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ROLE OF DORSAL RAPHE NUCLEUS AND VENTRAL TEGMENTAL AREA ON REWARD AND FEEDING BEHAVIORS IN MICE

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INDEX

PREFACE

Before presenting the work performed during my PhD program my main aim is to thankthe people that believed in me since I started my experience in the Neuroscience field. Firstly, wish to thank my family, my mother and my brother because they followed me during this path, too hard to take on in some moments in which I really thought to leave everything for coming back to my old, boring and easier life. I couldn't avoid to say thank you to my teacher Carla Cannizzaro because she is the first person that introduced me the neuroscience world, sharing with me her passion for the research. In the last two years of my PhD program, I performed my research in NIDA laboratories, under the supervision of the Dr. Antonello Bonci. Even if my previous work dealt with the role of stress reaction in alcohol addiction, when I moved in NIDA I spent about four months in learning optogenetic, anatomical and immunohistochemistry tools that, coupled with my already assessed behavioral knowledge, could permit me to perform an independent experimental research. In order to do that I supported a Senior Post Doc, Ross McDevitt, who, teaching me all these techniques, involved me in his project. This project coped with the characterization of Dorsal Raphe cell types responsible for reward processes. Even if we already published a paper on September 25, 2014" *Serotonergic versus non-serotonergic dorsal raphe projection neurons: differential participation in reward circuitry"* we are still working on it. I was also involved in another project on Parkinson's disease that copes with VGLUT2 role in the development of this pathology. In the last two months, looking forward my new path as post-doc in Bonci's Lab, I launched a new project on VTA role on feeding and rewarding behaviors.

THE HETEROGENEITY OF VENTRAL TEGMENTAL AREA NEURONS: PROJECTION FUNCTIONS IN A MOOD-RELATED CONTEXT

Dopaminergic neurons of the ventral tegmental area (VTA) play a central role in reward learning (Wise, 2004). Midbrain dopamine neurons located in the VTA play also a key role in several disorders including schizophrenia, drug addiction and mood disorders such as depression (Marinelli and White, 2000, Krishnan et al., 2007, Cao et al., 2010, Valenti et al., 2011, Chaudhury et al., 2013, Friedman et al., 2014). Even if classically the VTA was thought to consist of dopamine (DA) neurons (Yim and Mogenson, 1980, Grace and Onn, 1989), studies have shown that while the majority of cells in the VTA are dopaminergic (~70%), there are also small percentages of both GABA (~30%) and glutamatergic (~2–3%) neurons in this region (Yamaguchi et al., 2007, Nair-Roberts et al., 2008). Additionally, certain subpopulations of neurons have been shown to co-release two transmitters (Sulzer et al., 1998,Stuber et al., 2010, Tritsch et al., 2012). The advent of optogenetics has allowed for the dissection of neural circuits in both a cell-type and projection-specific manner (Lobo et al., 2010, Lammel et al., 2011, Chaudhury et al., 2013, Tye et al., 2013). Further studies in non-human primates suggested that phasic activation of DA neurons was found to serve more in denoting the occurrence in reward related-stimuli than actually mediating the hedonic effects of reward (Schultz, 1998b). More specifically, recordings in non-human primates performing an operant task demonstrated that DA neurons could be activated by conditioned, reward predicting stimuli (Schultz, 1998a). Occurrence of reward in the absence of a conditioned stimulus (CS) induces phasic activation of DA neurons. Further, it was seen that when a CS predicted the occurrence of reward phasic firing was elicited immediately following the CS prior to the onset of the reward. Finally, phasic activation of DA neurons occurs following a CS, however, in the failure of a reward, DA neurons are depressed at texact

expected time of the reward. Initially, many *in vitro* slice recording experiments, performed both in mice and rats, suggested that VTA DA neurons were a homogenous population (Ungless et al., 2001, Argilli et al., 2008, Chen et al., 2008,Stuber et al., 2008). Early *in vitro* electrophysiological studies, performed in Sprague-Dawley rats, classified DA neurons of the VTA as the primary population of neurons (Grace and Onn, 1989, Schultz, 1998a). However, later studies note that other populations of cells also exist within the VTA, GABAergic, as well as glutamatergic neurons (Nair-Roberts et al., 2008). The neurochemical identities of all of these neurons still remain uncharacterized. GABAergic neurons within the VTA of Sprague-Dawley rats, exhibit a large amount of heterogeneity with a large range of action potential durations and firing rates. (Margolis et al., 2012). They constitute approximately 15–20% of the entire neuronal population (Margolis et al., 2012) and synapse onto both DA and non-DA VTA neurons (Bayer and Pickel, 1991,Omelchenko and Sesack, 2009). Similar to DA VTA neurons, GABAergic VTA neurons may also play diverse roles in behavioral responses. Some neurons in the VTA of both Sprague-Dawley rats and VGLUT1 kockout mice, express vesicular glutamate transporter 2 (VGLUT2), a marker of glutamatergic neurons, and are 2–3% of the total neuronal population, being located primarily in the rostro-medial portion of the VTA (Fremeau et al., 2004, Nair-Roberts et al., 2008). All cells contain glutamate for their role in protein synthesis, however, for exocytotic release, the VGLUTs are required (Reimer and Edwards, 2004, Takamori, 2006). The VTA projects to many regions including the NAc, mPFC, and the amygdala (Wise and Bozarth, 1985).

While it has been established that the VTA-to-NAc circuit is a crucial element in the pathogenesis of stress-related disorders, other areas, such as the mPFC and amygdala are also known to affect these behaviors. Notably, the mPFC both receives innervations from the VTA and sends projections to the VTA and NAc, forming a regulatory feedback mechanism (Nestler and Carlezon, 2006).

MORPHOLOGICAL AND NEUROCHEMICAL CHARACTERIZATION OF DORSAL RAPHE NUCLEUS

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 primary synaptic inputs within and to the raphe are glutamatergic and GABAergic. The DRN is divided into three subfields, i.e., ventromedial (vmDR), lateral wings (lwDR) and dorsomedial (dmDR). Although 5-HT neurons have similar physiological properties, important differences exist between subfields. Non-5-HT neurons are indistinguishable from 5-HT neurons. Glutamate neurons, as defined by vGlut3 anti-bodies, are intermixed and co-localized with 5-HT neurons within all raphe subfields. Finally, the dendritic arbor of the 5-HT neurons is distinct between subfields. Previous studies regard 5-HT neurons as a homogenous population. Understanding the interaction of the cell properties of the neurons in concert with their morphology, local distribution of GABA and glutamate neurons and their synaptic input, reveals a more complicated and heterogeneous raphe. These findings leave an open question: how specific subfields can modulate behavior?

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; Rompre 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 (Gotestam 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 (Rompre and Miliaressis, 1987). These studies suggest that the DRN contains a population of non-serotonergic 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.

DOPAMINE ROLE IN REWARD FUNTION

Several fine points continue to be discussed in the literature. Is dopamine absolutely necessary for reward? Is dopamine more important for the expectancy of reward or for the impact of reward after it is delivered? Is the dopamine in nucleus accumbens more important for reward than the dopamine in other brain regions? Some of these recent issues are best resolved by consideration of the early literature. Studies involving pharmacological blockade of dopamine receptors have suggested a necessary role for dopamine in the reward function (Wise & Rompre´ 1989; Wise 2004). Dopamine is also important for the motivation to seek anticipated food or rather for the reinforcing effects of food once it has been earned and received (Berridge & Robinson 1998; Salamone & Correa 2002). Food rewards have both kinds of effect (Wise 1989, 2004). Dopamine antagonists impair learning (Wise & Schwartz 1981) and, by extinguishing them, previously learned (Wise et al. 1978a,b) instrumental responding for food. Several lines of study confirm that they do so by blunting reward function itself (Wise 1982, 2004a; Beninger 1983; Smith 1995) rather than, as has been suggested (Mason et al. 1980; Koob 1982; Tombaugh et al. 1982; Salamone 1986), by simply impairing performance capacity. The earliest evidence that dopamine plays an important role in motivational function was that brain stimulation and psychomotor stimulants were simply ineffective as reinforcers in animals treated with response-sparing doses of dopamine antagonists. Intravenous amphetamine and cocaine failed to maintain responding when tested under the influence of dopamine antagonists, despite evidence of adequate response capacity. Indeed, in this case animals respond at higher than normal rates before ceasing to respond following pretreatment with dopamine antagonists (Yokel & Wise 1975, 1976; de Wit & Wise 1977; Ettenberg et al. 1982). In the case of brain stimulation reward, responding is generally lower when animals are treated with dopamine antagonists; however, several conditions

reveal that the low response rates are due to ineffectiveness of the reinforcer and not incapacitation of the animal. First, responding decreases progressively, both within sessions and across sessions, in animals pretreated with dopamine antagonists (Fouriezos & Wise 1976; Fouriezos et al. 1978; Franklin 1978; Franklin & McCoy 1979).

The concept of reinforcement is, at its core, a concept of how stimulus (Pavlov 1928) and response (Thorndike 1933) associations are formed and how they serve as the basis of habit acquisition (Skinner 1938). Food does not serve as a normal reinforcer in animals pretreated with dopamine antagonists; such treatment causes, for example, a dose-dependent decrease in how quickly animals learn to lever-press for food (Wise & Schwartz 1981). Under pretreatment with low doses of the dopamine antagonist animals eventually reach the normal performance asymptote; however, they require more trials to do so. With higher pretreatment doses learning is slower and may not reach the same performance asymptote. With yet higher doses there is no evidence of learning.

