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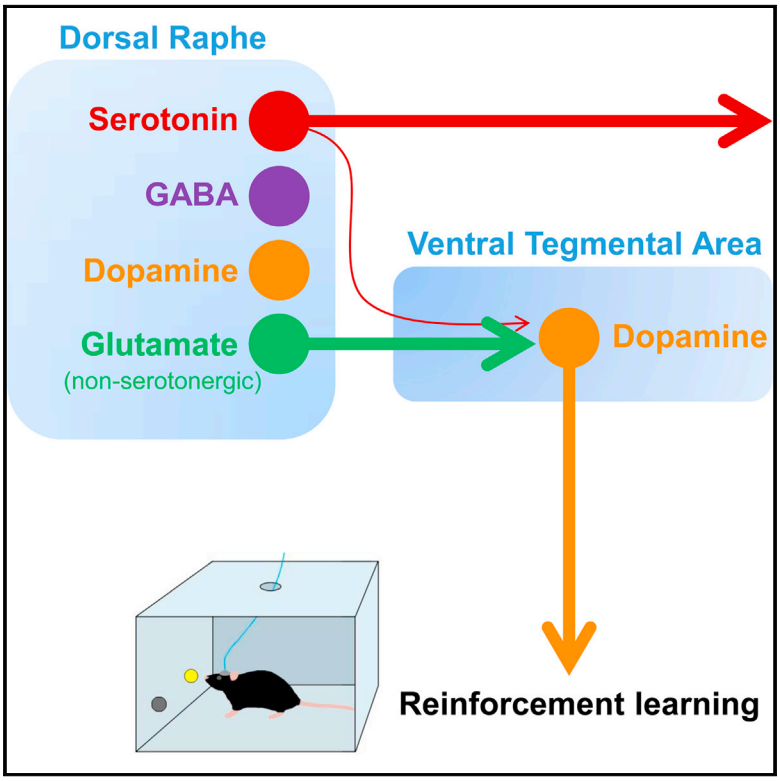
Serotonergic versus Nonserotonergic Dorsal Raphe Projection Neurons: Differential Participation in Reward Circuitry

Graphical Abstract



Highlights

Stimulation of nonserotonergic neurons in the DRN is highly rewarding

The majority of projections from DRN to VTA are nonserotonergic

Nonserotonergic DRN neurons provide glutamatergic input to VTA dopamine neurons

Optogenetic/pharmacological stimulation of DRN serotonin is not highly rewarding

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In Brief

The majority of the brain's serotonin neurons are found in the dorsal raphe nucleus, a brain region in which the influence over reward learning has proven enigmatic. Although drugs that enhance serotonin function have minimal abuse liability, laboratory rats will work vigorously to earn electrical stimulation of the dorsal raphe nucleus. In this study, McDevitt et al. explore nonserotonergic cell populations of the dorsal raphe nucleus, examining their ability to reinforce behavior and control the firing of midbrain dopamine neurons.

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Serotonergic versus Nonserotonergic Dorsal Raphe Projection Neurons: Differential Participation in Reward Circuitry

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SUMMARY

The dorsal raphe nucleus (DRN) contains the largest group of serotonin-producing neurons in the brain and projects to regions controlling reward. Although pharmacological studies suggest that serotonin inhibits reward seeking, electrical stimulation of the DRN strongly reinforces instrumental behavior. Here, we provide a targeted assessment of the behavioral, anatomical, and electrophysiological contributions of serotonergic and nonserotonergic DRN neurons to reward processes. To explore DRN heterogeneity, we used a simultaneous two-vector knockout/optogenetic stimulation strategy, as well as cre-induced and cre-silenced vectors in several cre-expressing transgenic mouse lines. We found that the DRN is capable of reinforcing behavior primarily via nonserotonergic neurons, for which the main projection target is the ventral tegmental area (VTA). Furthermore, these nonserotonergic projections provide glutamatergic excitation of VTA dopamine neurons and account for a large majority of the DRN-VTA pathway. These findings help to resolve apparent discrepancies between the roles of serotonin versus the DRN in behavioral reinforcement.

INTRODUCTION

Dopaminergic neurons of the ventral tegmental area (VTA) play a central role in reward learning (Wise, 2004). Whole-brain mapping studies have found the greatest density of VTA-projecting neurons to reside in the dorsal raphe nucleus (DRN) (Phillipson, 1979; Watabe-Uchida et al., 2012). The DRN contains the largest group of serotonin neurons in the brain and supplies the vast majority of ascending serotonergic projections (Jacobs and Azmitia, 1992). The role of the DRN in reinforcement learning is unclear, with literature suggesting both excitatory and inhibitory functions. For example, electrical stimulation of the DRN is sufficient to vigorously reinforce instrumental behavior in rats (Corbett and Wise, 1979; Margules, 1969; Rompré and Miliaressis, 1985; Simon et al., 1976; Van Der Kooy et al., 1978). In contrast, drugs that selectively elevate levels of serotonin, the major neurotransmitter output of the DRN, possess very low abuse liability in humans and are not self-administered in laboratory animals (Götestam and Andersson, 1975; Griffiths et al., 1976; Zawertailo et al., 1995).

A recent study provided evidence that optogenetic stimulation of serotonergic DRN cell bodies is capable of reinforcing instrumental behavior (Liu et al., 2014). However, a majority of the rewarding effects of electrical DRN stimulation act through fibers with refractory periods that are too rapid to be of serotonergic origin (Rompré and Miliaressis, 1987). These studies suggest that the DRN contains a population of nonserotonergic fibers capable of reinforcing behavior to a greater degree than serotonin-producing neurons. However, it is not known whether these fibers originate from neurons within the DRN or instead represent axons of distal cell bodies projecting to or through the DRN.

Given recent demonstrations that DRN projection neurons are heterogeneous and include serotonergic, dopaminergic, GABAergic, and nonserotonergic glutamate populations (reviewed in Vasudeva et al., 2011), we explored the participation of these populations in reward circuitry and reinforcement learning. By testing self-stimulation behavior, anterograde/retrograde tracing, and electrophysiology, we found that the DRN reinforces behavior preferentially through nonserotonergic neurons, which make up the majority of the DRN-VTA pathway and produce strong glutamatergic excitation of VTA dopamine neurons.

RESULTS

Stimulation of Dopamine, but not Serotonin, Reinforces Instrumental Behavior

To test whether the release of serotonin produces reward-related behavior, we examined the ability of the serotonin-releasing

