CHARACTERIZING THE VENTRAL SUBICULUM OUTPUT PROJECTION TO THE
NUCLEUS ACCUMBENS SHELL

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Thesis directed by Assistant Professor Jason Aoto

ABSTRACT

The ventral subiculum serves as the primary output structure of the ventral hippocampus and is comprised of two types of electrophysiologically distinct excitatory neurons called regular- and burst-spiking cells. Regular spiking and burst spiking neurons project to downstream brain regions which includes the nucleus accumbens. The nucleus accumbens is a brain region that controls drug-induced reward seeking and potently regulates dopamine levels in the brain. Dysfunction of the ventral subiculum - nucleus accumbens circuit has been strongly linked to drug addiction and schizophrenia - two disorders that are thought to result from dopamine imbalance. Despite the importance of this circuit to mental health and addiction, our current understanding of the connectivity between these two regions is lacking. Here, through the use of novel circuit tracing methods we showed that the ventral subiculum provides robust excitatory input to the nucleus accumbens. This input primarily formed presynaptic boutons within the shell of the nucleus accumbens. In addition, we characterized the proportion of regular spiking and burst spiking neurons that make up this projection. We showed that the ventral subiculum projection to all outputs consists of 60% burst spiking and 40% regular spiking neurons while nucleus accumbens shell specific output projections contained 30% burst spiking and 70% regular spiking neurons. This information provides the basis for future cell-type specific investigations within this circuit.

The form and content of this abstract are approved. I recommend its publication.

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LIST OF ABBREVIATIONS

A2A: Adenosine 2A receptor
AAV: Adeno-associated virus
ADP: After spike depolarization
AHP: After spike hyperpolarization
AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AVPV: Anteroventral periventricular nucleus
BLA: Basolateral amygdala
BS: Burst spiking
CA1: Cornu ammonis 1
CA3: Cornu ammonis 3
CAV: Canine adenovirus
ChR2: Channel rhodopsin 2
Cre: Cre recombinase
D1R: Dopamine 1 receptor
D2R: Dopamine 2 receptor
DA: Dopamine
DAPI: 4’6-diamidino-2-phenylindole
DG: Dentate gyrus
dHipp: Dorsal hippocampus
DIO: Double inverted orientation, flanking Lox-P sites
DMEM: Dulbecco’s modified eagle medium
EGF: Epidermal growth factor
FBS: Fetal bovine serum
FLEX/FRT: Flp excision switch, flanking FRT sites
Flp: Enhanced flp recombinase
GABA: γ-aminobutyric acid
GluA2: Glutamate receptor subunit A2
HBS: HEPES buffered saline
HEK-293: Human embryonic kidney cell line
LB: Luria broth
LNS: Laminin-nectin-sex hormone binding globulin
MSN: Medium spiny neuron
NAc: nucleus accumbens
NAcC: nucleus accumbens core
NAcS: nucleus accumbens shell
NMDAR: N-methyl-D-aspartate receptor
NRXN: Neurexin
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
PFA: Paraformaldehyde
RS: Regular spiking
TeNT: Tetanus toxin
TTC: Tetanus toxin C-fragment
vHipp: Ventral hippocampus
vSub: ventral subiculum
VTA: Ventral tegmental area
WGA: Wheat germ agglutinin
CHAPTER I
INTRODUCTION TO THE VENTRAL SUBICULUM-NUCLEUS ACCUMBENS SHELL CIRCUIT

Drug addiction is a debilitating mental illness with few effective treatments (NIDA, 2016; NIDA 2017). Addictive drugs provide strong feelings of reward and pleasure that reinforce drug taking behavior (Yokel and Wise, 1975; Gardner, 2011). Users will move from occasional recreational use to habitual compulsive use. The brain’s reward center becomes sensitive to the drug stimulus dampening the effect of natural rewards. The addictive drugs do this through action on dopamine (DA) levels in the brain (DiChiara & Imperato, 1988; Nestler, 2005; Diana, 2011). Enhancement of dopaminergic signaling is a common property shared by drugs of abuse (Bonci et al., 2003). Under the influence of the drug, there is an increase in DA which causes euphoric feelings; however, through chronic use these euphoric feelings are diminished and drug taking becomes an effort to maintain higher levels of circulating DA. This effort begins to require higher and higher amounts of drug causing tolerance and dependence. Without the drug, the brain enters a DA impoverished state which precipitates further drug seeking and taking (Gardner, 2011). In addition, drug associated actions and drug associated cues also contribute to compulsive drug seeking and taking (Jones & Bonci, 2005). The behavioral effects seen in drug addiction are the result of changes in the main reward center in the brain: the mesolimbic DA system.