While the concept of reinforcement is most frequently used to explain response learning (Thorndike 1933; Skinner 1935; Hull 1937), it was first used in relation to stimulus learning (Pavlov 1928). Stimulus learning is now known to contribute significantly to response learning (Rescorla & Solomon 1967; Bindra 1972) and dopamine is thought to play a role in both (Wise 1989). Most studies of the reinforcing efficacy of food reward deal with the ability of the reward to maintain rather than to establish instrumental behaviour; without reinforcement both stimulus associ ations (Pavlov 1928) and response associations (Skinner 1933) extinguish. When well-trained animals are tested under the influence of dopamine antagonists, food loses the ability to maintain normal responding. Whereas normal responding is initiated, responding slows progressively both within sessions and across sessions (Wise et al. 1978b; Dickinson et al. 2000). Similar progressive loss, both within and across trials, can be seen in the ability of food to

maintain free feeding (Wise & Raptis 1986). The response slowing resembles what is seen in extinction conditions (when the normal reward is withheld), and is generally interpreted as a reflection of the impoverishment or 'devaluation' of food reward in the dopamine-impaired animal (Wise et al. 1978a,b; Xenakis & Sclafani 1981, 1982; Geary & Smith 1985). Few alternative hypotheses have been offered to explain the progressive response deficits seen when animals are tested under conditions of dopamine blockade. There is the suggestion that the progressive deficit might reflect a susceptibility to fatigue (or some other progressive within-trial performance impair- ment) caused by dopamine antagonists. This hypothesis can be ruled out from a variety of findings. First, the deficits are not only progressive within-trials; responding decreases progressively across repeated tests that are spaced days apart, with normal levels of responding between the days when the dopamine antagonist is given (Fouriezos et al. 1978; Wise et al. 1978b; Wise & Raptis 1986). Second, animals trained under intermittent dopamine blockade, like animals under intermittent reinforcement, respond more, not less, when tested for habit strength during extinction trials (Ettenberg & Camp 1986).

INVOLVEMENT OF VTA DOPAMINE NEURONS IN FEEDING AND REWARDING BEHAVIOR

The lateral hypothalamus (LH) has long been implicated by lesion and electrical stimulation studies in feeding and reward (Anand and Brobeck, 1951; Olds, 1956; Margules and Olds, 1962; Hoebel, 1969). While the cells of the LH have been traditionally assumed to integrate feeding-related signals (Saper et al., 2002; Berthoud and Munzberg, 2011), it has not been clear whether LH cells of origin or medial forebrain bundle fibers of passage were responsible for the disruptions of feeding and reward caused by lateral hypothalamic lesions (Ungerstedt, 1971; Stricker and Zigmond, 1974) or the induction of feeding and reward by electrical stimulation (Ranck, 1975; Bielajew et al., 2000). Recent optogenetic studies have now confirmed that activation of GABAergic projections to the VTA from LH cells of origin is sufficient to induce feeding (Jennings et al., 2015; Nieh et al., 2015), food-seeking (Jennings et al., 2015; Nieh et al., 2015) and reward (Jennings et al., 2015). However, electrical stimulation studies indicate that stimulation in the ventral tegmental area as well as in the lateral hypothalamus can induce both feeding and reward. Moreover, paired-pulse stimulation studies confirm that the reward and feeding involve fibers that connect the two regions and have similar refractory periods, conduction velocities, and anatomical alignment within the medial forebrain bundle (Shizgal et al., 1980; Bielajew and Shizgal, 1982; Gratton and Wise, 1988a, b). These common characteristics could suggest (but do not confirm) that the same fiber population is involved in both effects. This suggestion fits with the optogenetic finding that both effects can be induced by activation of a GABAergic projection to the VTA (Jennings et al., 2015; Nieh et al., 2015). It is well established that DA has a crucial role in both compulsive drug and food seeking behaviors. Motivation and Reinforcing aspects are key components of

these behaviors but how are they dependent on modulation of dopaminergic system is still an open question.CertainlyLateral Hypothalamus (LH) can play a crucial role in the modulation of both compulsive drug and food seeking behaviors. Indeed, studies on experimental rodent models and humans have shown that stimulation of LH is rewarding (Olds J., 1958; Heath RG, 1972; Hess WR, 1957) since it is able to induce compulsive-like behavior.The absolute refractory periods for the fibers mediating LH brain stimulation reward and feeding range from 0.4 to about 1.2 msec (Yeomans JS, 1978-Wise R, 1988). Animals don't show behavioral improvement when C-T pulse intervals are increased between 0.6 - 0.7 msec (Wise R,1988). It suggests that there are two populations of fibers:

- ✓ VERY FAST (refractory periods between 0.4 - 0.6 msec)
- ✓ SLOWER (RP ranging between 0.7 and 1.2 msec).

AIMS

The goal of my PhD program was firstly to assess the characterization of Dorsal Raphe cell types responsible for reward processes and even if we already published a paper on September 25, 2014 *" Serotonergic versus non-serotonergic dorsal raphe projection neurons: differential participation in reward circuitry"* we are still working on it. Particularly 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 non-serotonergic neurons, which make up the majority of the DRN-VTA pathway and produce strong glutamatergic excitation of VTA dopamine neurons. Nevertheless this already assessed information was for me and my work team a start point for the development of a new project that aims to clarify glutamate population role on reward circuitry and reinforcement learning using optogenetic approach in two cre-expressing transgenic mouse lines:SERT cre and Vglut 3 cre (project in collaboration with Ross McDevitt). Given that the DNR-VTA projection looks to be responsible of reward processes, in my mind are risen new open questions:

- ✓ Is the LH-VTA gabaergic stimulation involved in compulsive or rewarding aspect of feeding?
- ✓ Does the VTA act as a role player in feeding?
- ✓ Can the compulsive and rewarding feeding be regulated by specific DAergic neuronal populations receiving distinct LH projections or by a cluster of DAergic neurons acting in a frequency dependent manner?

EXPERIMENTAL PROCEDURES OF DNR PROJECT

Animals

Adult (8+ weeks) male and female mice were housed with food and water available *ad libitum*. Mice were housed on a 12/12 hour light cycle with lights on at 7:00 AM. All experiments except intravenous self-administration were carried out during the animals' light cycle. Wild-type C57Bl6/J mice were ordered from Jackson Laboratories (Bar Harbor, ME); 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 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. 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 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 National Institutes of Health 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 naïve 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; Wageningen, Netherlands) 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 minutes. 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 (Rompre 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 non-serotonergic DRN cells. 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 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; St Albans, VT) for 45 minutes 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 hour. A nose poke into the active port resulted in a 3- second train of laser pulses (30mW for midline DRN stimulation, 2 × 15mW 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 second stimulation period had no consequence and were not counted towards the active nose poke total.

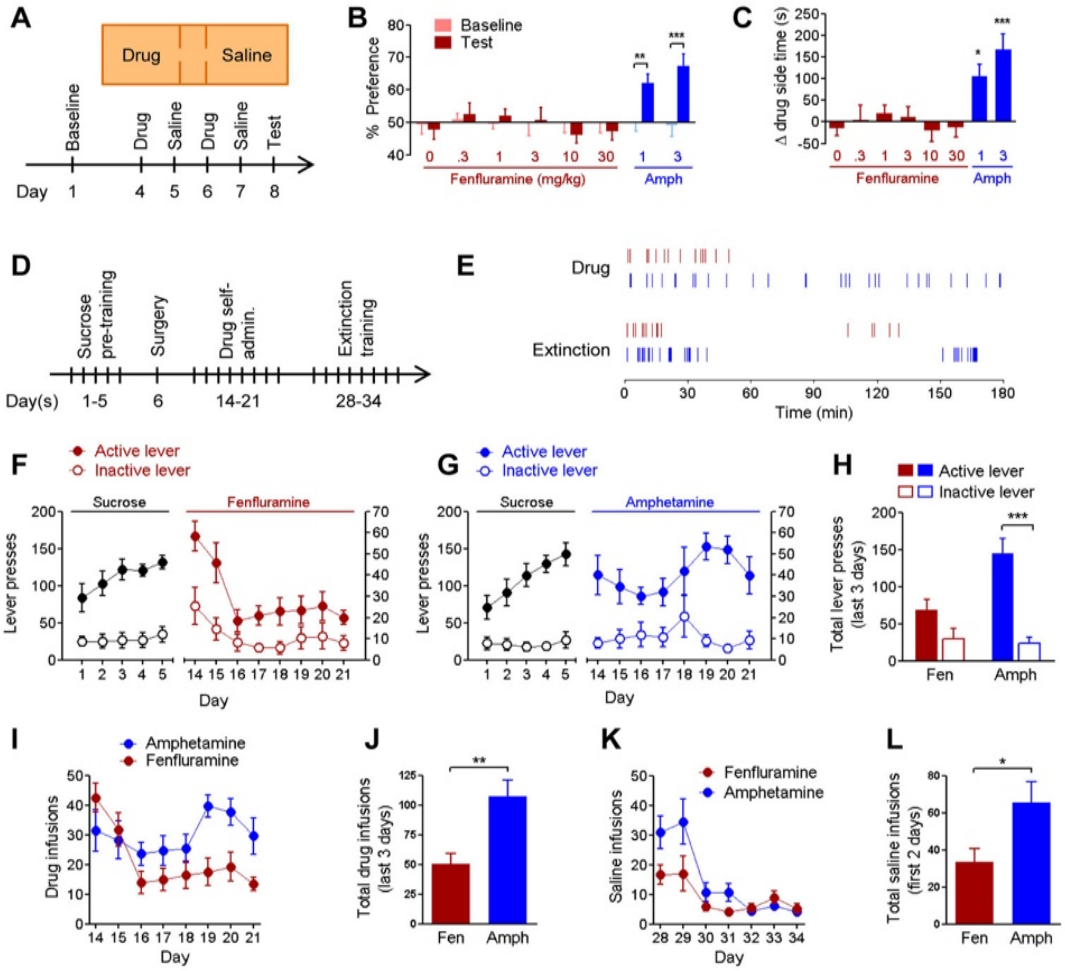
Electrophysiology

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.

