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

Authors

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Short Title: VTA dopamine modulates IPN activity to control anxiety
Background

Dopamine (DA) is hypothesized to modulate anxiety-like behavior although the precise role of DA in anxiety behaviors and the complete anxiety network in the brain have yet to be elucidated. Recent data indicate dopaminergic projections from the ventral tegmental area (VTA) innervates the interpeduncular nucleus (IPN), but how the IPN responds to dopamine (DA) and what role this circuit plays in anxiety-like behavior is unknown.

Methods

We expressed a genetically encoded GPCR-activation-based-DA sensor in mouse midbrain to detect DA in IPN slices using fluorescence imaging combined with pharmacology. Next, we selectively inhibited or activated VTA→IPN DAergic inputs via optogenetics during anxiety-like behavior. We utilized a biophysical approach to characterize DA effects on neural IPN circuits. Site-directed pharmacology was used to test if DA receptors in the IPN can regulate anxiety-like behavior.

Results

DA was detected in mouse IPN slices. Silencing/activating VTA→IPN DAergic inputs oppositely modulated anxiety-like behavior. Two neuronal populations in the ventral IPN (vIPN) responded to DA via D1 receptors (D1R). vIPN neurons were controlled by a small population of D1R neurons in the caudal IPN (cIPN) that directly respond to VTA DAergic terminal stimulation and innervate the vIPN. IPN infusion of a D1R agonist and antagonist bidirectionally controlled anxiety-like behavior.
Conclusions

VTA DA engages D1R-expressing neurons in the cIPN that innervate vIPN thereby amplifying the VTA DA signal to modulate anxiety-like behavior. These data identify a DAergic circuit that mediates anxiety-like behavior through unique IPN microcircuity.

Keywords

Anxiety, Circuitry, Dopamine, Interpeduncular Nucleus, Ventral Tegmental Area, Medial Habenula
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(8,9,10,11). 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
withdrawal(15,16,17,18,19). The habenulo-interpeduncular pathway also contributes to regulating baseline anxiety-like behavior(20,21), although the mechanism(s) involved, particularly in the IPN, are not clearly understood.

We recently described a mesointerpeduncular circuit in which VTA DAergic neurons project to the neighboring IPN(22). While the DAergic neuron-rich VTA is largely associated with increased motivation towards novelty, reinforcement, and positive affective state, the IPN is a brain region governing reduced motivation towards familiarity, as well as aversion, and negative affective state(19,22,23,24,25,26,27).

Thus, general activity in these two regions promotes opposing behaviors suggesting the mesointerpeduncular circuit could act as an important balancing point governing motivation and anxiety-like behavior. Indeed, previously we showed that stimulating this pathway with optogenetic tools could shift the motivational aspects of familiar stimuli interactions and enhance their salience as if they were novel(22). Here, we provide a comprehensive understanding on the mechanistic connection between the VTA and IPN and how endogenous DA released from this circuitry contributes to anxiety-associated behaviors.

Materials and Methods

Animals

All experiments followed the guidelines for care and use of laboratory animals provided by the National Research Council, and with approved animal protocols from the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. C57Bl/6J (#000664), GAD2-Cre (#010802), Chat-Cre (#006410), DAT-
Cre (#006660), Chat-ChR2 (#014546), DRD1-Cre (#028298), and Drd1a-tdTomato (#016204) mice were obtained from The Jackson Laboratory, bred in the UMMS animal facility and used in behavioral, optogenetic and biophysical experiments as indicated. Cre lines were crossed with C57Bl/6J mice and only heterozygous animals carrying one copy of the Cre recombinase gene were used for experimental purposes. Mice were housed together in cages of no more than five animals and kept on a standard 12 h light/dark cycle (lights ON at 7 A.M.) with ad libitum access to food and water. Three to four weeks before experimentation, subject mice were kept under a reverse 12 h light/dark cycle (lights ON at 7 P. M.) for at least 5 days before any behavioral testing

**Viral Preparation**

Optogenetic plasmids were packaged into AAV serotype 2 (AAV2) viral particles by the UMMS Viral Vector Core. GRAB\(_{DA2m}\) is derived from GRAB\(_{DA1m}\), with additional mutations in cpEGFP. GRAB\(_{DA2m}\) has ~3-fold improvement in the maximal ∆F/F0 and similar apparent affinity (EC\(_{50}\) ~90 nM). Detailed characterization of GRAB\(_{DA2m}\) will be published elsewhere. GRAB\(_{DA2m}\) was packaged into AAV9 by Vigene Biosciences. Additional Materials and Methods can be found in Supplementary Materials.

