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Chemogenetic Stimulation of Electrically Coupled Midbrain GABA Neurons in Alcohol Reward and Dependence

Stephanie Suzette Pistorius
Brigham Young University

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Stephanie Suzette Sandoval Pistorius

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Master of Science
Neuroscience

Scott C. Steffensen, Chair
Sterling N. Sudweeks
Jeffrey G. Edwards

Department of Physiology and Developmental Biology

Brigham Young University

ABSTRACT

Chemogenetic Stimulation of Electrically Coupled Midbrain
GABA Neurons in Alcohol Reward and Dependence

Stephanie Suzette Sandoval Pistorius
Department of Physiology and Developmental Biology, BYU
Master of Science
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The prevailing view is that enhancement of dopamine (DA) transmission in the mesolimbic system leads to the rewarding properties of alcohol. The mesolimbic DA system, which plays an important role in regulating reward and addiction, consists of DA neurons in the midbrain ventral tegmental area (VTA) that innervate the nucleus accumbens (NAc). It is believed that VTA DA neurons are inhibited by local gamma-aminobutyric acid (GABA) interneurons that express connexin-36 (Cx36) gap junctions (GJs). We have previously demonstrated that blocking Cx36 GJs suppresses electrical coupling between VTA GABA neurons and reduces ethanol intoxication and consumption suggesting that electrical coupling between mature VTA GABA neurons underlies the rewarding properties of ethanol.

The aim of this study was to further investigate the role of VTA GABA neurons expressing Cx36 GJs in regulating DA neuron activity and release and mediating ethanol effects on VTA GABA neurons. To this end, we customized a Designer Receptor Exclusively Activated by Designer Drugs (DREADDs) viral vector to target VTA GABA neurons expressing Cx36 GJs in order to chemogenetically modulate their activity. In order to more conclusively demonstrate the role of this sub population of VTA GABA neurons in regulating DA neural activity and release we used electrophysiology to characterize the electrical changes that occur in VTA DA and GABA neurons when Cx36-expressing VTA GABA cells were selectively activated. In addition, we evaluated the effects of activation of VTA GABA neurons on brain stimulation reward and alcohol consumption in ethanol naive and dependent mice.

Results indicate that there are two populations of GABA neurons in the VTA, one that is GAD65+/Cx36+ and one that is GAD67+/Cx36-. Activation of Cx36+ VTA GABA neurons by clozapine-n-oxide (CNO) in mice injected with Gq DREADD activated VTA DA neurons and subsequent DA release in the NAc, suggesting that Cx36-containing GABA neurons are inhibiting non-Cx36 GABA neurons to disinhibit DA neurons. In hM3Dq animals, CNO administration provided a rewarding stimulus in the conditioned pace preference paradigm, and reduced consumption in the drink-in-the-dark ethanol consumption paradigm in dependent and naïve mice. A better understanding of the circuitry of the mesolimbic DA system is key to understanding the mechanisms that lead to addiction and may ultimately lead to improved therapies for substance abuse.

Keywords: GABA, dopamine, connexin-36, gap junction, chemeogenetics

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INTRODUCTION

Significance

Drug abuse has drastic effects on society. Economically, drug abuse is costly to our nation. Costs associated with public damage, health care costs and loss of workplace productivity related to drug abuse adds up to over $700 billion a year ("Trends and Statistics," 2015). The estimated costs of excessive alcohol consumption annually in the U.S. is $223.5 billion (Bouchery, Harwood, Sacks, Simon, & Brewer, 2011). Excessive drinking not only harms the alcoholic but also others around them. Amongst drugs of abuse, alcohol causes the most harm to individuals other than the abuser (Nutt, King, Phillips, & Independent Scientific Committee on, 2010). However, not much is known about the mechanisms involved in alcohol dependence. As such, a major goal of basic research on drug dependence is to understand the neural basis of drug use and the pathological progression to dependence. The compulsion to consume alcohol stems from its positive reinforcing properties, including its anxiolytic and euphoric effects, as well as its negative reinforcing properties, including the physical and psychological withdrawal symptoms that result from abstinence (Koob, Rassnick, Heinrichs, & Weiss, 1994). The pathway involved in modulating both the positive and negative reinforcing properties of alcohol is the mesocorticolimbic dopamine (DA) system.

The Mesolimbic Reward Pathway in the Brain

The mesocorticolimbic DA pathway is composed of DA neurons in the ventral tegmental area (VTA) of the midbrain that project to the nucleus accumbens (NAc; Figure 1) and prefrontal cortex (PFC) (Wise, 2004). This pathway is involved in anticipation of reward and in directing attention to rewarding stimuli, learned behavior, and drug addiction (Fields, Hjelmstad,

Margolis, & Nicola, 2007; Ikemoto, 2007). Current dogma suggests that enhancement of DA transmission in target areas of the mesocorticolimbic system underlies reward (Wise, 2008). It is theorized that the activity of VTA DA neuron activity is locally controlled by gamma-aminobutyric acid (GABA) interneurons in the VTA (Figure 1A). The neuroadaptations that occur in the mesolimbic DA system in association with drug dependence, including alcohol, may

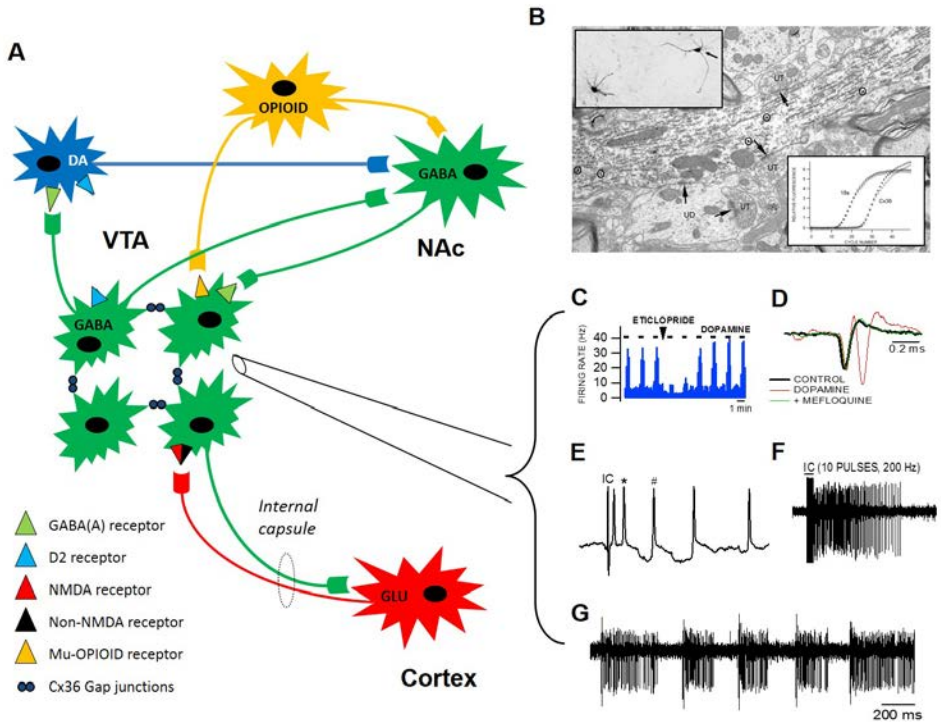

Figure 1: Mesolimbic Dopamine System Hodology: Evidence for Electrical Coupling Between VTA GABA Neurons. (A) Dopamine (DA) neurons in the VTA project to the nucleus accumbens (NAc) via the medial forebrain bundle. GABA neurons in the NAc project back to VTA GABA neurons via the direct pathway. Glutamate neurons in the pre-frontal cortex project to VTA GABA and DA neurons via the internal capsule. VTA GABA neurons form an electrical network. (B) Evidence for gap junctions between VTA GABA neurons. Inset left shows dye coupling between VTA GABA neurons when only one is labeled. Inset right shows that VTA GABA neurons express Cx36 mRNA. The electron micrograph shows that VTA GABA neurons from a mature animal that express mu-opioid receptors and that some of the synapses are non-vesicular and likely gap junctions (GJs). (C) In situ iontophoretic application of DA markedly enhances the firing rate of VTA GABA neurons with block by the D2R antagonist eticlopride. (D) Dopamine enhancement of firing rate also couples nearby VTA GABA neuron spikes which are blocked by the Cx36 GJ blocker mefloquine. (E) Stimulation of the internal capsule couples spikes recorded intracellularly in vivo. (F) High frequency stimulation of the IC induces multiple spike discharges that are blocked by mefloquine (data not shown). (G) VTA GABA neurons evince rhythmic discharges during transitions from sleep to wakefulness that are blocked by mefloquine (data not shown)

result from plasticity associated with inhibitory GABA in the VTA.