RESULTS

Pharmacological stimulation of dopamine but not serotonin release reinforces behavior

A, Mice (*n*=9–18/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, Percent of time spent in drug-paired chamber on baseline and test days. Repeated measures ANOVA (drug x 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 vs. saline. D, Self-administration experiment in a separate cohort of mice. After pre-training 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/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,G, daily lever-press counts during sucrose pre-training (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 x 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 last three days drug access, ** p<0.01. K, Daily counts of infusions of saline during extinction training. L, total number of saline infusions during the first two days of extinction, * p<0.05. Group data are presented here and in subsequent figures as mean ± SEM.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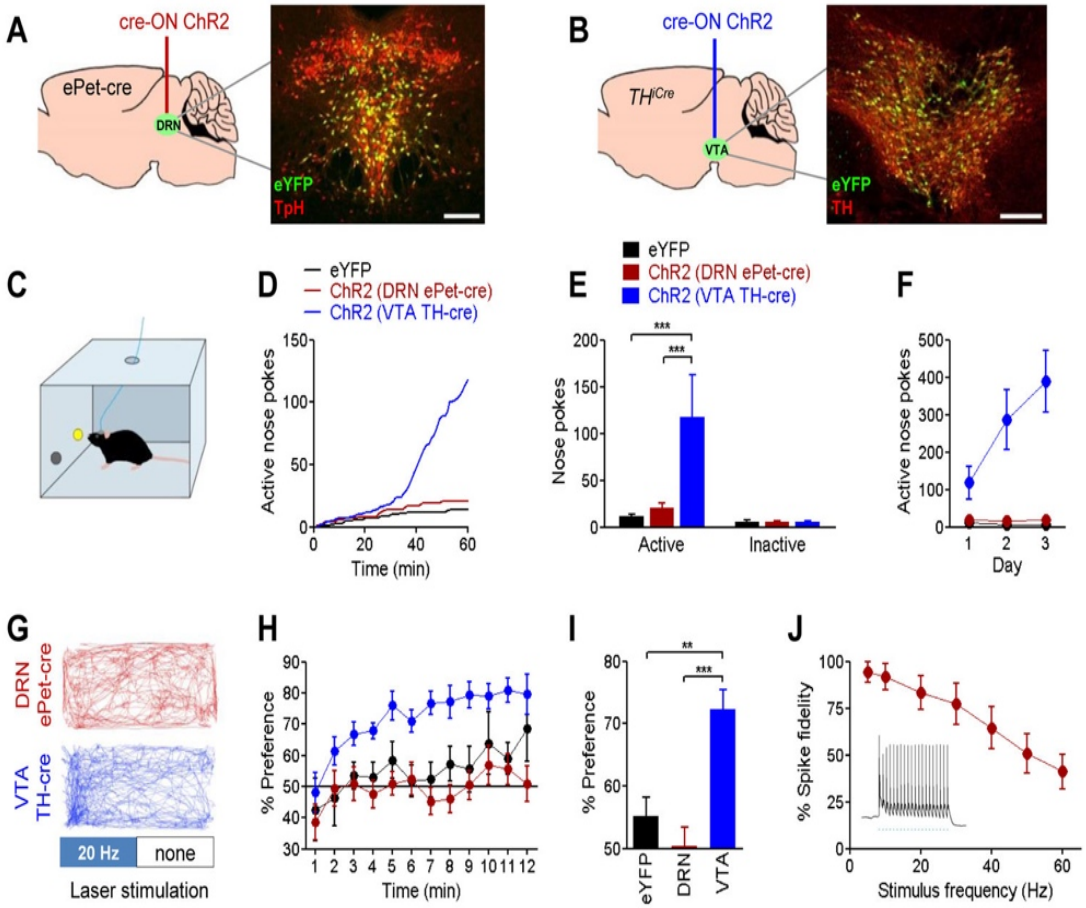
Optogenetic stimulation of VTA dopamine but not DRN serotonin cell bodies reinforces behavior

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. 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 = 200 μm. C, Mice were trained to nose-poke into an active port to receive 3- second trains of 20 Hz laser stimulation; nose-pokes into an inactive port were not reinforced. D,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 x port) interaction $F_{(2,86)}$=8.317, p<0.001; *** p<0.001 post-hoc. F, Active nose poke responding on three 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 percent of time spent in the laser-paired half of the chamber. I, Overall preference for laser-paired side during 12- minute 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.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Active nose pokes
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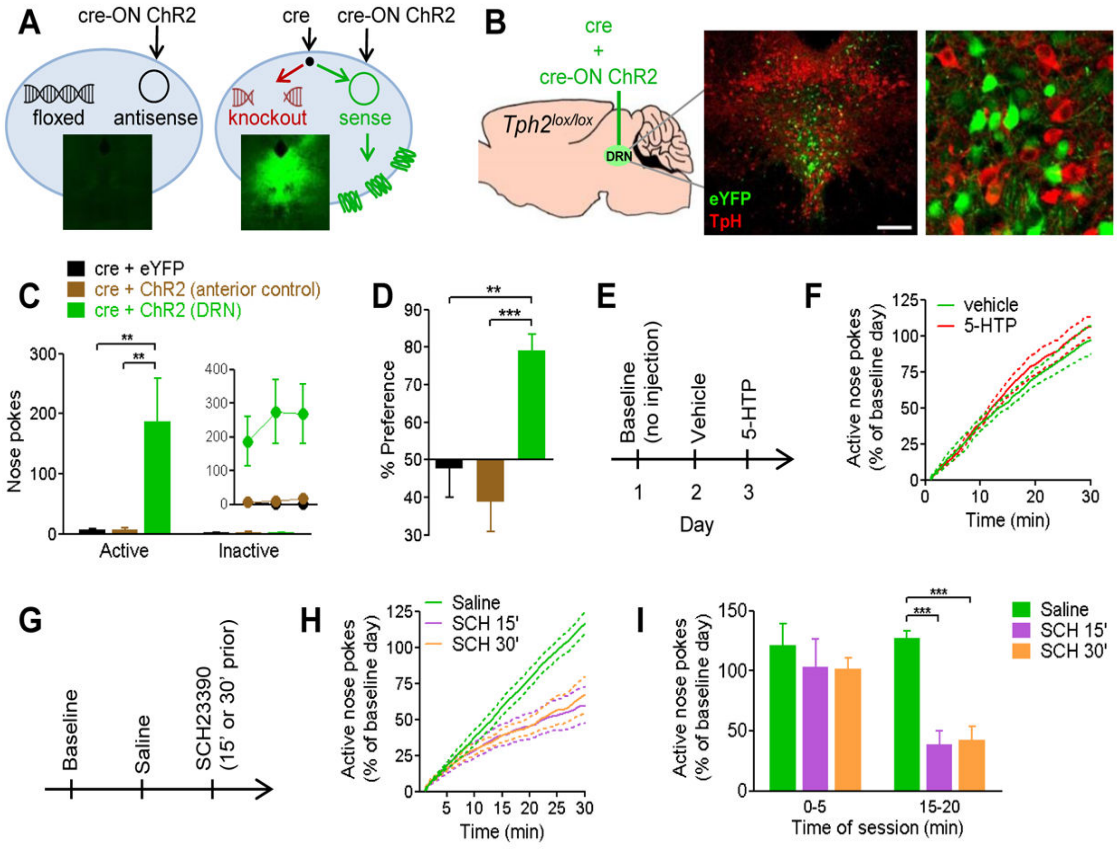
DRN ePet-cre
VTA TH-cre
20 Hz
none
Laser stimulation
H
% Preference
Time (min)
I
% Preference
eYFP
DRN
VTA
J
% Spike fidelity
Stimulus frequency (Hz)

Optogenetic stimulation of DRN cell bodies reinforces behavior in a dopamine-dependent, serotonin-independent manner

