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3 **Periaqueductal efferents to dopamine and GABA**
4 **neurons of the VTA**

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19 **Abstract**

20 Neurons in the periaqueductal gray (PAG) modulate threat responses and nociception.
21 Activity in the ventral tegmental area (VTA) on the other hand can cause reinforcement
22 and aversion. While in many situations these behaviors are related, the anatomical
23 substrate of a crosstalk between the PAG and VTA remains poorly understood. Here
24 we describe the anatomical and electrophysiological organization of the VTA-
25 projecting PAG neurons. Using rabies-based, cell type-specific retrograde tracing, we
26 observed that PAG to VTA projection neurons are evenly distributed along the rostro-
27 caudal axis of the PAG, but concentrated in its posterior and ventrolateral segments.
28 Optogenetic projection targeting demonstrated that the PAG-to-VTA pathway is
29 predominantly excitatory and targets similar proportions of I_h -expressing VTA DA and
30 GABA neurons. Taken together, these results set the framework for functional analysis
31 of the interplay between PAG and VTA in the regulation of reward and aversion.

32 Introduction

33 The periaqueductal gray (PAG) is a heterogeneous midbrain structure that is critical for the
34 endogenous modulation of nociception and for the expression of defensive behaviors [1]. These
35 functions have been shown to be mediated by neurons anatomically segregated in the
36 longitudinal columns corresponding to the dorsolateral (dl), lateral (l) and ventrolateral (vl)
37 subdivisions of the PAG [2]. The PAG is also the major site of action for opioid analgesia [3,4].

38 Anatomical tracing studies have described ascending and descending projections from the PAG
39 to a variety of brain structures [5]. Among these projection targets is the ventral tegmental area
40 (VTA), a major component of the brain reward system [6]. While it is primarily known for its
41 role in reward prediction, and positive reinforcement [7,8], the VTA has also been implicated
42 in the modulation of nociception and in the expression of fear and aversive responses [9–12].

43 The engagement of VTA dopamine- (DA) and *gamma*-aminobutyric acid (GABA)-releasing
44 neurons with PAG afferents through both symmetric and asymmetric synaptic contacts has
45 been demonstrated with rabies-assisted retrograde tracing and ultrastructural immunoelectron
46 microscopic analyses [13,14].

47 It remains elusive, however, whether the input neurons providing these VTA afferents spatially
48 segregate within specific PAG columns, potentially associating the PAG-to-VTA pathway with
49 specific anti-nociceptive or defensive functions. Moreover, evidence for an
50 electrophysiologically functional connection, and knowledge of its excitatory or inhibitory
51 effect onto VTA DA and GABA neurons is still lacking. To this end, the present study will
52 describe the rostro-caudal distribution of VTA-projecting neurons across the PAG columns,
53 and will test whether these neurons exhibit a preferential excitatory or inhibitory effect on DA
54 and GABA neurons of the VTA.

55 **Materials and methods**

56 **Animals**

57 Experiments were performed on DAT-Cre [15] and GAD65-Cre mice [16] of both sexes. All
58 animal procedures were performed in accordance with the authors' university animal care
59 committee's regulations.

60 **Injection procedures**

61 All stereotaxic intracranial injections were performed under isoflurane anesthesia (2-5%,
62 Attane) using glass capillary pipettes connected to a microinjection pump (Narishige) at a rate
63 of ~ 100 nl min⁻¹. The coordinates used were (from bregma, in mm): AP -3.4, ML ± 0.5 ,
64 DV -4.3 for VTA injections; AP -4.0, ML ± 0.3 , DV -2.6 for PAG injections. For retrograde
65 tracing experiments, 300 nl of a 1:1 mixture of AAV8-CAG-DIO-RG and AAV5-EF1a-DIO-
66 TVA-mCherry was injected unilaterally in the VTA, followed 2 weeks later by the injection of
67 800 nl of RVΔG-EnvA-EGFP at the same coordinates. For patch clamp experiments, animals
68 were bilaterally injected with AAV5-EF1a-DIO-mCherry in the VTA and with AAV2-hsyn-
69 ChR2-EYFP in the PAG (all viruses from the UNC Vector Core Facility).