Gap Junctions

We and others have shown that VTA GABA interneurons are known to express connexin-36 (Cx36) gap junctions (GJs; Figure 1A-G) (D. W. Allison et al., 2006; Allison et al., 2011b; Belluardo et al., 2000; Condorelli, Belluardo, Trovato-Salinaro, & Mudo, 2000; S. C. Steffensen et al., 2011; S. C. Steffensen, Svingos, Pickel, & Henriksen, 1998). Gap junctions (GJs) are pores formed by connexin (Cx) subunits which enable the passage of current and small molecules between cells, thereby providing a means for intercellular communication in both the developing and mature nervous system (Bennett & Zukin, 2004). Two types of Cxs are ubiquitously expressed in mammalian neurons: Cx36, found mostly in GABA interneurons of the mature CNS (Belluardo et al., 2000; Condorelli et al., 2000) and Cx45, expressed throughout the nervous system during development and less so in the adult (Maxeiner et al., 2003). Gap junctions have been linked to the generation of high-frequency oscillations and GABA neuron networks may regulate these oscillations through GJs (Buzsaki & Chrobak, 1995; Galarreta & Hestrin, 2001; Tamas, Buhl, Lorincz, & Somogyi, 2000). Indeed, GJs exist in critical areas of the mesolimbic reward pathway in mature animals, including the VTA (D. W. Allison et al., 2006; Lassen et al., 2007) and NAc (Moore & Grace, 2002). The role of GJs in mediating or modulating reward is supported by only a few studies. For example, cocaine and methamphetamine self-administration produces region-specific and time-dependent changes in Cx36 expression in rats (McCracken, Hamby, et al., 2005; McCracken, Patel, Vrana, Paul, & Roberts, 2005), suggesting at least a correlative involvement of Cx36 GJs in drug reward. The Steffensen lab has published a handful of papers demonstrating Cx36 GJ-mediated electrical coupling between VTA GABA neurons and their role in alcohol and brain stimulation reward

(D. W. Allison et al., 2006; Allison et al., 2011b; Lassen et al., 2007; S. C. Steffensen et al., 2011; S. C. Steffensen et al., 1998).

Selective Activation of Neurons by Chemogenetic Stimulation

Lesion and stimulation studies, while important tools for dissecting the pathways involved in behavior, lack the ability to selectively stimulate the neurons or pathways of interest. Thus, this study aimed to better characterize the role of electrical coupling between mature VTA GABA neurons in regulating DA activity and release, or vice versa, in the mesocorticolimbic DA system through chemogenetic selective stimulation of these neurons using designer receptors exclusively activated by designer drugs (DREADDs).

DREADDs are an adeno associated viral vector that contain the gene for an engineered G-protein coupled receptor that is exclusively activated by clozapine-N-oxide (CNO), a drug that has no mammalian receptor. G_i-DREADDs insert a mutated muscarinic acetylcholine receptor (mAChR) that is coupled to a G_i protein that silences cells by two mechanisms: (1) induction of hyperpolarization by $G_{\beta/\gamma}$-mediated activation of G-protein inwardly rectifying potassium channels (GIRKs) and (2) by inhibition of the presynaptic release of neurotransmitter (Vardy et al., 2015). G_i-DREADDs induce a modest hyperpolarization and a strong inhibition of axonal release of neurotransmitter (Roth, 2016). G_q-DREADDs, like G_i-DREADDs, use a mutated mAChR. This receptor is coupled to a G_q protein that leads to excitation of the host cell by mobilizing intracellular calcium (Roth, 2016). DREADDs target cells through a promoter for a protein that is included in the viral construct that allows for transcription of the viral vector when the target protein is present. Currently the human synapsin promoter (hSYN), Calcium/calmodulin-dependent protein kinase IIA (CaMKIIa), and glial fibrillary acidic protein (GFAP) are used to target neurons. The advantage of DREADDs chemogenetic stimulation over

other methods of selective modulation of neurons, like optogenetics stimulation which utilizes the insertion of bacterial channel rhodopsin-2 into the plasma membrane, is that the excitability of neurons is not prone to the permeability of the channel to second messengers like calcium. The disadvantage of DREADDs chemogenetic stimulation compared to optogenetic stimulation is its slow onset, long duration of effect and real-time control.

RATIONALE AND HYPOTHESES

As previously mentioned, GABAergic synaptic transmission is still regarded as one of the main factors underlying the intoxicating, rewarding, and withdrawal-related effects of ethanol (S.C. Steffensen, Schilaty, & Hedges, 2016). GABAergic projections to the VTA come from several regions including the NAc and the ventral pallidum. However, the primary inhibitory regulation of DA neurons is by GABAergic interneurons within the VTA (S.W. Johnson & R.A. North, 1992; S. C. Steffensen et al., 1998). Our published studies provide compelling evidence that VTA GABA neurons express Cx36 GJs, dye couple (D. W. Allison et al., 2006; Allison et al., 2011b; Lassen et al., 2007; S. C. Steffensen et al., 2011; S. C. Steffensen et al., 1998), and form part of a larger syncytium of GABA neurons in the reticular formation that are linked by Cx36 electrical synapses (Lassen et al., 2007). Some of the ultrastructural, immunohistochemical and electrophysiological evidence for Cx36 GJs and electrical coupling between VTA GABA neurons is shown in Figure 1 B-G. I explored the role of Cx36+ VTA GABA neurons in mediating reward seeking. By better understanding this, we will be able to modulate reward seeking and possibly identify the neurons responsible for dependence. In the prospectus, I proposed the following three hypotheses:

> Hypothesis 1: Activation of GABA neurons that contain Cx36 GJs in the VTA will result in a decrease in VTA DA neuron activity and corresponding DA release in the NAc.
>
> Hypothesis 2: Selective activation of electrically coupled VTA GABA neurons is aversive.
>
> Hypothesis 3: Activation of GABA neurons containing Cx36 gap junctions will drive alcohol self-administration and reward.

METHODS

Animal Subjects

Male C57BL6 (black) and glutamate-decarboxylase-67 (GAD-67)-green fluorescent protein knock-in on a CD-1 (white albino) mice (Tamamaki et al., 2003) will be bred and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For each methodology to be employed, animals will be treated in strict accordance with the Brigham Young University Animal Research Committee (IACUC) guidelines, which incorporate and exceed current NIH guidelines. The BYU IACUC has reviewed and approved the procedures detailed herein. Once weaned at PND 21, all mice will be housed in maximum groups of four and given ad libitum access to solid food and water and placed on a reverse light/dark cycle with lights ON from 8 PM to 8 AM. Any mice used in injection experiments will be anesthetized with isoflurane (5%), and injected IP with a sterile needle. Animals are returned to their home cages 30 minutes following the injection.

Stereotaxic Surgery

All surgeries were performed in P28-60 day old C57BL/6, GAD-GFP knock-in mice, and 220g-500g Wistar rats. Before surgery, animals were anesthetized with isofluorane (2-5%). A 10 uM Hamilton syringe/needle was inserted into the VTA (mice: A/P, -3.0; M/L, +0.5; D/V, -4.5 mm; rats: A/P, -6.0; M/L, +0.9; D/V, -8.0 mm from bregma). A volume of 0.5uL of AAV8.hCx36.hM3D(Gq)-mCherry.WPRE.rBG or AAV9.CB7.CI.mCherry.WPRE.rBG (Penn Vector Core, Philadelphia, PA) was injected into each hemisphere at a rate of 0.075uL/min using a microinjection pump (World Precision Instruments, Sarasota, FL). The cannula was left in place following the injection for 7-10 minutes to ensure diffusion. Animals were allowed to

recover and to allow expression of the novel receptor for 10-14 days before being used for experiments.