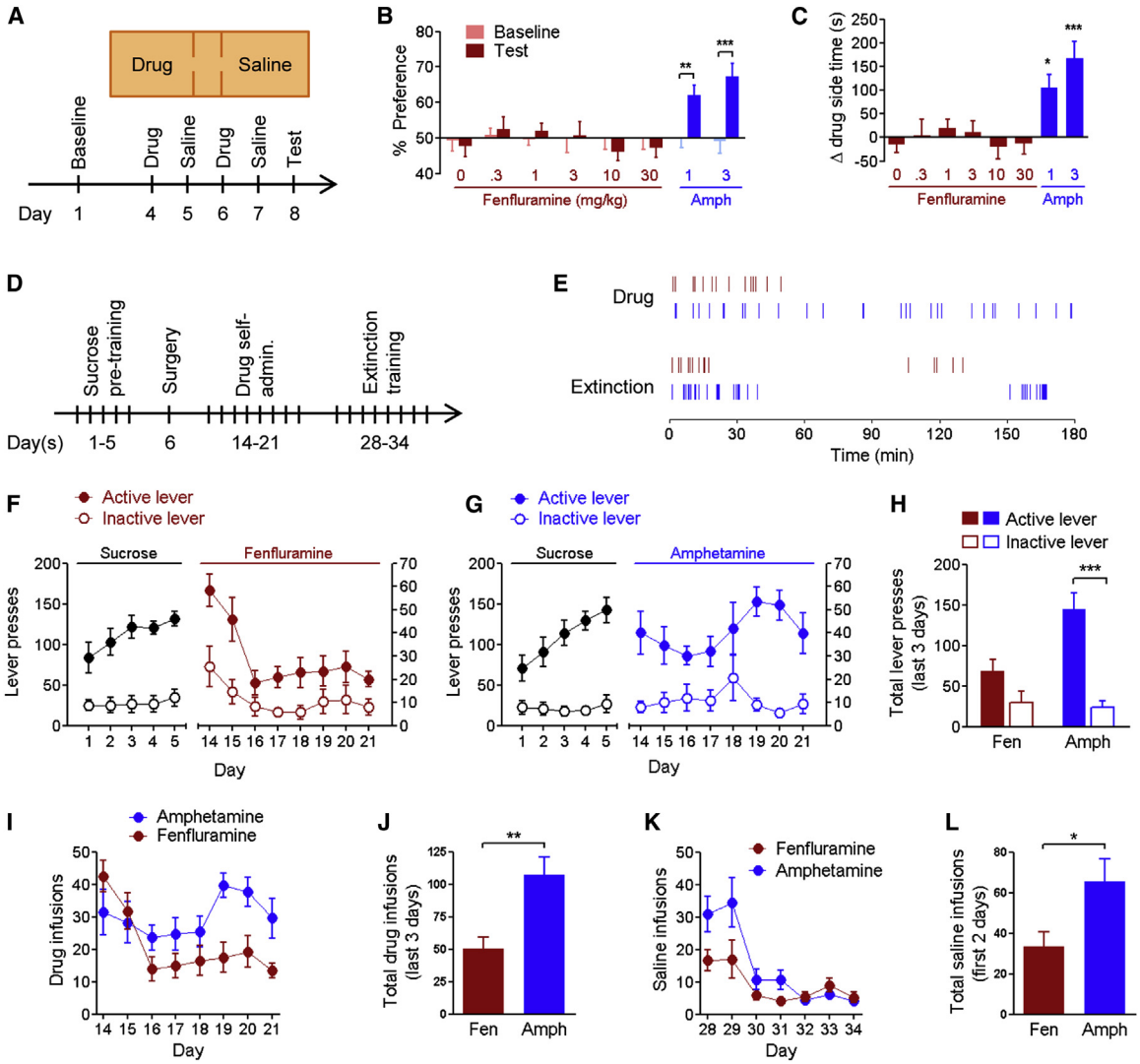

Figure 1. Pharmacological Stimulation of Dopamine but Not Serotonin Release Reinforces Behavior

(A) Mice (n = 9–18 per group) were conditioned with injections of the serotonin-releasing agent fenfluramine (0–30 mg/kg, i.p.) or the dopamine-releasing agent amphetamine (1–3 mg/kg, i.p.).

(B) Percentage of time spent in drug-paired chamber on baseline and test days. Repeated-measures ANOVA (drug × day) interaction $F_{(7,96)} = 4.166$, $p < 0.001$; **$p < 0.01$, ***$p < 0.001$ post hoc.

(C) Change in time spent on drug-paired chamber between test and baseline days. One-way ANOVA $F_{(7,96)} = 5.318$, $p < 0.0001$; *$p < 0.05$, ***$p < 0.001$ Dunnett's post hoc versus saline.

(D) Self-administration experiment in a separate cohort of mice. After pretraining for sucrose, mice were implanted with intravenous catheters and allowed to self-administer fenfluramine (0.03 mg/kg/infusion) or amphetamine (0.05 mg/kg/infusion) (n = 8 per group).

(E) Sample data from individual self-administration sessions demonstrating timing of infusions for mice with access to fenfluramine (red) and amphetamine (blue) and during the first day of extinction training.

(F and G) Daily lever-press counts during sucrose pretraining (left y axis) and drug self-administration (right y axis). These experiment phases are plotted on different scales because the number of maximally allowed rewards differed.

(H) Total number of lever presses during last three days of drug access. Two-way ANOVA (drug × lever) interaction $F_{(1,28)} = 8.095$, $p < 0.01$; ***$p < 0.001$ post hoc.

(I) Daily drug infusions during drug self-administration phase.

(J) Total number of drug infusions during the last 3 days of drug access, **$p < 0.01$.

(K) Daily counts of infusions of saline during extinction training.

(L) Total number of saline infusions during the first 2 days of extinction, *$p < 0.05$. Group data are presented here and in subsequent figures as mean ± SEM.

agent fenfluramine to elicit a conditioned place preference and to support self-administration in mice (Figure 1). Amphetamine, which is mechanistically similar to fenfluramine but acts preferentially on dopaminergic reuptake sites (Rothman and Baumann, 2009), was used as a positive control. In the conditioned place preference paradigm, none of the five doses of fenfluramine we

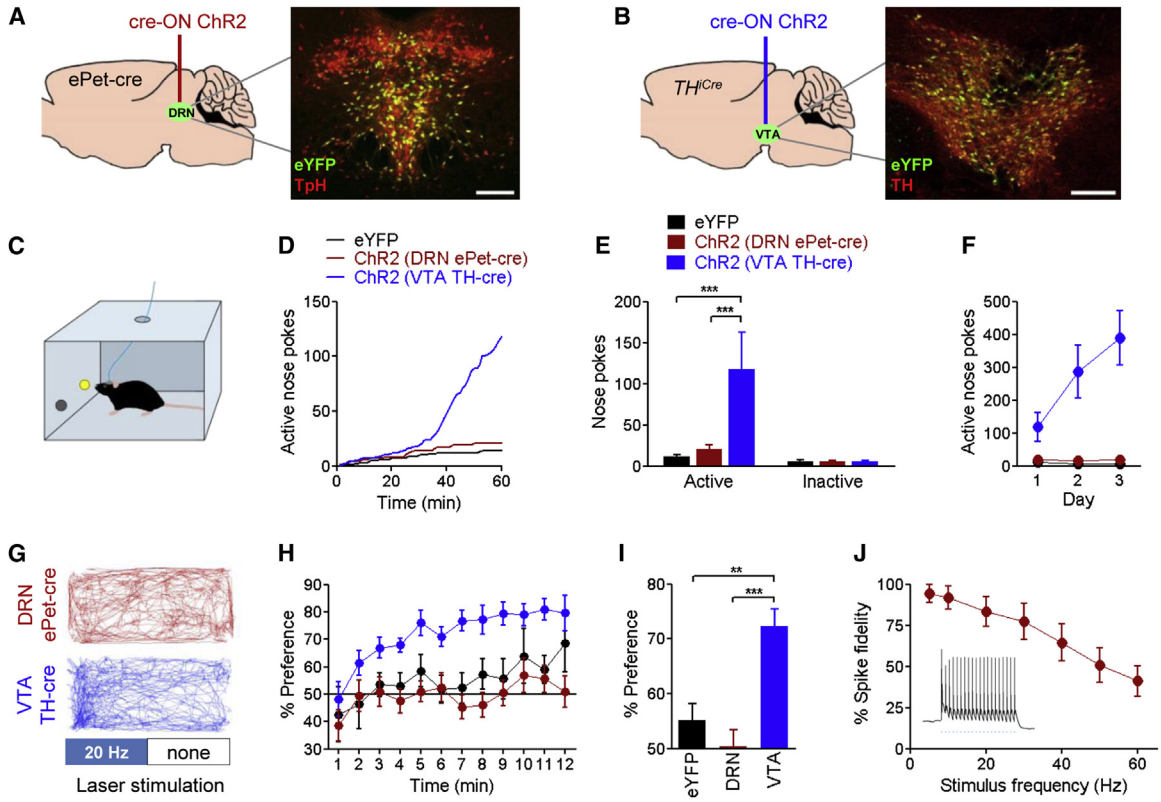

Figure 2. Optogenetic Stimulation of VTA Dopamine but Not DRN Serotonin Cell Bodies Reinforces Behavior

(A and B) Selective targeting of gene expression was achieved by injecting cre-induced ("cre-ON") vectors expressing ChR2-eYFP or eYFP alone into the (A) DRN of ePet-cre mice or (B) VTA of TH^{iCre} mice. The insets depict expression of eYFP (green), double labeled in red with tryptophan hydroxylase (TpH) for DRN tissue or tyrosine hydroxylase (TH) for VTA tissue. Scale bars represent 200 μm.

(C) Mice were trained to nose poke into an active port to receive 3 s trains of 20 Hz laser stimulation; nose pokes into an inactive port were not reinforced.

(D and E) Representative cumulative-activity graph and group mean nose pokes made in first behavioral session for VTA-dopamine (n = 11), DRN-serotonin (n = 18), and a combined control group expressing eYFP in DRN or VTA (n = 17). Two-way ANOVA (group × port) interaction $F_{(2,86)} = 8.317$, $p < 0.001$; ***$p < 0.001$ post hoc.

(F) Active nose poke responding on 3 consecutive days of testing.

(G) Mice underwent a real-time place preference task in which presence in one half of a chamber triggered continuous 20 Hz laser stimulation. Example tracks for a DRN serotonin stimulated mouse (top, red) and a VTA dopamine stimulated mouse (bottom, blue).

(H) Minute-by-minute percentage of time spent in the laser-paired half of the chamber.

(I) Overall preference for laser-paired side during 12 min session. One-way ANOVA $F_{(2,38)} = 12.05$, $p < 0.0001$; **$p < 0.01$, ***$p < 0.001$ post hoc.

