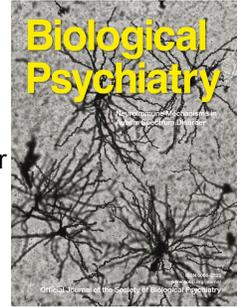# Journal Pre-proof

Midbrain dopamine controls anxiety-like behavior by engaging unique interpeduncular nucleus microcircuitry

Steven R. DeGroot, Rubing Zhao-Shea, Leeyup Chung, Paul M. Klenowski, Fangmiao Sun, Susanna Molas, Paul D. Gardner, Yulong Li, Andrew R. Tapper

Please cite this article as: DeGroot S.R., Zhao-Shea R., Chung L., Klenowski P.M., Sun F., Molas S., Gardner P.D., Li Y. & Tapper A.R., Midbrain dopamine controls anxiety-like behavior by engaging unique interpeduncular nucleus microcircuitry, *Biological Psychiatry* (2020), doi: https://doi.org/10.1016/j.biopsych.2020.06.018.

1

Midbrain dopamine controls anxiety-like behavior by engaging unique interpeduncular nucleus microcircuitry

4

Authors

Steven R. DeGroot[1, 2], Rubing Zhao-Shea[1], Leeyup Chung[1], Paul M. Klenowski[1], Fangmiao Sun[3,4], Susanna Molas[1], Paul D. Gardner[1], Yulong Li[3,4,5], Andrew R. Tapper[1*]

[1]Brudnick Neuropsychiatric Research Institute, Dept. of Neurobiology, University of Massachusetts Medical School, Worcester, MA, 01605, USA

[2]Graduate Program in Neuroscience, University of Massachusetts Medical School, Worcester, MA, 01605, USA

[3]State Key Laboratory of Membrane Biology, Peking University School of Life Sciences, 100871 Beijing, China

[4]PKU-IDG/McGovern Institute for Brain Research, 100871 Beijing, China

[5]Peking-Tsinghua Center for Life Sciences, 100871 Beijing, China

*Lead Contact, Correspondence to: andrew.tapper@umassmed.edu

17

Short Title: VTA dopamine modulates IPN activity to control anxiety

19

20 Background

21 Dopamine (DA) is hypothesized to modulate anxiety-like behavior although the precise

22 role of DA in anxiety behaviors and the complete anxiety network in the brain have yet

23 to be elucidated. Recent data indicate dopaminergic projections from the ventral

24 tegmental area (VTA) innervates the interpeduncular nucleus (IPN), but how the IPN

25 responds to dopamine (DA) and what role this circuit plays in anxiety-like behavior is

26 unknown.

27 Methods

28 We expressed a genetically encoded GPCR-activation-based-DA sensor in mouse

29 midbrain to detect DA in IPN slices using fluorescence imaging combined with

30 pharmacology. Next, we selectively inhibited or activated VTA→IPN DAergic inputs via

31 optogenetics during anxiety-like behavior. We utilized a biophysical approach to

32 characterize DA effects on neural IPN circuits. Site-directed pharmacology was used to

33 test if DA receptors in the IPN can regulate anxiety-like behavior.

34 Results

35 DA was detected in mouse IPN slices. Silencing/activating VTA→IPN DAergic inputs

36 oppositely modulated anxiety-like behavior. Two neuronal populations in the ventral IPN

37 (vIPN) responded to DA via D1 receptors (D1R). vIPN neurons were controlled by a

38 small population of D1R neurons in the caudal IPN (cIPN) that directly respond to VTA

39 DAergic terminal stimulation and innervate the vIPN. IPN infusion of a D1R agonist and

40 antagonist bidirectionally controlled anxiety-like behavior.

41 Conclusions

42 VTA DA engages D1R-expressing neurons in the cIPN that innervate vIPN thereby

43 amplifying the VTA DA signal to modulate anxiety-like behavior. These data identify a

44 DAergic circuit that mediates anxiety-like behavior through unique IPN microcircuitry.

45 **Keywords**

46 Anxiety, Circuitry, Dopamine, Interpeduncular Nucleus, Ventral Tegmental Area, Medial

47 Habenula

48

49

50

51

52

53

54

55

56

57

58

59

Introduction

Anxiety is a complex, multi-circuit behavioral phenomenon characterized by a prolonged sense of unease and heightened arousal in the absence of a direct threat(1). Persistent uncontrolled anxiety inappropriate to the level of threat underlies anxiety disorders which are often comorbid with depression and many other psychiatric disorders(2). Understanding the neurocircuitry that regulates anxiety is necessary to inform future anxiolytic therapy development.

Basal and stress-induced anxiety states are governed by brain regions that process emotions including prefrontal cortex (PFC), hippocampus, and extended amygdala(3). Each of these regions is regulated by modulatory input from dopamine (DA)-rich midbrain areas that are hypothesized to shape anxiety-like behavior(4,5,6,7), although the exact role of DA and how it drives behavior in response to anxiogenic stimuli are unknown. Emerging data implicate a much more understudied pathway that contributes to fear and anxiety-like behavior, the habenulo-interpeduncular axis(8,9,1011). This pathway consists of neurons in the medial habenula (mHb) that project to the interpeduncular nucleus (IPN)(12). While the mHb receives input from the septum, the IPN transmits forebrain input to the mid- and hindbrain resulting in the regulation of behavior(13,14). The majority of studies on the mHb→IPN circuit have focused on nicotine addiction-associated behaviors, where this pathway has been implicated in regulating drug intake and aversive, affective, as well as physical aspects of nicotine

4

80   withdrawal(15,16,17,18,19). The habenulo-interpeduncular pathway also contributes to

81   regulating baseline anxiety-like behavior(20,21), although the mechanism(s) involved,

82   particularly in the IPN, are not clearly understood.

83   We recently described a mesointerpeduncular circuit in which VTA DAergic neurons

84   project to the neighboring IPN(22). While the DAergic neuron-rich VTA is largely

85   associated with increased motivation towards novelty, reinforcement, and positive

86   affective state, the IPN is a brain region governing reduced motivation towards

87   familiarity, as well as aversion, and negative affective state(19,22,23,24,25,26,27).

88   Thus, general activity in these two regions promotes opposing behaviors suggesting the

89   mesointerpeduncular circuit could act as an important balancing point governing

90   motivation and anxiety-like behavior. Indeed, previously we showed that stimulating this

91   pathway with optogenetic tools could shift the motivational aspects of familiar stimuli

92   interactions and enhance their salience as if they were novel(22). Here, we provide a

93   comprehensive understanding on the mechanistic connection between the VTA and IPN

94   and how endogenous DA released from this circuitry contributes to anxiety-associated

95   behaviors.

96   **Materials and Methods**

97   **Animals**

98   All experiments followed the guidelines for care and use of laboratory animals provided

99   by the National Research Council, and with approved animal protocols from the

100   Institutional Animal Care and Use Committee of the University of Massachusetts

101   Medical School. C57Bl/6J (#000664), GAD2-Cre (#010802), Chat-Cre (#006410), DAT-

102 Cre (#006660), Chat-ChR2 (#014546), DRD1-Cre (#028298), and Drd1a-tdTomato

103 (#016204) mice were obtained from The Jackson Laboratory, bred in the UMMS animal

104 facility and used in behavioral, optogenetic and biophysical experiments as indicated.

105 Cre lines were crossed with C57Bl/6J mice and only heterozygous animals carrying one

106 copy of the Cre recombinase gene were used for experimental purposes. Mice were

107 housed together in cages of no more than five animals and kept on a standard 12 h

108 light/dark cycle (lights ON at 7 A.M.) with ad libitum access to food and water. Three to

109 four weeks before experimentation, subject mice were kept under a reverse 12 h

110 light/dark cycle (lights ON at 7 P. M.) for at least 5 days before any behavioral testing

111 **Viral Preparation**

112 Optogenetic plasmids were packaged into AAV serotype 2 (AAV2) viral particles by the

113 UMMS Viral Vector Core. $GRAB_{DA2m}$ is derived from $GRAB_{DA1m}$, with additional

114 mutations in cpEGFP. $GRAB_{DA2m}$ has ~3-fold improvement in the maximal $\Delta F/F_0$ and

115 similar apparent affinity (EC_{50}~90 nM). Detailed characterizaion of $GRAB_{DA2m}$ will be

116 published elsewhere. $GRAB_{DA2m}$ was packaged into AAV9 by Vigene Biosciences.