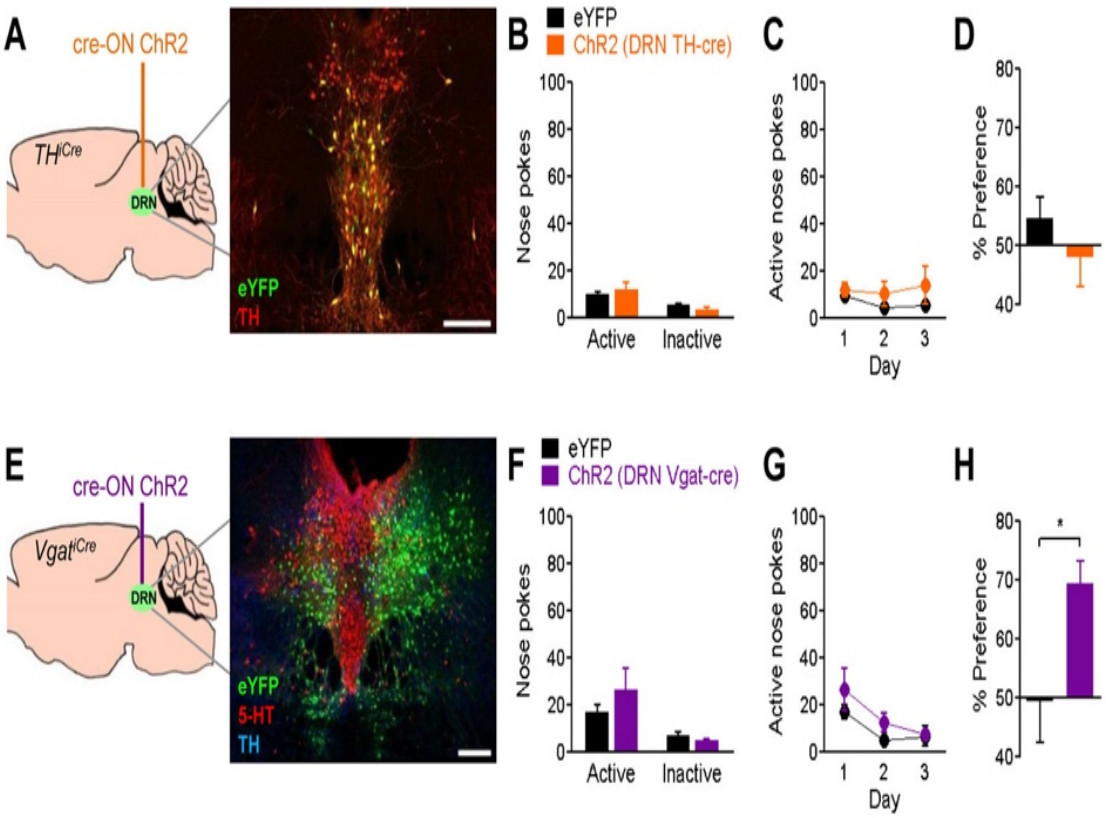
A, Left panel, schematic view of a cell transduced with cre-induced ("cre-ON") viral vector. In the absence of cre recombinase, viral plasmid DNA remains in antisense orientation and does not express functional protein. Inset, lack of eYFP signal in mouse injected with cre- ON ChR2-eYFP. Right panel, co-injection 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 co-injected with cre-expressing and cre-ON viral vectors. B, $Tph2^{lox/lox}$ mice were co-injected with viral vectors expressing cre and cre-ON ChR2-eYFP or eYFP into DRN. Insets, whole DRN (scale bar = 200 μm) and detail of non-overlapping 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 co-injected with cre and cre-ON ChR2 0.7mm anterior to the DRN. C, Nose pokes during first day of self-stimulation testing for non-serotonergic DRN stimulation (n=10), anterior controls (n=8), and eYFP controls (n=7). Two-way ANOVA (group x 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, Percent 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- hocs. E, Non-serotonin DRN stimulated mice (n=6) were tested for nose-poke optical self-stimulation 30 minutes 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-minute baseline session on day 1. G, Non-serotonin 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 minutes before testing. H, Cumulative-activity graph of active nose pokes in 30-minute sessions following injection of saline or SCH. I, Active nose pokes during 5-minute bins at the beginning or in the middle of test depicted in panel H. Individual data points were normalized to percent of responses during baseline day. Repeated-measures ANOVA (drug x epoch) interaction $F_{(2,15)}$=4.560, p<0.05; *** p<0.001 Dunnett's post-hoc vs saline. See also Figures S3 and S4.



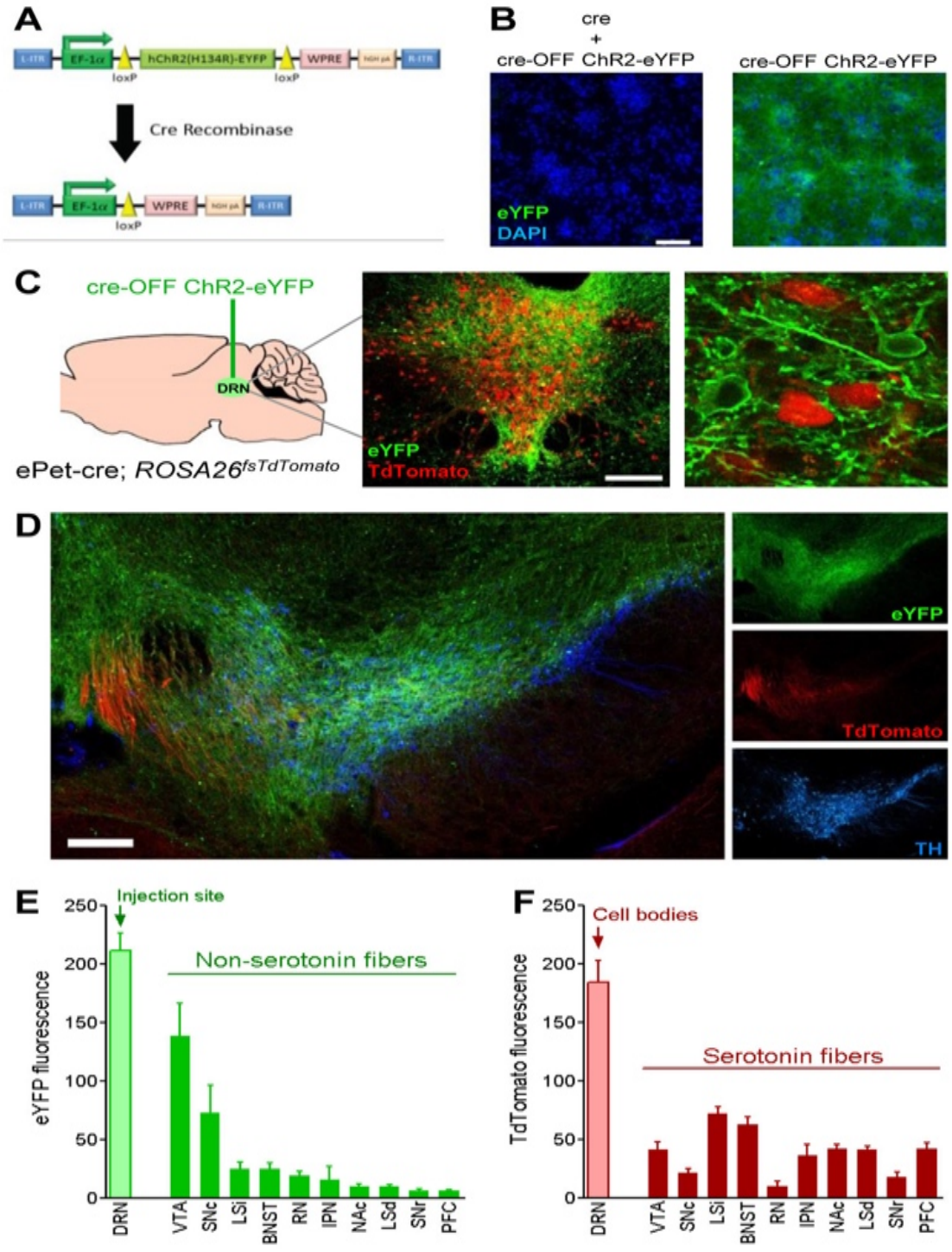
Optogenetic stimulation of dopaminergic or GABAergic DRN cell bodies fails to reinforce nose-poke self-stimulation

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. 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 three consecutive days of testing. D, Percent 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. Inset shows eYFP (green) cell bodies in the lateral DRN, which do not co-label for serotonin (5- HT, red) or tyrosine hydroxylase (TH, blue). Laser stimulation did not reinforce nose poke self-stimulation (F,G) but did induce a real-time place preference (H), $p<0.05$. Scale bars = 200 μm.


A
cre-ON ChR2
THiCre
DRN
eYFP
TH
B
eYFP
ChR2 (DRN TH-cre)
Nose pokes
Active
Inactive
C
Active nose pokes
Day
D
% Preference
E
cre-ON ChR2
VgatiCre
DRN
eYFP
5-HT
TH
F
eYFP
ChR2 (DRN Vgat-cre)
Nose pokes
Active
Inactive
G
Active nose pokes
Day
H
% Preference
*

