

# 1 Mesodiencephalic junction Gabaergic inputs are 2 processed separately from motor cortical inputs in the 3 basilar pons

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## 11 Abstract

12 The basilar pontine nuclei (bPN) receive inputs from the entire neocortex and constitute the main source  
13 of mossy fibers to the cerebellum. Despite their critical position in the cortico-cerebellar pathway, it  
14 remains unclear if and how the bPN process inputs. An important unresolved question is whether the bPN  
15 strictly receives excitatory inputs or also receives inhibitory inputs. In the present study, we identified the  
16 mesodiencephalic junction as a prominent source of GABAergic afferents to the bPN. We combined  
17 optogenetics and whole-cell patch clamp recordings and confirmed that the bPN indeed receives  
18 monosynaptic GABA inputs from this region. Furthermore, we found no evidence that these inhibitory  
19 inputs converge with motor cortex (M1) inputs at the single neuron level. We also found no evidence of  
20 any connectivity between bPN neurons, suggesting the absence of a local circuit. Finally, rabies tracings  
21 revealed that GABAergic MDJ neurons themselves receive prominent inputs from neocortical output  
22 neurons. Our data indicates that inhibition from the MDJ, and excitation from the neocortex remain  
23 separate streams of information through the bPN. It is therefore unlikely that inhibition in the bPN has a  
24 gating function, but rather shapes an appropriate output of the bPN during behavior.

25

## 26 Introduction

27 Motor control relies on brain-wide networks. Motor cortex directs voluntary movements (Guo et  
28 al. 2015) and the cerebellum coordinates movements (Manto et al. 2012). Reciprocal  
29 connections between these structures are necessary for proper motor control. Indeed, the  
30 cerebellum projects to motor cortex via the thalamus (Sawyer et al. 1994; Aumann 2002;  
31 Gornati et al. 2018), while motor cortex projects to the cerebellum via the pontine nuclei  
32 (Schwarz and Mock 2001; Kratochwil et al. 2017). This closed-loop connectivity enables forward  
33 and inverse models for motor control (Wolpert et al. 1998; Shadmehr and Krakauer 2008).  
34 Interestingly, other parts of neocortex and cerebellum are also connected (Kelly and Strick  
35 2003; Henschke and Pakan 2020; Pisano et al. 2021), enabling similar computational  
36 mechanisms for motor control and cognitive processes alike.

37 This places the pontine nuclei at the nexus of information transfer between neocortex and  
38 cerebellum. Indeed, the mossy fiber afferents from the basilar pontine nuclei (bPN) to the

39 cerebellum is one of the largest fiber tracts in the brain. Additionally, the bPN also receives  
40 inputs from numerous non-neocortical regions of the brain (Burne et al. 1981; Wiesendanger  
41 and Wiesendanger 1982; Kosinski et al. 1986; Mihailoff et al. 1988, 1989). These non-cortical  
42 and corticopontine afferents show a topographical organization with minimal regional overlap  
43 within the bPN (Leergaard and Bjaalie 2007; Proville et al. 2014; Kratochwil et al. 2017).  
44 Similarly, mossy fibers originating from the bPN project to specific zones in the cerebellum  
45 (Päällysaho et al. 1991; Mihailoff 1993; Odeh et al. 2005; Huang et al. 2013; Kratochwil et al.  
46 2017). Therefore, the bPN is often not considered to have an active role in integration of  
47 information, but is often considered to be a relay for information destined for the cerebellum.

48 Still, synaptic plasticity of inputs to the bPN has been described, suggesting a potential way of  
49 processing of inputs to bPN neurons (Möck et al. 1997), potentially shaping spiking activity in  
50 the bPN (Schwarz et al. 1997; Möck et al. 2006; Guo et al. 2021). Additionally, various extrinsic  
51 sources of inhibition to bPN neurons have been suggested (Border et al. 1986; Mihailoff and  
52 Border 1990; Möck et al. 1999), but these sources of GABAergic inputs to the bPN have never  
53 been physiologically confirmed or characterized, precluding conclusions about their function  
54 and integration in the cerebro-cerebellar circuit.

55 Here we identify the mesodiencephalic junction (MDJ) as the main source of GABAergic  
56 signaling to the bPN. This inhibition does not seem to interact with afferents from motor cortex,  
57 even though their projections overlap in the bPN. In contrast to the strongly depressing motor  
58 cortex inputs, GABAergic inputs from MDJ show remarkably little short-term depression.  
59 Finally, using rabies-tracing we show that pontine-projecting MDJ neurons receive prominent  
60 neocortical inputs, similar to bPN neurons themselves. These results suggest that the bPN  
61 contains separate streams for processing information from neocortex directly, and sign-inverted  
62 neocortical inputs.

63

## 64 **Materials & Methods**

### 65 **Animals**

66 Male and female wt C57BL/6J mice were used for acute slice experiments. Animals were housed  
67 socially (max. four per cage) and had ad libitum access to chow and water. All experimental  
68 procedures were approved by the Central Authority for Scientific Procedures on Animals and  
69 local animal welfare body of the VU University and VU University Medical Center (Amsterdam,  
70 Netherlands) and carried out in accordance with European and Dutch law.