70 **Electrophysiology on acute slices**

71 Coronal VTA slices (180 μ m) were prepared using a vibratome in ice-cold cutting solution
72 containing (in mM): NaCl 87, NaHCO₃ 25, KCl 2.5, MgCl₂ 7, NaH₂PO₄ 1.25, CaCl₂ 0.5,
73 glucose 25, and sucrose 75. Slices were incubated in the same solution for 20 min at 31°C
74 before being transferred to regular room-temperature artificial cerebrospinal fluid (aCSF),
75 containing (in mM): NaCl 119, NaHCO₃ 26.2, KCl 2.5, MgCl₂ 1.3, NaH₂PO₄ 1, CaCl₂ 2.5, and
76 glucose 11. After at least one hour for recovery, slices were transferred to the recording

77 chamber, superfused with aCSF at 2 ml/min. All solutions were constantly bubbled with 95%
78 O₂ and 5% CO₂. Postsynaptic currents were evoked by stimulating ChR2 with brief (4 ms) blue
79 light pulses using a 470nm LED mounted on the microscope and powered by an LED driver
80 under computer control. Kynurenic acid (kyn, 4 mM, Sigma) and picrotoxin (PTX, 200 μM,
81 Sigma) were added to the aCSF in order to block glutamate- and GABA-mediated currents,
82 respectively. All representative traces were made from averaging at least 20 consecutive
83 sweeps. Neurons were visually identified using an IR CCD camera mounted on an Olympus
84 BX45 microscope. Borosilicate glass pipettes at a resistance range of 2-4 MΩ were used for
85 recording. The internal solution used contained (in mM): K-gluconate 30, KCl 100, MgCl₂ 4,
86 creatine phosphate 10, Na₂ATP 3.4, Na₃GTP 0.1, EGTA 1.1, and HEPES 5. The calculated
87 reversal potential with this internal solution for Cl⁻ was -5mV and cells were voltage-clamped
88 at -70 mV. Currents were amplified, filtered at 2 kHz, digitized at 10 kHz, and saved on a hard
89 disk. The liquid junction potential was small (-4 mV) and traces were therefore not corrected.
90 Access resistance was monitored by a hyperpolarizing step of -4 mV at the onset of every
91 sweep, and the experiment was discarded if the access resistance changed by more than 20%.

92 **Histological procedures and imaging**

93 Mice were deeply anesthetized with pentobarbital (300 mg/kg i.p.) and transcardially perfused
94 with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA, Sigma).
95 Brains were removed, post-fixed in 4% PFA for 24 h at 4°C, and cut in 50 μm sections on a
96 vibratome. For tyrosine hydroxylase (TH) immunohistochemistry, brain sections were rinsed
97 in PBS (0.1 M) and incubated for 1 h at room temperature in a blocking solution containing
98 5% bovine serum albumin (Sigma) and 0.3% Triton X-100 (Axon Lab AG) in PBS. Sections
99 were then incubated overnight at +4° C with a rabbit anti-TH antibody (1:500; Millipore) in
100 blocking solution, then rinsed in PBS, incubated for 2 h at room temperature with an alexa-

101 conjugated goat anti-rabbit antibody (1:500; Invitrogen) in blocking solution. Sections were
102 then rinsed again in PBS and mounted on glass slides with Fluoroshield Mounting Medium
103 (Abcam). Confocal images were captured with a Nikon A1r Spectral scanning confocal
104 microscope, and then processed with ImageJ.

105 **Data analysis and statistics**

106 Equal regions of interest (ROIs) were selected for rabies-infected starter cell counts and then
107 averaged among all mice. Cell counts of retrogradely labeled input neurons in the PAG were
108 performed using Paxinos and Franklin's mouse brain atlas as reference to divide the PAG into
109 dorsolateral, lateral and ventrolateral segments, and into rostral (AP coordinates between -2.8
110 and -3.4), central (between -3.4 and -4.16) and caudal regions (between -4.16 to -5.0) [17]. All
111 data are expressed as mean \pm standard error of the mean, and statistical analysis was performed
112 with Igor, Microsoft Excel, and GraphPad Prism.