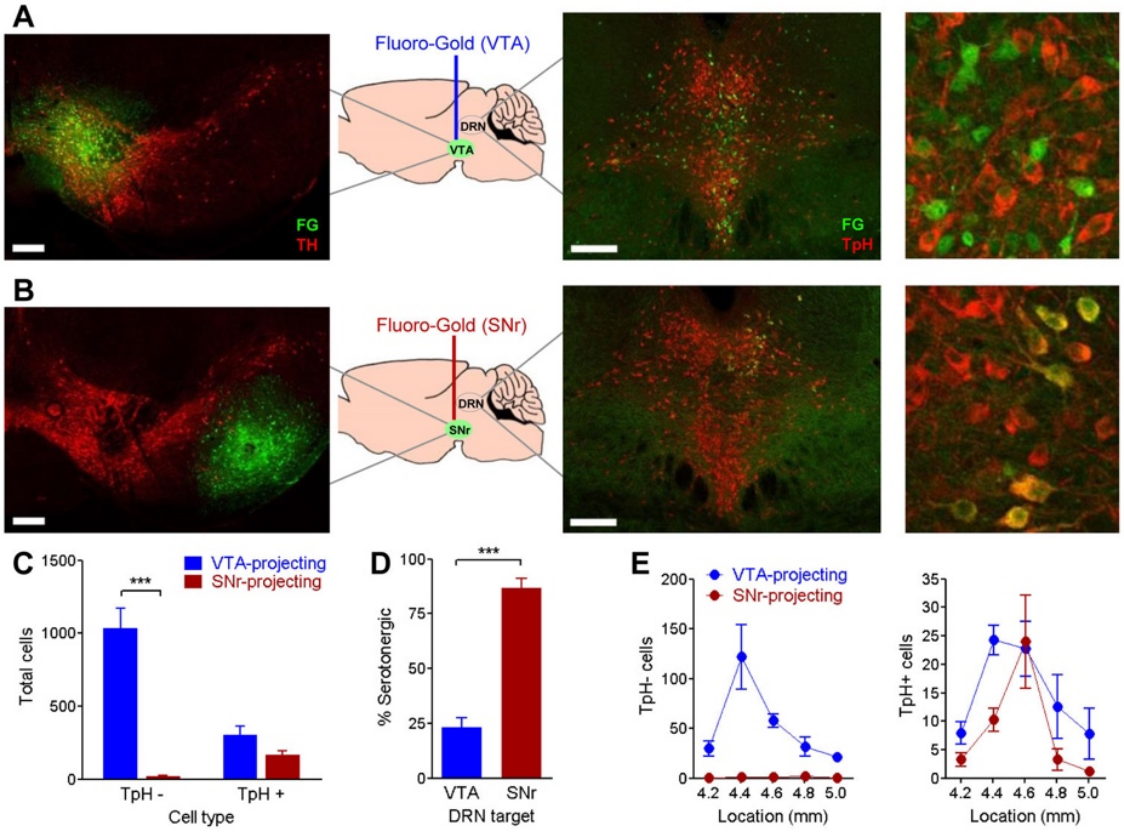
Unlike serotonergic neurons, non-serotonergic DRN neurons preferentially project to the VTA

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 co-transduced with a vector expressing cre recombinase (right); DAPI nuclear staining (blue) is unaffected. Scale bar = 100 μm. C, Transgenic mice co-expressing cre and TdTomato inserotonergic neurons (ePet-cre; $ROSA26^{fsTdTomato}$, n=4) were injected with cre-OFF ChR2-eYFP into the DRN. 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 non-serotonergic (green) axons are visible in the VTA, identifiable by tyrosine hydroxylase immunoreactivity (TH, blue). Scale bar = 200 μm. E,F Quantitation of (E) eYFP and (F) TdTomato fluorescence intensity in brain regions with conspicuous eYFP expression. Abbreviations: ventral tegmental area (VTA), substantia nigra pars compacta (SNc), intermediate portion of the lateral septum (LSi), bed nucleus of the stria terminalis (BNST), red nucleus (RN), interpeduncular nucleus (IPN), nucleus accumbens (NAc), dorsal portion of the lateral septum (LSd), substantia nigra reticulata (SNr), prefrontal cortex (PFC).


A
L-ITR
EF-1α
hChR2(H134R)-EYFP
WPRE
R-ITR
loxP
loxP
Cre Recombinase
B
cre
+
cre-OFF ChR2-eYFP
cre-OFF ChR2-eYFP
eYFP
DAPI
C
cre-OFF ChR2-eYFP
DRN
ePet-cre; ROSA26fsTdTomato
eYFP
TdTomato
D
eYFP
TdTomato
TH
E
Injection site
Non-serotonin fibers
eYFP fluorescence
250
200
150
100
50
0
DRN
VTA
SNc
LSi
BNST
RN
IPN
NAc
LSd
SNr
PFC
F
Cell bodies
Serotonin fibers
TdTomato fluorescence
250
200
150
100
50
0
DRN
VTA
SNc
LSi
BNST
RN
IPN
NAc
LSd
SNr
PFC

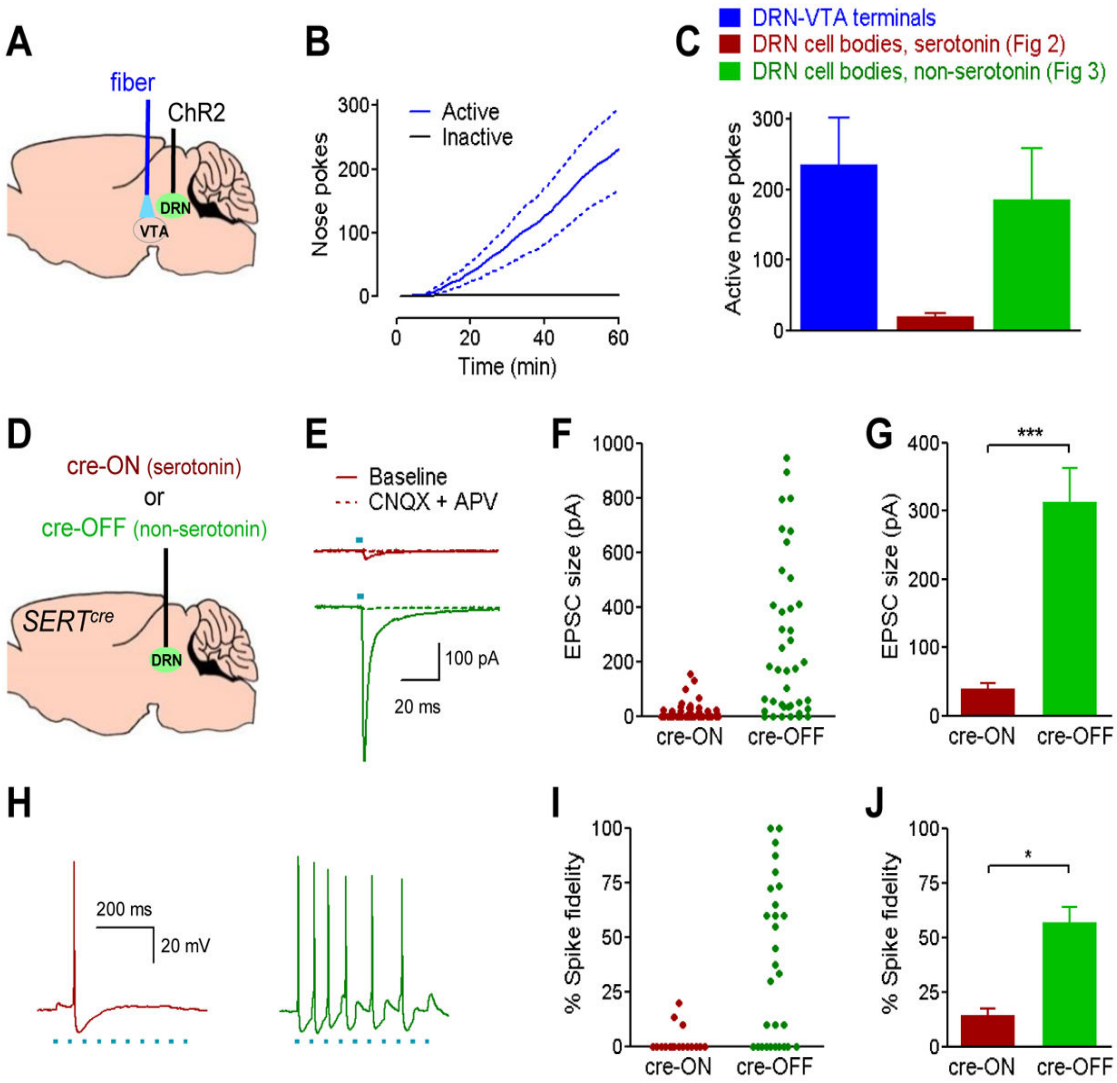
The majority of DRN cell bodies that project to VTA are non-serotonergic

The retrograde tracer Fluoro-Gold was iontophoretically infused into the (A) VTA or (B) substantia nigra reticulata (*n*=4/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 = 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 x 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. X-axis indicates location of DRN tissue, in millimeters posterior to bregma.


A
Fluoro-Gold (VTA)
DRN
VTA
FG
TH
FG
TpH
B
Fluoro-Gold (SNr)
DRN
SNr
C
VTA-projecting
SNr-projecting
***
Total cells
1500
1000
500
0
TpH -
TpH +
Cell type
D
***
% Serotonergic
100
75
50
25
0
VTA
SNr
DRN target
E
VTA-projecting
SNr-projecting
TpH- cells
200
150
100
50
0
4.2 4.4 4.6 4.8 5.0
Location (mm)
TpH+ cells
35
30
25
20
15
10
5
0
4.2 4.4 4.6 4.8 5.0
Location (mm)

DRN-VTA projections reinforce behavior and provide synaptic glutamatergic excitation of VTA dopamine neurons primarily via non-serotonergic projections

A, Mice (*n*=11) were injected with non-specific 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 is reconstituted from previous experiments stimulating serotonergic and non-serotonergic DRN cell bodies. D, Serotonergic and non- serotonergic 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 excitatory post-synaptic current (EPSC) resulting from stimulation of terminals of the serotonergic (top trace) or non-serotonergic (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 non-serotonin (right) pathways. I, Individual spike fidelity measurements; represented as percent of laser pulses during a 0.5 second, 20 Hz train that resulted in action potentials. J, Average spike fidelity in cells that responded to light with at least one action potential, $p<0.05$.