### 71 **Intracranial virus and tracer injections**

72 Microinjection needles were pulled from 3.5" borosilicate glass capillaries (Drummond SCI,  
73 USA) on a Sutter P-87 puller (Sutter, CA) and backfilled with mineral oil before virus solution  
74 was loaded. AAV9 viruses were purchased from Addgene (USA) syn.Chronos-GFP.WPRE.bGH  
75 and syn.ChrimsonR-tdTomato.WPRE.bGH were injected at  $4 \cdot 10^{12}$  vg/ml titer and  $1.5 \cdot 10^{12}$  vg/ml  
76 respectively. Retrograde AAV2 virus was purchased from University of Zurich vector core.  
77 AAV2r-hSyn1-chI-iCre-WPRE-SV40 was injected at a titer of  $7.9 \cdot 10^{12}$  vg/ml. Rabies virus  
78 (Rabies-SAD-dG-tdTomato) and AAV helper virus (rAAVdj-hsyn1-dlox-TVA-2A-EGFP-2a-  
79 oG(rev)-dlox-WPRE-bGhp(A)) were a generous gift from Klaus Conzelmann. All mice used for  
80 optogenetic experiments received intracranial virus injections at postnatal 21. For all surgeries,  
81 mice received Carprofen (5 mg/kg s.c.) and Buprenorphine (50 µg/kg s.c.) pre-operatively. A  
82 second Carprofen injection (5 mg/kg s.c.) was administered twenty-four hours post-surgery.  
83 Animals were kept under general anesthesia during surgery with Isoflurane (0.5% - 1%). Ear  
84 bars were placed to secure the skull, a small amount of Lidocaine cream was applied before  
85 placement. Local analgesia was applied by injecting a small volume of Lidocaine (2%)  
86 underneath the scalp before incising the skin. The scalp was cut and folded open to expose the  
87 skull, holes were drilled to access the injection sites, and virus was delivered via injection.  
88 (relative to bregma(Paxinos and Watson 1998) (in mm), M1: AP 1.30; ML 1.08L; DV 1.20, MDJ:  
89 AP -3.50; ML 0.50L; DV 3.00 Cerebellum: AP -6.2; ML 1.5R; DV 2.0 bPN: AP-4.0 ML 0.5L DV  
90 5.5). For optogenetic experiments, total volume of 500 nl was injected per site in steps of 50  
91 nl/min using a Nanoject II (Drummond SCI, USA) set to the 'slow' rate (23 nl/sec). The  
92 microinjection needle was left in place for 5 minutes before and after injection. Mice were  
93 sacrificed for acute slice experiments at least two weeks after viral injection to allow for  
94 adequate expression. For tracing experiments, total volumes between 10 and 100nl were  
95 injected per site and needles were left in place for 15 minutes before retraction. Retrobead  
96 transport was assessed after 14 days. For rabies tracing animals were injected with AAV to  
97 express cre, oG and TVA in one surgery. After 1 week rabies virus was injected, after which we  
98 waited another week before animals were perfused with 4% formaldehyde solution in 0.1M  
99 phosphate-buffered saline (PBS) for analysis.

### 100 **Acute slice preparation**

101 Acute slices were prepared for optogenetic experiments (sagittal orientation) and paired  
102 recordings (sagittal or coronal). Before decapitation, mice first received a lethal pentobarbital  
103 injection (120 mg/kg i.p.) and were perfused with ice cold N-Methyl- D -glucamine (NMDG)  
104 solution containing (in mM): NMDG 93, KCl 2.5, NaH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 30, HEPES 20, Glucose

105 25, sodium pyruvate 3, sodium ascorbate 5, MgSO<sub>4</sub> 10, CaCl<sub>2</sub> 0.5, adjusted to 315 mOsm ± 5 and  
106 pH 7.3. After decapitation, the brain was removed from the skull and sliced in the same  
107 oxygenated ice-cold NMDG solution. Brains were sliced using a ceramic blade (Campden  
108 Instruments ltd., England) and slices (250 μm) were collected in an oxygen-perfused brain slice  
109 chamber filled with a holding solution containing (in mM): NaCl 92, KCl 2.5, NaH<sub>2</sub>PO<sub>4</sub> 1.2,  
110 NaHCO<sub>3</sub> 30, HEPES 20, Glucose 25, sodium pyruvate 3, sodium ascorbate 5, MgSO<sub>4</sub> 10, CaCl<sub>2</sub>  
111 0.5, adjusted to 305 ± 5 mOsm. Slices were kept oxygenated at room temperature until the  
112 moment of recording.

113

### 114 **Acute slice whole-cell recordings**

115 During all acute slice experiments, whole-cell recordings were acquired at a temperature of 33 ±  
116 1 °C. Brain slices were placed in a bath continuously perfused with oxygenated ACSF containing  
117 (in mM): NaCl 125, KCl 2.5, NaH<sub>2</sub>PO<sub>4</sub> 1.25, NaHCO<sub>3</sub> 26, Glucose 25, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 1.3, adjusted  
118 to 305 ± 5 mOsm. Borosilicate glass capillaries were pulled to produce patch-pipettes with a  
119 resistance of 3 – 6 MΩ. For optogenetic experiments, patch-pipettes were filled with a cesium  
120 methanesulfonate-based pipette solution containing (in mM): CsMethanesulfonate 115, TEA 25,  
121 HEPES 10, EGTA 0.2, QX-314 Cl 5, NaCl 4, MgATP 2, Na<sub>3</sub>GTP 0.4, Na<sub>2</sub>Phosphocreatine 10. For  
122 paired patch-clamp experiments, patch-pipettes were filled with a potassium gluconate-based  
123 solution containing (in mM): KGluconate 135, KOH 31, NaCl 10, HEPES 10, EGTA 10, Na<sub>2</sub>ATP  
124 4, Na<sub>3</sub>GTP 0.4. Both internal solutions were adjusted to pH 7.2 and 310 mOsm. Biocytin  
125 (0.05%) was added to internal solution on the day of the experiment. Cells were loaded with  
126 biocytin during whole-cell patch clamp recordings and resealed at the end of the experiment.  
127 Slices were then transferred to paraformaldehyde (PFA, 4%) and fixed for at least 48 hours.