Chronic Intermittent Ethanol Exposure

Ethanol vapor chambers combined with ethanol drinking were used to establish ethanol dependence. We modified the vapor chamber system developed in the lab of Graeme Mason at Yale (Wang, Jiang, Du, & Mason, 2012). The six automated chamber system consisted of an air-pressurized, feedback-controlled ethanol flask with flow valves to each of three sealed chambers to regulate the flow of air (11 L/min) and concentration of alcohol to three of six chambers placed in a ventilation hood (Figure 2). A breathalyzer (Drager Alcotest 6510) was used in a feedback loop to regulate the concentration of ethanol. To validate ethanol exposure in our modified vapor chamber system, we calibrated three feedback flowmeter settings (1, 3, and 5 L/min) in a 3 L alcohol pressurized flask with breathalyzer values and BALs (Sigma Enzymatic method) obtained in a cohort of vapor exposed mice. We obtained breathalyzer values of 52.1 ± 4.2, 130.2 ± 4.8 and 225.6 ± 4.1 mg% (n=6) ethanol and BALs of 46.1 ± 3.7, 119.7 ± 2.9, and 234.9 ± 5.5 mg% (n=6) ethanol at feedback flowmeter flask settings 1, 3, and 5 L/min, respectively.

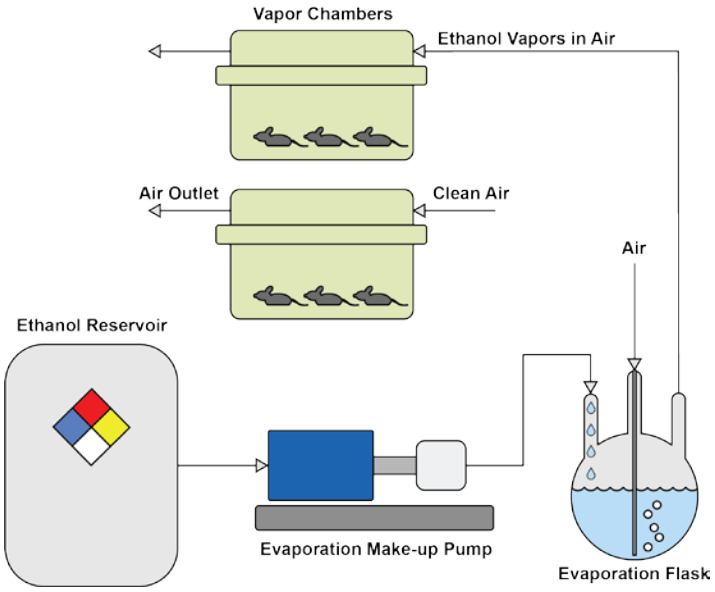

Figure 2: Diagrammatic Representation of the Vapor Chamber Apparatus Used in Administration of CIE.

Based on these calibrations, mice were exposed to feedback flowmeter settings corresponding to 200 mg% breathalyzer values for sixteen hours (1000-0200 hours) beginning in their dark cycle. In order to avoid overdosing the first week of CIE vapor exposure, mice were exposed to 2 hrs of vapor for two days, 4 hrs of vapor for two days, and 8 hrs of vapor for two days before exposing to 16 hrs vapor/day. Control animals were housed in three sealed chambers in the same ventilation hood, but only received air. Even at the 5 L/min feedback flowmeter level the air-exposed mice did not show any detectable alcohol above the 1 mg% detection limit of the breathalyzer or the BAL determination method.

Figure 3: The implementation of the Alcohol Vapor Chamber System.

Preparation of Brain Slices

All brain slice preparations were performed in P28-60 day old C57BL/6 and GAD-GFP knock-in mice. Brains were extracted via anesthetization with isoflurane (5%). Upon extraction, the brain was glued onto a cutting stage. The brain was then sectioned in room temperature (ACSF; in mM: 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 12 glucose, 1.5 $MgSO_4$, 2 $CaCl_2$) perfused with 95% O_2 / 5% CO_2. Targeting the VTA, horizontal slices (210μM thick) were placed in an incubation chamber containing ACSF perfused with 95% O_2 / 5% CO_2 for at least 30 minutes. After 30 minutes, brain slices were then placed in a recording tissue chamber with ACSF continuously flowing at physiological temperatures (35 °C).

Characterization of Neuron Types

GABA neurons were studied in GAD-GFP knock-in mice. In GAD-GFP knock-in mice, VTA GABA neurons were identified by a characteristic glow under fluorescence illumination. DREADD positive cells were confirmed through DREADD-mCherry fluorescence. Neurons that did not fluoresce were assumed to be DA neurons (D. W. Allison et al., 2006; Allison et al., 2011a; S. W. Johnson & R. A. North, 1992; Margolis, Lock, Hjelmstad, & Fields, 2006; S. C. Steffensen et al., 2011).

Immunohistochemisty

Following behavioral experiments, animals were anesthetized with isoflourane anesthesia (3%) and rapidly perfused with isotonic sterile saline followed by 4% paraformaldehyde. Following a sucrose gradient (10, 20, 30%), tissue was flash frozen and sectioned (35 um) on a cryostat. Slices were washed with PBS then blocked with 4% normal donkey serum in 0.1% TritonX-100 in 1% BSA in PBS (2 hours, RT) and then incubated overnight at 4 degrees Celsius with the primary antibody (anti-tyrosine hydroxylase, 1:400, ab113, Abcam Inc.). Sections were then washed again and incubated with Alexa Fluor 405 donkey anti-sheep (1:500, ab175676, Abcam) and washed again. Slices were then mounted on glass slides, covered in mounting medium, and images were acquired either using a confocal microscope or a fluorescence microscope.

Conditioned Place Preference

CPP experiments were performed on 250g-500g male Wistar rats. We have built a customized CPP chamber for rats and mice. The CPP apparatus consists of three adjacent conditioning compartments made out of acrylic separated by manual guillotine-type doors.

However, only two were used for the procedure. One of the compartments is equipped with walls with vertical stripes and the other with walls with horizontal stripes and the floors are of distinct texture, one smooth one rough. Piezoelectric transducers cemented under the floors sense locomotor activity and tremor based on a signal detection algorithm developed by the PI. First, animals were subjected to three 20 min pre-conditioning tests in order to determine any initial preference for one of the conditioning compartments. Typically, rodents prefer dark compartments about 80%. However, it is best not to have a strong preference. So, the chambers are made distinct enough so that preference is typically about 50%. The door was then inserted in the slots to create compartments. Each animal was conditioned with CNO (3mg/kg IP) in one of the compartments, and an equivalent volume of saline in the other compartment. CNO was injected 30 min before the conditioning session. The animals underwent 20 min conditioning sessions twice daily, once with CNO and once with saline separated by at least 8 hrs. Conditioning was repeated. Following 6 sequential conditioning days, animals were tested for place preference by allowing free access to both conditioning compartments for 60 min as in the three preconditioning trials.

Drink-in-the-Dark

To observe a behavioral correlate of escalating alcohol dependence, GAD GFP mice injected with a DREADD viral vector were trained and evaluated on a drink-in-the-dark (DID) two bottle choice self-administration test of either alcohol or CNO. Animals were removed from home cages three hours into the dark cycle, and placed individually in cages with bedding and food removed. During days 1-4 of DID, animals were given a saline injection equivalent to 3mg/kg CNO 20-30 minutes prior to drinking. On day 5, animals received a 3 mg/kg CNO injection 20-30 minute prior to drinking. They were then given two sipper tubes, with one

containing tap water and the other containing tap water and ethanol (20%). The mice were allowed to drink from the tubes for two hours in the dark, and were then returned to their home cages. Dependent animals received CNO on all 5 days of DID.

Cell-Attached, Voltage-Clamp Recording of Spike Activity in Brain Slices

Cell-attached studies used electrodes pulled from borosilicate glass capillaries (2.5-6MΩ) and then filled with a NaCl solution containing (in mM): 124 NaCl, 2 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 1.2 $MgSO_4$, 2 $CaCl_2$ adjusted to pH 7.4 with KOH. Positive pressure was applied to the electrode when approaching the neuron. By applying suction to the electrode, a seal (10MΩ – 1GΩ) was created between the cell membrane and the recording pipette. Spontaneous spike activity was then recorded in voltage-clamp mode with an Axon Instruments Multiclamp 700B amplifier. Spike activity was sampled at 10 kHz using an Axon 1440A digitizer, and collected and analyzed using pClamp10 software. Neurons were voltage-clamped at 0 mV throughout the experiment. A stable baseline recording of firing activity was obtained for 5-10 min before adding any substances.