113 Results

114 PAG neurons project to VTA DA and GABA neurons.

115 To reveal the distribution of PAG to VTA projecting neurons, we employed a cell-type specific,
116 virally assisted tracing approach using a rabies virus pseudotyped with the avian sarcoma and
117 leucosis virus envelope glycoprotein EnvA and lacking the rabies envelope glycoprotein gene
118 *rabG* (RVΔG-EGFP, [18]). First, we virally transfected the VTA of DAT-Cre or GAD65-Cre
119 mice with two Cre-dependent constructs encoding for the EnvA receptor TVA and RabG (DIO-
120 TVA-mCherry/RG), and followed up with the RVΔG-EGFP transfection two weeks later (Fig.
121 1A). Seven days later, infection of VTA DA and GABA neurons and their retrogradely labeled
122 afferents were observed (Fig. 1B). VTA neurons co-expressing RVΔG-EGFP and the DIO-
123 TVA-mCherry/RG constructs were identified as starter cells, from which the inputs were
124 monosynaptically tagged by the expression of RVΔG-EGFP alone (Fig. 1C). We then
125 visualized the cell bodies of these VTA projection neurons in the PAG. Given its extensive
126 rostro-caudal extension, we partitioned the PAG into three segments that represent the
127 columnar divisions as described in Paxinos and Franklin's mouse brain atlas [17]. The rostral
128 segment includes the PAG from the anteroposterior (AP) coordinates -2.8 and -3.4 (in mm,
129 from bregma). The central segment, subdivided in dorsolateral PAG (dIPAG) and lateral PAG
130 (lPAG), lies between AP -3.4 and -4.16. The caudal PAG, subdivided in dIPAG, lPAG and
131 ventrolateral PAG (vlPAG), extends from AP -4.16 to -5.0 (Fig. 1D). We observed that the
132 PAG projection neurons were sparse in the rostral PAG, and increasingly more dense in the
133 central and caudal PAG segments. We found that VTA DA- and GABA-projecting neurons are
134 similarly distributed along the PAG, with the exception for the caudal PAG, where more inputs
135 are provided to VTA GABA neurons (AP coordinate -4.6, DA vs GABA: 17.3 ± 4.4 vs $53.8 \pm$
136 11.3), despite a similar number of starter cells compared to DA neurons (DA vs GABA: 96.5

137 ± 18.3 vs 92.7 ± 5.6) (Fig. 1E). We then analyzed the intracolumnar distribution of VTA
138 projecting neurons (Fig. 1F). We found that both VTA DA and GABA inputs are similarly
139 scattered across the PAG columns, with the caudal ventrolateral and central lateral ones
140 providing the highest proportion of total PAG inputs (DA: caudal vIPAG, 38.8 ± 6.9 , central
141 IPAG, 31.3 ± 2.4 ; GABA: caudal vIPAG, 33.3 ± 3.4 , central IPAG, 32.7 ± 3.9). While some
142 inputs were observed to decussate from the contralateral side, the majority DA and GABA
143 inputs was similarly labeled in the ipsilateral hemisphere, indicating a moderate level of input
144 lateralization (DA vs GABA: 0.77 ± 0.03 vs 0.69 ± 0.02). Taken together, these results indicate
145 that both VTA DA- and GABA-projecting neurons from the PAG concentrate ipsilaterally and
146 preferentially in its lateral and ventrolateral subdivisions, in stark contrast with the dorsolateral
147 columns which provide less than 5% of the total PAG inputs (Fig. 1G).