Although they have different molecular signaling effects, all addictive drugs all activate the mesolimbic dopamine system that consists of the ventral tegmental area (VTA) and the nucleus accumbens (NAc). These structures make up the main reward system within the brain (Olds & Milner, 1954; Koob, 1992; Russo & Nestler, 2013). The initial use of an addictive drug causes an increase in dopamine (DA) concentrations in the VTA and the NAc (Gardner & David, 1999; Wise, 1993; Wise et al., 1995; Lüscher & Malenka, 2011). Nicotine increases firing of DA neurons through nicotinic receptors on the DA neurons (Maskos et al., 2005). Opioids, cannabinoids, and benzodiazepines decrease the activity of GABAergic interneurons within the VTA, indirectly raising
DA levels (Johnson & North, 1992; Tan et al. 2010). Cocaine, amphetamines, and ecstasy are known to target the dopamine transporter (DAT) which blocks reuptake of DA and causes an increase in DA in both the VTA and NAc (Sulzer et al., 2005). Importantly, psychostimulants have in common that they decrease dopaminergic neuron firing through D2R mediated auto inhibition; however, these drugs are able to exert their effects because there is still a net increase in DA being released. These changes in DA provide subjective feelings of reward and pleasure which provides a reinforcing effect mediated primarily by the NAc.

The NAc resides in the ventral striatum and is primarily involved in goal directed behavior (Mannella et al., 2013). This structure assigns motivational value to goals on the basis of internal and external stimuli to select a specific goal. It is able to encode an outcome value associated with a specific goal. In the case of drug addiction, a high motivational value is placed on the consumption of drugs to maintain the DA balance desired by the brain. The NAc is divided into two distinct sub-structures referred to as the NAc core (NAcC) or the NAc shell (NAcS) that serve different functions (DiChiara et al., 2004; Ito & Hayen, 2011). The NAc core receives signals from the basolateral amygdala (BLA) and para-hippocampal regions while the NAcS receives inputs from the BLA and the ventral subiculum of the hippocampus. Based on their different inputs they mediate different behaviors (Lintas et al., 2012). The NAc core controls reward and drug seeking behavior in response to discrete cues. The NAcS mediates reward and drug seeking in response to spatial/contextual information. Although both structures contribute to the pathophysiology of drug addiction, emphasis will be placed on the NAcS for the remainder of the introduction. This is because the NAcS receives input from the hippocampus which is thought to provide the drug user with environmental cues that may precipitate the activation of the NAcS and subsequently drive context induced relapse.

Although the NAcS receives numerous inputs, the strongest relative excitatory input is received from the ventral portion of the subiculum (vSub) (Lüscher and Malenka, 2011; Britt et al., 2012; Kahn et al., 2013). The subiculum as a whole serves as the primary output structure of the
hippocampus (Aggleton & Christiansen, 2015). It receives inputs from various structures, including the CA1 of the hippocampus (Amaral et al., 1991). Lesions of the subiculum in animals have indicated this structure as primarily involved in memory formation and contextual information processing (Taepavarapruk et al., 2014). This function indicates this structure as being involved in context induced relapse, a symptom of drug addiction.

Context induced relapse occurs during withdrawal from use of an addictive substance. A particular environmental context is able to elicit and increase in motivation to seek and take drugs. This environmental context has been previously linked to drug use and triggers the user to relapse. Previous research has connected the vSub-NAcS circuit to reinstatement of drug seeking behavior in response to an environmental stimulus. This circuit has been implicated in context induced relapse for a number of different addictive substances: cocaine, heroin, alcohol, and amphetamine (Bossert et al., 2007; Cruz et al., 2014; Bossert et al., 2016; Marchant et al., 2016). Following extinction of d-amphetamine self-administration in rats, Taepavarapruk et al. were able to initiate reinstatement of drug seeking behavior through electrical stimulation of the vSub-NAc projection circuit. This stimulation did not produce the same behavior in the presence of either D1 or D2 receptor antagonist in the NAc. In addition, pharmacologic inhibition of glutamate signaling via antagonism of either AMPAR or NMDAR has been shown to decrease drug seeking (Jones & Bonci, 2005). These combined results show a direct involvement of the vSub-NAcS in context induced drug relapse seen in drug addiction. This is likely due to synaptic changes in the circuit in response to chronic administration of a drug abuse and subsequent withdrawal from the substance.