Microdialysis and HPLC

Following completion of the CPP paradigm, male Wistar rats were anesthetized using isoflurane (1.5 – 2.0%) and implanted with a microdialysis probe (BR-2, Bioanalytical Systems, Inc., West Lafayette, IN, USA) in the NAc shell (NAcSh). The coordinates for the implanted probe were +1.7 AP, -0.8 ML, -8.0 DV. Artificial cerebral spinal fluid (aCSF) was perfused through the probe at a rate of 2 μL/min using a microinjection pump (A-99, Razel Scientific Instruments, Saint Albans, VT, USA). The aCSF was composed of 2 mM Na_2HPO_4, 150 mM NaCl, 3 mM Kcl, 1.4 mM $CaCl_2$, and 0.1 mM $MgCl_2$. Samples were collected and analyzed

using high pressure liquid chromatography (HPLC) every 20 minutes for 5 hours, with the first two hours occurring prior to injection with CNO (3 mg/kg, IP). Samples were assayed for DA content using a HPLC system (Dionex 3000, Thermo Fisher Scientific, Waltham, MA, USA). The mobile phase consisted of 75 mM NaH_2PO_4, 1.7 mM sodium octane sulfonate, 25 μM EDTA, 0.1% triethylamine, and 10-12% acetonitrile. The mobile phase flow rate was 0.5 mL/min. Separation of compounds was performed using a HR-80 column (Thermo Fisher Scientific). A coulometric detector (Coulochem III, Thermo Fisher Scientific) equipped with an analytical cell (5014B, Thermo Fisher Scientific) and a guard cell (5040, Thermo Fisher Scientific) was used for detection of DA. The first potential was set at -100 mV, and the second potential was set at +220 mV. The guard cell potential was +275 mV. DA content in the samples was expressed as percent of baseline DA content which was determined by averaging the first six collections.

In Vivo Single Unit Recordings

Following CPP, Single-unit potentials, discriminated spikes, and stimulation events in vivo were captured by National Instrument's NB-MIO-16 digital I/O and counter/timer data acquisition boards (Austin, TX) and processed by customized National Instruments LabVIEW software in Macintosh-type computers. Potentials were digitized at 20 kHz and 12-bit voltage resolution. For single-unit activity, all spikes were captured by computer and time stamped. Spontaneous firing rates were determined on- and off-line by calculating the number of events over a 5 min epoch, typically 5 min before and at specific intervals after drug injection. Peri-stimulus and interval-spike histograms were generated off-line using IGOR Pro (WaveMetrics, Lake Oswego, OR) analysis of the time-stamped data. The duration (msec) and extent (#events/bin) of post-stimulus permutation of ICPSDs was determined by rectangular integration

at specific time points on the peri-stimulus spike histogram using IGOR Pro analysis software. The minimum bin width for peri-stimulus spike histograms was 1.0 msec and the number of bins was 1000. These parameters allow for detection of all phases of pre- and post-stimulus spike activity.

Characterization of VTA GABA Neurons In Vivo

All neurons classified as VTA GABA neurons *in vivo* were located in the VTA, met the criteria established in previous studies for spike waveform characteristics and response to IC stimulation (D.W. Allison et al., 2006; S. C. Steffensen et al., 1998; Stobbs et al., 2004), and often were activated and spike-coupled by microelectrophoretic dopamine (DA; (Stobbs et al., 2004)). Presumed VTA GABA neurons were characterized by short-duration (<200 μsec; measured at half-peak amplitude of the spike), initially negative-going, non-bursting spikes, and were identified by the following IC stimulation criteria (S. C. Steffensen et al., 1998): Short latency (i.e., 2-5 msec) antidromic or orthodromic activation via single stimulation of the IC; and multiple spiking following high-frequency (10 pulses, 200 Hz) stimulation of the IC (ICPSDs; (D.W. Allison et al., 2006; Lassen et al., 2007; S. C. Steffensen et al., 1998; Stobbs et al., 2004). In all studies, stimulation was performed at a level that produced 50% maximum VTA GABA neuron ICPSDs. This was accomplished by determining the current needed to produce the maximum number of ICPSDs at 200 Hz and 10 pulses, and then adjusting the stimulus intensity until 50% ICPSDs were achieved.

Drug Preparation and Administration

Drugs used in vitro are made fresh in distilled water, added to ACSF, and superfused on brain slices: clozapine n oxide (20 μM; Hello Bio). Drugs used in injections are added to sterile 0.9% saline and injected IP: CNO (3.0 mg/kg; Hello Bio).

Statistical Analyses

All results were presented as raw mean values and percent control ± SEM. Results between groups were compared using a two-tailed unpaired *t* test or ANOVA. Experiments relying on variance in time or current were analyzed using mixed models ANOVA with post hoc t-test at individual points. Statistical significance required ≥ 95% level of confidence ($P \leq 0.05$). Analysis software includes Microsoft Excel and Igor Pro (Wavemetrics, Oswego, OR). Significance levels are indicated on graphs with asterisks *,**,*** and correspond to significance levels $P<0.05$, 0.01 and 0.001, respectively. Figures will be constructed with Igor Pro software.

RESULTS

CNO Activation of Cx36+ GABA Neurons

Our first goal was to confirm that we could use DREADDs to modulate fast firing neurons, such as the GABA neurons we planned to study (Steffensen et al, 1998). hM3Dq+ cells were identified using an mCherry filter and firing rate was measured. We performed loose patch experiments to test the effects of CNO on hM3Dq+ GABA neuron firing rate. We found that increasing doses of CNO resulted in increased firing rate in hM3Dq+ cells (Figure4). We saw the most robust effects with 20 μM CNO ($F_{(1,28)}$=17.89, p=0.0002). Therefore, 20 μM CNO was used for further patch clamp experiments.

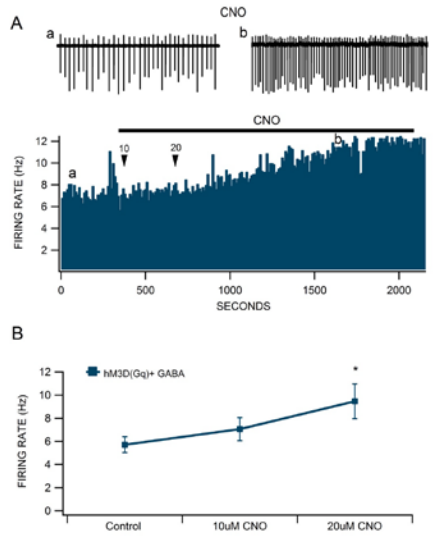

Figure 4: Firing Rate in VTA GABA Neurons Following CNO in Gq DREADD Injected Mice. (A) The ratemeter shows the firing rate of an hM3Dq+ VTA GABA neuron (traces in a,b recorded at times indicated on graph) recorded in a brain slice from a mouse infused with Cx36-hM3Dq DREADD. This representative neuron had a baseline firing rate of approximately 7.3 Hz. CNO markedly increased the firing rate of this VTA GABA neuron. All insets are representative 5 sec traces of GABA neuron spike activity recorded before (inset a) and after (inset b) 20 μM CNO. (B) CNO significantly increased the firing rate of hM3Dq+ VTA GABA neurons. Asterisks mark a significant difference between firing rate during control and 20 μM CNO.