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149 **VTA DA and GABA neurons receive a similar excitatory** 150 **input from the PAG.**

151 Because the PAG is a heterogeneous structure containing glutamate- as well as GABA-
152 releasing projection neurons [19,20], we sought to investigate the nature of the VTA inputs
153 from the PAG and test whether they exhibited a preferential targeting towards DA or GABA
154 neurons. We thus performed whole-cell patch clamp recordings on acute VTA slices from
155 animals transfected in the l/vIPAG with the light-sensitive cation channel ChR2 (Fig. 2A). VTA
156 DA or GABA neurons were visually identified by the Cre-dependent transfection of the red
157 fluorescent reporter mCherry in DAT-Cre or GAD65-Cre mice, respectively. Their
158 neurochemical identity was confirmed by the immunohistochemical co-localization (DAT-
159 Cre) or exclusion (GAD65-Cre) of the catecholaminergic marker tyrosine hydroxylase (TH)
160 (Fig. 2B). Consistently with the tracing results, both DA and GABA neurons received PAG

161 synaptic inputs with similar current amplitudes (DA vs GABA: 50.0 ± 11.2 pA vs 55.9 ± 14.2
162 pA) and connection rates (DA vs GABA: 40.2 ± 10.1 % vs 29.6 ± 5.0 %) (Fig. 2C). We chose
163 to employ a human synapsin (hsyn) promoter-driven ChR2 in order to transfect non-selectively
164 excitatory and inhibitory PAG projection neurons and record both excitatory inputs in the same
165 postsynaptic neurons. Light-evoked currents were then pharmacologically blocked with
166 glutamatergic or GABAergic antagonists in order to determine the proportion of excitatory and
167 inhibitory afferents to VTA DA and GABA neurons (Fig. 2D). The majority of PAG inputs to
168 either VTA DA and GABA neurons were abolished by the application of the AMPA/NMDA
169 antagonist kynurenic acid (kyn) (DA, $n = 16/17$; GABA, $n = 14/18$), whereas the remaining
170 kyn-resistant currents were blocked by bath application of the GABA_A blocker PTX (Fig. 2D-
171 E). This suggests that PAG projections to the VTA are predominantly excitatory and do not
172 show a preferential targeting towards either cell type. Finally, we tested whether basic
173 electrophysiological properties of connected cells could predict the strength of the synaptic
174 connection. We therefore analyzed the amplitude of the hyperpolarization-activated cation
175 current I_h but found no correlation with the postsynaptic current amplitude in either VTA DA
176 or GABA neurons. However, when we compared connected and non-connected neurons, we
177 observed a statistically significant higher I_h current in both cell types (DA, connected vs non-
178 connected: 91.3 ± 24.8 pA vs 45.3 ± 13.4 pA; GABA, connected vs non-connected: 40.7 ± 12.8
179 pA vs 19.8 ± 4.6 pA) (Fig. 2F), suggesting a higher expression of the I_h -mediating HCN
180 channels within the PAG-targeted VTA neurons. Altogether, these results describe a
181 predominantly glutamatergic PAG-to-VTA pathway equally targeting DA and GABA neurons,
182 and preferentially contacting neurons exhibiting a larger I_h amplitude.

183 Discussion

184 Our anatomical tracing data indicate that PAG afferents to the VTA project towards both DA
185 and GABA neurons, consistently with earlier observations in the rat VTA [14]. We describe
186 here the spatial organization of the PAG inputs and provide electrophysiological evidence of
187 functional PAG synapses similarly impinging on DA and GABA neurons of the mouse VTA.

188 Rabies-assisted circuit tracing has been used to obtain a brain-wide map of the afferents to
189 neurotransmitter-defined VTA neurons [13]. This study identifies the PAG as input. Our
190 findings expand on their results by providing a spatial profile of the location of the VTA
191 projection neurons along the rostral-caudal and dorso-ventral axes of the PAG. We found that
192 both VTA DA- and GABA-projecting PAG neurons were more concentrated in the central and
193 caudal PAG, this latter segment containing a higher number of inputs to VTA GABA neurons.
194 This comparison requires various controls. First, the absolute number of inputs depends on the
195 size of the population of VTA DA or GABA starter neurons. Therefore, we transfected a similar
196 number of DA and GABA starter cells in DAT-Cre and GAD-Cre mice, respectively, despite
197 DA neurons being more frequent than GABA neurons, in a 2:1 ratio [21]. Second, distinct
198 neuronal types might have different degrees of input convergence and different connectivity
199 rates. VTA GABA neurons receive more convergent inputs than DA neurons, thus adding a
200 confounding variable in the case of a starter cell-normalized comparison of the input neurons
201 between cell types [13]. For this reason we also analyzed the number of inputs in each
202 subregion normalized to the total PAG inputs to conclude that IPAG and vIPAG neurons
203 provide the majority of periaqueductal efferents to VTA DA and GABA neurons.