At the synaptic level, delicate cell type specific connectivity exists within the vSub-NAcS. The vSub projects two main types of excitatory neurons termed regular spiking (RS) or burst spiking (BS) (Staff et al., 2000; Menendez de la Prida et al., 2003; Eller et al, 2015). These cells are morphologically similar and have no distinct molecular markers making it difficult to elucidate what type of neuron is being observed; however these neurons differ in their electrophysiology which can
be used as an identifier of the cell-type. Supra-threshold current pulses in a RS neuron cause regularly spaced firing of action potentials. In response to slight depolarizing current, BS cells exhibit bursts of two to three action potentials (Stewart, 1997; Jarsky et al., 2008). Because of these electrophysiological differences, the RS and BS neurons transmit information differently and can have different downstream effects.

Evidence has shown that bulk output from the vSub is represented by 70% BS and 30% RS neurons (Kim and Spruston 2012); however, it is thought that these proportions may be output specific. Kim and Spruston (2012) investigated a number of vSub output projections and demonstrated output specific proportions of RS:BS neurons contained within the vSub. Looking within the vSub they showed a proximal-distal distribution of RS and BS neurons with more RS neurons residing in the proximal vSub. Through retrograde labeling, the authors showed NAc output within the proximal location of the vSub. This suggests a possible increase in the RS:BS ratio within the NAcS output as compared to the bulk output from the vSub. The vSub output projection to the NAcS has yet to be characterized.

These RS and BS neurons project excitatory signals from the vSub to the medium spiny neurons (MSNs) of the NAcS. MSNs make up 90-95% of the total neuron population of the NAc (Scofield et al., 2016). These MSNs are GABAergic and send signals to the VTA via either the direct or indirect pathway. The direct pathway is a projection from the NAc to the VTA whereas the NAc projections traveling via the indirect pathway send signals to the VTA via the ventral palladium (VP). The direct pathway MSNs are identified by their expression of the dopamine 1 receptor (D1R) while indirect pathway MSNs primarily express dopamine 2 receptor (D2R) (Gangarossa et al., 2013). Glutamatergic input from the vSub and dopaminergic signaling from the VTA converge at the MSNs. The two dopamine receptors of interest are metabotropic and have different functions in response to DA stimulation. The D1R is coupled to $G_{na,olf}$ G-protein which stimulates cAMP production and the subsequent downstream cascade including protein kinase A activation (Beaulieu & Gainetdinov,
Activation of the D2R inhibits the production of cAMP and the signaling cascade through the
\( G(\alpha_i/o) \) G-protein. The two types of MSNs exhibit different effects over drug-related behaviors
based on these different molecular effects. Literature indicates that D1R MSNs positively regulate
psychostimulant-induced behavioral and cell responses while D2R MSNs negatively regulate these
same behaviors. Similarly, selective optogenetic stimulation of either D1R MSNs or D2R MSNs had
opposing effects over cocaine reward with D1R activation enhancing the rewarding effects while
D2R activation attenuated these rewarding effects (Lobo et al. 2010).

Drug abuse and subsequent withdrawal alters the postsynaptic MSNs changing their
responses to the RS and BS neurons of the vSub. Major plasticity changes occur in the NAc in
response to the development of a drug addiction (Quintero, 2013; Lüscher & Malenka, 2011; Renteria
et al., 2017). Initial use of an addictive drug can alter the postsynaptic MSNs, but it is the effects that
occur during weeks of withdrawal that ultimately precipitate context-induced drug relapse. Pascoli et
al. (2014) investigated the plasticity at NAc MSNs in response to withdrawal. The authors showed
that one month following withdrawal, within vHipp inputs to the MSNs, there is an increase in
AMPA/NMDAR ratio and more GluA2 containing AMPARs are added to the surface. This makes
the cell more likely to fire in response to glutamate stimulation.

Changes in the AMPA receptor composition and surface expression contribute to this
excessive activation of the vSub-NAcS circuit in response to a specific cue which precipitates drug
seeking behavior. These alterations in AMPA receptor surface expression and subunit composition
require a concerted effort from multiple proteins. Receptor subunits must be properly trafficked to
and localized at the synapse. And the synapses must be connected in such a way to facilitate neuronal
communication. The question remains as to how these synapses are changing at the molecular level
which then drives larger, circuit level changes. To begin to interrogate the molecular changes,
genome wide association studies and single nucleotide polymorphism studies were conducted to
identify possible targets. One gene identified through these studies to be affected in drug addiction is
the Neurexin-3 gene (Hishimoto et al., 2007; Lachman et al., 2007). This was very interesting in that the neurexins are a large family of synaptic adhesion molecules. These synaptic adhesion molecules work to alter synaptic morphology and synaptic signaling indicating this gene might be contributing to the synaptic changes in the vSub-NAcS circuit.

