals of  
376 projections to the globus pallidus. Finally, the recently evidenced efferent projections of the STN  
377 neurons innervated by the CM/Pf onto the globus pallidus in addition to the SNr (Watson et al., 2021)  
378 might also be involved.

379 After having characterized the responses elicited in the SNr by optogenetic activation of neuronal  
380 subpopulations of CM/Pf distinguished on the basis of their connectivity with the striatum, the STN  
381 and the SNr, we aimed at examining whether and how the information processed through these  
382 pathways are integrated in the BG output structure. To do so, we examined the responses evoked by  
383 global activation of CM/Pf neuronal populations either electrical or by optogenetics. The response  
384 patterns were highly similar in the two paradigms, excluding major contribution, in the responses to  
385 electrical CM/Pf stimulation, of axonal reflex involving pathways collateralizing onto both CM/Pf and  
386 a BG component, as the projections from the cerebral cortex that branch in the CM/Pf and striatum  
387 (Paré and Smith, 1996). These responses were in most cases multiphasic, the more complex including  
388 4 components, namely two early excitations followed by an inhibition and a late excitation. Given the  
389 connectivity-based responses reported above, the early excitations are likely to result from the  
390 recruitment of the trans-subthalamic and direct nigral pathways, the inhibition from recruitment of  
391 the trans-striatal direct pathway (presumably reinforced by local inhibitory microcircuits following SNr  
392 activation or recruitment of globus pallidus), and the late excitation from the activation of the indirect  
393 trans-striatal pathway. Taken as a whole, these data suggest that, although CM/Pf projections to  
394 extrastriatal structures are weak in terms of density of innervation, as compared to the massive  
395 thalamostriatal pathway, they should not be considered as functionally less important, since they have  
396 the potential to shape the activity of SNr neurons. Previous work in monkey called for a reappraisal of  
397 the functional weight of extrastriatal CM/Pf projections based on the observation that their long  
398 arborization, although poorly branched, covered a relatively large portion of each BG nucleus (Tandé  
399 et al., 2006). Moreover, in the rat, axons from the CM/Pf have been shown to contact more proximal  
400 dendrites of STN neurons (Bevan et al., 1995), which places them in position to exert potent action

401 onto the contacted neurons. If this is a general feature of extrastriatal projections, thalamic axons  
402 could then exert powerful action on the different components of the BG network.

403 In conclusion, our study shows that the trans-striatal, trans-subthalamic and direct nigral pathways  
404 formed from CM/Pf neurons are functional and although more or less segregated can provide  
405 converging information onto SNr neurons. The responses to global CM/Pf activation in the SNr thus  
406 provide a read-out of the functionality of the different subcircuits by which information from the  
407 CM/Pf can be processed through the BG to regulate the network outflow, which might be useful to  
408 detect changes in these circuits occurring in different treatment or pathological conditions, as  
409 previously done for the cortically-driven BG circuits. Here, CM/Pf neuronal populations were activated  
410 artificially either together or separately based on their connectivity, and what remains to be  
411 determined is whether and when these different components of the thalamo-BG circuits are recruited  
412 under physiological conditions in connection with behavior. Recent evidence has been provided for  
413 converging thalamic and cortical information onto direct and indirect MSNs in the striatal matrix, with  
414 a predominance of the cortical input (Huerta-Ocampo et al., 2014). Moreover, distinct cortical-  
415 thalamic-striatal circuits through the CM/Pf have been revealed, placing this thalamic complex as a  
416 critical node that organizes limbic, associative and somatosensory information in the BG (Mandelbaum  
417 et al., 2019). Whether cortical and thalamic projections to STN neurons are also organized in such  
418 sophisticated circuits would need to be investigated. In addition, CM/Pf is in position to also provide  
419 direct information to the output structures of the network. Characterizing the information that flows  
420 within the BG through striatal and extrastriatal thalamic pathways is then a major issue that needs to  
421 be addressed to fully appreciate CM/Pf implication in BG functioning in physiological and pathological  
422 conditions.

423

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525



526 **FIGURE LEGENDS:**

527 **Figure 1.** Schematic of the strategy used to assess the functionality of the thalamostriatal,  
528 thalamosubthalamic and thalamonigral pathways. In three different sets of experiments, expression  
529 of WGA-Cre was achieved by viral delivery of mCherry-IRES-WGA-Cre into the striatum (1), the  
530 subthalamic nucleus (STN; 2) or the substantia nigra pars reticulata (SNr, 3) in Rosa26-LoxP-stop-ChR2-  
531 EYFP mice. ChR2 is expressed in the locally transduced neurons, and after transneuronal retrograde  
532 transport of WGA-Cre, in the connecting ones, including the CM/Pf projection neurons. Light was then  
533 delivered in the CM/Pf to activate the neuronal subpopulation connecting the virally-injected basal  
534 ganglia target, and the evoked responses were recorded in the SNr.