(J) Percent of laser pulses in a 20 pulse train resulting in action potentials in ChR2+ DRN serotonin cell bodies, recorded ex vivo in whole-cell current clamp. Inset, sample trace with 20Hz stimulation (see also Figures S1 and S2).

tested (0.3–30 mg/kg, intraperitoneally [i.p.]) produced a significant preference or aversion (Figures 1A–1C). In contrast, amphetamine (1 or 3 mg/kg, i.p.) elicited a strong preference. Similarly, mice that had self-administration access to amphetamine significantly lever pressed more for drug infusions than mice with access to fenfluramine (Figures 1D–1J). After 1 week of drug abstinence, each group of mice was also tested for extinction responding, in which lever presses resulted in saline infusions. Mice with previous access to amphetamine exhibited greater measures of drug seeking (Figures 1K and 1L).

Although serotonin release was not sufficient to strongly elicit reward-related behavior, it is possible that such behavioral effects require precisely timed neuronal activity within DRN serotonin neurons and/or corelease of other transmitters. To explore these possibilities, we used optogenetic stimulation with the light-activated ion channel channelrhodopsin-2 (ChR2) to activate neurons in vivo (Adamantidis et al., 2007) and test whether stimulation of serotonergic DRN cell bodies was sufficient to reinforce behavior (Figure 2A). Compared with classical electrical stimulation, this approach offers the advantages of restricting stimulation to genetically defined populations of cells without affecting fibers of passage or terminals (Tye and Deisseroth, 2012). As a positive control, we also tested stimulation of dopaminergic VTA cell bodies (Figure 2B). Selective targeting of ChR2 to these cells was achieved by stereotaxically injecting cre-inducible ("cre-ON") vectors expressing ChR2-eYFP or eYFP alone into the DRN of ePet-cre mice or the VTA of TH^{iCre} mice. These transgenic lines selectively express cre recombinase in serotonergic and catecholaminergic neurons, respectively. Nearly all (91.4%) of 1,827 total eYFP-positive DRN

neurons counted from eight ePet-cre animals also tested positive for tryptophan hydroxylase, an enzyme essential for serotonin synthesis. Similarly, 88.9% of 903 eYFP-positive neurons counted from 4 TH^{iCre} animals also tested positive for tyrosine hydroxylase, an enzyme necessary for dopamine synthesis. Behavior from DRN and VTA-injected eYFP control mice did not differ, and they were combined into a single group. Mice were trained in an operant task (Figure 2C), in which nose pokes into an active port resulted in 3 s trains of 20 Hz laser stimulation. While stimulation of VTA dopamine neurons strongly reinforced behavior in this task, stimulation of DRN serotonin neurons had no effect (Figures 2D–2F). We found the same pattern of results in a real-time place preference test (Figure 2G), in which presence of the mouse in one half of a chamber resulted in continuous 20 Hz stimulation. Stimulation of VTA dopamine neurons reinforced behavior in this task, whereas stimulation of DRN serotonin neurons did not (Figures 2H and 2I). Parametric variations of laser intensity, pulse frequency, and repeated days of training all failed to elicit statistically significant measures of reward or aversion in DRN serotonin stimulated mice (Figure S1). DRN serotonin neurons were capable of following 20 Hz optical stimulation with action potentials (Figures 2J and S2), consistent with previous demonstrations that electrical stimulation produces increasing levels of serotonin release at increasing frequencies up to and including 20 Hz (Sharp et al., 1989). Together with the fenfluramine experiments, these data demonstrate that behavioral measures of reinforcement that are easily elicited by stimulation of dopamine release are much less sensitive to serotonin.

Stimulation of Nonserotonergic DRN Neurons Reinforces Instrumental Behavior

We next performed an experiment collectively stimulating all nonserotonergic DRN output to test for nonserotonergic reward mechanisms. To do this, we performed a combined knockout/stimulation experiment in mice homozygous for a floxed allele of *Tph2*, the gene encoding the rate-limiting enzyme for serotonin synthesis. $Tph2^{lox/lox}$ mice were coinjected with a virus mixture containing a 1:10 ratio of a vector nonspecifically expressing cre recombinase and a second cre-ON vector expressing either ChR2-eYFP or eYFP alone (Figures 3A and 3B). Because cre-ON vector expression is dependent on cre recombinase, which is not endogenously expressed in these mice, only cells transduced by both viral vectors will express functional ChR2 protein (Figure 3A). Although all DRN neuron types (serotonin, glutamate, GABA, dopamine) will be capable of laser-activated stimulation, cre recombinase will excise the *Tph2* gene, preventing synthesis of serotonin without affecting other transmitters. Indeed, only 1.3% of 1,276 total eYFP-positive DRN neurons counted from three animals also labeled for tryptophan hydroxylase. Optical stimulation of the DRN in these mice strongly reinforced behavior in nose-poke and real-time place preference tasks (Figures 3C and 3D). To test whether restoration of serotonin would alter the rewarding properties of DRN stimulation, we administered 5-hydroxytryptophan, the missing intermediate in the serotonin synthesis pathway of ChR2-expressing neurons. To ensure an ability to detect increases or decreases in rewarding properties of stimulation, laser pulse widths were adjusted for each mouse to deliver pulses that were empirically determined to produce 50% of maximal behavioral responding (Figure S3). Nose-poke behavior was not affected by administration of 5-hydroxytryptophan (40 mg/kg, i.p.) 30 min prior to testing (Figures 3E and 3F), a dose and time point that restores serotonin function in *Tph2* knockout mice (Liu et al., 2011). Finally, to ensure that behavioral effects were anatomically specific to the DRN, we included a control group in which ChR2 expression was targeted anterior to the DRN. The site of injection for anatomical control ChR2 mice ranged from 2.9 to 3.8 mm posterior to bregma; injection sites for DRN-targeted ChR2 mice ranged from 3.9 to 4.4 mm posterior to bregma (mean difference of 0.7 mm). Optical stimulation of neurons anterior to the DRN did not reinforce behavior (Figures 3C and 3D). These results indicate that a population of DRN cell bodies is capable of driving reward-related behavior in a serotonin-independent fashion.

To examine whether these effects were dependent on dopamine signaling, we tested effects of the D1 antagonist SCH23390 (SCH) on optical self-stimulation behavior (Figure 3G). On consecutive days, mice were injected 15 or 30 min prior to testing with either saline or a dose of SCH that did not prevent locomotion (30 μg/kg; Figure S4). To reduce intersubject variability, data from each mouse were normalized to performance on the last day of baseline training. Regardless of injection timing, D1 receptor blockade significantly reduced nose-poke responses during the later period in the session, but not during the first 5 min (Figures 3H and 3I). This pattern of responding suggests a reduction in the rewarding effects of laser stimulation, rather than an impaired ability to perform the nose-poke response (Fouriezos and Wise, 1976).

Stimulation of Dopaminergic or GABAergic DRN Cell Bodies Do Not Reinforce Instrumental Behavior

The anterior DRN contains dopaminergic neurons that are involved in attention and arousal (Lu et al., 2006) that could underlie dopamine-dependent, serotonin-independent DRN reward. We tested this by injecting cre-ON vectors expressing ChR2-eYFP or eYFP alone into the DRN of TH^{iCre} mice (Figure 4A). In DRN tissue from five mice, 240 of 356 eYFP-positive cells double labeled for tyrosine hydroxylase (67.4%). While this specificity was lower than that obtained in the VTA, similar numbers have been reported for the specificity of GFP expression within the DRN of TH-GFP transgenic mice (Dougalis et al., 2012), suggesting that the tyrosine hydroxylase promoter may be active in cells expressing protein at levels below immunohistochemical detection. Nevertheless, stimulation of these cells failed to reinforce behavior in nose-poke or real-time place preference tasks (Figures 4B–4D), which indicates that activity of DRN dopamine neurons cannot account for the reward-related behaviors seen previously.