CNO Activation of VTA DA Neurons

Additionally, we evaluated the effects of CNO on putative DA neurons. Firing rate recording in this study were performed in voltage-clamp, cell-attached mode in order to avoid dialyzing the contents of the cells and disrupting the cytoplasm. Thus, any electrophysiological characteristics of VTA neurons based on current and spike dynamics under whole cell recording

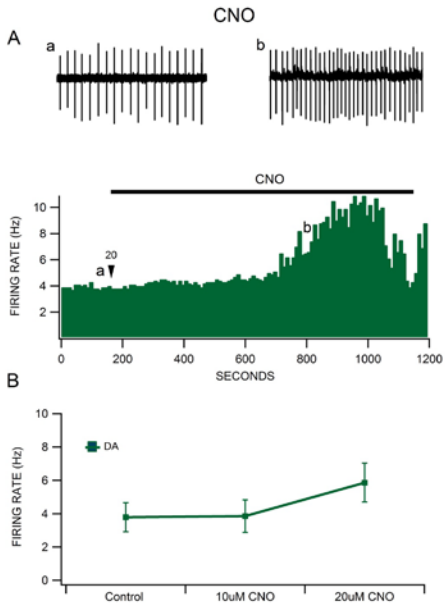

Figure 5: Firing Rate in VTA DA Neurons Following CNO. (A) The ratemeter shows the firing rate of a VTA DA neuron (traces in a,b recorded at times indicated on graph) recorded in a brain slice from a mouse infused with Cx36-hM3Dq DREADD. This representative neuron had a baseline firing rate of approximately 3.2 Hz. CNO increased the firing rate of this VTA DA neuron. All insets are representative 5 sec traces of DA neuron spike activity recorded before (inset a) and after (inset b) 20 μM CNO. (B) CNO increased the firing rate of VTA DA neurons.

conditions was not possible. Nonetheless, we present data on putative VTA DA neurons along with VTA GABA neurons, we compared their sensitivity in ethanol naïve GAD67 GFP CD-1 mice. Neurons in the VTA of GAD67 GFP mice that did not exhibit fluorescence, but were characterized by relatively slow, regular firing activity, were presumed to be DA neurons. Considering the previously held belief that VTA GABA neurons synapse on and inhibit local DA neurons (Figure 5), we expected to see a general decrease in DA firing activity. To our surprise, results show an increase in VTA DA firing rate following 20 μM CNO exposure (Figure 5). Considering these cells do not express the novel receptor and CNO has no endogenous receptor, these data suggest that there is an indirect connection between $Cx36^{+}$ GABA neurons and DA neurons. These results have yet to reach an acceptable level of significance ($F_{(1,18)}=2.93$, $p=0.10$). However, the trend suggests that there is an increase in DA activity.

In order to test for any possible effects of CNO on VTA DA neurons, we performed the same patch-clamp experiments on non-infused GAD GFP animals. Any cells that were not GFP^{+} were considered as DA neurons. Following CNO, firing rate in VTA DA neurons in a hM3Dq(-)

did not change (Figure 6). Further experiments are needed to be certain that CNO has no effect on VTA DA neurons on its own. However, when taking into consideration the percent increase of approximately 86% seen in hM3Dq+ VTA DA neuron, we are confident that CNO does not affect DA neuron activity in the absence of DREADDs. This is consistent with the inert properties of CNO and its lack of an endogenous receptor.

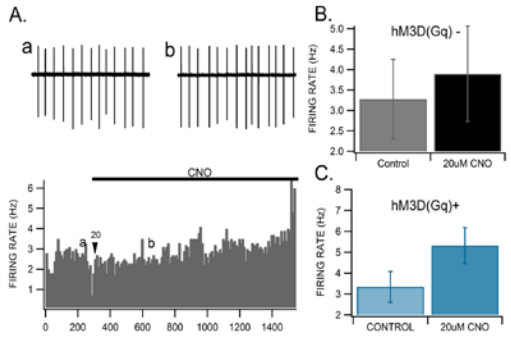

Figure 6: Firing Rate in VTA DA Neuron in GAD67 GFP Mice. (A)The ratemeter shows the firing rate of a VTA DA neuron (traces in a,b recorded at times indicated on graph) recorded in a mouse not infused with hM3Dq DREADD. This representative neuron had a baseline firing rate of approximately 3.3 Hz. CNO did not have an effect on firing rate. Insets are representative of 5 sec traces of neuron spike activity recorded before (a) and after (b) 20 μM CNO. (B) Graph illustrating that CNO does not increase DA firing rate in naïve animal. Control firing rate of 3.3 +/- 0.97 Hz. 20 M CNO of 3.9 +/- 1.2 Hz. (C)Graph representing the increase in DA firing rate in hM3Dq infused animals (Figure 5).

Expression of hM3Dq in VTA GABA Neurons

From initial electrophysiology studies, it became apparent that DREADD expression was not as anticipated. Using GAD GFP mice, we expected to observe co-localization of GAD GFP and hM3Dq. Interestingly, there was a small percentage of hM3Dq cells that co-express with GAD GFP cell, which are GAD 67+. Therefore, following data collection, animals were perfused and sections were taken for histological confirmation of DREADD expression within the VTA. In addition to confirming the accuracy of our injections, we performed immunohistochemistry for tyrosine hydroxylase (TH) in order to test for expression of the novel DREADD receptor in DA neurons. Figure 7 shows the level of expression in GAD GFP mice. From the IHC performed, we did not detect any expression of hM3Dq-mCherry in GAD 67+ and TH+ cells. This is consistent with our hypothesis that a subpopulation of VTA GABA neurons

express Cx36 GJs. Further studies will investigate the potential co-expression of GAD 65 and Cx36-hM3Dq-mCherry.

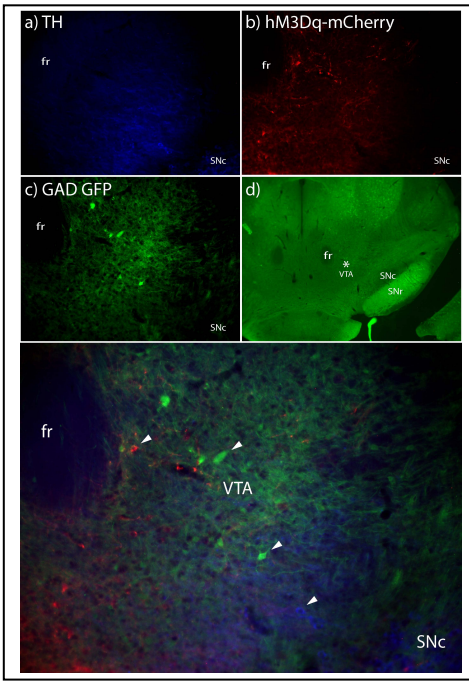

Figure 7: hM3Dq Expression in GAD67 GFP CD-1 Mice. (A)The VTA is identified in the coronal slice in-between the fasciculus retroflexus and substantia nigra pars compacta.(B) TH expression within the VTA allows visualization of VTA DA neurons. (C) hM3Dq expression within the VTA. (D) GAD67^{+} cells within the VTA. (E) A merged image of images B-D shows no co expression of GAD67^{+} neurons or TH^{+} neurons within the VTA. GAD65 neurons are not identifiable in these images.

We conducted many of our behavioral studies and all of our in vivo electrophysiology in hM3Dq infused rats. We had no reason to believe that the novel DREADD receptor would not express in rats but we confirmed hM3Dq expression nonetheless. Figure 8 shows the extent of hM3Dq expression in rats used for behavioral studies. Consistent with our IHC studies in the mouse, we did not observe co-expression of TH and hM3Dq-mCherry. These studies confirm that the effects measured throughout this study are resultant of Cx36+ VTA GABA neuron activation by CNO.

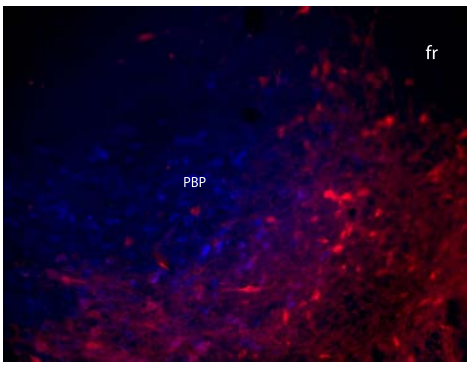

Figure 8: hM3Dq Expression in the Rat Brain. Following IHC, hM3Dq+ neurons are visualized in contrast to TH+ neurons. There is no apparent co-expression of Cx36 and TH.

Conditioned Place Preference to Measure Reward Following CNO

Conditioned place preference (CPP) is a paradigm that measures reward and reinforcement learning. In our initial studies, we found that CNO enhances VTA DA activity. Increased DA release is characteristic of drugs that are considered rewarding (Wise, 2008). In order to test for reward

resulting from CNO activation of Cx36+ VTA GABA neurons, we used a CPP paradigm where one side of the chamber was paired with a 3 mg/kg IP injection of CNO.