**Results**

**DA is released in the IPN.**

To test if endogenous DA release in the IPN occurs and may be involved in anxiety-like behavior, we expressed an enhanced genetically encoded GPCR-activated DA sensor (GRAB\(_{DA2m}\), see Methods) that changes in fluorescence upon DA binding, in the IPN of
C57Bl/6J mice using AAV-mediated gene delivery(28). To test appropriate function of GRAB\textsubscript{DA2m}, we prepared midbrain slices and measured changes in fluorescence in the IPN during bath application of neurotransmitter (Figure 1A). As expected, we recorded a robust increase in fluorescence intensity in response to 10 and 100 µM DA, with a lesser response to norepinephrine (NE) and no significant response to ACh, GABA, or glutamate (Figure 1A). To test if endogenous DA could be detected in IPN slices, we measured changes in fluorescence in response to the monoamine transporter substrate, amphetamine (Figure 1B). Bath application of amphetamine increased signal of GRAB\textsubscript{DA2m}, which was significantly blocked by preapplication of the D2 receptor antagonist, haloperidol indicating that the amphetamine-induced signal was mediated by GRAB\textsubscript{DA2m}. In addition, the amphetamine-induced signal persisted in the presence of the norepinephrine transporter inhibitor, desipramine, confirming that the signal was mediated by endogenous IPN DA release as opposed to NE (Figure 1C).

Together, these data indicate that endogenous DA is released in the IPN, a phenomenon which may be critical for regulating anxiety-like behavior.

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

A subpopulation of DAergic neurons in the VTA project to the IPN constituting a mesointerpeduncular pathway(18,22). To test if VTA→IPN axon terminals are the source of DA release and contribute to anxiety-like behaviors, we selectively expressed Cre-dependent halorhodopsin (NpHR)-eYFP in the VTA of DA transporter (DAT)::Cre mice via AAV2-mediated gene delivery and implanted fiber optic cannulas into the IPN to deliver yellow light (593nm, constant light, 20s on, 10s off, Figure 2A, S2) and photo-
inhibit VTA$^{DA}\rightarrow$IPN inputs during the elevated plus maze (EPM)(29, 30). VTA$^{DA}\rightarrow$IPN photo-inhibition resulted in a decrease in open arm time in the EPM compared to light-on eYFP controls (Figure 2B). VTA$^{DA}\rightarrow$IPN photo-inhibition had little effect on total arm entries compared to control conditions, suggesting normal locomotion in these animals. We also evaluated VTA$^{DA}\rightarrow$IPN photo-inhibition in the open field test (OFT) and observed a decrease in center time and no effect of photoinhibition on total activity (Figure 2C). To test the effect of activating VTA$^{DA}\rightarrow$IPN on open arm exploration, we selectively expressed Cre-dependent channelrhodopsin (ChR2)-eYFP in the VTA of DAT::Cre mice via AAV2-mediated gene delivery (Figure 2D, S1)(23). A fiber optic cannula was implanted targeting the IPN for blue-light stimulation of VTA$^{DA}\rightarrow$IPN inputs (473nm, 15 Hz, 20 ms/pulse, 5 s light-on, 5 s light off) during behavioral testing in the EPM. Photostimulation of VTA$^{DA}\rightarrow$IPN inputs significantly increased time spent in the open arms of the EPM compared to control mice expressing eYFP and receiving light stimulation, while having little effect on total arm entries compared to control conditions (Figure 2E). In the OFT, photostimulation of VTA$^{DA}\rightarrow$IPN increased time spent in the center compared to controls without significantly affecting total distance traveled (Figure 2F). To test if behavioral results obtained with our optogenetic approach could be due to silencing/activating of VTA DAergic neurons directly, we placed fiber optic cannulas dorsal to the IPN, targeting the VTA (Figure S2). Silencing VTA neurons increased open arm time in the EPM compared to eYFP controls (Figure S3A), an effect opposite to specific VTA$^{DA}\rightarrow$IPN inhibition, but did not significantly impact behavior in the OFT (Figure S3C); whereas activating VTA neurons with ChR2 did not significantly change open arm time in the EPM compared to eYFP controls (Figure S3B), but significantly
decreased center time in the OFT. Together, these data indicate that the VTA\textsuperscript{DA→IPN} pathway is a critical component of anxiety circuitry that, when engaged, drives reduced anxiety-like behavior.

Two neuronal populations in the ventral IPN differentially respond to DA via D1-like, but not D2-like, DA receptors.