Thus far, we have observed that a population of DRN neurons is capable of reinforcing behavior, but this effect is not seen with selective stimulation of serotonergic or dopaminergic cell types. This suggests that the rewarding effects of DRN stimulation are driven by GABAergic and/or glutamatergic neurons, the two remaining major DRN cell types. The DRN contains GABAergic projection neurons (Bang and Commons, 2012), which could

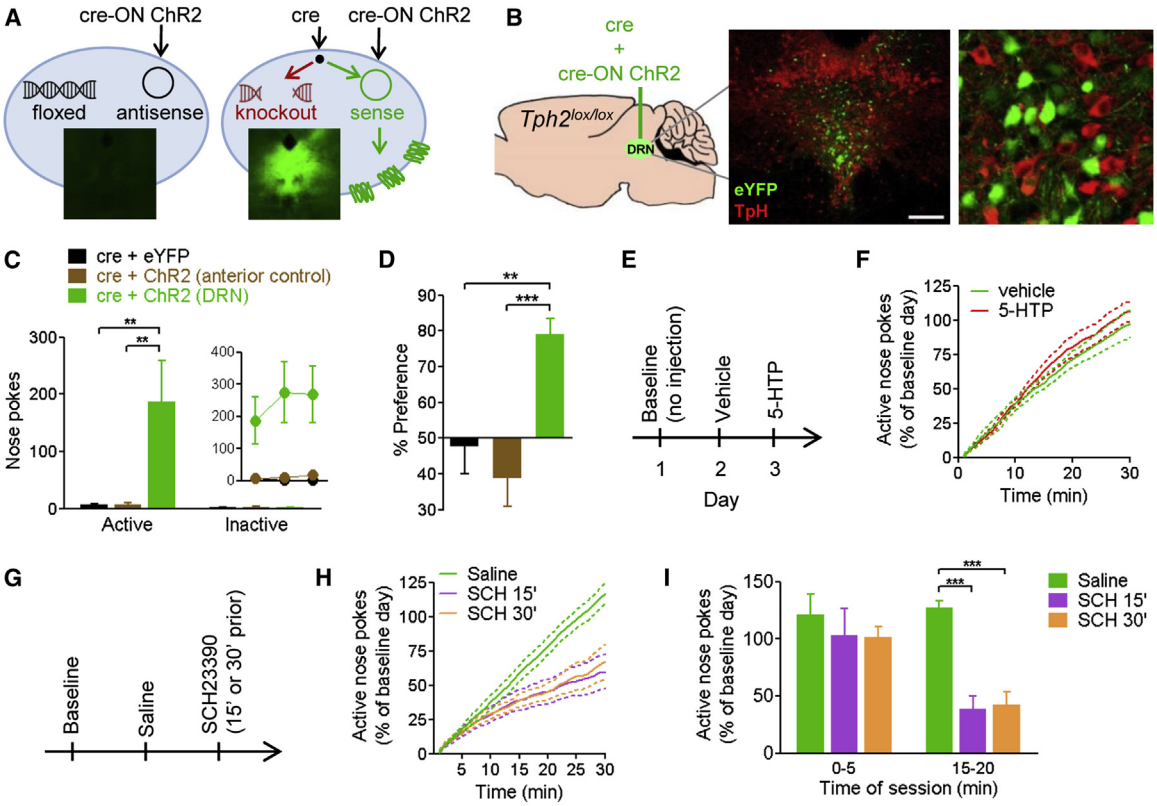

Figure 3. Optogenetic Stimulation of DRN Cell Bodies Reinforces Behavior in a Dopamine-Dependent, Serotonin-Independent Manner

(A) Left panel, schematic view of a cell transduced with cre-induced ("cre-ON") viral vector. In the absence of cre recombinase, viral plasmid DNA remains in antisense orientation and does not express functional protein. (Inset) Lack of eYFP signal in mouse injected with cre-ON ChR2-eYFP. (Right panel) Coinjection of cre-ON and cre-expressing viral vectors results in knockout of floxed genomic DNA and rearrangement of viral plasmid DNA into sense orientation, resulting in expression of ChR2-eYFP. (Inset) robust eYFP expression in mouse coinjected with cre-expressing and cre-ON viral vectors.

(B) *Tph2*$^{lox/lox}$ mice were coinjected with viral vectors expressing cre and cre-ON ChR2-eYFP or eYFP into DRN. (Insets) Whole DRN (scale bar, 200 μm) and detail of nonoverlapping expression of eYFP (green) and tryptophan hydroxylase (TpH, red). Thus, cells with ChR2 lack the enzyme necessary for serotonin synthesis. An additional anatomical control group was coinjected with cre and cre-ON ChR2-eYFP 0.7 mm anterior to the DRN.

(C) Nose pokes during first day of self-stimulation testing for nonserotonergic DRN stimulation (n = 10), anterior controls (n = 8), and eYFP controls (n = 7). Two-way ANOVA (group × nose port) interaction $F_{(2,44)} = 4.482$, $p < 0.05$; $^{**}p < 0.01$ post hoc. (Inset) Active nose pokes on 3 consecutive days of testing.

(D) Percentage of time spent on laser side in a real-time place preference task. One-way ANOVA $F_{(2,22)} = 11.24$, $p < 0.001$; $^{**}p < 0.01$, $^{***}p < 0.001$ post hoc.

(E) Nonserotonin DRN stimulated mice (n = 6) were tested for nose-poke optical self-stimulation 30 min after injection of vehicle or 5-hydroxytryptophan (5-HTP; 40 mg/kg i.p.), the intermediate in the serotonin synthesis pathway. 5-HTP is the product of the enzyme tryptophan hydroxylase, which is knocked out in ChR2-positive cells of these mice.

(F) Cumulative-activity graph of nose pokes in test sessions after injection of vehicle or 5-HTP. Individual data points were normalized to percent of nose pokes achieved during a 30 min baseline session on day 1.

(G) Nonserotonin DRN stimulated mice (n = 6) were tested after injection of the dopamine D1 receptor antagonist SCH23390 (SCH; 30 μg/kg, i.p.) at either 15 or 30 min before testing.

(H) Cumulative-activity graph of active nose pokes in 30 min sessions following injection of saline or SCH.

(I) Active nose pokes during 5 min bins at the beginning or in the middle of test depicted in (H). Individual data points were normalized to the percentage of responses during baseline day. Repeated-measures ANOVA (drug × epoch) interaction $F_{(2,15)} = 4.560$, $p < 0.05$; $^{***}p < 0.001$ Dunnett's post hoc versus saline (see also Figures S3 and S4).

promote reward-related behavior by disinhibiting mesolimbic circuitry. To test the ability of DRN GABA neurons to reinforce behavior, we injected cre-ON vectors expressing ChR2-eYFP or eYFP alone into the DRN of *Vgat*^{iCre} mice (Figure 4E). Out of 1,059 eYFP-positive neurons counted in DRN tissue from three mice, less than 1% of cells also labeled for tyrosine hydroxylase or serotonin. Stimulation of these neurons did not result in significant nose-poke behavior (Figures 4F and 4G), but it did induce a statistically significant real-time place preference (Figure 4H). To the extent that optical stimulation of DRN GABA neurons activates local inhibitory interneurons (Challis et al., 2013), this result is consistent with studies showing that local inhibition of the DRN can produce reward-related behavior in rats (Fletcher et al., 1993; Liu and Ikemoto, 2007). However, the lack of nose-poke behavior indicates that the rewarding effects of these neurons are minimal in the mouse, and they are not capable of driving the vigorous self-stimulation seen previously (Figure 3C).

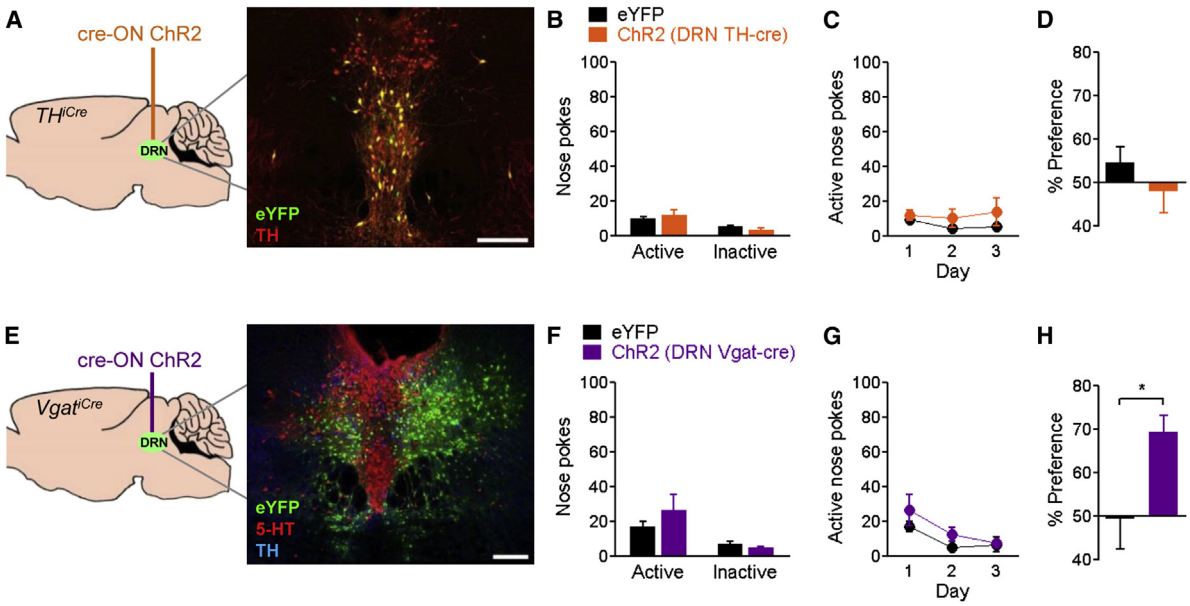

Figure 4. Optogenetic Stimulation of Dopaminergic or GABAergic DRN Cell Bodies Fails to Reinforce Nose-Poke Self-Stimulation

(A) DRN dopamine neurons were targeted by injecting cre-induced ("cre-ON") vectors expressing ChR2-eYFP (n = 10) or eYFP (n = 14) into the DRN of TH^{iCre} mice. The inset shows eYFP (green) double-labeled with tyrosine hydroxylase (TH, red).