### 128 **Optogenetic stimulation**

129 Optogenetic responses were evoked using a 4-channel LED system (DC4100 & LED4D114;  
130 Thorlabs inc., USA). Cells were voltage clamped at -70 and 0mV and screened for responses  
131 using full field 100 ms optical stimulation at all four wavelengths (405, 470, 505 & 590nm).  
132 When responses (EPSCs or IPSCs) were observed, the light source was restricted to a small  
133 beam (±100μm diameter) with high intensity (>100mW/mm<sup>2</sup>)(Jackman et al. 2014) to allow  
134 reliable axonal stimulation of afferents. Optical stimulation was delivered in trains of twenty  
135 pulses with a 10 second intertrain interval, and was alternated per sweep in a pseudorandom  
136 order (20 – 100 – 50 – 10Hz). All input characterizations are based on afferents expressing  
137 Chronos. A short negative voltage (50 ms, -10 mV) was injected at the start of each sweep to  
138 monitor access resistance throughout the experiment. Voltage clamp recordings were acquired  
139 at a 50.0 kHz sample rate with a 10 kHz low pass filter. Cells from optogenetic experiments were  
140 analyzed on the following conditions: (1) optical stimulation at 470 or 590 nm evoked a  
141 response at -70 mV or 0 mV holding potential; (2) at least nine sweeps per frequency were  
142 collected. Responses following stimulation were defined as optogenetically evoked inputs if they  
143 exceeded the threshold set at 2σ of the baseline. Responses that did not reach the computed  
144 threshold were not considered in the analysis. Response amplitudes were computed on averaged  
145 sweeps. The peak amplitude was detected within an eight-millisecond time window after each  
146 light pulse. Then, the response was determined by calculating the average maximum amplitude

147 over a one-millisecond time window of the peak amplitude. Baseline was defined as the average  
148 amplitude over a two-millisecond time window before optic stimulation.

### 149 **Paired recording**

150 Sagittal or coronal slices were prepared for paired recordings. Up to three neurons were  
151 recorded at the same time, and potential connections between neurons were probed by evoking  
152 spike trains successively in each neuron. Ten action potentials were evoked presynaptically  
153 using current injections of 2nA at 50Hz, followed by a single current injection after 500 ms.  
154 Cells were kept at or around resting membrane potential throughout recording to detect EPCs.  
155 Current clamp recordings were acquired at a 50.0 kHz sample rate with a 10 kHz low pass filter.  
156 We did not compensate for the liquid junction potential.

157 Cells from paired whole-cell patch clamp experiments were analyzed when: (1) stimulation  
158 evoked action potentials (APs); (2) cells did not have a negative leak current exceeding 500 pA;  
159 (3) recordings had a stable resting membrane potential; (4) at least fifteen sweeps were  
160 collected. To detect connections, we looked for EPSCs in the average postsynaptic response in  
161 the first 18ms after the AP to accommodate for mono- and disynaptic connections. Then, the  
162 postsynaptic response was determined by calculating the average amplitude over a one-  
163 millisecond time window of the peak amplitude.

### 164 **Pharmacology**

165 Gabazine (10  $\mu$ M) was bath-applied to inhibit post-synaptic GABA<sub>A</sub> responses. AMPA receptors  
166 were inhibited with 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10  $\mu$ M). Glycine receptors were  
167 inhibited by application of Strychnine (1  $\mu$ M). TTX (1  $\mu$ M) was applied to inhibit voltage-gated  
168 sodium channels. Voltage-gated potassium channels were inhibited with 4-Aminopyridine (4-  
169 AP, 100  $\mu$ M). All antagonists were bath-applied and perfused at least five minutes before the  
170 start of a recording.

### 171 **Histology**

172 For neurons recorded in vitro, slices were first washed in phosphate-buffered saline (PBS, 0.1 M,  
173 3x15') containing (in mM): NaCl 137, KCl 2.7, NaH<sub>2</sub>PO<sub>4</sub> 12, KH<sub>2</sub>PO<sub>4</sub> 1.8. Then, slices were  
174 permeabilized in Triton-X (PBS-T, 0.5%; 2h). Following permeabilization, slices were again  
175 washed in PBS (3x15') and stained with Streptavidin-Alexa 647 (1:500 in 0.5% PBS-T). Finally,  
176 slices were washed in PBS (4x15') and embedded in Mowiol (2%) on glass microscope slides for  
177 confocal imaging. Tracer-injected brains were washed in 0.1M PBS and embedded in 11% gelatin  
178 for whole-brain sectioning. 50-100 $\mu$ m sections were made on a Leica VS1000 vibratome and  
179 collected directly to glass slides (retrobead tracing, coronal slices), or in 3 jars per side (sagittal  
180 slices, 6 jars total per brain). Staining for GAD was performed on one jar per side of the brain,  
181 yielding 24-30 sections for analysis. Sections were washed 3x5' in PBS with 0.025% Triton-X,  
182 blocked for 30' (PBS+0.025%TX and 5% normal Donkey Serum), and subsequently incubated  
183 with GAD67 antibody O/N (1:500 MAB5406, Merck-Millipore) in PBS. The next day sections  
184 were washed 3x5' in PBS, incubated for 2 hours with Alexa647 secondary (Goat anti mouse,  
185 A21235, ThermoFisher), again washed 3x5' in PBS and mounted on slides with mowiol (2%).  
186 Cells recovered from in vitro recordings, and sections from rabies tracing experiments were  
187 imaged on a confocal microscope (Nikon), retrobead tracing was visualized on a epifluorescence  
188 microscope (Zeiss). Retrobead tracing was analyzed by first marking all labeled neurons via

189 cellcounter in matlab (<https://github.com/molgen.mpg.de/MPIBR/CellCounter>) and then aligning  
190 sections while the wholebrain tool in R (Fürth et al. 2018). Sections between B+3.0 and B-5.6  
191 were aligned to the Allen Brain Atlas.