There exists three classes of neurexins (NRXN1-3) (Ushkaryov et al., 1992; Ushkaryov & Südhof, 1993; Ushkaryov et al., 1994; Tabuchi & Südhof, 2002; Rowen et al., 2002). These genes have all been associated with neuropsychiatric illness suggesting a clear role mediating synaptic transmission. NRXN1 and NRXN2 have been associated with disorders such as autism and schizophrenia whereas NRXN3 has been associated with autism, schizophrenia, obesity, and drug addiction (Herd-Costa et al., 2009; Vaags et al., 2012). Each of the three neurexins comes in two main isoforms: the long α form or the short β form creating six unique neurexin proteins (NRXN1α-3α and NRXN1β-3β). Both α and β isoforms contain the same C-terminal end, but differ in their N-terminal sequences. α-Neurexins are longer and are composed of six laminin/neurexin/sex hormone-binding globulin-domains (LNS-domains) with three interspersed epidermal growth factor-like domains (EGF-domains). β-neurexins are shorter and lack five LNS-domains with the three EGF-like domains. In addition, neurexins are subjected to extensive alternative splicing with α-neurexins containing 5 splice sites (SS#1-5) while β-neurexins contain just SS#4 and SS#5. Alternative splicing at the SS#4 has been shown previously to serve a key role in determining protein contacts with the NRXNs (Aoto et al., 2013).

NRXN-3 has been associated with modifying AMPAR mediated signaling. Work by Aoto et al. (2016) has shown that selective knockout of both α and β NRXN-3 isoforms causes a drastic decrease in AMPAR mediated excitatory responses through an impairment of post-synaptic levels of AMPAR specifically within the hippocampus. The work suggests a role for NRXN-3 is to stabilize postsynaptic AMPAR (Aoto et al., 2015). Splice site variants, particularly inclusion or exclusion of SS#4, has been shown to also affect AMPAR. Specifically, inclusion of SS#4 within
NRXN-3 decreased AMPAR, but not NMDAR levels while enhancing AMPAR endocytosis. This all suggests that the presynaptic NRXN-3 may be playing a role in modifying the postsynaptic MSNs and changes in expression of variants may contribute to the pathophysiology contributing to context induced drug relapse. Knowledge of NRXN-3’s role in modifying synaptic connectivity and signaling is limited and its function within the vSub-NAcS has not been characterized.

Synaptic changes in the vSub-NAc drive drug addiction symptoms with a strong contribution to context induced drug relapse. The synaptic signaling between the RS and BS cells of the vSub and the D1R MSNs or D2R MSNs of the NAc is sensitive to the acquisition of a drug addiction. It is unclear as to how the presynaptic vSub input of RS and BS neurons projecting to the NAcS changes in response to cocaine administration. It is also unclear as to how these changes in upstream excitatory signaling contributes to changes in post-synaptic NAc MSNs. Because of input specificity these changes are circuit specific and require further investigation due to differences from the commonly seen cocaine-induced plasticity mechanisms. In addition, molecular mechanisms guiding these synaptic changes have not been elucidated. NRXN-3 represents a possible mechanism by which these synaptic changes are occurring. Even though NRXN-3 has been linked to drug addiction and likely plays a role in modifying this circuit, the functional consequences of NRXN-3 changes have yet to be explained. Investigation into the role of NRXN-3 in shaping the synaptic landscape in the vSub-NAcS circuit could provide vital insight into NAcS input specific changes in response to drug addiction.
CHAPTER II
MATERIALS AND METHODS

Animals. Male and female C57BL/6 mice were used. Mice were housed with same sex littermates (2-5 animals per cage) in a 12 hours lights on/off and given ad libitum access to food and water. Wild-Type mice were used for bulk-input experiments. Mice containing a cre-recombinase driven by the D1R promoter, described previously by Gong et al. 2007, were used for D1R/direct pathway experiments. Mice containing a cre-recombinase driven by the A2A promoter, described previously by Durieux et al. 2009, were used for D2R/indirect pathway experiments. Two types of transgenic mice were used for Neurexin experiments. Mice carrying a conditional knockout of both α and β neurexin-3 have been described by Aoto et al. 2015.