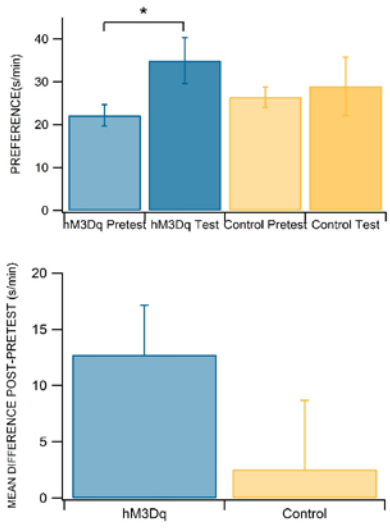

Figure 9: CPP Following CNO Conditioning.
Results suggest that following conditioning, there is a trend for animals showing a preference for the CNO-paired chamber. (A) During the pretest hM3Dq animals showed no preference for the CNO-paired chamber (22.2 +/- 2.5 s/min). hM3Dq animals showed an increase in time spent in the CNO-paired chamber following conditioning (34.9 +/- 5.3 s/min). Control animals spent 26.4 +/- 2.4 s/min in the CNO-paired chamber prior to conditioning and 28.9 +/-6.8 s/min in the CNO-paired chamber following conditioning. (B) This graph illustrates the difference (Post-Pre) between hM3Dq and control animals. hM3Dq animals spent 12.73 +/- 4.4 s/min more in the CNO-paired chamber after conditioning. Control animals spent 2.5 +/- 6.14 s/min more in the CNO-paired chamber following conditioning.

Consistent with our firing rate studies, our CPP studies suggest that CNO activation is rewarding. Reward is measured by the preference the animal has for one side of the chamber following conditioning, which is determined by the seconds the animals spend in the chamber per minute (s/min). Following 6 days of conditioning, the hM3Dq infused animals showed a slight preference for the CNO paired chamber compared to the control group albeit not statistically significant (Figure 9). Considering the trend between the mean difference in time spent in the CNO-paired chamber during the post-test compared to the pre-test, we believe that CNO may be rewarding. The trend suggests that a higher subject number should result in significance. Further studies are currently underway to increase the power of this study.

CNO Activation of VTA Neurons In Vivo

Following CPP studies, infused animals were used for in vivo single-unit recordings. These studies allowed for recording of real-time CNO effects on putative hM3Dq^{+} VTA GABA neurons. Consistent with data collected from our patch-clamp experiments, hM3Dq^{+} VTA GABA neurons were activated by CNO (Figure 10). After maintaining a stable signal for 5-10 minutes, 3 mg/kg CNO was administered via IP injection. On average, approximately 20 minutes following CNO, firing rate increased and immediately reached a firing rate of about 200% of the original baseline. This effect lasted for 2 hours. The increase seen in vivo is much greater than that observed in the slice and is unaccounted for but could be due to maintaining the integrity of local connections in vivo as compared to slice electrophysiology.

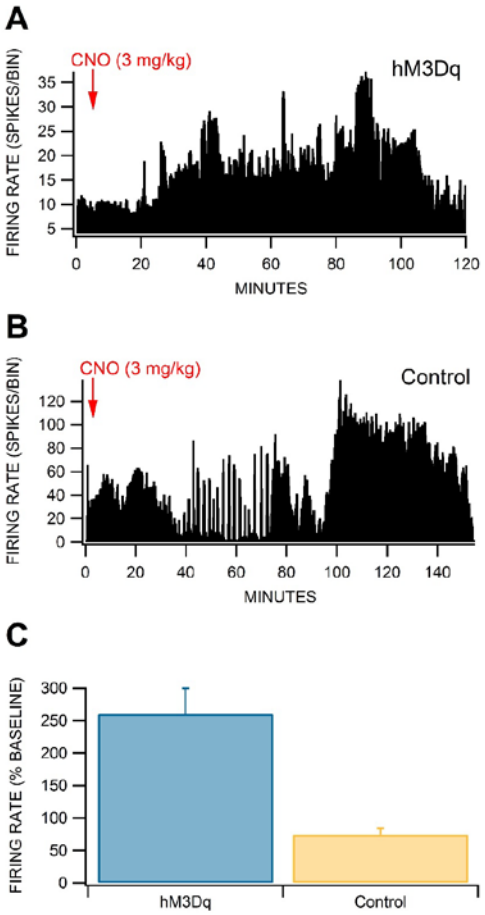

Figure 10: Chemogenetic Modulation of VTA GABA Neuron Firing Rate. (A) Representative ratemeter recording of a VTA GABA neuron recorded in an isoflurane-anesthetized rat. Administration of 3.0 mg/kg CNO IP in a rat injected with hM3Dq enhanced the firing rate of this neuron with a 20 min delay and for approximately 1 hr. (B) Representative ratemeter recording of a separate VTA GABA neuron in a rat injected with Control virus. Administration of CNO inhibited the firing rate of this neuron with a delay of 20-30 min and a duration of 1 hr. There appeared to be a rebound excitation. (C) In the average of 12 neurons to date, there were significant differences in CNO effects between hM3Dq-injected rats injected with hM3Dq vs Control.

CNO Activation Increases Dopamine Release in the Nucleus Accumbens

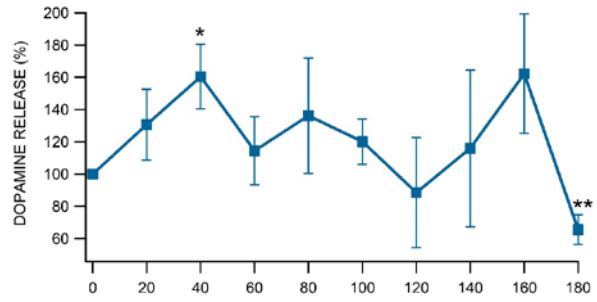

Figure 11: Dopamine Release in the NAc Following CNO. Dopamine was measured every 20 minutes. Control values were averages and are represented by 100% during minute 0. Despite large vaiations between animals, we saw a consistent increase in DA release 40 minutes after 3 mg/kg CNO; 142.9 +/- 3.6%. There was a robust decrease in CNO 180 minutes post CNO; 79.8 +/- 4.3%.

While our CPP studies have yet to yield statistically significant results, the data thus far suggest that CNO activation in $Cx36^{+}$ VTA GABA neurons is rewarding. We believed that this is due to an increase in DA release in the NAc due to activation of DA neurons in the VTA (Figure 5).

In order to test this theory, we used anesthetized microdialysis recordings to measure DA release in the NAc (Figure 11). All animals used in this study had previously undergone CPP. Samples were taken every 20 minutes and run through HPLC analysis to test for DA release. After two hours of control samples were taken, 3 mg/kg CNO was administered IP. In accordance with our in vivo experiments, we observed an increase in DA release around 20 minutes and saw a robust increase at about 40 minutes post CNO ($F_{(1,4)}$=141.7, p>0.001, n=3). We saw quite a bit of variability in our studies. With a larger sample size, we expect to see more consistent results. Of note is that 180 minutes after CNO a decrease in DA release within the NAc was observed ($F_{(1,4)}$=21.9, p=0.009, n=3). It is not clear why DA release decreases once CNO's effects are gone. This may be due to an increase in inhibition from $hM3Dq^{+}$ VTA GABA neurons following excitation by CNO leading to decrease DA neuron activity within the VTA.

Drink-in-the-Dark Behavioral Measure of Self-Administration

Previous studies from our lab have shown that animals will consume high levels of ethanol following ethanol exposure. Animals undergo 16-24 hours of withdrawal before allowed to self-administer ethanol. The withdrawal effects are thought to drive self-administration. We have shown that activating Cx36+ VTA GABA neurons results in not only an increase in VTA DA cell activity but also an increase in DA release resulting in reward. Using a DID paradigm we studied whether the reward following CNO in hM3Dq animals would block ethanol self-administration. Following three weeks of CIE, animals underwent DID. Animals were injected with 3 mg/kg CNO IP 20-30 minutes prior to drinking. Figure 12 shows that the hM3Dq group consumed less than the control group.