192 All display items show mean  $\pm$  SEM unless noted otherwise.

## 193 Results

194 To anatomically reveal cortico-cerebellar pathways that run via pontine nuclei, we first injected  
195 retrobeads into the cerebellum in p21 mice (**Figure 1**). Injections were done in the white matter  
196 of paravermal lobule 5 (**Figure 1A**, N=2) and retrograde labeling was photographed after 14  
197 days. As expected, retrograde labeling from the cerebellum could be observed in the inferior  
198 olivary nucleus, external cuneate nucleus, lateral reticular nucleus and basilar pontine nucleus  
199 (**Figure 1B**), but labeling was completely absent from cerebral cortex (**Figure 1B**). To  
200 investigate inputs to the basilar pontine nuclei, we injected retrobeads into the basilar pontine  
201 nuclei of p21 mice (**Figure 1C**, N=2). Injections were confined to the basilar pons, with minimal  
202 invasion of overlying structures (**Supplementary Figure 1**). We observed predominantly  
203 inputs from the ipsilateral side of the brain (**Figure 1G**), with almost all labeling (90% of  
204 retrogradely labeled neurons) in deep layers (**Figure 1F**) throughout the neocortex (**Figure**  
205 **1E**). Of other areas providing afferents to the bPN midbrain was most prominent (5%, with the  
206 mesodiencephalic junction, MDJ, being particularly prominent with 3% of brain-wide retrobead  
207 signal, **Figure 1H**), followed by thalamus and hypothalamus (3%), **Figure 1E**).

208 To characterize short-term plasticity of cortico-pontine synapses, we injected AAV to drive  
209 expression of Chronos-GFP in the M1 region to enable visualization and stimulation of M1-  
210 specific afferents in the bPN. Whole cell recordings from bPN neurons were made in sagittal  
211 slices, in which axons from M1 were stimulated with short pulses of blue light focused in a small  
212 spot (100  $\mu\text{m}$  diameter)  $>500\mu\text{m}$  away from the soma of the recorded neuron (**Figure**  
213 **2A,B**)(Jackman et al. 2014). All inputs evoked from motor cortex had a short delay to onset ( $2.4$   
214  $\pm 0.06$  ms), a fast rising phase and decay (10-90%:  $1.0 \pm 0.22$  ms and 90-10%:  $20 \pm 12$  ms,  
215 respectively), and were reduced to  $4.4 \pm 0.8\%$  of the original response by the AMPA and kainate  
216 receptor antagonist DNQX (10  $\mu\text{M}$ ; ACSF:  $20 \pm 5$  pA; DNQX:  $1.0 \pm 0.3$  pA;  $p < 0.001$ ;  $n = 4$   
217 neurons; **Figure 2C**), confirming that neocortical inputs to bPN are glutamatergic. During train  
218 stimulation we observed prominent short-term synaptic depression of M1 inputs across all  
219 frequencies, with more pronounced depression at higher frequencies and later in the stimulus  
220 train (**Figure 2E**,  $n=4$  cells in  $n=4$  animals). To check for possible opsin-specific influences on  
221 midbrain inputs, we repeated these experiments with ChrimsonR instead of Chronos expressed  
222 in M1. In these experiment we noticed that at higher stimulation frequencies Chrimson-evoked  
223 responses were more depressed after a train of stimuli (**Supplementary figure 2**). Since this  
224 is likely due to incomplete recovery of the channelrhodopsin variant (Klapoetke et al. 2014), we  
225 continued our characterization of short-term plasticity of cortico-pontine synapses using  
226 Chronos. In animals in which Chronos was expressed in motor cortex, synaptic responses  
227 recorded in the bPN were attenuated at 50 and 100Hz after a 20-pulse train stimulus to  $0.7 \pm$   
228  $0\%$  and  $0 \pm 0\%$  of initial amplitude respectively (steady-state, average of last five responses).  
229 After train stimuli at 10 and 20Hz, responses were attenuated to  $41 \pm 3\%$  and  $22 \pm 2\%$   
230 respectively. To confirm that neocortex makes monosynaptic connections to the bPN, we  
231 applied the voltage-gated sodium channel blocker tetrodotoxin (TTX) to block AP-generated  
232 neurotransmitter release, followed by the combined application of TTX and 4-aminopyridine (4-  
233 AP). As expected, optogenetically-evoked responses were virtually absent in the presence of TTX  
234 (reduced to  $2.2 \pm .2\%$  of the original response; ACSF:  $50 \pm 18$  pA; TTX:  $1.0 \pm 0.7$  pA;  $n=3$ ;  
235  $p < 0.001$ . Subsequent addition of 4-AP, which blocks voltage-gated potassium channels and  
236 prolongs optogenetically-evoked depolarization, recovered the synaptic responses ( $130 \pm 56\%$  of

237 amplitude in ACSF; TTX + 4-AP:  $50 \pm 28$  pA,  $n=3$ ;  $p=0.66$  vs ACSF; **Figure 2D**). These results  
238 show that motor cortex provides prominent, but strongly depressing monosynaptic  
239 glutamatergic inputs to bPN neurons.

240 Our retrograde tracing experiments (**Figure 1**) have shown that in addition to several  
241 neocortical regions, several subcortical brain regions provide inputs to the bPN. These  
242 subcortical brain regions might provide non-glutamatergic inputs, as has been suggested before  
243 (Border et al. 1986; Mihailoff et al. 1988, 1989; Mihailoff 1995). Furthermore, several studies  
244 have consistently reported the presence of GABAergic boutons in the bPN, suggesting a possible  
245 role of inhibitory signaling (Mihailoff and Border 1990). However, the exact source and  
246 potential role of these inputs has not been elucidated, nor electrophysiologically isolated and  
247 characterized. To investigate this in the bPN, we stained sections of mouse brain using an  
248 antibody against the enzyme Glutamate decarboxylase 67 (GAD67), which is present in somata  
249 and synapses of GABAergic neurons. In these animals we never observed GAD67+ somata, but  
250 we did observe prominent and numerous GAD67+ boutons (**Supplementary Figure 3A,B**;  $N$   
251 = 6 mice). Similarly, in GAD-GFP mice (Chattopadhyaya et al. 2004), we never observed GFP+  
252 somata, but we observed putative axons stained for GFP in the bPN (**Supplementary Figure**  
253 **3C**;  $N = 4$  mice). This indicates that there is a prominent extranuclear source of GABA to the  
254 bPN. Closer investigation of the afferent areas in midbrain revealed that the majority of inputs  
255 from midbrain arose from the mesodiencephalic junction (MDJ) (3% of all projections to the  
256 bPNm, figure 1H), an area intimately involved with the cerebellar circuit (Ruigrok 2004). Even  
257 though glutamatergic neurons of the MDJ project to the inferior olive (de Zeeuw et al. 1989;  
258 Ruigrok and Voogd 1995), these neurons are positioned intermixed with neurons that contain  
259 other neurotransmitters (De Zeeuw and Ruigrok 1994).