117 Additional Materials and Methods can be found in Supplementary Materials.

118 **Results**

119 **DA is released in the IPN.**

120 To test if endogenous DA release in the IPN occurs and may be involved in anxiety-like

121 behavior, we expressed an enhanced genetically encoded GPCR-activated DA sensor

122 ($GRAB_{DA2m}$, see Methods) that changes in fluorescence upon DA binding, in the IPN of

6

123   C57Bl/6J mice using AAV-mediated gene delivery(28). To test appropriate function of

124   GRAB$_{DA2m}$, we prepared midbrain slices and measured changes in fluorescence in the

125   IPN during bath application of neurotransmitter (Figure 1A). As expected, we recorded

126   robust increase in fluorescence intensity in response to 10 and 100 µM DA, with a

127   lesser response to norepinephrine (NE) and no significant response to ACh, GABA, or

128   glutamate (Figure 1A). To test if endogenous DA could be detected in IPN slices, we

129   measured changes in fluorescence in response to the monoamine transporter

130   substrate, amphetamine (Figure 1B). Bath application of amphetamine increased signal

131   of GRAB$_{DA2m}$, which was significantly blocked by preapplication of the D2 receptor

132   antagonist, haloperidol indicating that the amphetamine-induced signal was mediated

133   by GRAB$_{DA2m}$.   In addition, the amphetamine-induced signal persisted in the presence

134   of the norepinephrine transporter inhibitor, desipramine, confirming that the signal was

135   mediated by endogenous IPN DA release as opposed to NE (Figure 1C).

136   Together, these data indicate that endogenous DA is released in the IPN, a

137   phenomenon which may be critical for regulating anxiety-like behavior.

138   **DA VTA afferents in the IPN bidirectionally modulate anxiety-like behaviors.**

139   A subpopulation of DAergic neurons in the VTA project to the IPN constituting a

140   mesointerpeduncular pathway(18,22).   To test if VTA→IPN axon terminals are the

141   source of DA release and contribute to anxiety-like behaviors, we selectively expressed

142   Cre-dependent halorhodopsin (NpHR)-eYFP in the VTA of DA transporter (DAT)::Cre

143   mice via AAV2-mediated gene delivery and implanted fiber optic cannulas into the IPN

144   to deliver yellow light (593nm, constant light, 20s on, 10s off, Figure 2A, S2) and photo-

145   inhibit VTA^{DA}→IPN inputs during the elevated plus maze (EPM)(29, 30). VTA^{DA}→IPN

146   photo-inhibition resulted in a decrease in open arm time in the EPM compared to light-

147   on eYFP controls (Figure 2B). VTA^{DA}→IPN photo-inhibition had little effect on total arm

148   entries compared to control conditions, suggesting normal locomotion in these animals.

149   We also evaluated VTA^{DA}→IPN photo-inhibition in the open field test (OFT) and

150   observed a decrease in center time and no effect of photoinhibition on total activity

151   (Figure 2C). To test the effect of activating VTA^{DA}→IPN on open arm exploration, we

152   selectively expressed Cre-dependent channelrhodopsin (ChR2)-eYFP in the VTA of

153   DAT::Cre mice via AAV2-mediated gene delivery (Figure 2D, S1)(23). A fiber optic

154   cannula was implanted targeting the IPN for blue-light stimulation of VTA^{DA}→IPN inputs

155   (473nm, 15 Hz, 20 ms/pulse, 5 s light-on, 5 s light off) during behavioral testing in the

156   EPM. Photostimulation of VTA^{DA}→IPN inputs significantly increased time spent in the

157   open arms of the EPM compared to control mice expressing eYFP and receiving light

158   stimulation, while having little effect on total arm entries compared to control conditions

159   (Figure 2E). In the OFT, photostimulation of VTA^{DA}→IPN increased time spent in the

160   center compared to controls without significantly affecting total distance traveled (Figure

161   2F). To test if behavioral results obtained with our optogenetic approach could be due to

162   silencing/activating of VTA DAergic neurons directly, we placed fiber optic cannulas

163   dorsal to the IPN, targeting the VTA (Figure S2). Silencing VTA neurons increased

164   open arm time in the EPM compared to eYFP controls (Figure S3A), an effect opposite

165   to specific VTA^{DA}→IPN inhibition, but did not significantly impact behavior in the OFT

166   (Figure S3C); whereas activating VTA neurons with ChR2 did not significantly change

167   open arm time in the EPM compared to eYFP controls (Figure S3B), but significantly

168 decreased center time in the OFT. Together, these data indicate that the $VTA^{DA} \rightarrow IPN$

169 pathway is a critical component of anxiety circuitry that, when engaged, drives reduced

170 anxiety-like behavior.

171 **Two neuronal populations in the ventral IPN differentially respond to DA via D1-**

172 **like, but not D2-like, DA receptors.**

173 To determine DA responses in IPN neurons, we used electrophysiology in acute coronal

174 slices of C57Bl/6J mice. In cell-attached mode, we measured spontaneous action

175 potentials (spAPs) during a five-minute bath application of exogenous 10 µM DA (Figure

176 3). In the ventral IPN (vIPN), 18 out of 39 neurons responded to DA with an increase in

177 spontaneous action potential (spAP) frequency that reversed upon washout (designated

178 as "Type A" neurons, Figure 3A, B, C), while 17 out of 39 neurons responded to DA with

179 a decrease in spAP frequency that reversed upon washout (designated as "Type B"

180 neurons, Figure 3D, E, F). The remaining 4 neurons exhibited no obvious responses

181 (Figure 3G). To examine the physiological properties and current-voltage relationship of

182 these two types of vIPN neurons, we injected 100 pA to -100 pA current in -20 pA steps.

183 Type A and Type B neurons exhibited clear significant differences in their response to

184 current injection and input resistance (Figure 3H-I), with Type A neurons having a lower

185 input resistance compared to Type B neurons.

186 To test which DA receptors are required for DA-induced changes in spAP frequency in

187 the vIPN, DA was applied to Type A and Type B neurons in the absence and presence

188 of the D1-like receptor antagonist, SCH39166 (10 µM) or the D2-like receptor

189 antagonist, eticlopride (10 µM, Figure 4A-C). SCH39166, but not eticlopride,

190   significantly attenuated DA-mediated spAP frequency changes both in Type A and Type

191   B neurons, suggesting that DA acts through D1-like but not D2-like DA receptors in the

192   IPN. In addition, to further rule out D2 effects, we applied a D2-like DA receptor agonist,

193   quinpirole, to vIPN neurons and did not observe any changes in spAP frequency,

194   spontaneous excitatory post-synaptic current (spEPSC) frequency or amplitude (Figure

195   S4).

196   **DA modulates vIPN neurons via presynaptic DA receptors.**

197   To assess how D1-like DA receptors modulate vIPN neuron activity, we recorded from

198   Type A and B neurons under voltage-clamp and measured changes in excitatory input.

199   DA was bath applied and neurons were voltage-clamped at -70 mV to record spEPSCs.

200   Of note, DA failed to induce obvious inward or outward post-synaptic currents under

201   voltage-clamp (data not shown). However, DA increased spEPSC frequency in Type A

202   neurons while decreasing spEPSCs frequency in Type B neurons, with no effect on the

203   spEPSC amplitude in either neuron type, suggesting DA affects excitatory inputs via DA

204   receptors that are presynaptic (Figure 4D-I). The valence of spEPSC frequency was

205   also consistent with the DA-induced changes in spAP frequency observed in the two

206   vIPN neuron sub-types. In addition, when spEPSCs were blocked by NMDA and AMPA

207   receptor antagonists (20 µM AP-5 and 10 µM CNQX), the majority of vIPN neurons

208   ceased firing, suggesting that the change in spEPSC frequency induced by DA directly

209   causes the DA-induced change in spAP frequency (Figure S5). These findings indicate

210   that DA increases presynaptic excitatory transmission to Type A neurons and

211   decreases presynaptic excitatory transmission to Type B neurons.

cIPN neurons respond to afferent VTA DAergic terminal stimulation

212

213 To test if vIPN neurons respond to DAergic inputs from the VTA, we selectively

214 expressed Cre-dependent ChR2-eYFP in VTA DAergic neurons of DAT::Cre mice and

215 we recorded vIPN neuronal responses upon light-induced VTA^{DA}→IPN stimulation

216 (Figure 5; 20 Hz, 2 ms pulse width). VTA DAergic terminals were stimulated through the

217 microscope objective focused on the area around the recorded IPN neuron (Figure 5A).