A
fiber
ChR2
DRN
VTA
B
Active
Inactive
Nose pokes
300
200
100
0
0
20
40
60
Time (min)
C
DRN-VTA terminals
DRN cell bodies, serotonin (Fig 2)
DRN cell bodies, non-serotonin (Fig 3)
Active nose pokes
300
200
100
0
D
cre-ON (serotonin)
or
cre-OFF (non-serotonin)
SERTcre
DRN
E
Baseline
CNQX + APV
100 pA
20 ms
F
EPSC size (pA)
1000
800
600
400
200
0
cre-ON
cre-OFF
G
***
EPSC size (pA)
400
300
200
100
0
cre-ON
cre-OFF
H
200 ms
20 mV
I
% Spike fidelity
100
75
50
25
0
cre-ON
cre-OFF
J
*
% Spike fidelity
100
75
50
25
0
cre-ON
cre-OFF

DISCUSSION

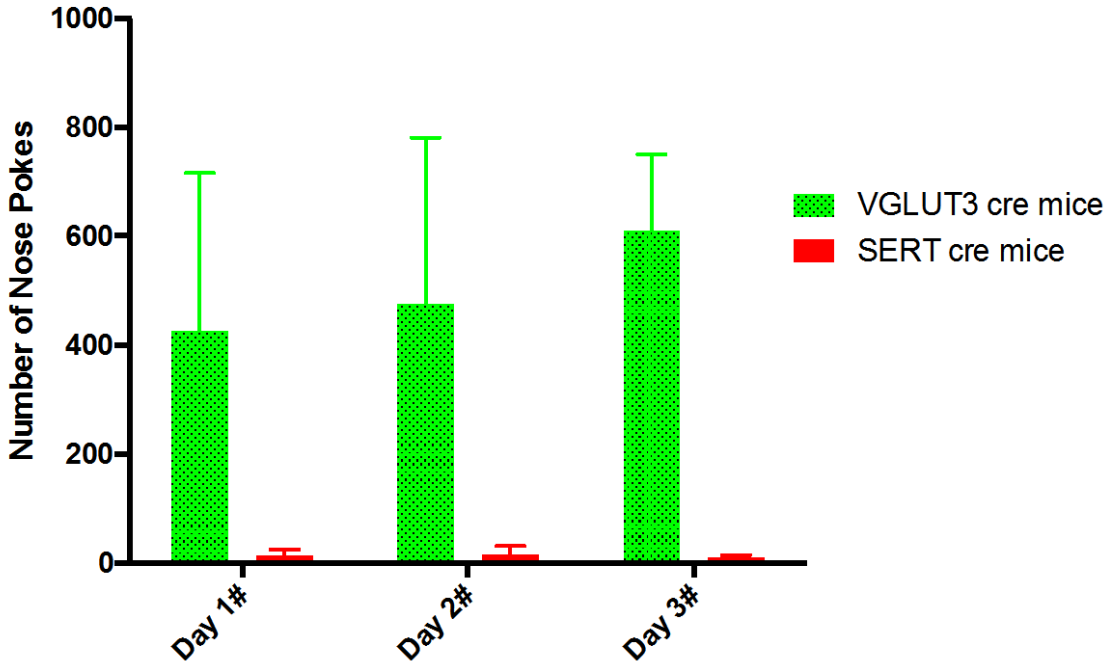
In the present study we found that stimulation of DRN cell bodies was capable of strongly reinforcing instrumental behavior, with a level of vigor comparable to direct stimulation of VTA dopamine neurons. Although serotonergic neurons are the largest population of projection neurons in the DRN, selective stimulation of these cells did not reinforce behavior. Rather, self-stimulation was preferentially elicited by targeting non-serotonergic 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 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 non-serotonergic glutamate neurons. Accordingly, *in vitro* stimulation of the non-serotonergic 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 magnitude of reward received (Inaba et al., 2013; Liu et al., 2014; Nakamura et al., 2008). We employed a variety of optogenetic and pharmacological methodologies 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 (Gotestam and Andersson, 1975; Griffiths et al., 1976), which we now extend to mice. In both fenfluramine and optogenetic place preference experiments, maximal trends towards rewarding effects were seen at low doses/frequencies, suggesting that serotonin may exert pro-reward 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 methodological differences between this study and our present work that likely afforded Liu *et al* greater sensitivity in detecting behavioral effects of laser stimulation which, in our experiments, produced non-significant 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 non-serotonergic neurons. Mice in our study vigorously nose-poked for optogenetic stimulation of non-serotonergic DRN neurons. This finding is consistent with reports that rats will respond for electrical DRN stimulation in a serotonin-independent manner (Margules, 1969; Rompre 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 which 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 (Rompre 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 non-serotonergic 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 Rompre, 1996), suggestive of the dense network of non-serotonergic fibers that we observed in the VTA. The rewarding properties of DRN stimulation were dependent upon 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 non-serotonergic 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 non- serotonergic 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 non-serotonergic neurons expressing vesicular glutamate transporter 3 (Commons, 2009; Hioki et al., 2010). Indeed, we observed that stimulation of the non-serotonergic 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 non-serotonergic 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 non-serotonergic 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 non-serotonergic projections. Our study raises important questions and open new avenues of investigation with respect to the role of this circuit in normal function and disease states. However we have preliminary data showing that VGLUT3 expressing neuron stimulation is responsible for rewarding effect.



Since October 2015, I started a new indipendent project that aims to clarify VTA DA neuron involvement on feeding and reward. Even if I'm at very beginning of this way, I already got some preliminary data that I have the pleasure to share with you. My experimental design is very simple, I just used DAT-cre mice injected with DIO ChR2 and fibers in VTA.

These mice underwent two experimental tests.

1. Self-photostimulation:

Analysis of nose-pokes self administration under increasing range of photostimulation frequencies.

2.Food seeking behavior:

Analysis of latency to approach the food and the time spent in the food zone throughout

the spatial location heat map with different frequency of photostimulation (5-10-20 and 40 Hz).

MATERIALS AND METHODS OF VTA PROJECT ON FEEDING AND REWARD

Animals

A total of 4 male DAT-IRES Cre mice were used in this study. Mice (weighing 20-30 g at the start of experiments) were housed in an animal vivarium maintained on a direct 12-h light-dark cycle (lights on at 7:00 am) and at a constant temperature of 23°C. They were kept undisturbed at least one week before the start of any experimental procedure and were handled and weighed daily in order to minimize handling stress during experiments. Food and water were provided ad libitum except during experimental sessions. Animal care and use were in accordance with institutional and international standards (National Research Council, 2011) and were approved by the National Institute on Drug Abuse Animal Care and Use Committee. All experiments were performed during the light phase of the diurnal cycle.

Surgical and intracerebral infusion procedures

Each mouse was anesthetized with 1-5% isofluorane, placed in a stereotaxic frame and the skull was exposed and leveled. Cre-inducible adeno-associated virus (AAV, serotype 1) coding for the light-sensitive protein channelrhodopsin-2 (ChR2) and enhanced yellow fluorescent protein (eYFP) or for eYFP alone, under control of the EF1α promoter were employed (NIDA IRP OTTC, Baltimore, MD). AAV1-EF1α- DIO-hChR2(H134R) -eYFP (ChR2 mice) was bilaterally injected into the VTA. The stereotaxic coordinates were: AP: -3,6 ML: ±1.6, DV: -4,9. Injections of 500 nl per side were made with a flow rate of 100 nl/min. Infusions were done using an UltraMicroPump with Micro 4 controller, 10 µl

Nanofil syringes and 35 G needles (WPI Inc., Sarasota, FL). The needle was left in place for additional 5 min to prevent reflux. Simultaneusly viral infusions, each mouse received twointracranial optic fiber (200 μm diameter, BFL37-200, Thorlabs, Newton, NJ) for VTA directed just dorsal to VTA. One or two stainless-steel screws and dental acrylic cement were employed to anchor optic the fiber to the skull. Animals were given the analgesic meloxicam (0.2 mg/kg) to prevent post-surgical pain or discomfort and were allowed at least 30 days of recovery before the beginning of any experimental manipulation. Body weight was measured daily after surgery.

Behavioral studies

Apparatus

Feeding studies were conducted in acrylic chambers (34 x 25 x 19 150 cm) containing regular bedding.

Optical intracranial self-stimulation (oICSS) studies were conducted in sound-attenuated operant chambers (Med Associates, St. Albans City, VT) equipped with two operant response levers, a house light and a cue light situated between the two levers. The system was monitored by MedPC software. Fiber optic cables were attached via FC/PC connector to 473 nm lasers (OEM/Opto Engine LLC, Midvale, UT) for photostimulation.