Plasmid Production. Plasmids for AAV were constructed using the Gibson Assembly Protocol (Gibson et al., 2009). Briefly, desired inserts are PCR amplified with 20-25 BP overlapping sequence (insert-insert overlap or insert-vector overlap). A vector containing the overlapping sequences is linearized by restriction digest. Both insert(s) and vector are incubated with a 5’ exonuclease, a DNA polymerase, and a DNA ligase (New England Biolabs™). Chemically competent DH5α E. coli cells (New England Biolabs™) were transformed with the plasmid construct and plated on LB agar plates with the appropriate antibiotic for selection. Plates were grown overnight at 37°C. Liquid cultures were made to produce large quantities of the desired plasmid.

Cell Culture. HEK-293T cells were solely used for virus production. Cells were grown at 37°C and 5% CO₂ in DMEM high glucose media with 4mM L-Glutamine, 4500mg/L Glucose, and sodium pyruvate (Hyclone™, Sigma Aldrich™). The DMEM was supplemented with 10% fetal bovine serum (FBS). 10 T-225 flasks of HEK-293T cells were cultured to 60% confluency prior to AAV infection.

Adeno-Associated Virus Production. HEK-293T cells were cultured to the proper confluency and then transfected with three AAV plasmids: AAV plasmid, pDJ, and pHelper. The AAV plasmid was produced via methods listed above. These plasmids were transfected using the calcium phosphate
protocol (Graham and van der Eb, 1973; Jordan et al., 1996). 150µg of each plasmid is mixed in a tube with 13.5 mL ddH$_2$O, and 1.5mL of 2.5M CaCl$_2$ (Sigma Aldrich™). This tube is slowly added to a second tube containing 15mL of 2X Hank’s balanced salts (HBS) with gentle vortexing. The mixture is incubated at room temperature for 10-15 minutes. Once DNA-CaPO$_4$ precipitate reaches an observable size, 3mL of the DNA solution is added to each of the 10 T-225 flasks containing HEK-293T cells.

HEK-293T cells were harvested two days post infection. Cultures were washed once with 1X PBS. 1XPBS+ 10mM EDTA was used to dissociate the cells from the flask. Cells were pelleted by centrifugation for 15 minutes at 1500g in a Sorvall™ Legend RT tabletop centrifuge. The supernatant was removed and the cell pellet was dissolved in Freezing Buffer (150mM NaCl, 20mM Tris [pH 8], 2mM MgCl$_2$). The solution was then exposed to three freeze thaw cycles using liquid nitrogen and a 37°C water bath. Benzonase nuclease was added to a final concentration of 50 units/mL and the solution was placed into a 37°C water bath for 30 minutes. Following the incubation, the solution was centrifuged at 3000g for 30 minutes. The supernatant was removed and added to the top of an iodixanol (Optiprep™) gradient. The iodixanol gradient was made in ultra-centrifuge tubes and consisted of 1 mL layers of different densities: 15% (15% iodixanol, 1XPBS, 1M NaCl, 1mM MgCl$_2$, 2.5mM KCl), 25% (25% iodixanol, 1XPBS, 1mM MgCl$_2$, 2.5mM KCl), 40%(40% iodixanol, 1XPBS, 1mM MgCl$_2$, 2.5mM KCl), and 60%(60% iodixanol, 1XPBS, 1mM MgCl$_2$, 2.5mM KCl) (All salts obtained from Sigma Aldrich™). Small amounts (<100µL) of 0.5% Phenol Red are added at different amounts to the different densities to allow visualization of the gradient. Ultra-centrifuge gradient tubes were placed in a Beckman™ Optima L-90K ultracentrifuge and spun at 63,500 RPM (~400,000g) for two hours at 4 °C. Following the spin, the 40% layer is removed and placed into 10 mL PBS+1mM MgCl$_2$. Centricron centrifugal Filter tubes MWCO 100,000 (Millipore™) were equilibrated with 5mL of PBS+1mM MgCl$_2$ by spinning at 3000g for 5 minutes. Virus solution was added to the equilibrated filter tubes and spun at 3000g for 1 hour. Tubes were checked every 20 minutes to monitor volume in
the filter. When the volume in the filter was reduced to <1 mL, 2mL of FBS free DMEM media was added to the tube and the spin is resumed. Following two additional washes with FBS free DMEM, the tube is spun until the virus volume in the filter is reduced to ~250µL. 4µL aliquots of virus were stored at -80°C.