260 To confirm that the MDJ is the source of GABAergic inhibition to the bPN, we injected AAV to  
261 express Chronos-GFP in this region. In acute slices we performed whole cell recordings in the  
262 bPN in the area that receives afferents from motor cortex. In these experiments we observed  
263 prominent outward currents in neurons held at 0 mV, with a short rise, and long decay ( $2.1 \pm$   
264  $0.36$  ms and  $140 \pm 46$  ms, respectively) when stimulating with light (**Figure 2F**). These inputs  
265 were reduced to  $9 \pm 19\%$  in the presence of the GABA receptor antagonist Gabazine (ACSF:  $20$   
266  $\text{pA} \pm 11$  pA; Gabazine:  $0.5 \pm 0.41$  pA;  $n = 10$  neurons;  $p < 0.001$ ; **Figure 2H**). But, we did not  
267 observe a change in holding current (ACSF:  $140 \pm 28$  pA vs Gabazine:  $160 \pm 33$  pA,  $n=10$   
268 neurons,  $p=0.27$ ), indicating that the inhibition from MDJ and in bPN neurons is  
269 predominantly phasic.

270 In contrast to glutamatergic synaptic inputs from neocortex, GABAergic synaptic input from  
271 MDJ neurons showed remarkably little short-term synaptic plasticity with intervals  $>20$  ms,  
272 even after a stimulation-train of 20 pulses we observed  $108 \pm 5\%$  and  $105 \pm 7\%$  of the initial  
273 amplitude for 10Hz and 20Hz stimulation trains respectively (**Figure 2J**). Only at frequencies  
274  $\geq 50$ Hz and towards the end of a train of pulses was the amplitude of responses depressed (to  $55$   
275  $\pm 7\%$  and  $14 \pm 7\%$  for 50 and 100Hz stimulus trains, respectively). Similar to responses evoked  
276 from M1 afferents, MDJ afferents showed enhanced short-term synaptic depression when we  
277 evoked responses from efferents that expressed the channelrhodopsin variant Chrimson  
278 (**Supplementary Figure 2**). To confirm that inputs from the MDJ are monosynaptic, we  
279 applied TTX followed by the combined application of TTX and 4-AP. Inputs from the MDJ are



280 completely blocked upon TTX application ( $5 \pm 3.9\%$  of the response in ACSF; ACSF:  $23 \pm 8$  pA  
281 vs TTX:  $1.5 \pm 0.61$  pA;  $p < 0.001$ ,  $n = 5$  neurons) and subsequently rescued in presence of 4-AP  
282 (to  $400 \pm 760\%$  of ACSF response; TTX +4-AP:  $50 \pm 23$  pA;  $p = 0.37$ ,  $n = 5$  neurons **Figure 2I**).

283 Our results thus far indicate the neurons in the bPN receive depressing excitatory input from  
284 neocortex and inhibitory input from the MDJ that undergoes very little short-term plasticity.  
285 These inputs could interact in several ways in the cerebro-cerebellar circuitry. First we  
286 investigated whether single neurons in the bPN receive inputs from both motor cortex and from  
287 MDJ. We therefore analyzed full field optical stimulation data of all neurons that received inputs  
288 from either M1 or MDJ (See methods). Neurons were clamped at  $-70$  mV and subsequently at  $0$   
289 mV to enable detection of both EPSCs and IPSCs, respectively, in the same neurons (**Figure**  
290 **3A-C**). Out of 53 recorded bPN neurons that responded to optogenetic stimulation, 62.26% (33  
291 out of 53) of neurons only received inputs from MDJ and 37.73% (20 out of 53) only received  
292 inputs from M1 **Figure 3D**). No neurons that responded to optic stimulation received inputs  
293 from both M1 and MDJ, suggesting that these afferents make synapses onto different neuron  
294 classes within the bPN. To test this, we compared several passive electrical properties between  
295 the two groups. However, we found no statistically significant differences in membrane  
296 resistance (M1:  $320 \pm 49$  M $\Omega$ ; midbrain:  $220 \pm 25$  M $\Omega$ ;  $p = 0.08$ ;  $n = 52$  neurons), membrane  
297 capacitance (M1:  $100 \pm 15$  pF; MDJ:  $108 \pm 8.2$ ;  $p = 0.86$ ;  $n = 52$  neurons) and membrane decay  
298 time constant (M1:  $1.18 \pm 0.08$  ms; MDJ:  $1.2 \pm 0.10$  ms;  $p = 0.96$ ;  $n = 52$  neurons) between these  
299 two groups, indicating that these neurons receive different inputs but do not represent separate  
300 classes of neurons (**Supplementary Figure 4**). Thus, our results show that convergence of  
301 inputs from M1 and MDJ in the bPN is at best remarkably rare. This overall segregation of  
302 excitatory and inhibitory streams suggests that it is unlikely that MDJ inputs modulate  
303 incoming motor inputs to bPN neurons.