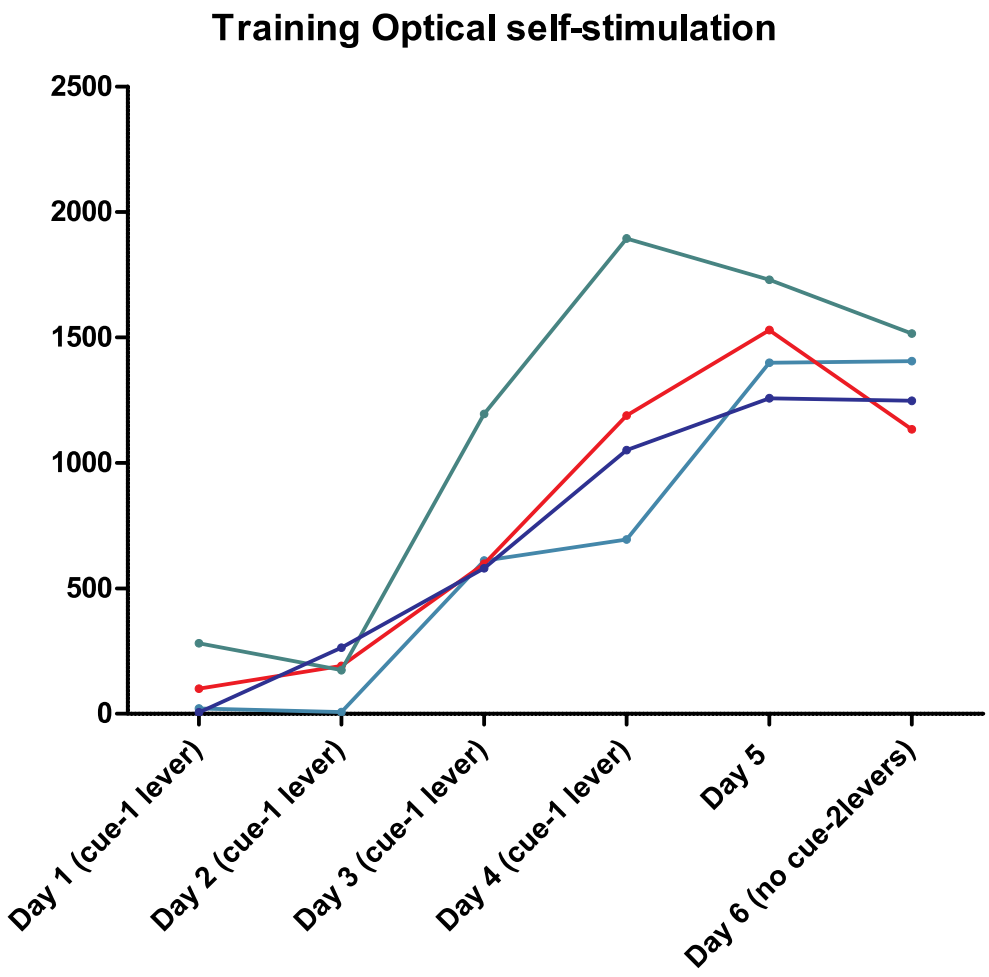
Feeding studies

ChR2 mice were habituated to the testing environment for three days before any experimental manipulation. On a given testday, each mouse was connected to a fiber optic cable and was placed in a chamber with a pre-weighed amount of food scattered in a cup

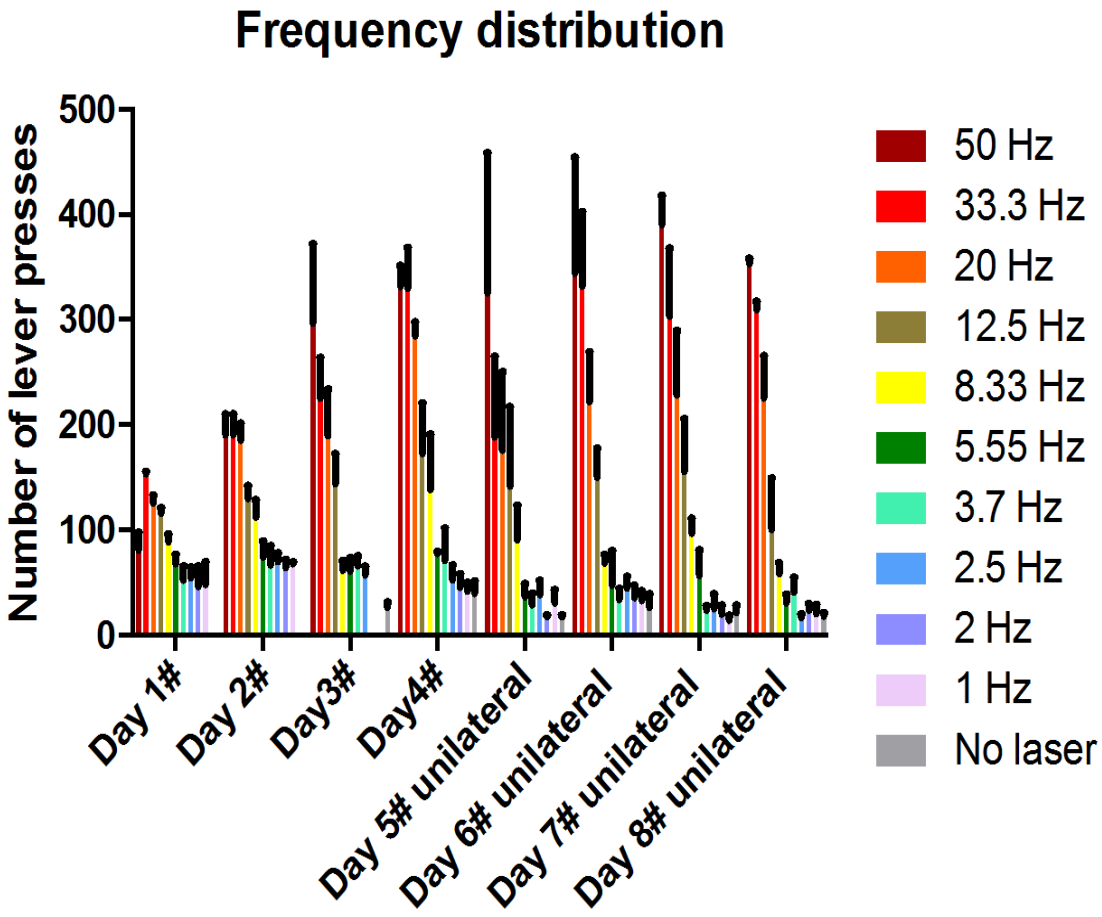
located in the corner of the chamber. Each session lasted 40min and was divided into ten 120-sec trials. During odd-numbered trials, the laser stimulation remained off for 120-sec. During even-numbered trials, continuous laser stimulation was given in 10msec pulses with laser intensity at the end of the fiber optics cable adjusted to 8 mW. Stimulation-induced feeding was assessed at four stimulation frequencies—5, 10, 20 and 50 Hz—on separate days and balanced sequence. Latency to start eating and amount eaten were recorded for each trial. Latency to pick up and bite cardboard or food and weights of the cardboard and food before and after testing were recorded.

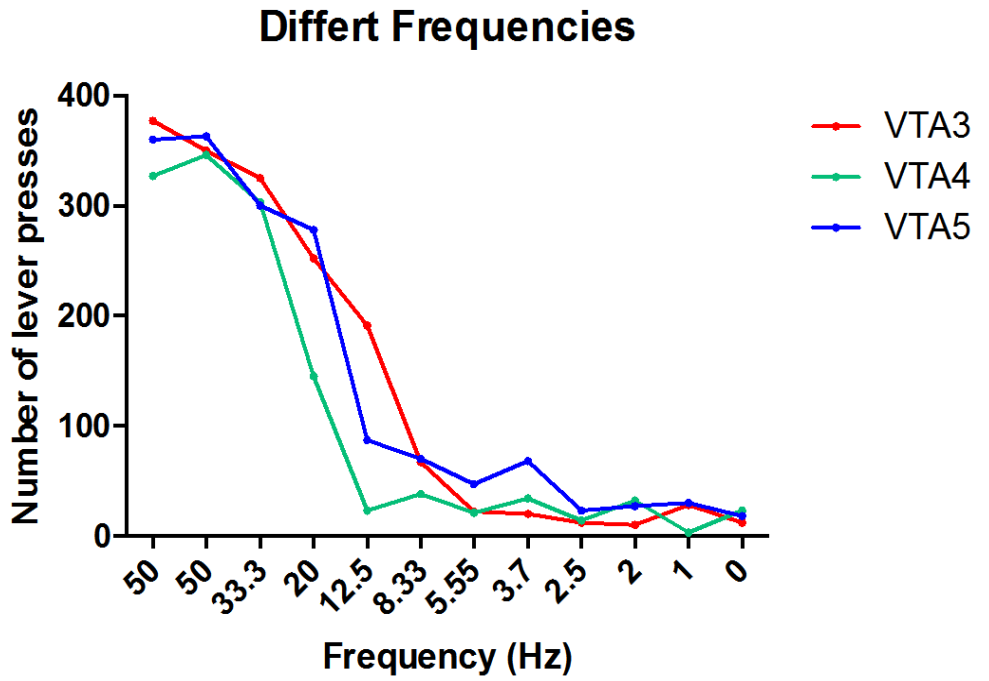
Optical intracranial self-stimulation studies