**In-vivo Virus Injections.** All procedures were performed in accordance with the Institutional Animal Care and Use Committee (IUCAC) guidelines. Male and female C57BL/6 mice, of the given genotype, were weened and injected at p21. Mice were anesthetized with an intraperitoneal injection of (250 mg/kg) tribromoethanol (Avertin™) and placed in a stereotaxic apparatus following a loss of tail/toe pinch response. Holes were drilled through the skull bone to the dura at the given coordinates: A/P from Bregma,-3.15; M/L from midline, +/-3.05; D/V from dura, -3.15 (vSub) or A/P, +1.7; M/L, +/-0.95; D/V, -3.85 (NAc). 500-700nL of virus solution was injected intracranially into both hemispheres at a rate of 15µL/hour. Injection needles remained in place following injection for five minutes prior to removal from the brain. Following injection, animals' scalp is sealed back up using 3M Health-Care vetbond™ surgical glue. A subcutaneous dose of 5 mg/kg carprofen (Carprieve™, Norbrook) was administered post-surgery and the animals recovered in the presence of a heating pad for 60-90 minutes.

**Histological Analysis of Injections.** Mice were anesthetized via inhalation of isoflurane until respiration slowed and deep anesthesia was reached. The chest cavity was exposed and a perfusion system was placed into the right ventricle of the heart. 15 mL of a 4% W/V Paraformaldehyde (PFA) (Electron Microscopy Sciences) in PBS was then perfused trans-cardially. Mice were decapitated and the whole brain was immediately removed. The brain was equilibrated in a 4% PFA, 30% sucrose solution (Sigma Aldrich™) at 4°C overnight. Fixed brains were washed with two times with 1X PBS and sliced into 50 µm sections using a Leica™ VT1000P vibratome. Slices were incubated in PBS for 20-30 minutes. Slices were then mounted onto Denville™ ultra-clear positively charged microscope slides with DAPI Fluoromount-G™ (Southern Biotech™). 10, 20, and 40X images of slices were
acquired using a Zeiss microscope (Zeiss-Axio Imager M2). Images were analyzed using Zen-2 Pro software (Zeiss™).

**Electrophysiology.** For bulk input experiments, brains were harvested from animals at p28-30. For cell-type specific experiments, brains were harvested from animals aged p56-p70. Mice were anesthetized via inhalation of isofluorane until respiration slowed. Animals were decapitated and the brain was immediately extracted and placed in ice cold cutting solution containing 85mM NaCl, 75mM sucrose, 2.5mM KCl, 1.3mM NaH2PO4, 24mM NaHCO3, 0.5mM CaCl2, 4mM MgCl2, 25mM D-Glucose (all salts were obtained from Sigma Aldrich™). The brain was horizontally sliced into 300 µm slices using a Leica™ VT1200 vibratome. Slices were equilibrated for 30 minutes at 31°C and then one hour at room temperature in artificial cerebrospinal fluid (aCSF) containing 126mM NaCl, 2.5mM KCl, 1mM NaH2PO4 monobasic, 26.2mM NaHCO3, 1.3mM MgSO4 heptahydrate, 11mM D-Glucose and saturated with 95%O2/5%CO2.

Slices were then transferred to the recording chamber, fixed to a modified microscope stage and allowed at least 30 minutes to equilibrate prior to recording. During recording experiments, slices were minimally submerged and superfused continuously with oxygenated aCSF (4.0 mL/min). A low power objective, 2.5X, was used to identify the vSub. A high-power water-immersion objective, 63X, with infrared video was utilized to visualize individual neurons for recording. Recording pipettes were fabricated from capillary glass (World Precision Instruments™) and exhibited tip resistances of 3+/−2 MΩ when filled with the intracellular solution described below containing. A multi-clamp 700B amplifier (Molecular Devices™) was used for current-clamp recordings. A current-step protocol was used consisting of -50, 0, 50, 100, 150, 200, 250, and 300 pA current injections. Current-clamp recordings were generated using Clamp-Fit™ version 10.5. For whole-cell recordings of synaptic currents in pyramidal neurons under current clamp, a current step protocol at the patch pipette saline solution was composed of 137mM K-Gluconate, 5mM KCl, 10mM HEPES (pH7.2), 10mM phosphocreatine (pH7.2), ATP-Mg2, and GTP-Na2 (pH adjusted to 7.4 and osmolarity adjusted to
280mosmol/l). AlexaFluor594™ was added to the pipette solution. We did not analyze cells that had access resistance of greater than 30 MΩ, baseline shift of greater than +/-15%, or resting membrane potential greater than -55 mV.