204 The anatomical organization may have functional consequences. Neurons in the PAG have
205 been proposed to subserve different behavioral functions – from opioid analgesia to modulation
206 of autonomic responses and defensive behaviors, depending on their columnar localization. For

207 instance, two distinct types of opioid-induced and opioid-independent pain suppression
208 mechanisms have been proposed to arise from the stimulation of the ventrolateral or lateral
209 columns of the PAG, respectively [2]. Analogously, two antagonistic threat responses, the
210 freezing and flight behaviors, were recently shown to be promoted by neurons within vIPAG
211 and dl/IPAG columns, respectively [22]. In light of these observations, it is tempting to
212 speculate that our results on the PAG inputs distribution pattern might reflect their involvement
213 in opioid analgesia and freezing responses. However, further experiments are needed to test
214 this hypothesis and to determine the physiological relevance of this projection to the VTA.

215 Both glutamate- and GABA-releasing output neurons have been described in the PAG [20,23].
216 We therefore tested whether there would be a neurotransmitter-specific organization these PAG
217 projections to either DA or GABA neurons of the VTA. However, our results did not support
218 this hypothesis, suggesting instead that the PAG provides an equal synaptic input to both cell-
219 types. Since the electrophysiological markers classically used to distinguish between VTA DA
220 and GABA neurons (e.g. action potential width, capacitance measures and presence of a
221 hyperpolarization-activated *I_h* current) are not reliable, in light of their variability and overlap
222 between the two cell types [24,25], we chose to employ a Cre-dependent genetic approach in
223 order to unequivocally identify DA and GABA neurons in DAT-Cre and GAD65-Cre mice,
224 respectively. In accordance with previous observations, both identified DA and GABA neurons
225 exhibited a variable range of *I_h* amplitudes [24]. Additionally, our results suggest that PAG
226 projections preferentially target neurons exhibiting larger *I_h* currents. It is plausible that these
227 differences reflect a higher expression, or a change in the subunit composition of the
228 hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, which mediate the *I_h*
229 currents and have been implicated in the reward system adaptations induced by drugs of abuse
230 [26,27].

231 Our findings indicate that PAG GABA neurons constitute a small fraction of the projection to
232 the VTA targeting the VTA neurons via GABA_A transmission. These results rely on non-
233 selective expression of ChR2 under the *hsyn* promoter in all PAG projection neurons. However,
234 the possibility that this promoter might differentially drive the expression of ChR2 among cell-
235 types seems unlikely. In comparison, a previous ultrastructural analysis of PAG axons within
236 the VTA reported that only 8% immunostained for GABA [14], although the authors note that
237 immunolabeling of electron microscopy samples may be prone to false negatives and,
238 consequently, the actual contribution of GABA neurons to the PAG input is likely to be higher.
239 Despite this possible underestimation, our electrophysiological results provide further support
240 to the observation of a small GABAergic participation in the PAG-to-VTA pathway. It should
241 be noted, however, that our findings do not take into account the potential presence of
242 inhibitory synapses expressing only the metabotropic GABA_B receptor, as it was recently
243 described for the NAc projections to VTA DA neurons [28].