To determine DA responses in IPN neurons, we used electrophysiology in acute coronal slices of C57Bl/6J mice. In cell-attached mode, we measured spontaneous action potentials (spAPs) during a five-minute bath application of exogenous 10 µM DA (Figure 3). In the ventral IPN (vIPN), 18 out of 39 neurons responded to DA with an increase in spontaneous action potential (spAP) frequency that reversed upon washout (designated as “Type A” neurons, Figure 3A, B, C), while 17 out of 39 neurons responded to DA with a decrease in spAP frequency that reversed upon washout (designated as “Type B” neurons, Figure 3D, E, F). The remaining 4 neurons exhibited no obvious responses (Figure 3G). To examine the physiological properties and current-voltage relationship of these two types of vIPN neurons, we injected 100 pA to -100 pA current in -20 pA steps. Type A and Type B neurons exhibited clear significant differences in their response to current injection and input resistance (Figure 3H-I), with Type A neurons having a lower input resistance compared to Type B neurons.

To test which DA receptors are required for DA-induced changes in spAP frequency in the vIPN, DA was applied to Type A and Type B neurons in the absence and presence of the D1-like receptor antagonist, SCH39166 (10 µM) or the D2-like receptor antagonist, eticlopride (10 µM, Figure 4A-C). SCH39166, but not eticlopride,
significantly attenuated DA-mediated spAP frequency changes both in Type A and Type B neurons, suggesting that DA acts through D1-like but not D2-like DA receptors in the IPN. In addition, to further rule out D2 effects, we applied a D2-like DA receptor agonist, quinpirole, to vIPN neurons and did not observe any changes in spAP frequency, spontaneous excitatory post-synaptic current (spEPSC) frequency or amplitude (Figure S4).

DA modulates vIPN neurons via presynaptic DA receptors.

To assess how D1-like DA receptors modulate vIPN neuron activity, we recorded from Type A and B neurons under voltage-clamp and measured changes in excitatory input. DA was bath applied and neurons were voltage-clamped at -70 mV to record spEPSCs. Of note, DA failed to induce obvious inward or outward post-synaptic currents under voltage-clamp (data not shown). However, DA increased spEPSC frequency in Type A neurons while decreasing spEPSCs frequency in Type B neurons, with no effect on the spEPSC amplitude in either neuron type, suggesting DA affects excitatory inputs via DA receptors that are presynaptic (Figure 4D-I). The valence of spEPSC frequency was also consistent with the DA-induced changes in spAP frequency observed in the two vIPN neuron sub-types. In addition, when spEPSCs were blocked by NMDA and AMPA receptor antagonists (20 µM AP-5 and 10 µM CNQX), the majority of vIPN neurons ceased firing, suggesting that the change in spEPSC frequency induced by DA directly causes the DA-induced change in spAP frequency (Figure S5). These findings indicate that DA increases presynaptic excitatory transmission to Type A neurons and decreases presynaptic excitatory transmission to Type B neurons.
cIPN neurons respond to afferent VTA DAergic terminal stimulation

To test if vIPN neurons respond to DAergic inputs from the VTA, we selectively expressed Cre-dependent ChR2-eYFP in VTA DAergic neurons of DAT::Cre mice and we recorded vIPN neuronal responses upon light-induced VTA$^{\text{DA}} \rightarrow$IPN stimulation (Figure 5; 20 Hz, 2 ms pulse width). VTA DAergic terminals were stimulated through the microscope objective focused on the area around the recorded IPN neuron (Figure 5A). Cell-attached mode was used to record spAPs. Interestingly, the majority of vIPN neurons failed to respond to VTA terminal optic stimulation (Figure S7A). Previously, using mice in which the fluorophore td-Tomato is under the control of the $DRD1$ (the gene encoding the DA D1 receptor) promoter (the Drd1a-tdTomato line(31)), we determined that D1 receptor expression is localized to soma in the caudal IPN (cIPN) while presumed terminal fields are localized to the vIPN (also see Figure S6A), raising the possibility that VTA$^{\text{DA}} \rightarrow$IPN DA innervation may be sub-region (i.e. cIPN) specific. In DAT$^{\text{Cre}}$::eYFP mice, we observed VTA DAergic inputs in the cIPN but not rostral IPN (Figures S6A, S6B) supporting this hypothesis. In addition, D1-TdTomato midbrain slices immuno-labeled for DAT revealed TdTomato-positive neurons in cIPN decorated with DAT-immunopositive puncta (Figure S6C). In contrast to vIPN neurons, light-evoked responses were observed in the cIPN matching the VTA$^{\text{DA}} \rightarrow$IPN innervation pattern. As compared to vIPN neurons, cIPN neurons exhibited a significantly higher input resistance and a different current-voltage relationship (Figure 5B, S7B) indicating a distinct cIPN sub-type that we refer to as “Type C”. In cIPN slices, a sub-population of Type C neurons responded to light stimulation of DAergic afferents with an increase in spAP frequency that was attenuated in the presence of SCH39166 (Figure 5C-E).
test the mechanism of light-evoked changes in AP frequency in Type C neurons, we
examined excitatory input, recording spEPSCs in response to light. Blue light failed to
evoke a change in either spEPSC frequency or amplitude, suggesting the effect of DA
on spAP frequency in this sub-population was due to post-synaptic D1 receptor
expression (Figure 5F-H). Moreover, we also observed a population of Type C neurons
that exhibited a light-induced decrease in spAP frequency, as well as, a reduction in
spEPSC frequency, that were likewise blocked by SCH39166 (Figure S8D-J). To gain
insight into localization of the DAergic neurons in the VTA that may project to the IPN,
we injected AAV2rg-hSyn-DIO-eGFP into different regions of striatum to label discreet
VTA DAergic neurons in DAT::Cre mice (Figure S8)(43-45). In mice in which paranigral
VTA DAergic neurons were labeled, we could trace projections into the cIPN (Figure
S8A); whereas, in mice in which paranigral neurons were not labeled, DAergic
VTA→IPN projections were less apparent (Figure S8B). Together, these data suggest
that a sub-population of Type C neurons in the cIPN that signal through D1 receptors,
may amplify the VTA DAergic input to other IPN neurons, for instance, vIPN Type A and
Type B neurons, and modulate their responses.