**Statistics:** Electrophysiology data was subjected to a two-tailed student’s T-test with a significance level set at p<0.05.
CHAPTER III
RESULTS

The Ventral Subiculum Sends Dense Projections that Terminate in the Nucleus Accumbens Shell

Drug addiction research has focused heavily on the mesolimbic dopamine (DA) system which consists of the Nucleus Accumbens (NAc) and the Ventral Tegmental Area (VTA) (Olds & Milner, 1954; Koob, 1992; Wise, 1993; Wise et al., 1995). Excessive activation of the NAc has been shown to precipitate drug seeking and taking and this is likely mediated through synaptic changes caused by excitatory inputs to the NAc. It is thought that glutamatergic input to the NAc provides important context and cue information that can influence the motivational value the NAc assigns to a particular behavior (Mannella et al., 2013). Changes that occur with inputs to the NAc can alter the motivational value assigned to a particular context thereby affecting the behavioral responses to that specific context.

Previous studies have shown that ventral hippocampus (vHipp) provides robust, excitatory, synaptic input into the nucleus accumbens shell (NAcS) and that these connections are important for context-dependent drug use and relapse (Taepavarapruk et al., 2014; Cruz et al., 2014; Bossert et al., 2016). However, these studies failed to delineate between the different sub-regions of the hippocampus and it is unclear which sub-region is responsible for communicating with NAcS. The subiculum is the major output of the hippocampus and is likely the key hippocampal subregion involved in connecting the vHipp with NAcS. In order to explore the contribution of vSub projections to NAcS, we first needed to identify stereotaxic coordinates to precisely infect the ventral subiculum. To do this, we performed a series of injections where we systematically adjusted the anterior-posterior (A-P), medial-lateral (M-L) and dorsal-ventral (D-V) coordinates. To identify the site of injection, we used an adeno-associated virus (AAV) that expresses the red fluorescent protein, mRuby, under the control of a human synapsin promoter (hSYN) (Figure 1A). The hSYN promoter restricts expression to neurons, effectively eliminating glial contamination (Kügler et al., 2003). We found that the injection coordinates (in mm):
A/P from Bregma,-3.15; M/L from midline, +/-3.05; D/V from dura, -3.15 resulted in reproducible infections of vSub, with minimal viral spillover into surrounding regions (Figure 1B). The precise targeting of vSub with in vivo stereotaxic injections of AAV now allowed us to use cutting-edge anterograde circuit tracing viruses to map the precise projection targets of vSub.

To identify if vSub projects to NAcS and to examine if vSub projection axons form synapses with neurons in the NAcS, we generated a viral construct that labels presynaptic boutons. Under the control of a human synapsin promoter, the viral transfer vector produces a single peptide that is comprised of eGFP-P2A-synaptophysin-mRUBY (AAV hSYNeGFP P2A synaptophysin-mRuby) (Figure 2A). The short P2A sequence is self-cleaving, which generates soluble eGFP and the fusion protein synaptophysin-mRuby (Szymczak et al., 2004; Luke et al., 2008; Wang et al., 2015). We used eGFP fluorescence to identify the exact injection site in vSub, while the synaptophysin-mRuby fusion localizes exclusively to presynaptic terminals because synaptophysin is a presynaptic vesicle-associated protein (Oh et al., 2014). We injected AAV hSYNeGFP P2A synaptophysin-mRuby into vSub and found abundant mRuby-positive puncta specifically in the NAcS (Figure 2B). We found minimal red fluorescence in the dorsal striatum or the nucleus accumbens core (NAcC) (Figure 2C). Thus, we concluded that the primary termination for vSub neurons that project to the nucleus accumbens is the NAcS. These results highlight the exquisite projection specificity of vSub output to the NAcS. Moreover, the selective connectivity of the vSub-NAcS circuit provides insight into why the NAcS has been implicated in context-dependent drug relapse.