218 Cell-attached mode was used to record spAPs. Interestingly, the majority of vIPN

219 neurons failed to respond to VTA terminal optic stimulation (Figure S7A). Previously,

220 using mice in which the fluorophore td-Tomato is under the control of the *DRD1* (the

221 gene encoding the DA D1 receptor) promoter (the Drd1a-tdTomato line(31)), we

222 determined that D1 receptor expression is localized to soma in the caudal IPN (cIPN)

223 while presumed terminal fields are localized to the vIPN (also see Figure S6A), raising

224 the possibility that VTA→IPN DA innervation may be sub-region (i.e. cIPN) specific. In

225 DAT^{Cre}::eYFP mice, we observed VTA DAergic inputs in the cIPN but not rostral IPN

226 (Figures S6A, S6B) supporting this hypothesis. In addition, D1-TdTomato midbrain

227 slices immuno-labeled for DAT revealed TdTomato-positive neurons in cIPN decorated

228 with DAT-immunopositive puncta (Figure S6C). In contrast to vIPN neurons, light-

229 evoked responses were observed in the cIPN matching the VTA→IPN innervation

230 pattern. As compared to vIPN neurons, cIPN neurons exhibited a significantly higher

231 input resistance and a different current-voltage relationship (Figure 5B, S7B) indicating

232 a distinct cIPN sub-type that we refer to as "Type C". In cIPN slices, a sub-population of

233 Type C neurons responded to light stimulation of DAergic afferents with an increase in

234 spAP frequency that was attenuated in the presence of SCH39166 (Figure 5C-E). To

235   test the mechanism of light-evoked changes in AP frequency in Type C neurons, we

236   examined excitatory input, recording spEPSCs in response to light. Blue light failed to

237   evoke a change in either spEPSC frequency or amplitude, suggesting the effect of DA

238   on spAP frequency in this sub-population was due to post-synaptic D1 receptor

239   expression (Figure 5F-H). Moreover, we also observed a population of Type C neurons

240   that exhibited a light-induced decrease in spAP frequency, as well as, a reduction in

241   spEPSC frequency, that were likewise blocked by SCH39166 (Figure S8D-J). To gain

242   insight into localization of the DAergic neurons in the VTA that may project to the IPN,

243   we injected AAV2rg-hSyn-DIO-eGFP into different regions of striatum to label discreet

244   VTA DAergic neurons in DAT::Cre mice (Figure S8)(43-45). In mice in which paranigral

245   VTA DAergic neurons were labeled, we could trace projections into the cIPN (Figure

246   S8A); whereas, in mice in which paranigral neurons were not labeled, DAergic

247   VTA→IPN projections were less apparent (Figure S8B). Together, these data suggest

248   that a sub-population of Type C neurons in the cIPN that signal through D1 receptors,

249   may amplify the VTA DAergic input to other IPN neurons, for instance, vIPN Type A and

250   Type B neurons, and modulate their responses.

251   **cIPN Type C neurons project to the vIPN to control activity of Type A and Type B**

252   **neurons.**

253   To test if D1-positive neurons in the cIPN project directly to the vIPN, we expressed

254   Cre-dependent ChR2-eYFP in the IPN of mice that express Cre under the control of the

255   DRD1 promoter (DRD1a::Cre mice) via AAV2-mediated gene delivery (Figure 6A).

256   eYFP signal was observed in cIPN neuronal soma and projections along the

257   cIPN→vIPN plane (Figure 6B). Stimulation of Type C terminals in the vIPN (20Hz, 2 ms

258   pulse width) resulted in a significant increase of spEPSC frequency in Type A neurons

259   and a significant decrease of spEPSC frequency in Type B neurons (Figure 6C, D, F,

260   G). These responses phenocopied the result of bath application of DA in 80% of vIPN

261   neurons as predicted by their input resistances (compare with Figure 4D-I).

262   Experiments were repeated in the presence of 1 µM TTX and 100 µM 4-AP to block

263   action potentials, and thus, block multi-synaptic responses(32). The changes in EPSC

264   frequency upon light stimulation were maintained in both Type A and B neurons

265   suggesting the D1 receptor-expressing Type C cIPN neurons project monosynaptically

266   to the vIPN (Figure 6E, H). In addition, combined GABA$_A$ and GABA$_B$ antagonists

267   saclofen (10 µM) and bicuculline (20 µM) blocked the light-evoked change in spEPSC

268   frequency in both Type A and B vIPN neurons suggesting Type C neurons release

269   GABA to modulate excitatory synapses in the vIPN (Figure 6I, J). As Type A and B

270   neurons are morphologically distinct and receive differential innervation from mHb

271   terminals (see Supplemental Results and Figure S9), which strongly innervate Type A

272   but not Type B neurons, and mHb terminals in the IPN are known to uniquely increase

273   excitatory transmission in response to activation of GABA$_B$ receptors(11,46,47), these

274   data suggest GABA bidirectionally modulates excitatory synapses on Type A and B

275   neurons through mHb and non-mHb excitatory inputs, respectively.

276   **Pharmacological manipulation of D1 receptors in the IPN bidirectionally**

277   **modulates anxiety-like behavior.**

278   To test if D1 signaling in the IPN modulates anxiety-like behavior, we implanted drug

279   infusion cannulas and delivered a D1 receptor agonist or antagonist into the IPN prior to

280   testing in the EPM and OFT assays (Figure 7). In the EPM and the OFT, intra-IPN

281   infusion of the D1 receptor agonist SKF82958 increased open arm time and increased

282   time in the center, respectively, compared to vehicle infusion, indicating an anxiolytic

283   effect of the drug. Conversely, the D1 receptor antagonist SCH39166 was anxiogenic,

284   reducing open arm time and time in the center compared to vehicle infusion (Figure 7B,

285   D). Neither drug affected the number of arm entries in the EPM, or total distance

286   traveled in the OFT (Figure 7C, E). Infusion of the D1-like receptor agonist and

287   antagonist directly into the VTA had little effect on anxiety-like behavior (Figure

288   S10B,D). However, VTA infusion of D1 drugs resulted in a depression of total arm

289   entries in the EPM (Figure S10C). The difference in locomotor effects and the lack of a

290   significant effect on anxiety-like behavior when the VTA was infused suggests behaviors

291   elicited from IPN infusions were not the result of off target effects from drug diffusion.

292   Overall, these results demonstrate that endogenous DA controls anxiety-like behavior

293   via anxiolytic D1 receptor signaling in the IPN.

294

Discussion

296   DA signaling has long been implicated in anxiety-like behavior presumably through

297   midbrain DA projection areas to the hippocampus, extended amygdala, and prefrontal

298   cortex, among other brain regions(4-6,34-36). Our data combining $GRAB_{DA}$ sensor

299   expression in the IPN with pharmacology and imaging revealed that endogenous DA is

300   released in the IPN.. Preventing IPN DA increases in vivo by silencing the VTA→IPN

301   input reduced both exploration of the EPM open arm and exploration of the center of the

302   OFT. Conversely, activating the input increased time spent in the EPM open arm and

303   exploration of the center of the OFT, suggesting that this IPN DA signal controls

304   anxiety-like behavior specifically by driving anxiolysis. Assays used to evaluate anxiety-

305   like behavior in mice including the EPM and OFT are multimodal and integrate two

306   opposite motivational drives: 1) behavioral avoidance and 2) novelty seeking(29).

307   Mice will be driven to explore the open arms of the EPM or center of the OFT because

308   they are novel but also avoid exploration because they are elevated or open and without

309   protection from predation. Thus, the read-out or expression of anxiety-like behavior

310   relies upon the strength of these two motivational drives. Interestingly, previous studies

311   implicate the habenulo-interpeduncular pathway in behavioral avoidance and

312   aversion(10,16,19); whereas, we have also discovered that the IPN and associated

313   circuitry is also critically involved in signaling familiarity, reducing motivation to explore

314   novelty to control novelty preference(22). Our data indicate that VTA input and IPN DA

315   may provide a signal that either reduces avoidance behavior to allow expression of

316   reduced anxiety-like behavior or increase motivation to explore novelty. Future studies

317   will focus on how the IPN integrates anxiety and novelty signals to drive exploratory

318   behavior.

319   Activation of DAergic IPN inputs stimulates a small sub-population of dopaminoceptive

320   neurons expressing the D1 receptor located predominantly in the caudal portion of the

321   IPN. Through retrograde tracing, our data suggest that a sub-population of accumbens

322   shell-projecting VTA DA neurons in the paranigral region may preferentially project into

15

323   the IPN to innervate cIPN neurons, although we cannot rule out that DAergic neurons in

324   other regions of the VTA, or other brain areas, also may contribute to this circuit, an

325   issue that will require further experimentation.