(B) Nose pokes in the first day of testing.

(C) Active nose pokes on 3 consecutive days of testing.

(D) Percentage of time spent on laser side in real-time place preference task.

(E) DRN GABA neurons were targeted by injecting cre-ON ChR2-eYFP (n = 8) or eYFP (n = 4) into the DRN of $Vgat^{iCre}$ mice. The inset shows eYFP (green) cell bodies in the lateral DRN, which do not colabel for serotonin (5-HT, red) or tyrosine hydroxylase (TH, blue).

(F–H) Laser stimulation did not reinforce nose poke self-stimulation (F and G) but did induce a real-time place preference (H); *$p < 0.05$. (Scale bars, 200 μm).

Distribution of Serotonergic and Nonserotonergic DRN Projections

To explore potential interactions of the DRN with mesolimbic reward circuitry, we examined the ascending projections of both serotonergic and nonserotonergic DRN neurons. We constructed a cre-OFF viral vector (Figure 5A) containing a loxP-flanked ChR2-eYFP coding region, thereby expressing eYFP in the absence, but not in the presence, of cre recombinase (Figure 5B). To visualize serotonergic and nonserotonergic projections within the same mice, we injected cre-OFF ChR2-eYFP vector into the DRN of mice containing the serotonergic ePet-cre transgene and the cre-induced TdTomato fluorophore (ePet-cre; $ROSA26^{fsTdTomato}$; Figure 5C). In these double transgenic mice, serotonergic cre-expressing neurons should express TdTomato but not eYFP. All other transduced neurons within the DRN should express only eYFP. Tissue was collected beginning at 12 weeks after surgery to allow sufficient time for protein to fill distal processes. Red and green fluorescence within the DRN segregated into separate populations of cre-positive (serotonergic) and cre-negative (nonserotonergic) DRN cells (Figure 5C). Nonserotonergic (eYFP) projections were seen emanating rostroventrally from the DRN; this tract split bilaterally and entered the VTA, where it overlapped strongly with tyrosine hydroxylase-positive cell bodies of the lateral VTA (Figure 5D). This was the strongest site of eYFP expression outside of the DRN (Figure 5E). Faint eYFP expression was seen in a limited number of structures in the anterior brain, including the dorsolateral bed nucleus of the stria terminalis, lateral septum, and nucleus accumbens. In contrast, serotonergic (TdTomato) projections did not exhibit the same dramatic degree of variability (Figure 5F). At the level of the VTA, serotonergic projections appeared predominantly as a dense bundle of ascending fibers, located medially to the dopaminergic cells. These findings indicate that nonserotonergic neurons of the DRN project robustly to the dopamine-rich region of the lateral VTA.

To determine whether nonserotonergic, VTA-projecting neurons reside within the traditional boundaries of the DRN, we iontophoretically infused the retrograde tracer Fluoro-Gold into the VTA or, for comparison, into the substantia nigra reticulata. In VTA-infused mice, strong retrograde labeling was observed medially within the DRN, concentrated at midrostral levels (Figures 6A and 6E). Very little was seen in the median raphe nucleus, similar to previous reports (Watabe-Uchida et al., 2012). Mice infused with tracer into the substantia nigra reticulata showed retrograde labeling that was tightly restricted to a small group of neurons in the dorsal DRN (Figure 6B). While these neighboring pathways both originated within the boundaries of the DRN, the number of nonserotonergic DRN neurons projecting to the VTA vastly outnumbered those projecting to the substantia nigra reticulata (Figures 6C and 6D). Thus, retrograde and anterograde measures indicate that the DRN-VTA circuit is a robust pathway comprised of primarily nonserotonergic neurons, whose major output is the VTA.

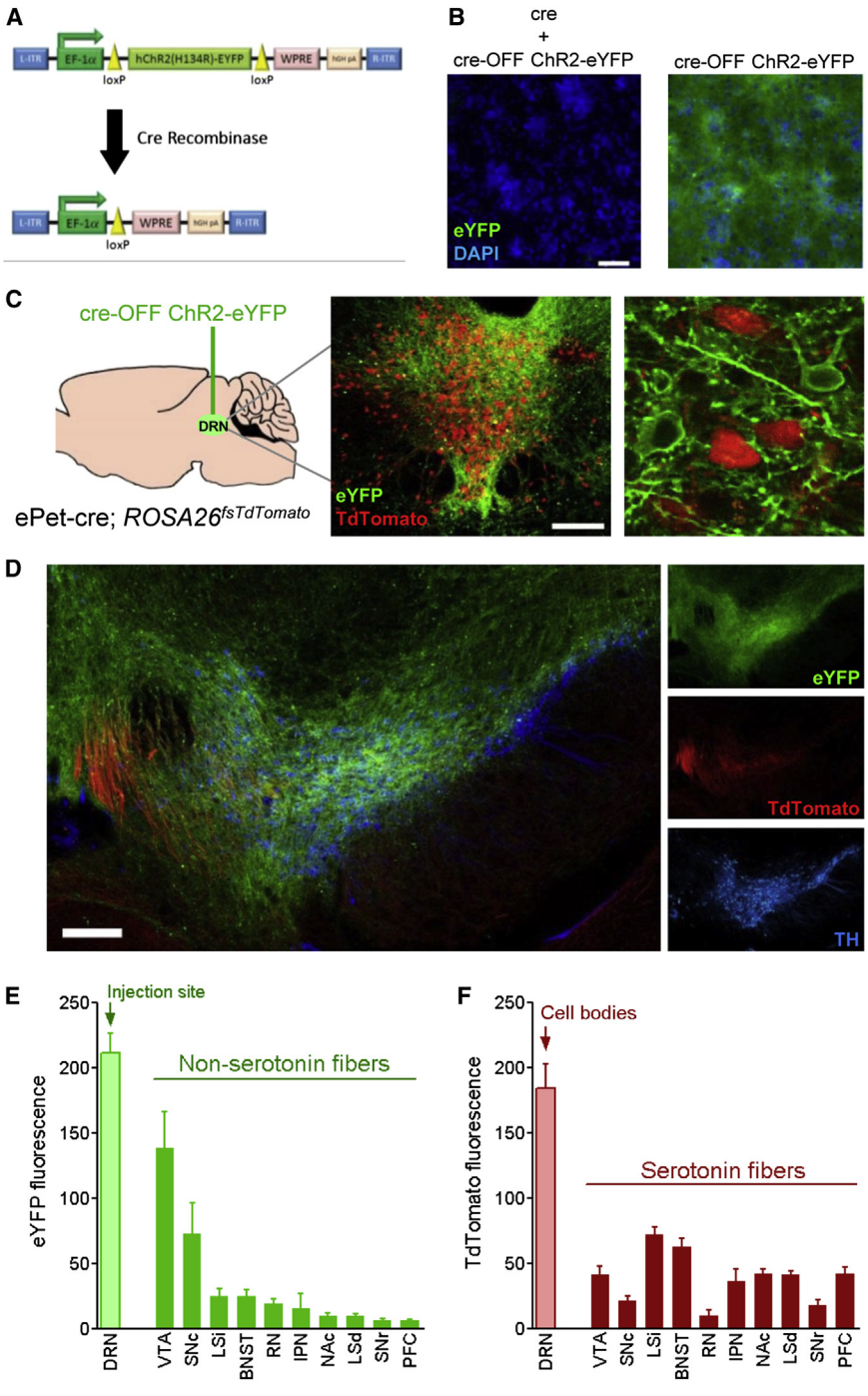

Figure 5. Unlike Serotonergic Neurons, Nonserotonergic DRN Neurons Preferentially Project to the VTA

(A) Schematic of cre-silenced ("cre-OFF") DNA construct containing loxP-flanked ChR2-eYFP coding region.