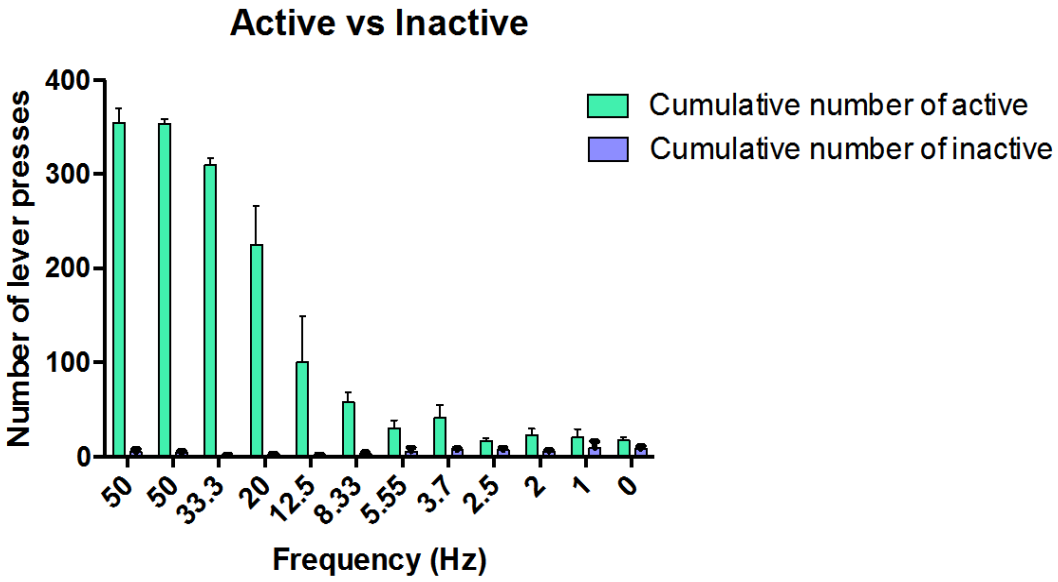
ChR2 mice were connected to the fiber optic cable and laser, and were placed in operant chambers equipped with two response levers (left and right) for daily 30-min self-stimulation testing. During the training each session (60 minutes) began with illumination of the house light, which remained on for the entire session. A lever press ("active" lever) activated a cue light above and caused a 2-sec train of 33,3 Hz photostimulation (10mW, 10 ms) followed by a 3 sec "time-out" during which stimulation was not available. The cue light remained illuminated until the end of the time-out. Responses on the other ("inactive") lever were not rewarded. During the first 4 days of training, the rightlever was designated as the active lever; on day 5 and 6 the left inactive lever was introdused and the cue was removed.



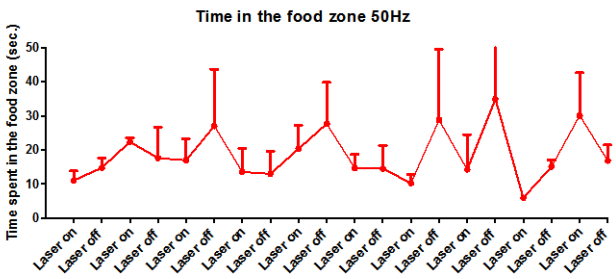
After 6 days of training at 33,3Hz, mice were tested daily at 8 frequencies (No laser, 1, 2, 2.5, 3.7, 5.55, 8.33, 12.5, 20, 33.3, and 50 Hz). Each session was formed by 10 trials of 10 minutes, in which we tested one frequency for time in decreasing order.During the first four days the stimulation was bilateral then I switchedwith unilateral, but unlucky I missed an animal during the experiment. Obviously I didn't do any statistical analysis on this staff because I have just a very small animal group and I need to compare the results with a control group injected with DIO ChR2 in LH and fibers in VTA. I already did all the stereotaxic surgeries but I'm still waiting for the expression of the virus. Nevertheless, these preliminary data show that the favorite frequencies for the reward are the highers.

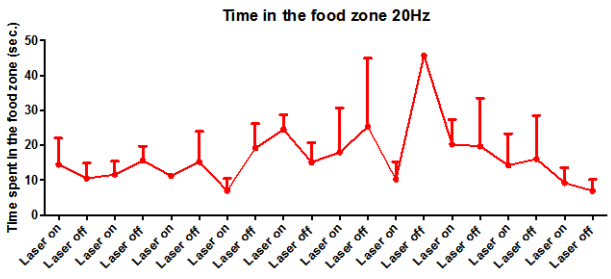

Frequency distribution
Number of lever presses
500
400
300
200
100
0
Day 1#
Day 2#
Day3#
Day4#
Day 5# unilateral
Day 6# unilateral
Day 7# unilateral
Day 8# unilateral
50 Hz
33.3 Hz
20 Hz
12.5 Hz
8.33 Hz
5.55 Hz
3.7 Hz
2.5 Hz
2 Hz
1 Hz
No laser





Stimulating the VTA DA neurons I didn't see any feeding behavior and just a very small effect on locomotion was observed, how the following data show.


Time in the food zone 50Hz
Time spent in the food zone (sec.)
0
10
20
30
40
50
Laser on
Laser off


Time in the food zone 20Hz
Time spent in the food zone (sec.)
0
10
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40
50
Laser on
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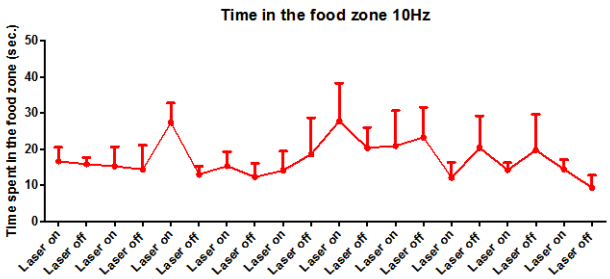
Time in the food zone 10Hz
Time spent in the food zone (sec.)
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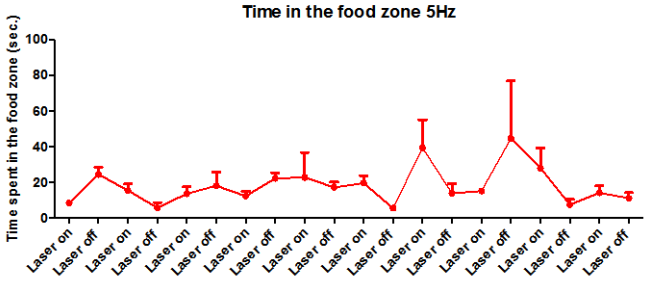
10
20
30
40
50
Laser on
Laser off


Time in the food zone 5Hz
Time spent in the food zone (sec.)
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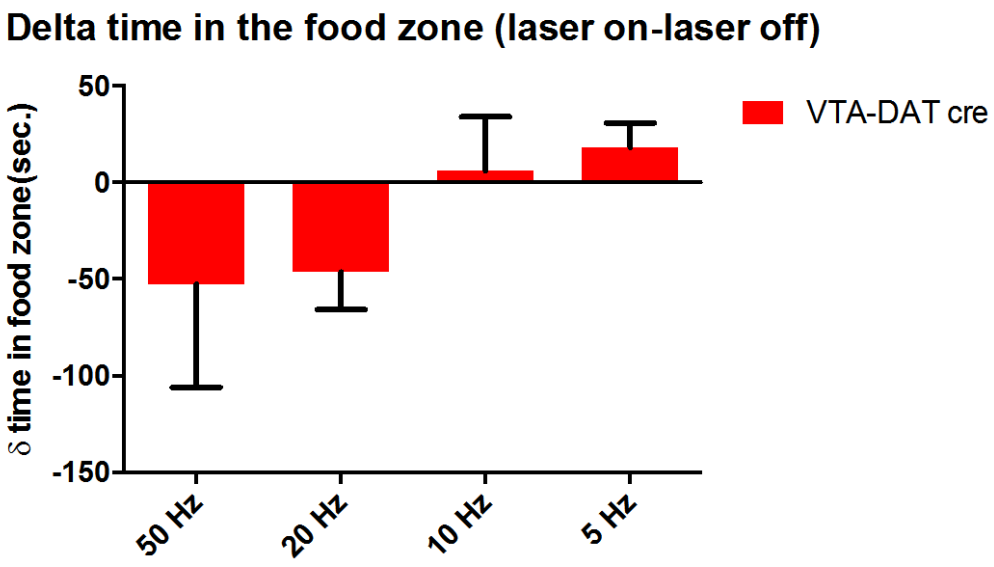
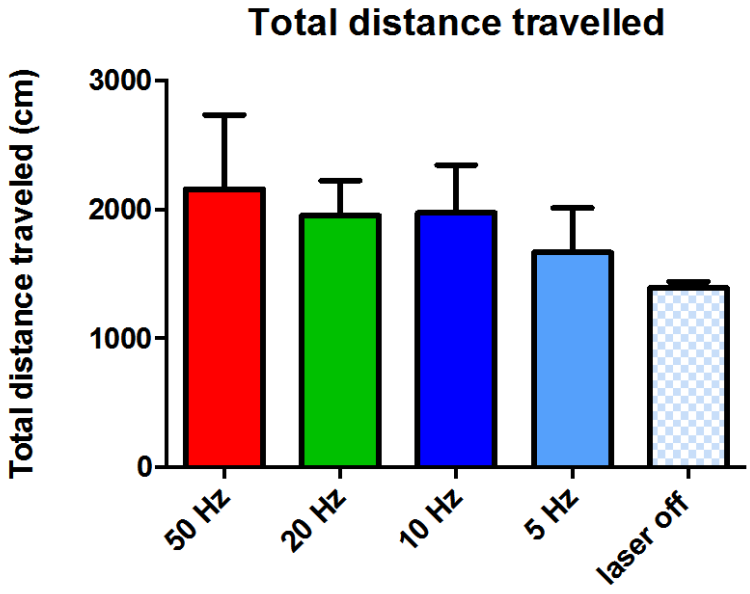
20
40
60
80
100
Laser on
Laser off





At this point my hypothesis is that DA cells in VTA are not involved in feeding and as consequence I wish to investigate the role of glutamatergic and gabaergic neurons on this behavior.

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