326   Remarkably, cIPN neurons, through a microcircuit spanning the vIPN, amplify the DA

327   signal ultimately controlling anxiety-like behavior. Indeed, the vast majority of vIPN

328   neurons respond to exogenous DA in midbrain slices (35 out of 39) presumably through

329   D1 receptor-expressing Type C terminals which modulate excitatory input to vIPN

330   neurons. One potential caveat with our results is that we used a D1 antagonist, SCH

331   39166, to block DA effects in midbrain slices. While SCH 39166 is selective for D1/D5

332   receptors, it can also block D2-like receptors at higher concentrations and may also be

333   a low affinity antagonist at 5HT2 receptors(48). However, the concentration used in our

334   experiments was similar to that of other studies examining D1-receptor mediated

335   responses in rodent brain slices(49-51) and effects of DA signaling we observed in the

336   IPN persisted in the presence of a D2 antagonist. In addition, the D1 antagonist not

337   only blocked effects of bath application of DA, but also responses observed by specific

338   optic activation of VTA DAergic terminals in the IPN. The effect of exogenous DA

339   application on vIPN neuronal activity was phenocopied by direct optogenetic activation

340   of D1-expressing terminals in vIPN, supporting a DA signal-amplifying micro-circuit.

341   Thus, what at first glance would appear to be a modest connection between VTA and

342   cIPN, through this amplification step, transmits activity to the majority of neurons in the

343   ventral portion of the nucleus to control behavior.

344   The microcircuit controlling activity of vIPN neurons is unique in that it consists of two

345   morphologically distinct neurons, Type A and Type B, which both receive GABAergic

16

346   innervation from cIPN Type C neurons but act oppositely in response to GABA. Type A

347   neurons are excited by activation of Type C terminals via increased glutamate release;

348   whereas Type B neurons are inhibited by activation of Type C terminals via decreased

349   glutamate release (Figure 8). Interestingly, Type A neurons are robustly controlled by

350   mHb excitatory inputs that are activated by GABA via excitatory GABA$_B$ receptors on

351   mHb terminals(11,33). Type B neurons, on the other hand, are weakly innervated by

352   the mHb, thus, it is likely that GABA reduces excitatory input from other, unidentified

353   excitatory afferents that express inhibitory GABA receptors. In the future, it will be

354   necessary to apply additional circuit mapping approaches to identify this excitatory IPN

355   input. Ultimately, engaging this microcircuit either through optogenetic stimulation of

356   VTA→IPN inputs or through infusion of D1 receptor agonist increases Type A neuronal

357   activity while decreasing Type B neuronal activity to reduce anxiety-like behavior.

358   In summary, our data indicate that VTA DAergic input to the IPN mediates anxiety-like

359   behavior by activating D1-expressing neurons in the cIPN. This small population of

360   dopaminoceptive neurons amplify VTA DA input by projecting to and innervating vIPN

361   through mHb glutamatergic inputs to bidirectionally control anxiolysis. Thus, we have

362   identified a critical component of the neural network contributing to affective state

363   through DAergic signaling that engages a unique IPN microcircuit.

364   **Author Contributions**

365   S.R.D., R.Z, L.C., P.M.K., and S.M. conducted the experiments. Y.L. provided the

366   GRAB$_{DA}$ sensors. S.R.D., R.Z., L.C., P.M.K., F.S., P.D.G. Y.L. and A.R.T. designed the

367   experiments. S.R.D. and A.R.T. wrote the paper with input from all co-authors.

17

Acknowledgments

Declaration of Interests

The authors report no biomedical financial interests or potential conflicts of interest.

Data Availability

The data presented in this study are available from the corresponding author upon reasonable request.

Figure Legends

Figure 1. Dopamine sensing in the IPN. (A) Left, experimental strategy for functional verification of GRAB$_{DA2m}$ in midbrain slices. Middle, heat map of IPN GRAB$_{DA2m}$

18

387　responses to 2 min bath application of neurotransmitter, applied at t=2 min. Right,

388　summed average of maximal responses of bath application of neurotransmitter (One

389　way ANOVA: $F_{(5, 31)}$ = 89.6, p = 0.0001; Bonferroni's multiple comparisons test:

390　****p<0.0001). **(B)** Top, heat map of IPN GRAB$_{DA2m}$ responses to ACSF (n=10),

391　amphetamine (AMPH, n=16), or AMPH following pre-application of haloperidol (HALO,

392　n=6). AMPH applied at t=10 min until the end of recording. Bottom, summed average of

393　maximal responses from top panel (One way ANOVA: $F_{(2, 29)}$ = 35.2, p = 0.0001;

394　Bonferroni's multiple comparisons test: ****p<0.0001). **(C)** Top, heat map of IPN

395　GRAB$_{DA2m}$ responses to ACSF (n=6), desipramine (DPA, n=6), AMPH (n=6), or AMPH

396　following pre-application of DPA (n=6). Bottom, summed average of maximal responses

397　from top panel (One way ANOVA: $F_{(3, 20)}$ = 29.7, p = 0.0001; Bonferroni's multiple

398　comparisons test: ****p<0.0001).

399

400　**Figure 2. VTA→IPN DA input controls anxiety-like behavior. (A)** Diagram of strategy

401　for halorhodopsin experiments. **(B)** Open arm time (left) and total arm entries (right)

402　during *in vivo* NpHR inhibition of VTA terminals in the IPN of light-on NpHR (n=13) and

403　light-on eYFP (n=11) DAT^{Cre} animals. (Unpaired two-tailed t-test: p=0.0006. Mean ±

404　SEM.) **(C)** OFT activity during *in vivo* NpHR inhibition of VTA terminals in the IPN.

405　Representative heat map of mouse position (top). Graphs of center time (bottom left)

406　and total distance traveled (in cm, bottom right, n=11, 14, eYFP and NpHR,

407　respectively, Unpaired t-test with Welch's correction: p=0.023. Mean ± SEM.). See

408　Figure S2 for canula placement. **(D)** Top, diagram of strategy for channelrhodopsin

409　experiments (also see Figure S2). **(E)** Open arm time (left) and total arm entries (right)

during *in vivo* 15 Hz stimulation of ChR2-expressing VTA terminals in the IPN for light-

on eYFP (n=10) and light-on ChR2 (n=10) groups. (Unpaired t-test: p=0.0038. Mean ±

SEM.) **(F)** OFT activity during *in vivo* 15 Hz stimulation of ChR2-expressing VTA

terminals in the IPN. Representative heat map of mouse position (top). Graphs of center

time (bottom left) and total distance traveled (bottom right). See Figure S2 for canula

placement. (n=12, 10 eYFP and NpHR, respectively, Unpaired t-test: p=0.0028. Mean ±

SEM.)

Figure 3. DA modulates neuronal activity in two vIPN neuron sub-populations.
(A) Representative cell-attached trace from a Type A neuron in response to DA and **(B)**
AP frequency of Type A neurons at baseline, during the last minute of DA application,
and after washout (Friedman test: Friedman statistic$_{(2, 34)}$ = 19.13, p<0.0001. ***p<0.0001
compared to baseline, Dunn's multiple comparison test. Mean ± SEM.). **(C)** Time
course of drug application in Type A neurons. **(D)** Representative cell-attached trace of
a Type B neuron (top) in response to DA and **(E)** AP frequency of Type B neurons at
baseline, during the last minute of DA application, and after washout (Friedman
statistic$_{(2, 32)}$ = 22.81, p≤0.0001. ****p<0.0001 compared to baseline, Dunn's multiple
comparison test. Mean ± SEM.) **(F)** Time course of drug application in Type B neurons.
(G) Diagram of a coronal section of the IPN with approximate locations of Type A
neurons (blue circles) and Type B neurons (red circles). Neurons without a response to
DA are depicted as green circles. Location taken from digital images of the recording
pipette in the slice after each recording. Representative traces of Type A **(H)** and B **(I)**
current-voltage relationships in response to 20 pA current injection steps. Traces are to
scale with each other. **(J)** Input resistance of Type A and B neurons calculated from the

20

433 0 to -20 pA step from traces in (D) and (E). (n = 10 and 12, respectively, unpaired t-test

434 with Welch's correction: ***p=0.0003. Data presented as mean ± SEM.) **(K)** Current

435 voltage relationship. (Two-way ANOVA: Significant cell-type x current step interaction,

436 $F_{(10, 218)}$=5.07, p = 0.0001. Bonferroni's multiple comparisons test: **p≤0.01, ***p≤0.001,

437 ****p≤0.0001. Mean ± SEM.)