304 Nonetheless, there is a distinct possibility that inputs from M1 and MDJ can interact in the MDJ  
305 via a local network between bPN neurons. One study has suggested that short-range interactions  
306 between neurons in the bPN are absent, but this dataset only comprised of 20 tested pairs and  
307 only probed connections within a short range from each other (Möck, Schwarz, 2006). To  
308 investigate this issue over a longer distance, we recorded from a total 125 pairs (i.e. 250  
309 unidirectional connections) spaced up to  $500\mu\text{m}$  apart, in slices cut in both the coronal ( $n = 168$ )  
310 and sagittal ( $n = 82$ ) orientation to avoid confounding effects of slice orientation (Shinoda et al.  
311 1992) (**Figure 3E**). In these sampled pairs we never detected evidence of mono- or disynaptic  
312 contacts between neurons. Therefore, it is unlikely that M1 and MDJ inputs interact at the level  
313 of either single neurons or within a local circuit, but rather that M1 and MDJ inputs are  
314 processed separately

315 We have thus far shown that bPN neurons receive GABAergic inputs from the MDJ and that at  
316 the level of the bPN these inputs do not interact with inputs from M1. Furthermore, this  
317 inhibition is predominantly mediated phasically rather than via a tonic current. Therefore, it is  
318 unlikely that input from the MDJ induce a dampening or level-setting effect on neurons of the  
319 bPN (Silver 2010). Another possible role for inhibition is the gating of information (Crowley et  
320 al. 2009; Geborek et al. 2013). However, in that case one would expect that inputs interact at the  
321 level of the bPN, which is not in line with our data. A final possibility is that input from the MDJ  
322 represents a separate stream of information through the pons to the cerebellum. This possibility

323 could explain recent reports that distinct pontine neuron populations either increase or decrease  
324 their firing rates during a voluntary reaching and grabbing task (Guo et al. 2021). If bPN-  
325 projecting MDJ neurons are indeed recruited during movement, we expect that they receive  
326 prominent inputs from the neocortex. We investigated this possibility by using monosynaptic  
327 rabies tracing (Wickersham et al. 2006).

328 To map inputs to neurons in the MDJ that provide inputs to the bPN, we first checked whether  
329 we could trace connections from neocortex, through bPN to cerebellum. Indeed, by injecting a  
330 retrograde virus into cerebellum to express cre in bPN neurons, followed by viruses to express  
331 TVA and rabies glycoprotein in bPN, we could visualize rabies-infected neurons in neocortex  
332 after injections with rabies virus in bPN (**Supplementary Figure 5**). We then injected  
333 retrograde AAV in the bPN to express Cre in all afferent areas to bPN. Subsequent injections  
334 with AAVs to express TVA and optimized G protein were made into the MDJ, followed by  
335 pseudotyped rabies virus one week later. In these experiments (**Figure 4A**, n=3 mice) we  
336 observed starter neurons in the MDJ that were GAD-positive (**Figure 4B**). As expected, in the  
337 bPN we could observe many axon terminals from starter neurons that contained labeling from  
338 the rabies virus, and were GAD-positive (**Figure 4C**). This confirmed that MDJ GAD-positive  
339 neurons indeed make contacts in the bPN. In the neocortex of these mice we could observe  
340 labeling of deep neocortical pyramidal neurons (**Figure 4D**). These results show that neurons  
341 in the MDJ that provide afferents to the bPN receive inputs from neocortex.

342

343

## 344 **Discussion**

345 We show that the bPN receives prominent inputs from neocortex and from MDJ. Using whole  
346 cell recordings and optogenetic stimulation we show that inputs from neocortex are  
347 glutamatergic and strongly depressing, while inputs from MDJ are GABAergic, but show  
348 remarkably little short-term depression. Interestingly, M1 and MDJ inputs do not interact at the  
349 single neuron level, nor via a network of synaptic connections in the bPN, and thus represent  
350 separate streams of information through the bPN. Finally, using Rabies-virus tracing we show  
351 that MDJ neurons that project to the bPN receive prominent input from neocortical output  
352 neurons. Thus, our results show and characterize an unknown connection from neocortex to  
353 bPN, which could provide sign-inversed inputs from neocortex to cerebellar granule cells.

## 354 **The source of inhibitory afferents to the bPN**

355 The source of inhibition to the bPN has been unclear for a long time. Several afferent nuclei such  
356 as the zona incerta, anterior pretectal nucleus, cerebellar nuclei, prerubral area, the medullary  
357 formation (Border et al. 1986) and even neurons in the bPN itself (Border and Mihailoff 1985,  
358 1990; Brodal et al. 1988) have been suggested to provide GABAergic inputs. In functional  
359 studies, inhibition of bPN neurons has been observed during behavior (Guo et al. 2021), and  
360 inhibition in bPN neurons has been observed in vitro after stimulation of the cerebral peduncle  
361 and the tegmentum (Möck et al. 1997). Indeed, in our present experiments, we observed  
362 prominent GABAergic inputs from MDJ, a group of neurons located in the tegmentum that  
363 receives prominent inputs from neocortex (De Zeeuw et al. 1998; Kubo et al. 2018).  
364 Furthermore, we have found no evidence that local interneurons are present in the bPN, nor  
365 that any neuron in the bPN makes local connections (see also Möck *et al.*, 2006). GABAergic  
366 inhibition to bPN neurons therefore seems to be completely extrinsic. However, we can't exclude  
367 that other sources than MDJ inputs may contribute to inhibition in the bPN

## 368 **bPN network architecture**

369 The bPN are considered to integrate incoming motor and sensory information from the  
370 neocortex at the single cell level (Potter et al. 1978). Indeed, some neurons in the bPN respond  
371 only to movement whereas others are responsive to multiple modalities such as movement and  
372 cue (Guo et al. 2021), but this might also represent integration in neocortex. Although the  
373 precise extent of convergent streams in the bPN remains an important unanswered question,  
374 based on anatomical tracing data it is suggested that convergence of excitatory afferents from  
375 different regions to single bPN neurons is likely (Mihailoff et al. 1988; Lee and Mihailoff 1990;  
376 Schwarz and Thier 1999; Leergaard 2003; Leergaard and Bjaalie 2007) It is all the more striking  
377 then that we did not find any convergence of excitatory cortical and inhibitory MDJ inputs in the  
378 bPN. Although we cannot unequivocally rule out the presence synaptically connected bPN  
379 neurons, we expect that such connections would be too sparse to hold a substantial functional  
380 role.