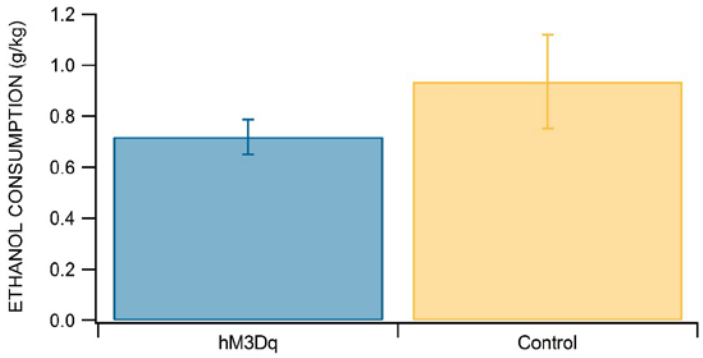

Figure 12: Ethanol Self-Administration Following Chronic Ethanol Exposure. DREADD infused animals consumed less ethanol following a 3 mg/kg injection of CNO prior to DID. hM3Dq animals consumed an average of 0.72 +/- 0.07 mg/kg. The control animals consumed an average of 0.94 +/- 0.18 mg/kg.

To test whether CNO would block drinking after acute exposure to ethanol we allowed ethanol naïve animals 10-14 days post virus infusion to self-administer ethanol two hours a day for 5 days. On days 1-4, animals received a saline injection equivalent to 3 mg/kg CNO prior to drinking. On the fifth day, animals received a 3 mg/kg CNO injection prior to drinking. On

average, there was no significant difference in drinking between the hM3Dq and control groups on days 1-4 (Figure 13). On the fifth day, hM3Dq animals drank slightly less than the average of days 1-4. Albeit, not yet significant ($F_{(1,18)}$=2.57, p=0.13). There is, however, a significant difference between the amount of ethanol consumed by the hM3Dq and the control group on day 5. hM3Dq animals drank significantly less ethanol than the control group ($F_{(1,29)}$=5.07, p=0.03). We were not anticipating such an increase in ethanol consumption by the control group on the fifth day. This result suggests that CNO may not be as inert as originally thought.

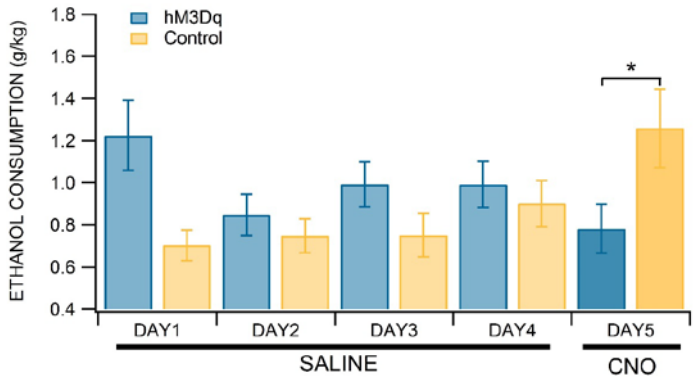

Figure 13: Ethanol Self-Administration in Ethanol Naïve Animals. (A) Animals previously infused with hM3Dq or control were allowed to freely consume ethanol for five days. On days 1-4 there was no difference in ethanol consumption. The average ethanol consumption for days 1-4 was 1.15 +/- 0.09 g/kg for hM3Dq animals and 0.95 +/- 0.08 g/kg for control animals. On day five, following 3 mg/kg CNO IP injection, hM3Dq animals consumed .77 +/- 0.15 g/kg ethanol and the control group consumed 2.04 +/- .23 g/kg ethanol.

DISCUSSION

The overall aim of this study was to investigate the role of $Cx36^+$ VTA GABA neurons in mediating reward seeking. Despite our initial hypothesis being incorrect, we describe rewarding characteristics of activating $Cx36^+$ VTA GABA neurons as well as neuronal activity within the VTA resulting from chemogenetic stimulation of $Cx36^+$ VTA GABA neurons by CNO in mice injected with hM3Dq DREADD into the VTA. Additionally, we provide evidence in support of subpopulations of VTA GABA neurons. There are ambiguities associated with the identification of DA neurons in GAD GFP mice, as 5% of the neurons in the VTA are neither GABA nor DA neurons (Margolis, Toy, Himmels, Morales, & Fields, 2012), and some GABA and DA neurons express GAD65 (Merrill, Friend, Newton, Hopkins, & Edwards, 2015). Thus, while VTA GABA neurons in GAD GFP mice can be unequivocally identified as GAD67 neurons, caution must be taken in the identification of DA neurons in this study. However, since DA neurons represent 65% and GABA neurons 30% of VTA neurons (Margolis et al., 2012), it is highly likely that the GFP^- neurons evaluated here for CNO effects were DA neurons. Most of the $Cx36^+$ neurons in the VTA were not $GAD67^+$! However, as mentioned above, some DA neurons express GAD65. Unfortunately, GAD65 immunohistochemistry is problematical for this and other technical reasons.

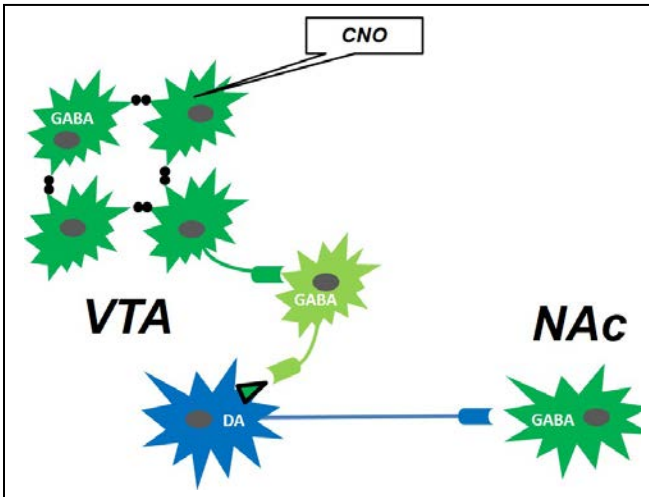

Figure 14: Modified Model of VTA Circuitry Demonstrating Two Populations of VTA GABA Neurons. CNO activates $GAD65^+/Cx36^+$ GABA neurons which inhibit $GAD67^+/Cx36^-$ GABA neurons thereby exciting DA neurons in the VTA and DA release in the NAc.

Notwithstanding these vagaries, we propose that CNO activation of GAD65+/Cx36+ neurons inhibits GAD67+ neurons and ultimately disinhibits VTA DA neurons (Figure 14).

Previous studies conducted in Cx36 KO mice showed that Cx36 KO mice consume less ethanol than WT animals (Steffensen et al., 2011). When taking our firing rate studies into consideration, this effect may be due to an increase in DA neuron activity, which results in an increase in DA neurotransmitter release. We speculate that the animals are not consuming as much ethanol due to elevated levels of DA following CNO administration. This suggests that blocking Cx36 GJs in these cells may lead to an increase in cell activity. Future studies might use mefloquine, a Cx36 GJ antagonist, with Gi-coupled DREADDs to decrease $Cx36^{+}$ neuron activity in order to test whether blocking the GJ alone will lead to similar results as the Gq-coupled DREADDs.

It has been well established that the VTA is a heterogenous brain structure (German, 1993; Nair-Roberts, 2008). However, there have not been many studies characterizing the differences in potential subpopulations of VTA GABA neurons. From our studies, we believe that there is a subpopulation of VTA GABA neurons that express Cx36 GJs. Considering that the majority of the cells that expressed our Cx36 specific DREADD virus were not co-labeled with our GAD67 GFP cells in mice used from our transgenic mouse line, we have reason to believe that Cx36 GJs are expressed in mostly GAD 65^{+} GABA neurons (figure 12). Further studies need to be conducted in order to confirm this hypothesis.

Our firing rate studies suggest that the $Cx36^{+}$ subpopulation of VTA GABA neurons modulates another subpopulation of GABA interneurons which directly synapse on DA neurons within the VTA. It is not clear whether the $Cx36^{+}$ GABA neurons modulate local GABA interneurons or those within the NAc which then synapse on VTA DA neurons. Further studies

would measure changes in neuron activity in hM3Dq-negative VTA GABA neurons following CNO exposure. Based on our hypothesis, we would expect to see a decrease in activity due to inhibition from hM3Dq+ VTA GABA neurons. One limitation to our slice electrophysiology studies is that CNO does not wash out. Studies have shown that even after 2 hours of recording CNO's effects were still present. This disadvantage kept us from seeing a reversal of our effect. However, we are confident that the effects seen are due to CNO superfusion.