438 **Figure 4. vIPN neurons respond to DA through presynaptic D1-like but not D2-like**

439 **DA receptors. (A)** Schematic of experiment. Dotted lines indicate approximate

440 positions where coronal slice was cut (left). Neurons were recorded from a coronal slice

441 of the vIPN (right). **(B)** Averaged normalized spAP frequency of Type A neurons in

442 response to DA in the absence and presence of the D1-like receptor antagonist

443 SCH39166 (10 µM, top) or the D2-like receptor antagonist Eticlopride (10 µM, bottom).

444 (n=6, One-way ANOVAs: (Top) $F_{(2, 10)}$ = 19.6, p=0.0003; SCH39166: $F_{(2, 10)}$ = 0.1435,

445 p=0.8680; (Bottom) $F_{(2, 10)}$ = 6.492, p=0.016; Eticlopride: $F_{(2, 10)}$ = 9.23, p=0.0054. Data

446 presented as mean ± SEM.) *p<0.05, **p<0.01, ***p<0.001 compared to baseline. **(C)**

447 Averaged normalized spAP frequency of Type B neurons in response to DA in the

448 absence and presence of the D1-like receptor antagonist SCH39166 (10 µM, top) or the

449 D2-like receptor antagonist eticlopride (10 µM, bottom, n=6, One-way ANOVAs: (Top)

450 $F_{(2, 12)}$ = 8.593, p=0.0048; SCH39166: $F_{(2, 12)}$ = 1.852, p=0.1991; (Bottom) $F_{(2, 10)}$ = 17.86,

451 p=0.0005; $F_{(2, 10)}$ = 25.79, p=0.0001). **p<0.01, ***p<0.001 compared to baseline, Mean

452 ± SEM. **(D)** Representative whole-cell patch clamp traces of spEPSCs in a Type A

453 neuron before, during, and after DA application. **(E)** Type A spEPSC frequency at

454 baseline, during last minute of DA application, and after washout (n=10, Friedman test:

455 **p≤0.01, Friedman statistic$_{(2, 18)}$ = 9.6., p=0.0075). ** p<0.01 Dunn's test compared to

456 baseline. Data are mean ± SEM. **(F)** Type A spEPSC amplitude at baseline, during last

457 minute of DA application, and after washout. (n=10, One-way ANOVA: $F_{(2, 18)}$ = 0.3592,

458 p=0.7031). Data are mean ± SEM. **(G)** Representative whole-cell patch clamp traces of

459 spEPSCs in a Type B neuron before, during, and after DA application. **(H)** Type B

460 spEPSC frequency at baseline, during last minute of DA application, and after washout

461 (n=7, One-way ANOVA: $F_{(2, 12)}$ = 14.47, p=0.0008). *p < 0.05 compared to baseline.

462 Data are mean ± SEM. **(I)** Type A spEPSC amplitude at baseline, during last minute of

463 DA application, and after washout (One-way ANOVA: $F_{(2, 12)}$ = 0.6047, p=0.5621. Data

464 are mean ± SEM).

465 **Figure 5. Optogenetic stimulation of VTA→IPN DAergic terminals modulate cIPN**

466 **neurons via D1 receptors. (A)** Schematic of experiment**.** Cre-dependent ChR2-eYFP

467 was expressed in putative DAergic neurons of the VTA in DAT[Cre] mice via AAV2-

468 mediated gene delivery (left). Neurons in the cIPN were recorded in coronal slices while

469 optogenetically stimulating the DRD1 expressing terminals (right). **(B)** Representative

470 whole-cell current-clamp traces from a cIPN neuron in response to 20 pA current

471 injection steps from +100 to -40 pA. Compare to Figure 2A and B. **(C)** Representative

472 traces of a cIPN neuron that responded to VTA terminal stimulation with an increase in

473 firing rate. **(D)** spAP frequency of Type C neurons that responded to light stimulation

474 with an increase in spAP frequency (n=10, One-way ANOVA: $F_{(2, 18)}$ = 5.59, p=0.013).

475 *p<0.05 compared to Light-off control. Data presented as mean ± SEM. **(E)** spAP

476 frequency of cIPN neurons from (D) during 10 µM SCH39166 application (Friedman

477 statistic$_{(2, 16)}$ = 5.35, p=0.07). Data presented as mean ± SEM. **(F)** Representative trace

478 of EPSC frequency from a cIPN neuron that increased its spAP frequency in response

22

479   to VTA terminal stimulation. **(G)** In cIPN neurons that increased their spAPs, spEPSC

480   frequency was not significantly affected. (One-way ANOVA: $F_{(2, 10)}$ = 0.1732, p=0.8435).

481   Data presented as mean ± SEM. **(H)** In cIPN neurons that increased their spAPs,

482   spEPSC amplitude was not significantly affected. (One-way ANOVA: $F_{(2, 10)}$ = 2.106,

483   p=0.1725). Data presented as mean ± SEM.

484   **Figure 6. cIPN Type C putative D1 receptor-expressing neurons project to the**

485   **vIPN and modulate Type A and Type B neuronal activity via GABA. (A)** Schematic

486   of experiment. Cre-dependent ChR2-eYFP was expressed in putative DRD1-expressing

487   neurons of the cIPN in DRD1::Cre mice via AAV2-mediated gene delivery (left).

488   Neurons in the vIPN were recorded in coronal slices while optogenetically stimulating

489   the DRD1 expressing terminals (right). **(B)** Sagittal slice showing Cre-dependent eYFP

490   (green) from a (DRD1)::Cre mouse. cIPN neurons send projections rostrally to the vIPN.

491   **(C)** Representative whole-cell patch clamp traces of Type A neuron EPSCs before,

492   during and after 20 Hz stimulation of cIPN terminals in the presence of TTX and 4-AP.

493   **(D)** Type A EPSC response to 20 Hz terminal stimulation. (n=8, One-way ANOVA: $F_{(2,}$

494   $_{14)}$ = 20.8, p=0.0001). ***p<0.001 compared to Light-off control. Data presented as

495   mean ± SEM. **(E)** Type A response to DRD1-Cre terminal stimulation in the presence of

496   AP blockers. The response was "monosynaptic" (n=14, Friedman test: Friedman

497   statistic$_{(2,26)}$ = 24.57, p<0.0001). **p<0.01 compared to Light-off control. Data presented

498   as mean ± SEM. **(F)** Representative whole-cell patch clamp traces of Type B neuron

499   EPSCs before, during and after stimulation of cIPN terminals in the presence of TTX

500   and 4-AP. **(G)** Type B response to 20 Hz terminal stimulation (n=7, One-way ANOVA: F

501   $_{(2, 12)}$ = 4.4, p=0.037). *p<0.05 compared to Light-off control. Data presented as mean ±

502 SEM. **(H)** Type B response to 20 Hz DRD1-Cre terminal stimulation in the presence of

503 AP blockers. The connection was monosynaptic (n=9, One-way ANOVA: $F_{(2, 16)}$ = 6.58,

504 p=0.0082). **p<0.01 compared to Light-off control. Data presented as mean ± SEM. **(I)**

505 Normalized EPSC frequency of a Type A neuron before, during, and after cIPN DRD1-

506 Cre terminal stimulation in the presence of 1 μM TTX and 100 μM 4-AP. The

507 experiment was repeated with the addition of bath-applied 20 μM Bicuculline and 100

508 μM Saclofen to block $GABA_A$ and $GABA_B$ receptors, respectively. (n=7, One-way

509 ANOVAs: $F_{(2, 12)}$ = 10.08, p=0.0027; $GABA_{A+B}$ receptor antagonists: $F_{(2, 12)}$ = 1.539, p=

510 0.2542). **p<0.01 compared to Light-off control. Data are presented as mean ± SEM.

511 **(J)** Normalized EPSC frequency of a Type B neuron before, during, and after cIPN

512 DRD1-Cre terminal stimulation in the presence of 1 μM TTX and 100 μM 4-AP. The

513 experiment was repeated with the addition of bath-applied 20 μM Bicuculline and 100

514 μM Saclofen to block $GABA_A$ and $GABA_B$ receptors, respectively. (n=5, One-way

515 ANOVA: $F_{(2, 8)}$ = 7.437, p=0.015; $GABA_{A+B}$ receptor antagonists: $F_{(2, 8)}$ = 3.458, p=

516 0.0827). *p<0.05 compared to Light-off control. Data are presented as mean ± SEM.