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

To test if D1-positive neurons in the cIPN project directly to the vIPN, we expressed
Cre-dependent ChR2-eYFP in the IPN of mice that express Cre under the control of the
DRD1 promoter (DRD1a::Cre mice) via AAV2-mediated gene delivery (Figure 6A).
eYFP signal was observed in cIPN neuronal soma and projections along the
clPN→vlPN plane (Figure 6B). Stimulation of Type C terminals in the vlPN (20Hz, 2 ms pulse width) resulted in a significant increase of spEPSC frequency in Type A neurons and a significant decrease of spEPSC frequency in Type B neurons (Figure 6C, D, F, G). These responses phenocopied the result of bath application of DA in 80% of vlPN neurons as predicted by their input resistances (compare with Figure 4D-I).

Experiments were repeated in the presence of 1 µM TTX and 100 µM 4-AP to block action potentials, and thus, block multi-synaptic responses(32). The changes in EPSC frequency upon light stimulation were maintained in both Type A and B neurons suggesting the D1 receptor-expressing Type C clPN neurons project monosynaptically to the vlPN (Figure 6E, H). In addition, combined GABA<sub>A</sub> and GABA<sub>B</sub> antagonists saclofen (10 µM) and bicuculline (20 µM) blocked the light-evoked change in spEPSC frequency in both Type A and B vlPN neurons suggesting Type C neurons release GABA to modulate excitatory synapses in the vlPN (Figure 6I, J). As Type A and B neurons are morphologically distinct and receive differential innervation from mHb terminals (see Supplemental Results and Figure S9), which strongly innervate Type A but not Type B neurons, and mHb terminals in the IPN are known to uniquely increase excitatory transmission in response to activation of GABA<sub>B</sub> receptors(11,46,47), these data suggest GABA bidirectionally modulates excitatory synapses on Type A and B neurons through mHb and non-mHb excitatory inputs, respectively.

Pharmacological manipulation of D1 receptors in the IPN bidirectionally modulates anxiety-like behavior.
To test if D1 signaling in the IPN modulates anxiety-like behavior, we implanted drug infusion cannulas and delivered a D1 receptor agonist or antagonist into the IPN prior to testing in the EPM and OFT assays (Figure 7). In the EPM and the OFT, intra-IPN infusion of the D1 receptor agonist SKF82958 increased open arm time and increased time in the center, respectively, compared to vehicle infusion, indicating an anxiolytic effect of the drug. Conversely, the D1 receptor antagonist SCH39166 was anxiogenic, reducing open arm time and time in the center compared to vehicle infusion (Figure 7B, D). Neither drug affected the number of arm entries in the EPM, or total distance traveled in the OFT (Figure 7C, E). Infusion of the D1-like receptor agonist and antagonist directly into the VTA had little effect on anxiety-like behavior (Figure S10B,D). However, VTA infusion of D1 drugs resulted in a depression of total arm entries in the EPM (Figure S10C). The difference in locomotor effects and the lack of a significant effect on anxiety-like behavior when the VTA was infused suggests behaviors elicited from IPN infusions were not the result of off target effects from drug diffusion. Overall, these results demonstrate that endogenous DA controls anxiety-like behavior via anxiolytic D1 receptor signaling in the IPN.