## 381 **Difference in short-term plasticity between afferents**

382 We show that MDJ GABAergic and glutamatergic cortical inputs are markedly different in their  
383 short-term plasticity. Cortical inputs show clear synaptic depression across all tested  
384 frequencies, which is particularly strong at relatively high frequencies. Conversely, MDJ inputs  
385 undergo little synaptic plasticity at all except for slight depression towards the end of a pulse  
386 train at higher stimulation frequencies. These differences are important, since synaptic  
387 depression or potentiation plays an important role in shaping the activity of neurons (Silver  
388 2010). Layer 5 neurons provide the output from neocortex to bPN (Tervo et al. 2016), and  
389 respond with changes in firing rate up to 50Hz during movement (Park et al. 2021; Guo et al.  
390 2021). According to our electrophysiological data, signals from neocortex below 50Hz will  
391 undergo limited short-term depression, and thus can be faithfully transmitted to bPN neurons.  
392 Indeed, during reaching, bPN neurons show modulations of their firing rates in line with activity  
393 in layer 5 of neocortex (Guo et al. 2021). Interestingly, GABAergic inputs from the MDJ undergo  
394 very little short-term depression. It is important to note that our estimates of short-term  
395 depression were obtained from optogenetic stimulations, which might induce artificial synaptic  
396 depression (Jackman et al. 2014). Indeed, Chrimson, a slower variant of channelrhodopsin  
397 showed more pronounced depression than Chronos, a fast variant (Klapoetke et al. 2014). Still,  
398 for frequencies up to 20Hz, both Chrimson and Chronos-mediated stimulation yielded very  
399 comparable results, indicating that depression in glutamatergic connections from neocortex  
400 probably are not an artifact from optogenetic stimulation.

#### 401 **Potential roles of inhibitions in the bPN (gating vs. gain setting vs. enrichment)**

402 It remains unclear by which mechanism inhibition in the bPN contributes to voluntary motor  
403 control. Although our findings do not decisively point to one single mechanism, we are able to  
404 rule out several hypotheses. Our rabies tracings suggest that GABAergic MDJ afferents to the  
405 bPN could be recruited by cortical activation. This possibly explains why optogenetic  
406 stimulation of the cortex either induces an increase or a decrease in the firing rate of bPN  
407 neurons (Guo et al. 2021). Although complete optogenetic silencing of the bPN disturbs  
408 movement (Wagner et al. 2019; Guo et al. 2021), our data show that inhibition from the MDJ  
409 specifically avoids bPN neurons that receive inputs from motor cortex. Therefore, we consider it  
410 unlikely that feedforward inhibition from the MDJ serves as a general gating mechanism  
411 (Crowley et al. 2009; Geborek et al. 2013). Furthermore, the phasic nature of GABA signaling in  
412 the bPN suggests a timing-dependent mechanism rather than continuous response gain  
413 adjustment (Silver 2010). It is therefore likely that the MDJ provides the MDJ with a negative  
414 signal based on neocortex input to MDJ. In this arrangement the bPN would transmit one  
415 direct, positive signal based on direct corticopontine inputs, and one negative signal based on  
416 cortico-MDJ-pontine inputs. This would greatly enrich the inputs that are provided to the input  
417 layer of the cerebellar cortex, which would support cerebellar learning (Chabrol et al. 2015;  
418 Cayco-Gajic et al. 2017; Straub et al. 2020).

419  
420

421 **Acknowledgements**

422 This work was supported by an NWO vernieuwingsimpuls VENI grant 016.Veni.171.056. The  
423 authors thank J.C. Lodder, A.J. Timmerman, T.S. Heistek and J Wortel for their excellent  
424 technical assistance. The authors also thank S. Abirashid, V Zucconi Galli Fonseca and J.K.W. de  
425 Vries for help with experiments and F.J. Meye and R.A.H. Adan for help with designing the  
426 rabies virus experiment. EnvA-complemented rabies virus (SAD-dG-tdTomato) was a generous  
427 gift from K.K. Conzelmann (DFG project-ID 118803580-SFB 870). pAAV-Syn-Chronos-GFP and  
428 pAAV-syn-ChrimsonR-tdT were a generous gift from Edward Boyden.

429 **Author Contributions**

430 L.W. designed the study. A.K. performed and analyzed electrophysiological experiments. L.W.  
431 performed and analyzed anatomical experiments. All authors checked data analysis. A.K. and  
432 L.W. wrote the manuscript. All authors critically revised the manuscript.

433

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595

596 **Figure legends**

597 **Figure 1: The basilar pontine nuclei are intermediate between the cerebral cortex**  
598 **and the cerebellum.**

599 (A) Schematic representation of a retrobead injection in cerebellum. (B) Examples of retrobead-  
600 labeled neurons in the inferior olivary nucleus (IO), lateral reticular nucleus (LRN), external  
601 cuneate nucleus (eCU), basilar pontine nuclei (bPN), and the absence of labeled neurons in  
602 primary sensory cortex (S1), and primary motor cortex (S1). Scale bar represents 50  $\mu\text{m}$ . (C)  
603 Schematic representation of a retrobead injection in the bPN. (D) Example retrograde labeling  
604 after injections in bPN. Scale bar represents 500  $\mu\text{m}$ . (E) Quantification of retrograde labeling in  
605 cerebrum, diencephalon, midbrain, pons and other areas. Shown is the average and individual  
606 data points. (F) Retrograde labeling in neocortex is predominantly found in deeper layers (layer  
607 5 and 6a), consistent with the location of extratelencephalic projection neurons. (G) Retrograde  
608 labeling can be predominantly found ipsilateral. (H) Of the prominent inputs from midbrain,  
609 MDJ provides the most prominent input to bPN.