517

518 **Figure 7. Manipulation of D1 receptors in the IPN controls anxiety-like behaviors.**

519 **(A)** Diagram of experiment (left). Representative image of guide canula track in slice

520 immune-labeled with TH antibody (green, also see Figure S11F). **(B)** Quantification of

521 open arm time in the EPM between D1-like DA receptor agonist (n=10, SKF82598 0.7

522 μg/μl, infused 0.3 μl, 210 ng), antagonist (n=13, SCH39166 35 ng/μl, infused 0.3 μl,

523 10.5 ng), or saline control (n=15). (One-way ANOVA with repeated measures: $F_{(2, 35)}$=

524   11.43, p = 0.0002). *p≤0.05, ***p≤0.001. Data presented as mean ± SEM. **(C)** Total arm

525   entries in the EPM after drug infusion. (One-way ANOVA with repeated measures: $F_{(2, 35)}$

526   = 0.6541, p=0.5261). Data presented as mean ± SEM. **(D)** Representative heat map

527   of mouse position in the OFT after infusion of drug into the IPN (left). Quantification of

528   center time in the OFT (right). (n=10, 9, 8 for saline, agonist, antagonist, respectively,

529   One-way ANOVA with repeated measures: *p≤0.05, **p≤0.01, $F_{(2, 24)}$ = 8.558. Mean ±

530   SEM.) **(E)** Quantification of total distance moved in the OFT. There was no significant

531   difference between groups. (One-way ANOVA with repeated measures: $F_{(2, 24)}$ = 1.437,

532   p=0.2574). Data are presented as mean ± SEM.

533   **Figure 8. Circuit model for DA signal amplification in the IPN.** Circles represent

534   neurons, the lines originating from the circles represent axons and the triangles

535   represent terminals. The terminals are set so that the side of the triangle opposite the

536   axon faces its presumed target. Each color represents a unique population of neurons.

537

538

539

540

541

542

543

544

545

546

547

548

549

References

551 1 Lieb, R. Anxiety disorders: clinical presentation and epidemiology. *Handb Exp
552   Pharmacol*, 405-432 (2005).
553 2 Chisholm, D., Sweeny, K., Sheehan, P., Rasmussen, B., Smit, F., Cuijpers, P. *et al.*
554   Scaling-up treatment of depression and anxiety: a global return on investment analysis.
555   *The lancet. Psychiatry* **3**, 415-424, doi:10.1016/S2215-0366(16)30024-4 (2016).
556 3 Calhoon, G. G. & Tye, K. M. Resolving the neural circuits of anxiety. *Nat Neurosci* **18**,
557   1394-1404, doi:10.1038/nn.4101 (2015).
558 4 Zweifel, L. S., Fadok, J. P., Argilli, E., Garelick, M. G., Jones, G. L., Dickerson, T. M. *et
559   al.* Activation of dopamine neurons is critical for aversive conditioning and prevention of
560   generalized anxiety. *Nat Neurosci* **14**, 620-626, doi:10.1038/nn.2808 (2011).
561 5 Jennings, J. H., Sparta, D. R., Stamatakis, A. M., Ung, R. L., Pleil, K. E., Kash, T. L. *et
562   al.* Distinct extended amygdala circuits for divergent motivational states. *Nature* **496**,
563   224-228, doi:10.1038/nature12041 (2013).
564 6 Refojo, D., Schweizer, M., Kuehne, C., Ehrenberg, S., Thoeringer, C., Vogl, A. M. *et al.*
565   Glutamatergic and dopaminergic neurons mediate anxiogenic and anxiolytic effects of
566   CRHR1. *Science* **333**, 1903-1907, doi:science.1202107 [pii]10.1126/science.1202107
567   (2011).

568 7 Berry, A. S., White, R. L., Furman, D. J., Naskolnakorn, J. R., Shah, V. D., D'Esposito,
569   M. *et al.* Dopaminergic mechanisms underlying normal variation in trait anxiety. *J
570   Neurosci*, doi:10.1523/JNEUROSCI.2382-18.2019 (2019).
571 8 Jesuthasan, S. Fear, anxiety and control in the zebrafish. *Dev Neurobiol*,
572   doi:10.1002/dneu.20873 (2011).
573 9 Okamoto, H. & Aizawa, H. Fear and anxiety regulation by conserved affective circuits.
574   *Neuron* **78**, 411-413, doi:10.1016/j.neuron.2013.04.031 (2013).
575 10 Soria-Gomez, E., Busquets-Garcia, A., Hu, F., Mehidi, A., Cannich, A., Roux, L. *et al.*
576   Habenular CB1 Receptors Control the Expression of Aversive Memories. *Neuron* **88**,
577   306-313, doi:10.1016/j.neuron.2015.08.035 (2015).
578 11 Zhang, J., Tan, L., Ren, Y., Liang, J., Lin, R., Feng, Q. *et al.* Presynaptic Excitation via
579   GABAB Receptors in Habenula Cholinergic Neurons Regulates Fear Memory
580   Expression. *Cell* **166**, 716-728, doi:10.1016/j.cell.2016.06.026 (2016).

581   12   Molas, S., DeGroot, S. R., Zhao-Shea, R. & Tapper, A. R. Anxiety and Nicotine
582   Dependence: Emerging Role of the Habenulo-Interpeduncular Axis. *Trends Pharmacol*
583   *Sci* **38**, 169-180, doi:10.1016/j.tips.2016.11.001 (2017).
584   13   Shibata, H., Suzuki, T. & Matsushita, M. Afferent projections to the interpeduncular
585   nucleus in the rat, as studied by retrograde and anterograde transport of wheat germ
586   agglutinin conjugated to horseradish peroxidase. *J Comp Neurol* **248**, 272-284,
587   doi:10.1002/cne.902480210 (1986).
588   14   Lima, L. B., Bueno, D., Leite, F., Souza, S., Gonçalves, L., Furigo, I. C. *et al.* Afferent
589   and efferent connections of the interpeduncular nucleus with special reference to circuits
590   involving the habenula and raphe nuclei. *J Comp Neurol* **525**, 2411-2442,
591   doi:10.1002/cne.24217 (2017).
592   15   Fowler, C. D., Lu, Q., Johnson, P. M., Marks, M. J. & Kenny, P. J. Habenular alpha5
593   nicotinic receptor subunit signalling controls nicotine intake. *Nature* **471**, 597-601,
594   doi:nature09797 [pii]10.1038/nature09797 (2011).

595   16   Fowler, C. D. & Kenny, P. J. Nicotine aversion: Neurobiological mechanisms and
596   relevance to tobacco dependence vulnerability. *Neuropharmacology* **76 Pt B**, 533-544,
597   doi:10.1016/j.neuropharm.2013.09.008 (2014).
598   17   Zhao-Shea, R., Liu, L., Pang, X., Gardner, P. D. & Tapper, A. R. Activation of
599   GABAergic neurons in the interpeduncular nucleus triggers physical nicotine withdrawal
600   symptoms. *Curr Biol* **23**, 2327-2335, doi:10.1016/j.cub.2013.09.041 (2013).
601   18   Zhao-Shea, R., DeGroot, S. R., Liu, L., Vallaster, M., Pang, X., Su, Q. *et al.* Increased
602   CRF signalling in a ventral tegmental area-interpeduncular nucleus-medial habenula
603   circuit induces anxiety during nicotine withdrawal. *Nature communications* **6**, 6770,
604   doi:10.1038/ncomms7770 (2015).
605   19   Wolfman, S. L., Gill, D. F., Bogdanic, F., Long, K., Al-Hasani, R., McCall, J. G. *et al.*
606   Nicotine aversion is mediated by GABAergic interpeduncular nucleus inputs to
607   laterodorsal tegmentum. *Nature communications* **9**, 2710, doi:10.1038/s41467-018-
608   04654-2 (2018).
609   20   Yamaguchi, T., Danjo, T., Pastan, I., Hikida, T. & Nakanishi, S. Distinct roles of
610   segregated transmission of the septo-habenular pathway in anxiety and fear. *Neuron* **78**,
611   537-544, doi:10.1016/j.neuron.2013.02.035 (2013).
612   21   Pang, X. Liu, L., Ngolab, J., Zhao-Shea, R., McIntosh, J. M., Gardner, P. D. *et al.*
613   Habenula cholinergic neurons regulate anxiety during nicotine withdrawal via nicotinic
614   acetylcholine   receptors.   *Neuropharmacology*   **107**,   294-304,
615   doi:10.1016/j.neuropharm.2016.03.039 (2016).
616   22   Molas, S., Zhao-Shea, R., Liu, L., DeGroot, S. R., Gardner, P. D., Tapper, A. R. A
617   circuit-based mechanism underlying familiarity signaling and the preference for novelty.
618   *Nat Neurosci* **20**, 1260-1268, doi:10.1038/nn.4607 (2017).
619   23   Tsai, H. C., Zhang, F., Adamantidis, A., Stuber, G. D., Bonci, A., de Lecea, L. *et al.*
620   Phasic firing in dopaminergic neurons is sufficient for behavioral conditioning. *Science*
621   **324**, 1080-1084, doi:1168878 [pii]10.1126/science.1168878 (2009).