Discussion

DA signaling has long been implicated in anxiety-like behavior presumably through midbrain DA projection areas to the hippocampus, extended amygdala, and prefrontal cortex, among other brain regions(4-6,34-36). Our data combining GRAB DA sensor expression in the IPN with pharmacology and imaging revealed that endogenous DA is
released in the IPN.. Preventing IPN DA increases in vivo by silencing the VTA→IPN
input reduced both exploration of the EPM open arm and exploration of the center of the
OFT. Conversely, activating the input increased time spent in the EPM open arm and
exploration of the center of the OFT, suggesting that this IPN DA signal controls
anxiety-like behavior specifically by driving anxiolysis. Assays used to evaluate anxiety-
like behavior in mice including the EPM and OFT are multimodal and integrate two
opposite motivational drives: 1) behavioral avoidance and 2) novelty seeking(29).
Mice will be driven to explore the open arms of the EPM or center of the OFT because
they are novel but also avoid exploration because they are elevated or open and without
protection from predation. Thus, the read-out or expression of anxiety-like behavior
relies upon the strength of these two motivational drives. Interestingly, previous studies
implicate the habenulo-interpeduncular pathway in behavioral avoidance and
aversion(10,16,19); whereas, we have also discovered that the IPN and associated
circuitry is also critically involved in signaling familiarity, reducing motivation to explore
novelty to control novelty preference(22). Our data indicate that VTA input and IPN DA
may provide a signal that either reduces avoidance behavior to allow expression of
reduced anxiety-like behavior or increase motivation to explore novelty. Future studies
will focus on how the IPN integrates anxiety and novelty signals to drive exploratory
behavior.

Activation of DAergic IPN inputs stimulates a small sub-population of dopaminoceptive
neurons expressing the D1 receptor located predominantly in the caudal portion of the
IPN. Through retrograde tracing, our data suggest that a sub-population of accumbens
shell-projecting VTA DA neurons in the paranigral region may preferentially project into
the IPN to innervate cIPN neurons, although we cannot rule out that DAergic neurons in
other regions of the VTA, or other brain areas, also may contribute to this circuit, an
issue that will require further experimentation.

Remarkably, cIPN neurons, through a microcircuit spanning the vIPN, amplify the DA
signal ultimately controlling anxiety-like behavior. Indeed, the vast majority of vIPN
neurons respond to exogenous DA in midbrain slices (35 out of 39) presumably through
D1 receptor-expressing Type C terminals which modulate excitatory input to vIPN
neurons. One potential caveat with our results is that we used a D1 antagonist, SCH
39166, to block DA effects in midbrain slices. While SCH 39166 is selective for D1/D5
receptors, it can also block D2-like receptors at higher concentrations and may also be
a low affinity antagonist at 5HT2 receptors(48). However, the concentration used in our
experiments was similar to that of other studies examining D1-receptor mediated
responses in rodent brain slices(49-51) and effects of DA signaling we observed in the
IPN persisted in the presence of a D2 antagonist. In addition, the D1 antagonist not
only blocked effects of bath application of DA, but also responses observed by specific
optic activation of VTA DAergic terminals in the IPN. The effect of exogenous DA
application on vIPN neuronal activity was phenocopied by direct optogenetic activation
of D1-expressing terminals in vIPN, supporting a DA signal-amplifying micro-circuit.

Thus, what at first glance would appear to be a modest connection between VTA and
cIPN, through this amplification step, transmits activity to the majority of neurons in the
ventral portion of the nucleus to control behavior.

The microcircuit controlling activity of vIPN neurons is unique in that it consists of two
morphologically distinct neurons, Type A and Type B, which both receive GABAergic
innervation from cIPN Type C neurons but act oppositely in response to GABA. Type A neurons are excited by activation of Type C terminals via increased glutamate release; whereas Type B neurons are inhibited by activation of Type C terminals via decreased glutamate release (Figure 8). Interestingly, Type A neurons are robustly controlled by mHb excitatory inputs that are activated by GABA via excitatory GABA$_B$ receptors on mHb terminals(11,33). Type B neurons, on the other hand, are weakly innervated by the mHb, thus, it is likely that GABA reduces excitatory input from other, unidentified excitatory afferents that express inhibitory GABA receptors. In the future, it will be necessary to apply additional circuit mapping approaches to identify this excitatory IPN input. Ultimately, engaging this microcircuit either through optogenetic stimulation of VTA→IPN inputs or through infusion of D1 receptor agonist increases Type A neuronal activity while decreasing Type B neuronal activity to reduce anxiety-like behavior.