610 **Figure 2: Optogenetic stimulation of neocortical and mesodiencephalic afferents**  
611 **to the bPN.**

612 (A) Schematic overview of intracranial virus injection in M1. (B) Schematic overview of the  
613 experimental patch-clamp approach (left), and post-hoc recovered and stained neuron (cyan)  
614 showing GFP+ fibers from M1 (Yellow). (C) neocortical inputs can be effectively blocked with an  
615 antagonist against the AMPA and kainate receptor. Example trace (left) and quantification  
616 (right). (D) Neocortex makes monosynaptic contacts to bPN. Example trace (left) and  
617 quantification (right). (E) Neocortical inputs are strongly depressing, especially at high  
618 frequencies. (F) Schematic overview of intracranial virus injection in MDJ. (G) Schematic  
619 overview of experimental approach (left) and a recovered neuron (cyan) with GFP+ fibers from  
620 MDJ (Yellow) (H) MDJ inputs can be effectively blocked with an antagonist against the GABA<sub>A</sub>  
621 receptor. Example trace (left) and quantification (right). (I) MDJ makes monosynaptic contacts  
622 to bPN. Example trace (left) and quantification (right). (J) MDJ inputs show very limited short-  
623 term depression, even at higher frequencies.

624 **Figure 3: The bPN receives monosynaptic GABAergic inputs from midbrain**

625 (A) Schematic overview of intracranial virus injection configuration in midbrain (Chronos) and  
626 M1 (Chrimson) (left), or inverse (right) (B) Single neurons either receive input from neocortex,  
627 or from mesodiencephalic junction, but not from both. Shown are two example neurons  
628 responsive to blue light, but not to yellow light. (D) Quantification of inputs from neocortex and  
629 from mesodiencephalic junction. (E) Experimental setup whole-cell patch clamp recordings in  
630 coronal (top) and sagittal (bottom) sections of bPN with example paired recordings of bPN  
631 neurons in coronal slice (top) and sagittal slice (bottom). Presynaptic neurons are indicated in  
632 black, postsynaptic neurons in grey. Right: Distances of probed connections measured between  
633 pre-synaptic neuron (black square, middle) and post-synaptic neuron (purple). Teal markers  
634 indicate reciprocal distances and are point-mirrored to purple markers. Dorsoventral and  
635 lateromedial orientation as shown in (A). Far right: Soma-to-soma distance distribution of all

636 probed pairs for coronal (top) and sagittal (bottom) experiments. D: Dorsal; V: Ventral; L:  
637 Lateral; M: Medial.

638 **Figure 4: Rabies tracing of inputs to mesodiencephalic junction neurons that**  
639 **project to bPN**

640 (A) Schematic representation of virus injections. (B) GABAergic rabies tracing starter neurons in  
641 mesodiencephalic junction were labeled with GFP from helper plasmids for rabies tracing.  
642 GAD67 staining (Cyan), Helper plasmids (Yellow), Rabies virus (Red). (C) GAD+ terminals from  
643 starter neurons can be observed in bPN. (D) Rabies tracing revealed prominent deep layer  
644 labeling in neocortex. Scale bars are 50  $\mu\text{m}$ .

645

646 **Supplementary figures**

647 **Supplementary figure 1: Injection locations for retrobead tracing from bPN.**

648 Two mice were injected with retrobeads in their bPN (top and bottom). Shown are the locations  
649 where tracer was injected with the atlas superimposed.

650 **Supplementary figure 2: Optogenetic stimulation of ChrimsonR and Chronos  
651 expressing MDJ and M1 afferents**

652 MDJ inputs (left) were stimulated via Chronos (blue) or Chrimson (Orange) in separate  
653 experiments. When stimulating with Chrimson more pronounced synaptic depression can be  
654 observed compared with Chronos. Especially at higher frequencies (>50Hz) and at the end of  
655 the train (second column) this is more pronounced. M1 inputs (right) were stimulated in a  
656 similar manner via Chronos and Chrimson, resulting in comparable results.

657 **Supplementary Figure 3: GAD staining and GAD-GFP mice show the absence of  
658 GABAergic neurons, but the presence of GABAergic fibers In bPN**

659 (A) GAD67 staining of sections of mice show that there are no GABAergic neurons in the bPN of  
660 mice. Compare bPN with areas with known prominent GABAergic neurons (IPR, IPC, MM).  
661 RtTg: Reticulotegmental nucleus of the pons; IPC: Caudal subnucleus of the Interpeduncular  
662 Nucleus; IPR: Rostral subnucleus of the Interpeduncular Nucleus; MM: Medial Mammillary  
663 Nucleus. (B) Enlarged view of bPN showing prominent GABAergic fibers. (C) GAD-GFP mice  
664 show that there is absence of GABAergic neurons in bPN, but GABAergic fibers can be  
665 distinguished.

666 **Supplementary Figure 4: Electrophysiological characterization of passive  
667 membrane properties of MDJ and M1-receiving bPN neurons**

668 Membrane resistance, membrane time-constant and capacitance show no systematic difference  
669 between groups of MDJ and M1 input-receiving neurons. Horizontal lines represent single  
670 neurons, box plot shows median, 25<sup>th</sup> and 75<sup>th</sup> percentile. Bars represent 10<sup>th</sup> and 90<sup>th</sup> percentile.

671 **Supplementary Figure 5: Rabies tracing of inputs to bPN.**

672 (A) Schematic representation of injection sites for AAV-Cre (grey), AAVs with TVA and  
673 glycoprotein (yellow) and EnveA-Rabies (red). (B) After injection of rabies virus in bPN,  
674 prominent labeling of deep layer pyramidal neurons was observed, scale bar 50  $\mu$ m. (C) In bPN  
675 starter neurons (Yellow) could be found together with afferents to bPN (Red). GAD67 staining  
676 (Cyan) indicates that some afferents, but none of the starter neurons were GABAergic. (D)  
677 Enlargement of the area shown with a white box in (C) indicating overlap between GAD staining  
678 and some afferents, but none of the starter neurons. Shown is a summed stack (left column)  
679 through with orthogonal view (right column) at the location indicated with the black arrowheads  
680 in the bottom stack. Scale bar 5  $\mu$ m. (E) In cerebellar cortex, clear mossy fibers from bPN starter  
681 neurons could be found. Scale bar 50  $\mu$ m. (F) In MDJ neuron could be observed that were  
682 positive for rabies virus, and positive for GAD67, indicating a GABAergic input from MDJ to  
683 bPN.