622   24   Adamantidis, A. R., Adamantidis, A. R., Tsai, H. C., Boutrel, B., Zhang, F., Stuber, G. D.,
623   Budygin, E. A. *et al.* Optogenetic interrogation of dopaminergic modulation of the
624   multiple   phases   of   reward-seeking   behavior.   *J Neurosci* **31**,   10829-10835,
625   doi:10.1523/JNEUROSCI.2246-11.2011 (2011).
626   25   Chaudhury, D., Walsh, J. J., Friedman, A. K., Juarez, B., Ku, S. M., Koo, J. W. *et al.*
627   Rapid regulation of depression-related behaviours by control of midbrain dopamine
628   neurons. *Nature* **493**, 532-536, doi:10.1038/nature11713 (2013).

629 26 Tye, K. M., Mirzabekov, J. J., Warden, M. R., Ferenczi, E. A., Tsai, H. C., Finkelstein, J.
630   *et al.* Dopamine neurons modulate neural encoding and expression of depression-
631   related behaviour. *Nature* **493**, 537-541, doi:10.1038/nature11740 (2013).

632 27 Gunaydin, L. A., Grosenick, L., Finkelstein, J. C., Kauvar, I. V., Fenno, L. E., Adhikari, A.
633   *et al.* Natural neural projection dynamics underlying social behavior. *Cell* **157**, 1535-
634   1551, doi:10.1016/j.cell.2014.05.017 (2014).

635 28 Sun, F., Zeng, J., Jing, M., Zhou, J., Feng, J., Owen, S. F. *et al.* A Genetically Encoded
636   Fluorescent Sensor Enables Rapid and Specific Detection of Dopamine in Flies, Fish,
637   and Mice. *Cell* **174**, 481-496 e419, doi:10.1016/j.cell.2018.06.042 (2018).

638 29 Walf, A. A. & Frye, C. A. The use of the elevated plus maze as an assay of anxiety-
639   related behavior in rodents. *Nature protocols* **2**, 322-328, doi:10.1038/nprot.2007.44
640   (2007).

641 30 Gradinaru, V., Zhang, F., Ramakrishnan, C., Mattis, J., Prakash, R., Diester, I. *et al.*
642   Molecular and cellular approaches for diversifying and extending optogenetics. *Cell* **141**,
643   154-165, doi:10.1016/j.cell.2010.02.037 (2010).

644 31 Ade, K. K., Wan, Y., Chen, M., Gloss, B. & Calakos, N. An Improved BAC Transgenic
645   Fluorescent Reporter Line for Sensitive and Specific Identification of Striatonigral
646   Medium Spiny Neurons. *Frontiers in systems neuroscience* **5**, 32,
647   doi:10.3389/fnsys.2011.00032 (2011).

648 32 Petreanu, L., Mao, T., Sternson, S. M. & Svoboda, K. The subcellular organization of
649   neocortical excitatory connections. *Nature* **457**, 1142-1145, doi:10.1038/nature07709
650   (2009).

651 33 Ren, J., Qin C., Hu, F., Tan, J., Qiu, L., Zhao, S. *et al.* Habenula "cholinergic" neurons
652   co-release glutamate and acetylcholine and activate postsynaptic neurons via distinct
653   transmission modes. *Neuron* **69**, 445-452, doi:S0896-6273(10)01088-3
654   [pii]10.1016/j.neuron.2010.12.038 (2011).

655 34 Liu, J., Perez, S. M., Zhang, W., Lodge, D. J. & Lu, X. Y. Selective deletion of the leptin
656   receptor in dopamine neurons produces anxiogenic-like behavior and increases
657   dopaminergic activity in amygdala. *Mol Psychiatry* **16**, 1024-1038,
658   doi:10.1038/mp.2011.36 (2011).

659 35 Kim, S. Y., Adhikari, A., Lee, S. Y., Marshel, J. H., Kim, C. K., Mallory, C. S. *et al.*
660   Diverging neural pathways assemble a behavioural state from separable features in
661   anxiety. *Nature* **496**, 219-223, doi:10.1038/nature12018 (2013).

662 36 Zhong, P., Vickstrom, C. R., Liu, X., Hu, Y., Yu, L., Yu, H. G. *et al.* HCN2 channels in the
663   ventral tegmental area regulate behavioral responses to chronic stress. *eLife* **7**,
664   doi:10.7554/eLife.32420 (2018).

665 37 Shevtsova, Z., Malik, J. M., Michel, U., Bahr, M. & Kugler, S. Promoters and serotypes:
666   targeting of adeno-associated virus vectors for gene transfer in the rat central nervous
667   system in vitro and in vivo. *Exp Physiol* **90**, 53-59 (2005).

668 38 Zhao-Shea, R., Liu, L., Soll, L. G., Improgo, M. R., Meyers, E. E., McIntosh, J. M., *et al.*
669   Nicotine-mediated activation of dopaminergic neurons in distinct regions of the ventral
670   tegmental area. *Neuropsychopharmacology* **36**, 1021-1032, doi:npp2010240
671   [pii]10.1038/npp.2010.240 (2011).

672 39 Klenowski, P. M., Fogarty, M. J., Belmer, A., Noakes, P. G., Bellingham, M. C., Bartlett,
673   S. E. Structural and functional characterization of dendritic arbors and GABAergic
674   synaptic inputs on interneurons and principal cells in the rat basolateral amygdala. *J
675   Neurophysiol* **114**, 942-957, doi:10.1152/jn.00824.2014 (2015).

676 40 Sholl, D. A. Dendritic organization in the neurons of the visual and motor cortices of the
677   cat. *Journal of anatomy* **87**, 387-406 (1953).

678 41   Klenowski, P. M. *et al.* Increased Synaptic Excitation and Abnormal Dendritic Structure
679   of Prefrontal Cortex Layer V Pyramidal Neurons following Prolonged Binge-Like
680   Consumption of Ethanol. *eNeuro* **3**, doi:10.1523/ENEURO.0248-16.2016 (2016).

681 42   Harris, K. M. & Kater, S. B. Dendritic spines: cellular specializations imparting both
682   stability and flexibility to synaptic function. *Annu Rev Neurosci* **17**, 341-371,
683   doi:10.1146/annurev.ne.17.030194.002013 (1994).

684 43   Beier, K. T., Steinberg, E. E., DeLoach, K. E., Xie, S., Miyamichi, K., Schwarz, L. et al.
685   Circuit Architecture of VTA Dopamine Neurons Revealed by Systematic 1084 Input-
686   Output Mapping. Cell 162, 622-634, doi:10.1016/j.cell.2015.07.015 (2015). 1085

687 44   Lerner, T. N., Shilyansky, C., Davidson, T. J., Evans, K. E., Beier, K. T., Zalocusky, K. A.
688   et al. Intact-Brain Analyses Reveal Distinct Information Carried by SNc 1086 Dopamine
689   Subcircuits. Cell 162, 635-647, doi:10.1016/j.cell.2015.07.014 (2015). 1087

690 45   Menegas, W., Akiti, K., Amo, R., Uchida, N. & Watabe-Uchida, M. Dopamine neurons
691   1088 projecting to the posterior striatum reinforce avoidance of threatening stimuli. Nat
692   Neurosci 1089 21, 1421-1430, doi:10.1038/s41593-018-0222-1 (2018).

693 46   Koppensteiner P., Melani R., Ninan I. A cooperative mechanism involving Ca2+-
694   permeable AMPA receptors and retrograde activation of GABAB receptors in interpeduncular
695   nucleus plasticity. *Cell Rep.* 2017 Aug 1; 20(5): 1111–1122. doi: 10.1016/j.celrep.2017.07.013

696 47   Melani R., Von Itter R., Jing D., Koppensteiner P., Ninan I. Opposing effects of an
697   atypical glycinergic and substance P transmission on interpeduncular nucleus plasticity.
698   *Neuropsychopharmacology.* 2019 Sep;44(10):1828-1836. doi: 10.1038/s41386-019-0396-6.
699   Epub 2019 Apr 20.

700 48   McQuade R.D., Duffy R.A., Anderson C.C., Crosby G., Coffin V.L., Chipkin R.E., Barnett
701   A. [3H]SCH 39166, a new D1-selective radioligand: in vitro and in vivo binding analyses. *J
702   Neurochem.* 1991 Dec;57(6):2001-10.