In summary, our data indicate that VTA DAergic input to the IPN mediates anxiety-like behavior by activating D1-expressing neurons in the cIPN. This small population of dopaminceptive neurons amplify VTA DA input by projecting to and innervating vIPN through mHb glutamatergic inputs to bidirectionally control anxiolysis. Thus, we have identified a critical component of the neural network contributing to affective state through DAergic signaling that engages a unique IPN microcircuit.

**Author Contributions**

S.R.D., R.Z, L.C., P.M.K., and S.M. conducted the experiments. Y.L. provided the 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 experiments. S.R.D. and A.R.T. wrote the paper with input from all co-authors.
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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}
responses to 2 min bath application of neurotransmitter, applied at t=2 min. Right, summed average of maximal responses of bath application of neurotransmitter (One way ANOVA: $F_{(5, 31)} = 89.6$, $p = 0.0001$; Bonferroni’s multiple comparisons test: ****$p<0.0001$).

(B) Top, heat map of IPN GRAB$_{DA2m}$ responses to ACSF ($n=10$), amphetamine (AMPH, $n=16$), or AMPH following pre-application of haloperidol (HALO, n=6). AMPH applied at t=10 min until the end of recording. Bottom, summed average of maximal responses from top panel (One way ANOVA: $F_{(2, 29)} = 35.2$, $p = 0.0001$; Bonferroni’s multiple comparisons test: ****$p<0.0001$).

(C) Top, heat map of IPN GRAB$_{DA2m}$ responses to ACSF (n=6), desipramine (DPA, n=6), AMPH (n=6), or AMPH following pre-application of DPA (n=6). Bottom, summed average of maximal responses from top panel (One way ANOVA: $F_{(3, 20)} = 29.7$, $p = 0.0001$; Bonferroni’s multiple comparisons test: ****$p<0.0001$).

Figure 2. VTA→IPN DA input controls anxiety-like behavior. (A) Diagram of strategy for halorhodopsin experiments. (B) Open arm time (left) and total arm entries (right) during in vivo NpHR inhibition of VTA terminals in the IPN of light-on NpHR (n=13) and light-on eYFP (n=11) DAT$^{Cre}$ animals. (Unpaired two-tailed t-test: p=0.0006. Mean ± SEM.) (C) OFT activity during in vivo NpHR inhibition of VTA terminals in the IPN. Representative heat map of mouse position (top). Graphs of center time (bottom left) and total distance traveled (in cm, bottom right, n=11, 14, eYFP and NpHR, respectively, Unpaired t-test with Welch’s correction: p=0.023. Mean ± SEM.). See Figure S2 for canula placement. (D) Top, diagram of strategy for channelrhodopsin 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
0 to -20 pA step from traces in (D) and (E). (n = 10 and 12, respectively, unpaired t-test with Welch’s correction: ***p=0.0003. Data presented as mean ± SEM.) (K) Current voltage relationship. (Two-way ANOVA: Significant cell-type x current step interaction, $F_{(10, 218)}=5.07$, $p = 0.0001$. Bonferroni’s multiple comparisons test: **p≤0.01, ***p≤0.001, ****p≤0.0001. Mean ± SEM.)

**Figure 4.** vIPN neurons respond to DA through presynaptic D1-like but not D2-like DA receptors. (A) Schematic of experiment. Dotted lines indicate approximate positions where coronal slice was cut (left). Neurons were recorded from a coronal slice of the vIPN (right). (B) Averaged normalized spAP frequency of Type A neurons in response to DA in the absence and presence of the D1-like receptor antagonist SCH39166 (10 µM, top) or the D2-like receptor antagonist Eticlopride (10 µM, bottom). (n=6, One-way ANOVAs: (Top) $F_{(2, 10)} = 19.6$, $p=0.0003$; SCH39166: $F_{(2, 10)} = 0.1435$, $p=0.8680$; (Bottom) $F_{(2, 10)} = 6.492$, $p=0.016$; Eticlopride: $F_{(2, 10)} = 9.23$, $p=0.0054$. Data presented as mean ± SEM.) *p<0.05, **p<0.01, ***p<0.001 compared to baseline. (C) Averaged normalized spAP frequency of Type B neurons in response to DA in the absence and presence of the D1-like receptor antagonist SCH39166 (10 µM, top) or the D2-like receptor antagonist eticlopride (10 µM, bottom, n=6, One-way ANOVAs: (Top) $F_{(2, 12)} = 8.593$, $p=0.0048$; SCH39166: $F_{(2, 12)} = 1.852$, $p=0.1991$; (Bottom) $F_{(2, 10)} = 17.86$, $p=0.0005$; $F_{(2, 10)} = 25.79$, $p=0.0001$). **p<0.01, ***p<0.001 compared to baseline, Mean ± SEM. (D) Representative whole-cell patch clamp traces of spEPSCs in a Type A neuron before, during, and after DA application. (E) Type A spEPSC frequency at baseline, during last minute of DA application, and after washout (n=10, Friedman test: **p≤0.01, Friedman statistic_{(2, 18)} = 9.6., p=0.0075). ** p<0.01 Dunn’s test compared to
baseline. Data are mean ± SEM. (F) Type A spEPSC amplitude at baseline, during last minute of DA application, and after washout. (n=10, One-way ANOVA: F_{(2, 18)} = 0.3592, p=0.7031). Data are mean ± SEM. (G) Representative whole-cell patch clamp traces of spEPSCs in a Type B neuron before, during, and after DA application. (H) Type B spEPSC frequency at baseline, during last minute of DA application, and after washout (n=7, One-way ANOVA: F_{(2, 12)} = 14.47, p=0.0008). *p < 0.05 compared to baseline. Data are mean ± SEM. (I) Type A spEPSC amplitude at baseline, during last minute of DA application, and after washout (One-way ANOVA: F_{(2, 12)} = 0.6047, p=0.5621. Data are mean ± SEM).