244 **Conclusion**

245 Neurons of the VTA are known to be a major target of addictive drugs [29], and their activity
246 has been associated with both rewarding and aversive behaviors [12,30]. Similarly, PAG
247 neurons have been implicated in the expression of opioid-induced analgesia and in the
248 modulation of fear responses [1]. The anatomical and electrophysiological data presented here
249 thus expand on the existing knowledge of the connectivity between VTA and PAG neurons,
250 providing a substrate for future investigations on their interaction during motivationally
251 relevant behaviors.

252 **Acknowledgements**

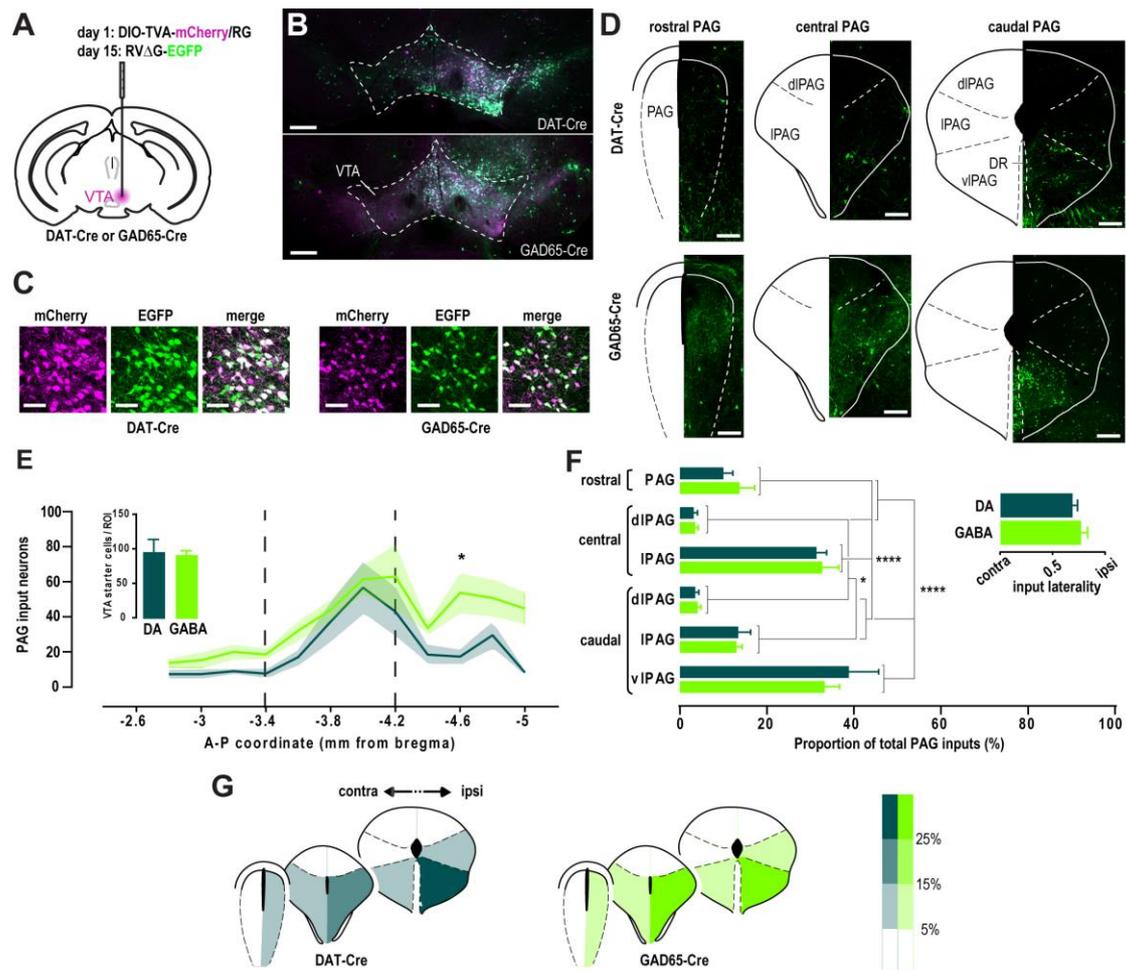
253 We thank all members of the Lüscher laboratory for stimulating discussions and valuable
254 comments on the manuscript.