703 49   Aosaki T., Kiuchi k., Kawaguchi Y. Dopamine D1-Like Receptor Activation Excites Rat
704   Striatal Large Aspiny Neurons In Vitro. J Neurosci. 1998 Jul 15; 18(14): 5180–5190. doi:
705   10.1523/JNEUROSCI.18-14-05180.1998

706 50   Nimitvilai S., Brodie M.S. Reversal of Prolonged Dopamine Inhibition of Dopaminergic
707   Neurons of the Ventral Tegmental Area. J Pharmacol Exp Ther. 2010 May; 333(2): 555-563.
708   doi: 10.1124/jpet.109.163931

709 51   Liu S., Plachez C., Shao Z., Puche A., Shipley M.T. Olfactory Bulb Short Axon Cell
710   Release of GABA and Dopamine Produces a Temporally Biphasic Inhibition–Excitation
711   Response in External Tufted Cells. J Neurosci. 2013 Feb 13; 33(7): 2916–2926. doi:
712   10.1523/JNEUROSCI.3607-12.2013
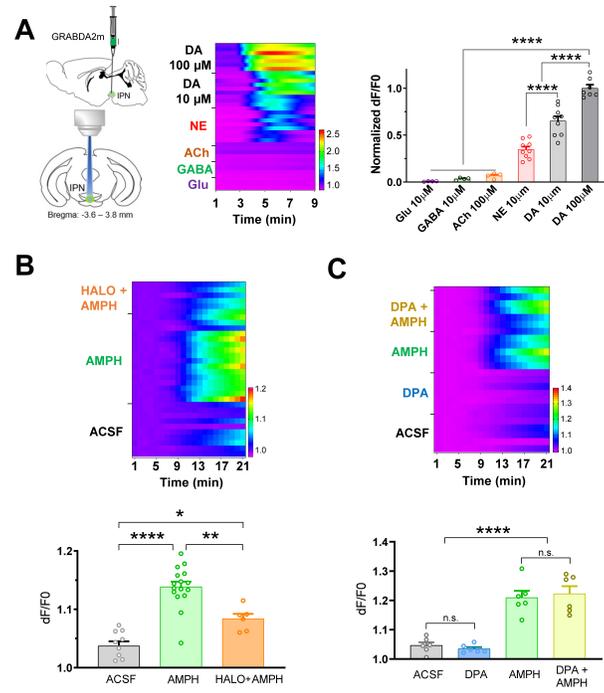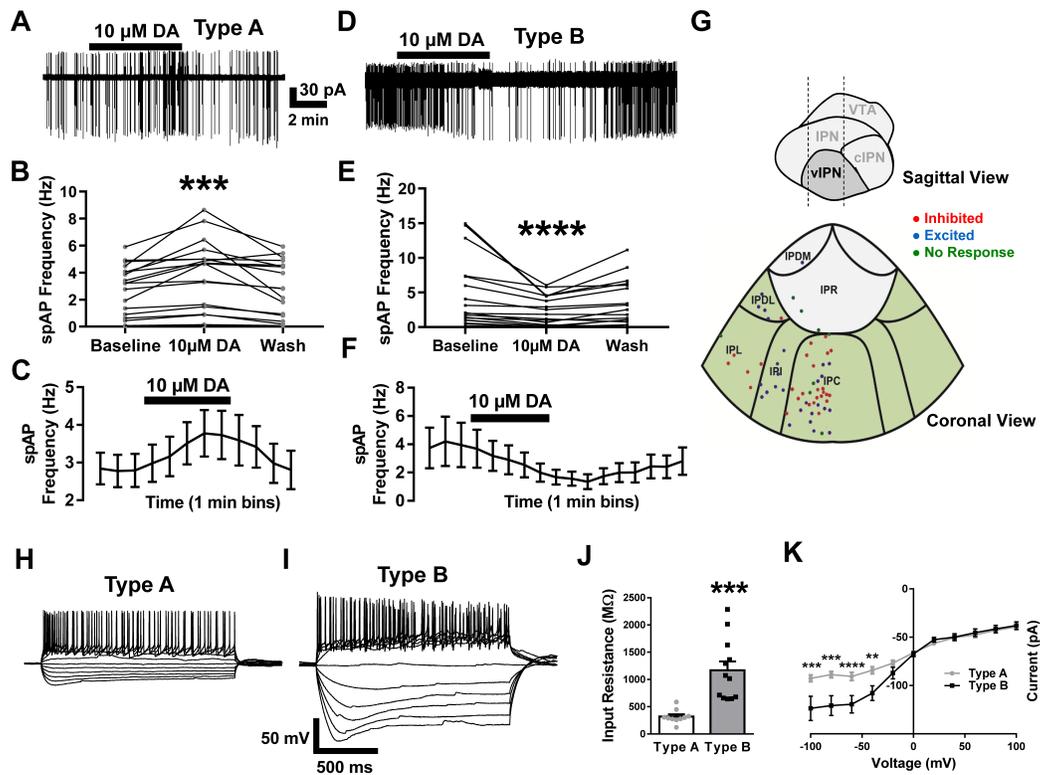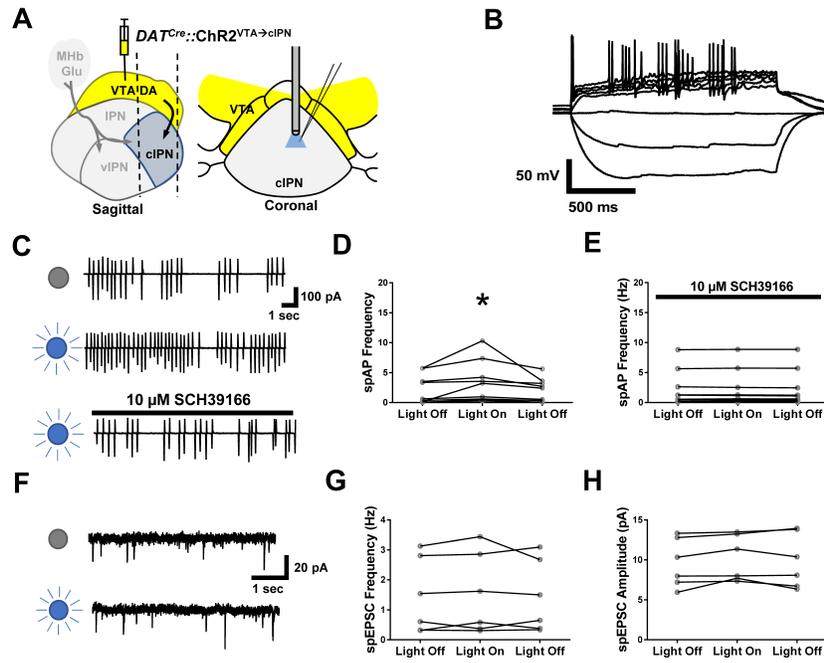
713

714

715

Figure 1

Figure 3.

Figure 5.

Figure 6.

A

*DRD1^{Cre}::*ChR2^{cIPN→vIPN}

MHb
Glu

VTA/D

IPN

cIPN

vIPN

sagittal

VTA

IPR

vIPN

coronal

B

vIPN

cIPN

eYFP

50 μm

C   **Type A**

Light Off

Light On

Light Off

20 pA

2 Sec

D

spEPSC Frequency (Hz)

***

Light: Off   On   Off

E   1 μM TTX + 100 μM 4-AP

spEPSC Frequency (Hz)

**

Light: Off   On   Off

F   **Type B**

Light Off

Light On

Light Off

G

spEPSC Frequency (Hz)

*

Light: Off   On   Off

H   1 μM TTX + 100 μM 4-AP

spEPSC Frequency (Hz)

**

Light: Off   On   Off

I   1 μM TTX + 100 μM 4-AP

Normalized spEPSC Frequency

**

GABA_{A+B} Antagonist

Light: Off   On   Off   Off   On   Off

J   1 μM TTX + 100 μM 4-AP

Normalized spEPSC Frequency

*

GABA_{A+B} Antagonist

Light: Off   On   Off   Off   On   Off

Figure 2

Figure 4.

Figure 8.

VTA DAergic neurons

• DA

DRD1 expressing GABAergic Type C neurons

Glutamatergic neurons
Net release activated by GABA
(MHb dominant)

Glutamatergic neurons
Net release inhibited by GABA
(MHb sparse)

Type A neurons

Type B neurons

↑ Activation

↓ Inhibition

Anxiety-like Behaviors

Figure 7

A

B

C

D

E