(B) Transduction of primary cultured rat neurons with cre-OFF ChR2-eYFP viral vector produces eYFP fluorescence (left) that is abolished in cells cotransduced with a vector expressing cre recombinase (right); DAPI nuclear staining (blue) is unaffected. The scale bar represents 100 μm.

(C) Transgenic mice coexpressing cre and TdTomato in serotonergic neurons (ePet-cre; *ROSA26*^{fsTdTomato}, n = 4) were injected with cre-OFF ChR2-eYFP into the DRN. The inset depicts whole DRN tissue (scale bar, 200 μm) and detail demonstrating segregation of TdTomato and eYFP fluorescence into separate populations of cells.

(D) Serotonergic (red) and nonserotonergic (green) axons are visible in the VTA, identifiable by tyrosine hydroxylase immunoreactivity (TH, blue). The scale bar represents 200 μm.

(E and F) Quantitation of (E) eYFP and (F) TdTomato fluorescence intensity in brain regions with conspicuous eYFP expression. SNc, substantia nigra pars compacta; LSi, intermediate portion of the lateral septum; BNST, bed nucleus of the stria terminalis; RN, red nucleus; IPN, interpeduncular nucleus; NAc, nucleus accumbens; LSd, dorsal portion of the lateral septum; SNr, substantia nigra reticulata; PFC, prefrontal cortex.

Nonserotonergic DRN-VTA Pathway Mediates Behavioral Reinforcement and Excites VTA Dopamine Neurons

To determine if selective stimulation of DRN-VTA projections was sufficient to reinforce behavior, we targeted ChR2 expression to the DRN of wild-type mice and implanted optical fibers in the VTA (Figure 7A). Mice vigorously nose poked for stimulation of the DRN-VTA pathway (Figures 7B and 7C), indicating that DRN-VTA projections are sufficient to drive reward-related behavior. The lack of rewarding effects from stimulation of DRN serotonin, dopamine, and GABA cell bodies strongly suggests that this rewarding DRN-VTA pathway contains a glutamatergic component. To compare glutamatergic contributions of serotonergic and nonserotonergic DRN-VTA projections, we used cre-ON and cre-OFF vectors to express ChR2-eYFP in *SERT*^{cre} mice (Figure 7D). *SERT*^{cre} and ePet-cre mouse lines demonstrate similar selectively for expression in serotonergic neurons, as 90.3% of 651 total eYFP-positive cells from five *SERT*^{cre} mice injected with cre-ON eYFP virus were found to double label with tryptophan hydroxylase. However, preliminary results in TdTomato reporter mice suggested greater penetrance of expression in serotonergic populations with the *SERT*^{cre} line (data not shown); therefore, we used these mice in electrophysiology experiments to ensure that cre-OFF viral expression was limited to nonserotonergic neurons. We obtained whole-cell patch clamp recordings in horizontal slices containing VTA tissue beginning 12 weeks after surgery. Stimulation of the nonserotonin pathway produced excitatory

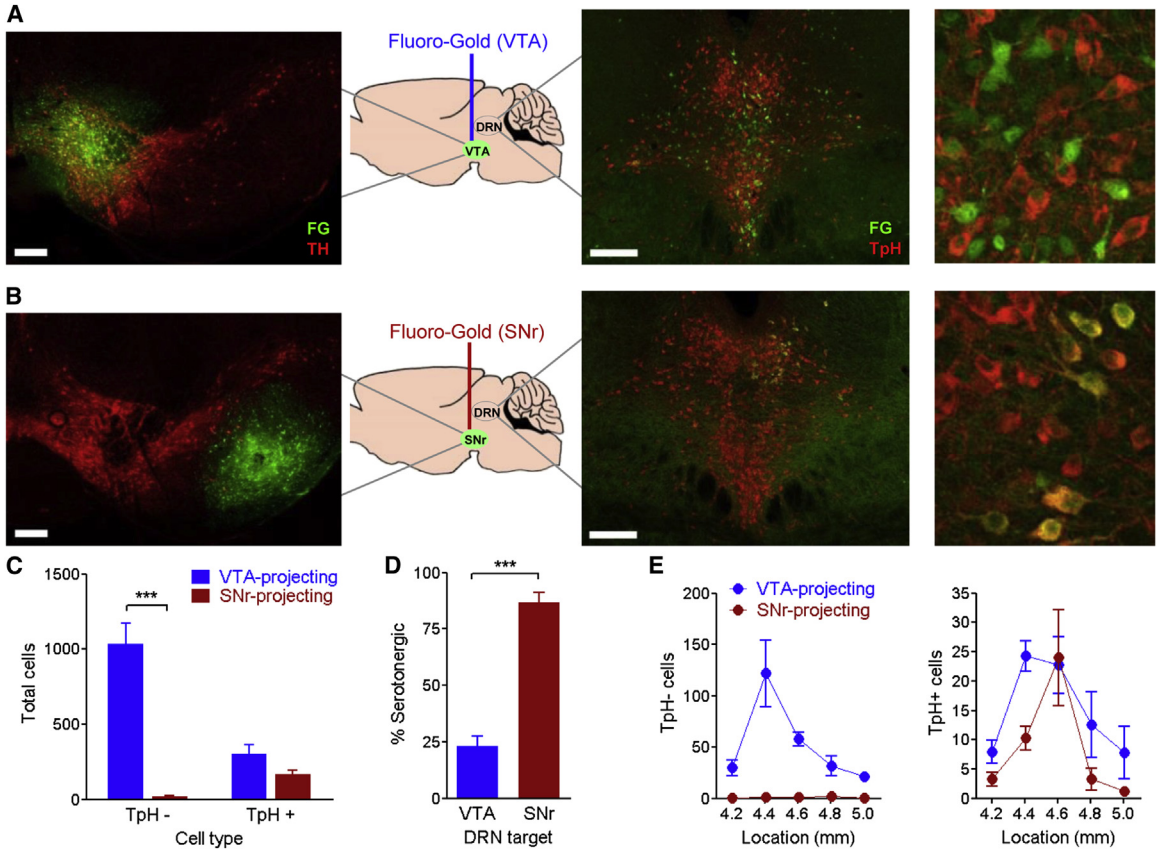

Figure 6. The Majority of DRN Cell Bodies that Project to VTA Are Nonserotonergic

(A and B) The retrograde tracer Fluoro-Gold was iontophoretically infused into the (A) VTA or (B) substantia nigra reticulata (n = 4 per group). (Left panels) Fluoro-Gold (green) at infusion site, double labeled with tyrosine hydroxylase (TH) to label dopamine neurons (red). (Right panels) Retrograde-labeled cells in DRN, double labeled with tryptophan hydroxylase (TpH) to label serotonin neurons (red). Scale bars represent 200 μm.

(C) Number of Fluoro-Gold-labeled cells in DRN tissue from mice injected with Fluoro-Gold in VTA or substantia nigra reticulata. Fluoro-Gold cells were grouped by presence or absence of tryptophan hydroxylase double label (TpH^+, TpH^-). Two-way ANOVA (region × TpH label interaction) $F_{(1,12)} = 34.11$, $p < 0.0001$; ***$p < 0.001$ post hoc.

(D) Percent of Fluoro-Gold labeled cells double-labeling for tryptophan hydroxylase. ***$p < 0.0001$.

(E) Number of TpH^- (left) and TpH^+ (right) Fluoro-Gold labeled cells across the rostrocaudal axis of the DRN. The x axis indicates location of DRN tissue, in millimeters posterior to bregma.

postsynaptic currents (EPSCs) in 34 of 43 cells tested (79.1%; Figure 7F), with an average latency of 1.84 ± 0.15 ms. Stimulation of the serotonin pathway produced EPSCs in 21 of 47 cells tested (44.7%), with an average latency of 2.51 ± 0.11 ms. The rapid latency and low jitter of current response times suggest monosynaptic connections. The magnitude of EPSCs produced by stimulation of the nonserotonin pathway was significantly larger than those produced by the serotonin pathway (Figure 7G). Bath application of selective α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) antagonists completely abolished evoked currents (Figure 7E). When light-responsive cells were tested for action potentials in current clamp (Figure 7H), stimulation of the nonserotonin pathway produced action potentials in 19 of 28 cells tested (67.9%; Figure 7I). The serotonin pathway only produced spikes in 3 of 17 cells (17.6%). In addition, spike fidelity was greater in response to optical stimulation of the nonserotonin pathway (Figure 7J). These data demonstrate that nonserotonergic, glutamatergic DRN neurons directly target VTA dopamine neurons and are highly efficacious in enhancing their rate of firing.