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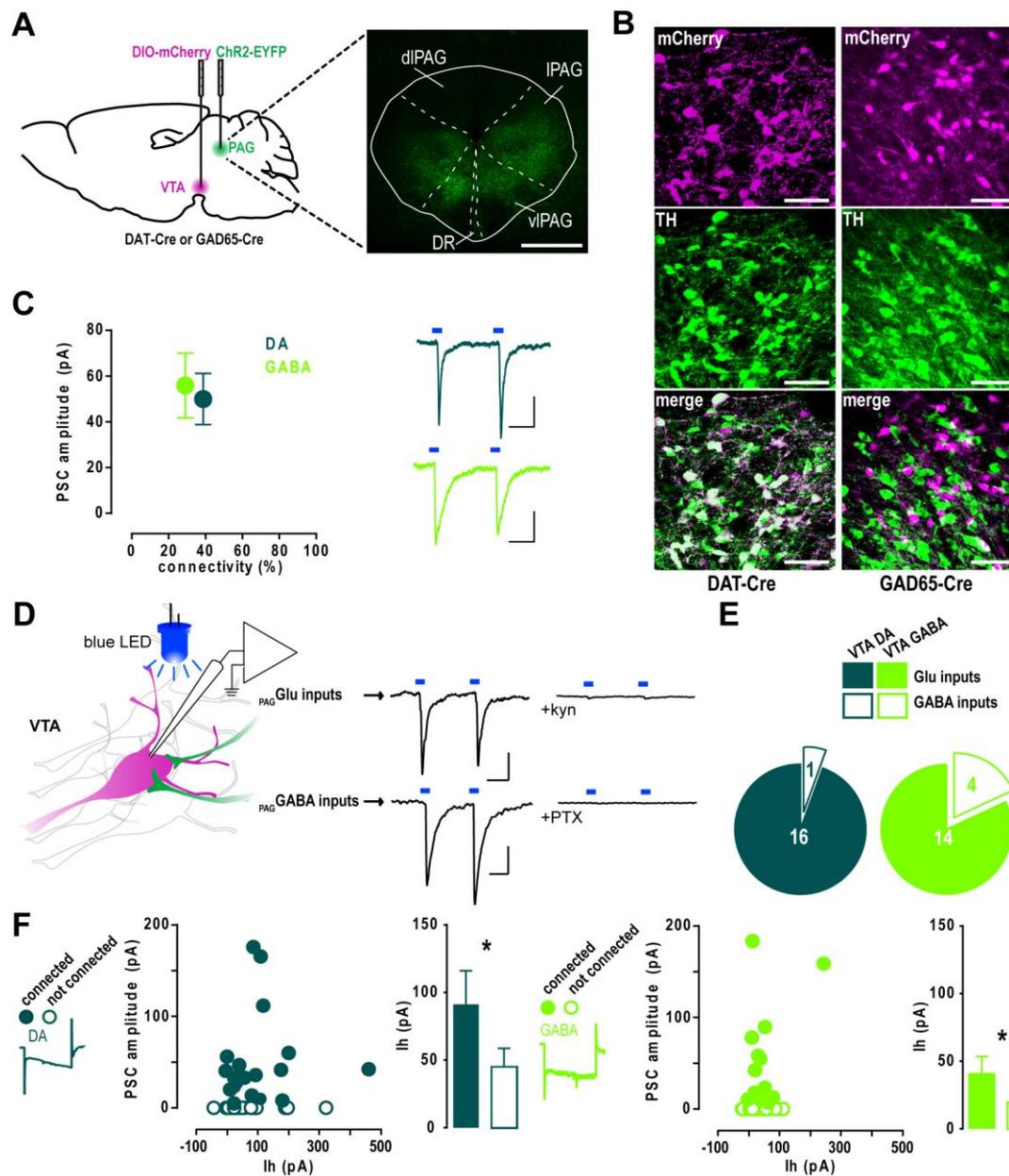
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338