Figure 5. Optogenetic stimulation of VTA→IPN DAergic terminals modulate cIPN neurons via D1 receptors. (A) Schematic of experiment. Cre-dependent ChR2-eYFP was expressed in putative DAergic neurons of the VTA in DAT^{Cre} mice via AAV2-mediated gene delivery (left). Neurons in the cIPN were recorded in coronal slices while optogenetically stimulating the DRD1 expressing terminals (right). (B) Representative whole-cell current-clamp traces from a cIPN neuron in response to 20 pA current injection steps from +100 to -40 pA. Compare to Figure 2A and B. (C) Representative traces of a cIPN neuron that responded to VTA terminal stimulation with an increase in firing rate. (D) spAP frequency of Type C neurons that responded to light stimulation with an increase in spAP frequency (n=10, One-way ANOVA: F_{(2, 18)} = 5.59, p=0.013). *p<0.05 compared to Light-off control. Data presented as mean ± SEM. (E) spAP frequency of cIPN neurons from (D) during 10 μM SCH39166 application (Friedman statistic_{(2, 16)} = 5.35, p=0.07). Data presented as mean ± SEM. (F) Representative trace of EPSC frequency from a cIPN neuron that increased its spAP frequency in response
to VTA terminal stimulation. (G) In cIPN neurons that increased their spAPs, spEPSC frequency was not significantly affected. (One-way ANOVA: $F_{(2, 10)} = 0.1732, p=0.8435$).

Data presented as mean ± SEM. (H) In cIPN neurons that increased their spAPs, spEPSC amplitude was not significantly affected. (One-way ANOVA: $F_{(2, 10)} = 2.106, p=0.1725$). Data presented as mean ± SEM.

Figure 6. cIPN Type C putative D1 receptor-expressing neurons project to the vIPN and modulate Type A and Type B neuronal activity via GABA. (A) Schematic of experiment. Cre-dependent ChR2-eYFP was expressed in putative DRD1-expressing neurons of the cIPN in DRD1::Cre mice via AAV2-mediated gene delivery (left). Neurons in the vIPN were recorded in coronal slices while optogenetically stimulating the DRD1 expressing terminals (right). (B) Sagittal slice showing Cre-dependent eYFP (green) from a (DRD1)::Cre mouse. cIPN neurons send projections rostrally to the vIPN. (C) Representative whole-cell patch clamp traces of Type A neuron EPSCs before, during and after 20 Hz stimulation of cIPN terminals in the presence of TTX and 4-AP. (D) Type A EPSC response to 20 Hz terminal stimulation. ($n=8$, One-way ANOVA: $F_{(2, 14)} = 20.8, p=0.0001$). ***$p<0.001$ compared to Light-off control. Data presented as mean ± SEM. (E) Type A response to DRD1-Cre terminal stimulation in the presence of AP blockers. The response was “monosynaptic” ($n=14$, Friedman test: Friedman statistic$_{(2,26)} = 24.57, p<0.0001$). **$p<0.01$ compared to Light-off control. Data presented as mean ± SEM. (F) Representative whole-cell patch clamp traces of Type B neuron EPSCs before, during and after stimulation of cIPN terminals in the presence of TTX and 4-AP. (G) Type B response to 20 Hz terminal stimulation ($n=7$, One-way ANOVA: $F_{(2, 12)} = 4.4, p=0.037$). *$p<0.05$ compared to Light-off control. Data presented as mean ± SEM.
SEM. **(H)** Type B response to 20 Hz DRD1-Cre terminal stimulation in the presence of AP blockers. The connection was monosynaptic (n=9, One-way ANOVA: $F_{(2, 16)} = 6.58$, p=0.0082). **p<0.01 compared to Light-off control. Data presented as mean ± SEM. **(I)** Normalized EPSC frequency of a Type A neuron before, during, and after cIPN DRD1-Cre terminal stimulation in the presence of 1 µM TTX and 100 µM 4-AP. The experiment was repeated with the addition of bath-applied 20 µM Bicuculline and 100 µM Saclofen to block GABA_A and GABA_B receptors, respectively. (n=7, One-way ANOVAs: $F_{(2, 12)} = 10.08$, p=0.0027; GABA_A+B receptor antagonists: $F_{(2, 12)} = 1.539$, p=0.2542). **p<0.01 compared to Light-off control. Data are presented as mean ± SEM.**