In order to study the Cx36+ subpopulation of VTA GABA neurons, we needed to customize a DREADDs viral vector to selectively target Cx36+ neurons. We accomplished this by using a hCx36 promoter in place of either hSyn or CAMKII promoters for our plasmid. This task was made difficult by the immense size of the hCx36 promoter. While we were able to locate a truncated Cx36 promoter from Dr. Florent Allagnat at the Université de Lausanne, we were unable to test the specificity of this promoter for our purposes. Furthermore, there is not much known about the extent of Cx36 expression in the adult brain. Expression of Cx36 GJs is believed to increase following brain injury, particularly following inflammation (Belousov & Fontes, 2013). An increase in Cx36 GJ expression in neurons that do not normally express this protein would interfere with the aims of his study.

Of note are a few concerns with CNO itself. There is some debate about the role of CNO in activating the DREADDs receptor. This is because there have been a number of studies that suggest that CNO is back-transformed to clozapine in guinea pigs and humans (Chang et al., 1998; Jann, Lam, & Chang, 1994; Lin, McKay, & Midha, 1996). This is currently under debate as there have also been studies that do not show back-transformation to clozapine in mice and rats (Jann et al., 1994; Wess, Nakajima, & Jain, 2013). While clozapine has a greater affinity for the DREADDs receptor (Armbruster, Li, Pausch, Herlitze, & Roth, 2007), clozapine has

endogenous receptors and is prescribed as an anti-psychotic suggesting that CNO may have indirect effects on receptors other than DREADDs receptors. In order to account for this, we used a sham virus for our control groups and administered CNO to both the experimental and control groups.

Overall, these studies provide intriguing insight into the circuitry of the mesolimbic dopamine system. Additionally, the implications of this study extend to providing information about the micro-circuitry within the VTA.

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CURRICULUM VITAE

Stephanie Suzette Sandoval Pistorius

EDUCATION

Master's of Science, Neuroscience — Brigham Young University 2015-17

Bachelor's of Science, Neuroscience — Brigham Young University 2008-15

Minor in Chemistry

RESEARCH EXPERIENCE

Graduate Research Assistant — Scott Steffensen Lab, Brigham Young University 2015 — 2017
Neurophysiological pharmacology lab studying mesocorticolimbic dopamine system.

Research Intern — Judith Walters Lab, National Institutes of Neurological Disorders and Stroke 2015
Neurphysiological pharmacology lab studying basal ganglia-thalamocorticol networks.

Undergraduate Research Assistant — Scott Steffensen Lab, Brigham Young University 2009 — 2015

TEACHING EXPERIENCE

Physiology Lab Adjunct Instructor — Utah Valley University 2016

Biology Lab Adjunct Instructor — Utah Valley University 2016

Physiology Lab Instructor — Brigham Young University 2016

French Teacher — Brigham Young University 2013-2014

PUBLICATIONS

Steffensen, S.C., Shin, S.I., Nelson, A.C., Pistorius, S.S., Williams, S.D., Woodward, T.J., Park, H.J., Friend, L., Gao, M., Gao, G., Taylor, D.H., Olive, M.F., Edwards, J.G., Sudweeks, S.N., Buhlman, L.M., McIntosh, J.M., Wu, J. *a6Subunit-containing nicotinic receptors mediate low-dose ethanol effects on ventral tegmental area neurons and ethanol reward.* Addiction Biology. (2017) Submitted.

Nelson, Ashley; Williams, Stephanie; Pistorius, Stephanie; Park, Hyun Jung; Woodward, Taylor; Payne, Andrew; Obray, James; Shin, Samuel; Mabey, Jennifer; Steffensen, Scott. *Functional Shift in GABA(A) Receptors on Ventral Tegmental Area GABA Neurons by Acute and Chronic Ethanol.* Alcoholism: Clinical and Experimental Research. (2017) Submitted.

Allison, D.W., Wilcox, R.S., Ellefsen, K.L., Askew, C.E., Hansen, D.M., Wilcox, J.D., Sandoval, S.S., Eggett, D.L., Yanagawa, Y. and Steffensen, S.C. *Mefloquine effects on ventral tegmental area dopamine and GABA neuron inhibition: A physiologic role for connexin-36 gap junctions.* Synapse (2011) 65:804-8131.

CONFERENCE PRESENTATIONS AND ABSTRACTS

Pistorius, S.S., Obray, J.D., and Steffensen, S.C. *Fighting for our futures: An effort to increase early career scientists' involvement in science policy.* Soc. Neurosci Absts (2016)

Pistorius, S.S., Nelson, A.C., Woodward, T.J., Park, H.Y., Bair, S.D., Matthews, D.M., and Steffensen, S.C. *Functional switch in GABA(A) receptors on VTA GABA neurons by acute and chronic ethanol involves brain-derived neurotropic factor*. Soc. Neurosci Absts. (2016)

Nelson, A.C., Woodward, T.J., Park, H.Y., Pistorius, S.S., Bair, S.D., Matthews, D.M., and Steffensen, S.C. *Glutamate NMDA receptor-mediated plasticity in the ventral tegmental area by acute ethanol*. Soc. Neurosci Absts. (2016)

Nelson, A.C., Woodward, T.J., Park, H.Y., Shin, S.I., Pistorius, S.S., Bair, S.D., Payne, A.J., and Steffensen, S.C. *Glutamate NMDA receptor-mediated plasticity in the ventral tegmental area by acute ethanol*. Research Society on Alcoholism. (2016)

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Park, H.Y., Nelson, A.C., Woodward, T.J., Shin, S.I., Pistorius, S,S., Bair, S.D., and Steffensen, S.C. *Functional switch in GABA(A) receptors on VTA GABA neurons by acute and chronic ethanol*. Soc. Neurosci Absts (2015)

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Pistorius, S.S., Bermudez Cabrera, H.C., Delaville, C. and Walter, J.R. (2015) *Exploring the role of the globus pallidus and its inhibitory projections to the subthalamic nucleus in the generation of high beta oscillatory activity in the parkinsonian rat. Summer Poster Day, National Institutes of Health, Bethesda, MA.*

Nelson, A.C., Woodward, T.J., Park, H.J., Hedges, D.M., Pistorius, S.S., Steffensen, S.C. (2015) *Functional switch in GABA(A) receptors on VTA GABA Neurons by Chronic Ethanol*. *Marylou Foulton Conference, Brigham Young University, Provo, UT.*

Shin, S.I., Mabey, J.K., White, D.N., Sandoval, S.S., Nielson, C.A., Schilaty, N.D., Taylor, D.N., Sudweeks, S.N., Edwards, J.G., McIntosh, J.M., Wu, J. and Steffensen, S.C. *Ethanol inhibits GABA neurons in the ventral tegmental area and dopamine release in the nucleus accumbens via presynaptic α6 nicotinic receptors on GABA terminals*. Soc. Neurosci Absts 38 (2013) 60.08

Wilcox, R.S., Larsen, B.R., Sandoval, S.S., Allison, D.W. and Steffensen, S.C. *Long-term depression of GABAergic synapses on GABA neurons after a single ethanol exposure*. Soc. Neurosci. Absts 36 (2010) 473.19

HONORS AND AWARDS

Graduate Student Society Research Presentation Award, BYU	2016
Scholar, Hispanic Scholarship Fund	2016
Early Career Policy Ambassador, Society for Neuroscience	2016
Hinckley Endowed Chair Travel Award, BYU	2015
Exceptional Summer Student Award, National Institutes of Health	2015
of Research and Creative Activities Mentored Research Grant, BYU	2010
Dean of Students Enriched Environment Scholarship, BYU	2008-15

LEADERSHIPAND SERVICE

President, BYU Science Policy Club	2016-2017
PDBio Department Representative, BYU Graduate Student Council	2015-2017
Coach, Utah Special Olympics	2016
Editor-in-Chief, *Chiasm* BYU Undergraduate Journal of Neuroscience	2012-2014
Service Missionary, Canada Montreal Mission	2011-2012
Managing Editor, *Chiasm* BYU Undergraduate Journal of Neuroscience	2011
Volunteer, Brain Awareness Week	2009-2011, 2013