DISCUSSION

In the present study, we found that stimulation of DRN cell bodies was capable of strongly reinforcing instrumental behavior, with a level of vigor comparable to direct stimulation of VTA dopamine neurons. Although serotonergic neurons are the largest population of projection neurons in the DRN, selective stimulation of these cells did not reinforce behavior. Rather, self-stimulation was preferentially elicited by targeting nonserotonergic DRN neurons, which we showed to comprise the majority of DRN-VTA projections. Furthermore, stimulation of the DRN-VTA pathway was sufficient to fully reinforce instrumental

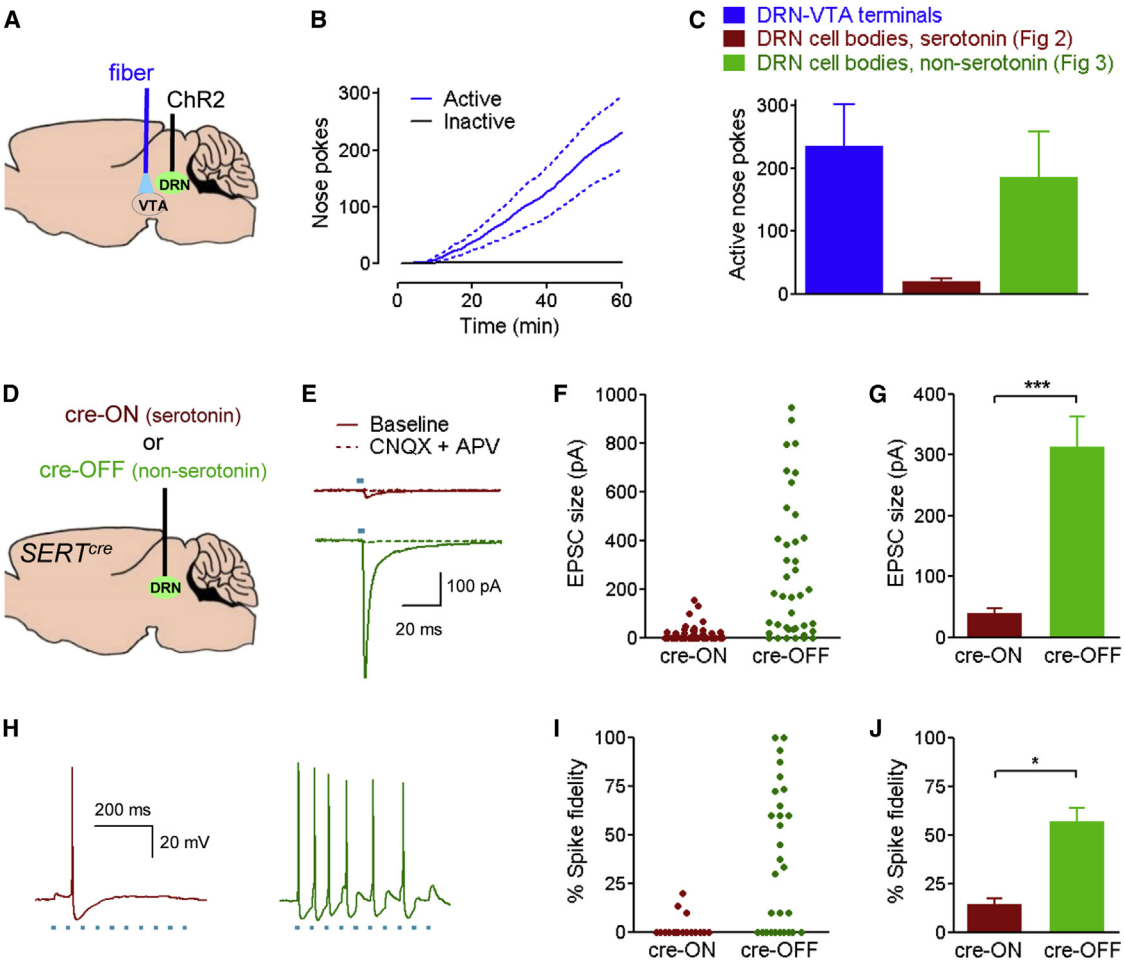

Figure 7. DRN-VTA Projections Reinforce Behavior and Provide Synaptic Glutamatergic Excitation of VTA Dopamine Neurons Primarily via Nonserotonergic Projections

(A) Mice (n = 11) were injected with nonspecific ChR2 viral vector in the DRN and implanted with fiber optic cables in the VTA.

(B) Cumulative activity graph of nose pokes into active and inactive ports on the first day of training. Total number of responses was greater into the active port ($p < 0.01$).

(C) Total number of active nose pokes on day 1 from DRN-VTA mice. For comparison, data are reconstituted from previous experiments stimulating serotonergic and nonserotonergic DRN cell bodies.

(D) Serotonergic and nonserotonergic DRN projections were targeted by injecting cre-induced ("cre-ON") (n = 6) or cre-silenced ("cre-OFF") (n = 4) vectors expressing ChR2-eYFP in *SERT*^{cre} mice.

(E) Representative voltage-clamp traces of VTA dopamine neurons showing optically evoked glutamatergic EPSC resulting from stimulation of terminals of the serotonergic (top trace) or nonserotonergic (bottom trace) DRN-VTA pathway.

(F) EPSC amplitudes in response to optical stimulation. Graph includes cells that did not respond to light (plotted as 0 pA).

(G) Average amplitude of light-responsive EPSCs, ***$p < 0.0001$.

(H) Representative current-clamp traces of a VTA dopamine neuron spiking in response to 20 Hz laser stimulation of DRN-VTA serotonin (left) or nonserotonin (right) pathways.

(I) Individual spike fidelity measurements; represented as percent of laser pulses during a 0.5 s, 20 Hz train that resulted in action potentials.

(J) Average spike fidelity in cells that responded to light with at least one action potential, *$p < 0.05$.

learning. Because self-stimulation was not supported by DRN dopaminergic or GABAergic cell bodies, our observations suggest that the rewarding effects of DRN stimulation are mediated by nonserotonergic glutamate neurons. Accordingly, in vitro stimulation of the nonserotonergic DRN-VTA pathway increased VTA dopamine neuron firing rates and produced monosynaptic glutamatergic currents that were substantially larger than those elicited by stimulating the serotonergic pathway.

The DRN contains the largest group of serotonergic neurons in the brain, a subset of which encode information about the magnitude of the reward received (Inaba et al., 2013; Liu et al., 2014; Nakamura et al., 2008). We employed a variety of optogenetic and pharmacological methods to test the possibility that such activity drives reinforcement learning. In all cases, the results of our experiments did not support this conclusion. This is consistent with reports that rats and primates do not

self-administer serotonergic drugs (Götestam and Andersson, 1975; Griffiths et al., 1976), which we now extend to mice. In both fenfluramine and optogenetic place preference experiments, maximal trends toward rewarding effects were seen at low doses/frequencies, suggesting that serotonin may exert proreward effects in an inverted U-shaped manner. A recent study demonstrated that optogenetic stimulation of serotonergic DRN neurons in mice can reinforce a variety of instrumental tasks (Liu et al., 2014). There were several methodologic differences between this study and our present work that likely afforded Liu et al. greater sensitivity in detecting behavioral effects of laser stimulation that, in our experiments, produced nonsignificant trends toward the same direction of effect. Nevertheless, using identical behavioral and optical parameters across experiments, we found that reinforcement learning was preferentially supported in this region by a population of nonserotonergic neurons.

Mice in our study vigorously nose poked for optogenetic stimulation of nonserotonergic DRN neurons. This finding is consistent with reports that rats will respond for electrical DRN stimulation in a serotonin-independent manner (Margules, 1969; Rompré and Miliaressis, 1987; Simon et al., 1976; but see Van Der Kooy et al., 1978). Although electrical self-stimulation literature provides the foundation for our understanding of brain reward circuitry, interpretation of this work is inherently limited by the fact that electrical stimulation of brain tissue excites both cell bodies and axonal fibers. In fact, action potentials are preferentially induced in axons, due to a far greater surface density of sodium channels (Nowak and Bullier, 1998). This issue is of particular importance because the DRN is bordered by dense fiber tracts; furthermore, it receives strong projections from several brain regions that are each individually sufficient to support reward learning, including the lateral hypothalamus, laterodorsal tegmental nucleus, and medial prefrontal cortex (Britt et al., 2012a; Kempadoo et al., 2013; Lammel et al., 2012; Lee et al., 2003). By using optogenetic methodology, we are able to negate the influence of stimulating fibers and localize the reward-relevant neuronal cell bodies.