**(J)** Normalized EPSC frequency of a Type B neuron before, during, and after cIPN DRD1-Cre terminal stimulation in the presence of 1 µM TTX and 100 µM 4-AP. The experiment was repeated with the addition of bath-applied 20 µM Bicuculline and 100 µM Saclofen to block GABA_A and GABA_B receptors, respectively. (n=5, One-way ANOVA: $F_{(2, 8)} = 7.437$, p=0.015; GABA_A+B receptor antagonists: $F_{(2, 8)} = 3.458$, p=0.0827). *p<0.05 compared to Light-off control. Data are presented as mean ± SEM.

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

**(A)** Diagram of experiment (left). Representative image of guide canula track in slice immune-labeled with TH antibody (green, also see Figure S11F). **(B)** Quantification of open arm time in the EPM between D1-like DA receptor agonist (n=10, SKF82598 0.7 µg/µl, infused 0.3 µl, 210 ng), antagonist (n=13, SCH39166 35 ng/µl, infused 0.3 µl, 10.5 ng), or saline control (n=15). (One-way ANOVA with repeated measures: $F_{(2, 35)}=$
11.43, p = 0.0002). *p ≤ 0.05, ***p ≤ 0.001. Data presented as mean ± SEM. (C) Total arm entries in the EPM after drug infusion. (One-way ANOVA with repeated measures: F(2, 35) = 0.6541, p=0.5261). Data presented as mean ± SEM. (D) Representative heat map of mouse position in the OFT after infusion of drug into the IPN (left). Quantification of center time in the OFT (right). (n=10, 9, 8 for saline, agonist, antagonist, respectively, One-way ANOVA with repeated measures: *p ≤ 0.05, **p ≤ 0.01, F(2, 24) = 8.558. Mean ± SEM.) (E) Quantification of total distance moved in the OFT. There was no significant difference between groups. (One-way ANOVA with repeated measures: F(2, 24) = 1.437, p=0.2574). Data are presented as mean ± SEM.

Figure 8. Circuit model for DA signal amplification in the IPN. Circles represent neurons, the lines originating from the circles represent axons and the triangles represent terminals. The terminals are set so that the side of the triangle opposite the axon faces its presumed target. Each color represents a unique population of neurons.
References


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


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Figure 3.

A 10 μM DA Type A

B Sagittal View

C Baseline 10μM DA Wash

D 10 μM DA Type B

E Inhibited

F Excited

G No Response

H Coronal View

I

J

K
Figure 5.

A

B

C

D

E

F

G

H

10 μM SCH39166

100 pA

1 sec

20 pA

1 sec

50 mV

100 pA

1 sec

500 ms

sEPSC Amplitude (μA)

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Figure 2
Figure 4.

A

C57BL/6

VTA

vIPN

sagittal

cIPN

vIPN

coronal

B

Baseline 10 µM DA 10 µM SCH39166

Normalized spAP Frequency

10 µM Eticlopride

C

Baseline 10 µM DA 10 µM SCH39166

Normalized spAP Frequency

10 µM Eticlopride

D

Type A

Baseline

10 µM DA

Wash

E

spEPSC Frequency (Hz)

Baseline 10 µM DA Wash

F

spEPSC Amplitude (pA)

Baseline 10 µM DA Wash

G

Type B

Baseline

10 µM DA

Wash

H

spEPSC Frequency (Hz)

Baseline 10 µM DA Wash

I

spEPSC Amplitude (pA)

Baseline 10 µM DA Wash
Figure 8.

Anxiety-like Behaviors
Figure 7