339 **Figure 1. Spatial distribution of VTA-projecting PAG inputs.** **A**, Schematic of the
 340 unilateral rabies injection protocol. **B-C**, Confocal images at low (**B**) and high (**C**)
 341 magnification showing the expression of TVA-mCherry (magenta) and RVΔG-EGFP
 342 (green) in the VTA seven days after the last injection. Scale bars, 500 μm (**B**) and 50
 343 μm (**C**). **D**, RVΔG-EGFP-expressing retrogradely labeled input neurons across the
 344 rostral, central and caudal PAG segments. Scale bars, 200 μm. **E**, Average number of
 345 inputs to VTA DA (DAT, $n = 5$) and GABA (GAD, $n = 5$) neurons along the rostro-
 346 caudal axis of the PAG (two-way ANOVA: no interaction between the cell type factor
 347 and AP coordinate factor, $F_{11,120} = 0.7027$, $p > 0.05$; main effect of cell type, $F_{1,120} =$
 348 18.2 , $p < 0.0001$; main effect of AP coordinate, $F_{11,120} = 6.31$, $p < 0.0001$; Bonferroni
 349 post-hoc test, * $p < 0.05$). Dashed lines denote the boundaries between rostral, central
 350 and caudal PAG. Inset shows the average number starter cells per ROI in DAT-Cre
 351 and GAD65-Cre mice (two-tailed t test: no difference between genotypes, $p > 0.05$).
 352 **F**, Relative contribution of different PAG subregions to the total inputs to VTA DA and
 353 GABA neurons (two-way ANOVA: no interaction between the cell type factor and
 354 subregion factor, $F_{5,48} = 0.5013$, $p > 0.05$; no main effect of cell type, $F_{1,48} < 0.0001$, p
 355 > 0.05 ; main effect of subregion, $F_{5,48} = 42.85$, $p < 0.0001$; Bonferroni post-hoc test, *
 356 $p < 0.05$, **** $p < 0.0001$). Inset shows the degree of lateralization of the PAG inputs
 357 (two-tailed t test: no difference between genotypes, $p > 0.05$). **G**, Color-coded
 358 representation of the relative input contribution of ipsilateral and contralateral PAG
 359 subregions.



360

361 **Figure 2. PAG afferents equally target VTA DA and GABA neurons.** **A**, Left,
 362 schematic of the injection protocol for patch clamp experiments. Right, example of
 363 image of ChR2-EYFP infection in the PAG. Scale bar, 500 μm . **B**, High magnification
 364 confocal images showing the colocalization or exclusion of mCherry and TH in DAT-
 365 Cre or GAD65-Cre mice, respectively. Scale bars, 50 μm . **C**, Mean amplitude of the
 366 light-evoked postsynaptic currents in VTA DA ($n = 47$) and GABA ($n = 62$) neurons
 367 plotted against the percentage of connected neurons (Mann Whitney U test: no
 368 difference in amplitudes, $p > 0.05$; Fisher's exact test: no difference in connectivity, p
 369 > 0.05). Scale bars, 20 ms, 20 pA. **D**, Left, schematic of the patch clamp experiments:
 370 whole-cell recordings were performed from mCherry-expressing VTA neurons while
 371 PAG afferents inputs were light-stimulated (left). Right, excitatory currents were
 372 blocked with kynurenic acid (kyn), while kyn-resistant inhibitory currents were blocked
 373 with picrotoxin (PTX). Scale bars, 20 ms, 20 pA. **E**, Proportion of kyn-sensitive
 374 glutamate inputs and PTX-sensitive GABA inputs in VTA DA ($n = 17$) and GABA ($n =$

375 18) neurons (Fisher's exact test: no difference between cell types, $p > 0.05$). **F**, Mean
376 light-evoked current amplitude plotted against I_h amplitude (Spearman's rank
377 correlation: no correlation between the variables, $r = 0.2702$, $p > 0.05$) and comparison
378 of I_h between connected and non-connected VTA DA (left) or GABA neurons (right)
379 (Mann Whitney U test: * $p < 0.05$).