Electrical mapping studies indicate that rewarding sites in the brain are not restricted to the DRN, but extend rostrally in a continuous band before bifurcating laterally and merging with the VTA (Rompré and Miliaressis, 1985). We found that stimulation of cell bodies rostral to the DRN did not produce behavioral measures of reward, although we did observe efferent fibers of DRN neurons in an identical pattern to the rewarding region described. Thus, the rewarding effects of electrical stimulation in this region are likely mediated by activation of axonal fibers originating from nonserotonergic cell bodies in the DRN. Furthermore, two-electrode collision experiments within this region suggest that the reward-relevant axons are highly branched between VTA and DRN (Boye and Rompré, 1996), suggestive of the dense network of nonserotonergic fibers that we observed in the VTA. The rewarding properties of DRN stimulation were dependent on dopamine receptor activation. Although the DRN contains dopaminergic cell bodies (Dougalis et al., 2012; Lu et al., 2006), stimulation of these cells did not evoke reward-related behavior, suggesting action on mesolimbic dopamine circuitry. Accordingly, we found that nonserotonergic DRN neurons primarily project to the VTA, with comparatively sparse projections to the nucleus accumbens and other forebrain structures. Furthermore, stimulation of the DRN-VTA pathway was sufficient to fully reproduce the rewarding effects of DRN cell body stimulation. Although other projection targets may contribute, these findings suggest that the DRN is capable of driving reinforcement learning primarily through its projection to the VTA.

Because individual stimulation of serotonergic, GABAergic, and dopaminergic DRN cell bodies failed to reinforce behavior, we infer that the rewarding effects seen in our nonserotonergic stimulation experiment were mediated through a distinct population of cell bodies. The largest remaining population of cells, accounting for approximately 10% of DRN neuronal cell bodies, are nonserotonergic neurons expressing vesicular glutamate transporter 3 (Commons, 2009; Hioki et al., 2010). Indeed, we observed that stimulation of the nonserotonergic DRN-VTA pathway produced strong monosynaptic glutamatergic currents and drove spiking activity in VTA dopamine neurons. Comparatively weak currents were observed following stimulation of the serotonergic DRN-VTA pathway. Because direct excitation of VTA dopamine neurons is sufficient to powerfully reinforce instrumental learning (Witten et al., 2011), it seems reasonable to propose that the rewarding effects of nonserotonergic DRN stimulation were driven, at least in part, by a glutamatergic DRN-VTA mechanism. However, the DRN is also noted to contain several peptidergic cell types, including corticotropin-releasing factor and substance P (Valentino and Commons, 2005). Although these peptides are aversive when administered intracerebroventricularly (Cador et al., 1992; Elliott, 1988), we cannot rule out the possibility that these or other DRN cell types contribute to reinforcement learning. With these caveats in mind, the most parsimonious interpretation of the data presented is that this population of nonserotonergic glutamate neurons is highly efficacious in driving reward-related behavior.

It has been shown that the DRN sends projections to mesolimbic circuitry, with the VTA receiving notably stronger innervation than nucleus accumbens (Vertes, 1991). Retrograde studies mapping whole-brain inputs to the VTA have noted the DRN as a major input (Geisler et al., 2007; Phillipson, 1979; Watabe-Uchida et al., 2012). None of these reports, however, examined the serotonergic composition of this projection. Early studies of DRN anatomy led to the view that nearly all of its projection neurons are serotonergic (reviewed in Jacobs and Azmitia, 1992). We have quantitatively compared the composition of the DRN-VTA projections using anterograde, retrograde, and electrophysiological techniques. All three approaches supported the same conclusion: the majority of this pathway consists of nonserotonergic projections. Our study raises important questions and opens new avenues of investigation with respect to the role of this circuit in normal function and disease states.

EXPERIMENTAL PROCEDURES

Animals

Adult (8+ weeks) male and female mice were housed with food and water available ad libitum. Mice were housed on a 12/12 hr light cycle with lights on at 7:00 a.m. All experiments except intravenous self-administration were carried out during the animals' light cycle. Wild-type C57Bl6/J mice were ordered from Jackson Laboratories; transgenic mice were bred in house.

Transgenic expression of cre recombinase was achieved in serotonin neurons using ePet-cre (Scott et al., 2005) or *Sl6a4*$^{cre/+}$ mice, referred to herein as *SERT*^{cre} (Zhuang et al., 2005). ePet-cre mice were considered advantageous for behavioral experiments (Figure 2) because *SERT*^{cre} mice are heterozygous knockouts for the serotonin transporter, a manipulation that alters basal extracellular serotonin levels (Mathews et al., 2004) and could possibly confound behavioral data. Additionally, ePet-cre mice do not demonstrate ectopic cre expression during early development like the *SERT*^{cre} line (Scott et al., 2005; Zhuang et al., 2005) and were therefore used to selectively induce recombination in serotonergic neurons for genetic fluorescent labeling (Figure 5). Dopaminergic and GABAergic neurons were targeted using *TH*$^{iCre/+}$ (Lindeberg et al., 2004) and *Vgat*$^{iCre/+}$ (Vong et al., 2011) mice. Deletion of serotonin synthesis was carried out in *Tph2*$^{lox/lox}$ mice (Wu et al., 2012). Cre-mediated fluorescence was produced using *ROSA26*$^{fsTdTomato/+}$ mice, which carry a floxed stop cassette preceding a coding region for the TdTomato gene (Madisen et al., 2010). All lines were backcrossed onto a C57Bl6/J background. Mice were surgically injected with viral vectors (Table S1) and implanted with fiber optic cables (Britt et al., 2012b), the details of which are described in Supplemental Experimental Procedures. All animal procedures were approved by the National Institute on Drug Abuse's animal care and use committee and carried out in accordance with NIH Guide for the Care and Use of Laboratory Animals.

Optogenetic Real-Time Place Preference

Mice were placed in a 24 × 36 cm plastic chamber with walls of opposite halves identified by horizontal or vertical stripes and a small 0.5 cm barrier on the floor at the division site. A cohort of naive wild-type mice tested in these chambers did not display a preference for either side (51.8% ± 2.6% preference for horizontally striped half; n = 6). Mouse movement was tracked with Ethovision computer software (Noldus), and presence in the randomly assigned laser-paired half resulted in 15 mW 473nm laser stimulation at 20 Hz, with 5 ms pulses. Mice remained in the chamber for 12 min. Electrical self-stimulation studies indicate that the reward substrate within the DRN is increasingly responsive to higher stimulation frequencies, with reward thresholds occurring within the range of 13–40 Hz, depending on electrode placement (Rompré and Miliaressis, 1985). ChR2 protein is capable of inducing action potentials in a variety of neuron types up to 20 Hz, above which spike fidelity is less reliable (Tye and Deisseroth, 2012). Therefore, in the present study, 20 Hz optogenetic stimulation was used to drive action potentials in serotonergic and nonserotonergic DRN cells (Figure S2). Although serotonergic DRN neurons typically have baseline firing rates below 5 Hz, they were recently shown to briefly fire at 20–30 Hz during a reward task (Liu et al., 2014). DRN serotonin neurons are capable of following extrinsic 20 Hz stimulation without entering depolarization block, as assessed with whole-cell patch clamp (Figure S2) and in vivo microdialysis (Sharp et al., 1989).

Optogenetic Nose-Poke Self-Stimulation

One week after the place preference task, mice were allowed to self-stimulate by performing a nose-poke instrumental response (Stuber et al., 2011). On a habituation day, mice were placed in operant chambers (Med Associates) for 45 min with ports closed off to prevent access. Mice were then given access to ports for 3 days of testing. Mice were placed in the chamber for 1 hr. A nose poke into the active port resulted in a 3 s train of laser pulses (30 mW for midline DRN stimulation, 2 × 15 mW for bilateral VTA stimulation) at 20 Hz with 5 ms pulses, accompanied by dimming of the house light and an auditory cue. Nose pokes during the 3 s stimulation period had no consequence and were not counted toward the active nose poke total.

Electrophysiology

Details of procedures and recipes can be found in supplemental methods. Briefly, cells within 250 μm slices of tissue containing DRN or VTA were recorded in whole-cell patch clamp using a potassium gluconate-based internal solution. VTA dopamine cells were identified by morphology, tonic spike rate, and presence of a hyperpolarization-induced I_h current, which can be a reasonable predictor of dopaminergic identity in mice (Margolis et al., 2006; Wanat et al., 2008; Zhang et al., 2010). Cells were optically stimulated with 473 nm laser light, directed at tissue through a fiber optic cable submerged in the bath and aimed at the region of interest.

ACCESSION NUMBERS

The Addgene accession number for the cre-OFF ChR2-eYFP plasmid reported in this paper is 50834.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.08.037.

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