535 **Figure 2.** Optogenetic strategy to activate subpopulations of CM/Pf neurons distinguished on the basis  
536 of their connectivity with BG components: Example of the thalamostriatal neurons. **A**,  
537 Channelrhodopsin expression in the CM/Pf as revealed by EYFP immunodetection following injection  
538 of AAV carrying the EF1 $\alpha$ ::mCherry-IRES-WGA-Cre cassette in the striatum (maximum intensity  
539 projection, 40  $\mu$ m depth). A strong labelling was observed in the ipsilateral CM/Pf (**A1** and **A3** at higher  
540 magnification) but not in the contralateral side (**A2**). **B1**, Relationship between EYFP expressing  
541 neurites and VGLUT1 expressing axon terminals (single confocal section). **B2** and **B3**, higher  
542 magnification of the boxed area in **B1** showing the absence of EYFP immunolabelling in VGLUT1 axon  
543 terminals. **B4**, Colocalization analysis of EYFP and VGLUT1 fluorescence confirms that the two markers  
544 are spatially segregated. **C**, Efficiency of the optogenetic strategy. Raw trace recording of a CM/Pf  
545 neuron (**C1**) whose firing was driven by 1 ms-blue light pulses (10 Hz). The cell body of this recorded  
546 neuron juxtacellularly labeled with neurobiotin (NBT, red) also shows EYFP immunolabelling (green)  
547 indicating that it actually expressed ChR2 (**C2**). fr: *fasciculus retroflexus*.

548 **Figure 3.** Patterns of responses evoked in the SNr by the photoactivation of CM/Pf neurons projecting  
549 to the striatum. Raster plots and PSTH illustrating examples of evoked responses that consisted in  
550 either an inhibition followed by an excitation (**A**) or a single inhibition (**B**). **C**, Population PSTH of the

551 overall responses evoked in SNr neurons (n = 18). PSTHs, generated from 140 stimulation trials, are  
552 aligned on the light pulse delivery (vertical blue line) and the light shaded color represents SEM. **D**,  
553 Characteristics of the responses evoked.

554 **Figure 4.** Patterns of responses evoked in the SNr by the photoactivation of CM/Pf neurons projecting  
555 to the STN. **A**, Raster plot and PSTH of an evoked response consisting in an excitation followed by an  
556 inhibition. **B**, Population PSTH of the responses evoked in SNr neurons (n = 8) expressed as in Figure 3.  
557 **C**, Characteristics of the responses evoked.

558 **Figure 5.** Patterns of responses evoked in SNr by the photoactivation of CM/Pf neurons projecting to  
559 the SNr. The evoked responses consisted in early excitations (**A**) that were often followed by an  
560 inhibition (**B**). **C**, Population PSTH of the responses evoked in SNr neurons (n = 14) expressed as in  
561 Figure 3. **D**, Characteristics of the responses evoked.

562 **Figure 6.** Patterns of responses evoked in SNr cells by CM/Pf electrical stimulation. **A-E**, Representative  
563 PSTHs illustrating the diversity of the responses recorded in individual SNr neurons; red lines indicate  
564 the time of electrical shock delivery. **F**, Pie-chart illustrating the percentage of each type of response  
565 exhibited by the overall responding cells (n = 54).

566 **Figure 7.** Representative photomicrographs of the typical mCherry expression patterns in frontal  
567 sections at the level of the CM/Pf (**A**), the cerebral cortex (**B**) and the striatum (**C**) of a transgenic  
568 CaMKII<sup>cre/+</sup> mice following AAV-ChR2-mCherry virus injection into the CM/Pf. mCherry expression is  
569 revealed by immunostaining with an antibody against RFP (red). The right panels (scale 10 µm) are  
570 magnification of the boxed areas in the corresponding left panels (scale 100 µm). Both labelled cell  
571 bodies and neurites were observed in the CM/Pf (**A1, A2**) whereas the cerebral cortex (**B1, B2**) and the  
572 striatum (**C1, C2**) contained only axonal fibers. Note the very sparse cortical staining compared with  
573 the dense striatal labelling. cc: *corpus callosum*; fr: *fasciculus retroflexus*; STR: striatum.

574 **Figure 8.** Patterns of responses evoked in SNr cells by CM/Pf photostimulation. **A-E**, Representative  
575 PSTHs illustrating the diversity of the responses evoked in distinct SNr cells and their similarity with  
576 those induced by CM/Pf electrical stimulation (see Fig. 6); Blue lines indicate the time of light flash (1  
577 ms) delivery. **F**, Pie-chart of the percentage of each type of response in the overall responding cells (n  
578 = 41).

579 **Figure 9.** Confocal image of retrogradely labelled cell bodies in the CM/Pf after Alexa Fluor 488-CTB  
580 injection into the STN (green) combined with Alexa Fluor 594-CTB injection into the SNr (magenta). **A**,  
581 merged image; **B**, **C**, single channels. The dually labelled cells are indicated by arrows. Scale bar: 20  
582  $\mu\text{m}$ .

583

584 **Table 1.** Characteristics of the responses evoked on SNr cells by electrical or optical stimulation of  
 585 the CM/Pf.

	Early excitation			2 <sup>nd</sup> excitation			Inhibition		Late excitation		
	L (ms) (N)	D (ms) (N)	Nb Sp (N)	L (ms) (N)	D (ms) (N)	Nb Sp (N)	L (ms) (N)	D (ms) (N)	L (ms) (N)	D (ms) (N)	Nb Sp (N)
Electrical stim. (54)	4.4±0.4 (27)	6.1±0.7 (27)	40.0±6.2 (27)	10.6±0.6 (26)	6.6±0.6 (26)	39.9±5.0 (26)	14.8±1.0 (37)	18.4±2.2 (37)	29.4±2.6 (22)	20.3±4.5 (22)	62.1±17.2 (22)
Optical stim. (41)	4.3±0.3 (19)	7.2±0.7 (19)	60.7±10.6 (19)	8.4±0.9 (11)	6.6±0.7 (11)	52.4±11.4 (11)	20.1±1.0 (36)	20.5±1.4 (36)	42.7±3.1 (8)	18.5±2.8 (8)	39.0±8.3 (8)

586