**Regular Spiking and Burst Spiking Neurons Project in Distinct Proportions to the Nucleus Accumbens Shell**

The vSub is comprised of two types of glutamatergic projection neurons, termed regular- and burst-spiking (Staff et al., 2000; Menendez de la Prida et al., 2003). These two classes of projection neurons are currently only identified by their electrophysiological spiking patterns because they are
morphologically similar and molecularly uncharacterized. To date, there are no known selective molecular markers between RS and BS neurons that allows for cell-type specific visualization via modern genetic techniques. The current method of identification of these two populations requires analysis of their firing of action potentials. With brief somatic current injections, RS neurons fire a single action potential while the BS neurons fire 2-3 action potentials at high frequency (Stewart, 1997; Jarsky et al., 2008) (Figure 3). These distinct firing patterns have been shown to have significant functional implications. RS and BS neurons are hypothesized to convey information about the nature of stimuli through these differences in firing properties (Cooper, 2002). Burst spiking cells are thought to convey the importance of new stimuli by converting brief input into long-lasting and strong output to activate connected neurons. By contrast, regular spiking cells do not amplify input, instead, they are thought to encode for normal stimuli. These two cell types ultimately influence postsynaptic plasticity by scaling the presynaptic calcium concentration to increase presynaptic release probability and/or increase the likelihood that the postsynaptic cell will fire an action potential. Through bursts of APs, BS neurons dramatically increase the influx of calcium into the postsynaptic cells as compared to the RS neurons. This allows the BS neurons to exert a stronger influence over the postsynaptic landscape than that of RS neurons. This suggests there exists a delicate balance of RS:BS neurons projecting from the vSub to the NAcS and these two populations encode valuable and discreet information. Thus, it is essential to identify the distribution and proportion of these two functionally and electrophysiologically distinct neurons that participate in the vSub-NAcS circuit. The functional characterization of the cellular identities of subicular input into NAcS will provide insight into how reward-relevant contextual information is encoded. Studies thus far analyzing the vSub output projection to the NAcS have suffered from a lack of sub-structure specificity and less than optimal tracing methods that were detrimental to neuronal health. For our experiments we required healthy cells for an impartial sampling of the projection neurons, accurate electrophysiological assessment, and subsequent single cell RNA-sequencing experiments.
In order to investigate the distribution and proportion of RS and BS neurons that innervate the NAcS, we first needed to define coordinates to deliver retrograde circuit tracing viruses specifically into the shell of NAc. Using a similar approach as was used for the vSub, we performed systematic injections of AAV-hSYN-mRuby until we identified coordinates that reproducibly targeted the NAcS (Figure 4A). The injection shown in Figure 4B displays a more local injection of the virus based upon the lower volume used. The white and black circles over the coronal image show the site of infection. There was some spread into the ventricle due to the fluid contained within the ventricles. For stereotaxic injections into the NAcS we adapted known coordinates to A/P, +1.7; M/L, +/-0.95; D/V, -3.85. With these coordinates we could now perform retrograde tracing to identify vSub neurons projecting to the NAcS.

A number of methods of retrograde circuit tracing have been well characterized (Nassi et al., 2015). Two methods: modified rabies virus and canine adenovirus type 2 (CAV-2) have been used extensively. Unfortunately, both of these viral retrograde tracers had major impediments that warranted the use of other methods. Although the modified rabies virus provides robust trans-synaptic retrograde tracing, this virus has well documented toxic effects (Kuypers & Ugolini, 1990; Inoue et al., 2004; Callaway, 2008; Ohara et al., 2013). This requires experiments to be completed within two weeks and severely compromises the health of labeled cells. The CAV-2 also provides robust trans-synaptic retrograde labeling, but this virus is extremely difficult to obtain and nearly impossible to modify it to suit the needs of this experiment (Kremer et al., 2000; Hnasko et al., 2006; Salinas et al. 2009; Zussy & Salinas, 2014). Therefore, we did not use either of these methods to retrogradely label vSub neurons within the NAcS projection.

In order to achieve retrograde tracing with maximal efficiency and minimal toxicity, we took advantage of the recently developed recombinant adeno-associated viruses (rAAVs, also called AAV2). It has been shown to carry robust retrograde functionality, up to two orders of magnitude increase in retrograde transport over existing variants, and efficiency comparable to synthetic tracers (Tervo et al.,
However, to our knowledge, whether it produces significant toxic effects to infected cells has not been tested.

We first asked, if efficient retrograde tracing can be achieved using rAAV-retro system with minimal toxic effects. We constructed AAV2-retro-hSYN-mRuby and carried out stereotaxic injections into the NAcS of P21 wild type mice (Figure 5A and 5B). We harvested the brains for the assessment of retrograde tracing and the health of the labeled neurons one week after the injection. Using epi-fluorescence imaging, high levels of labeling indicated by mRuby was observed throughout vSub only one week after injection (Figure 5C). The mRuby was largely concentrated in the soma and proximal dendrites of the projection neurons. This labeling allows for the future quantification of projection neurons in the neuronal population and gross morphological assessment of these neurons.

Next, we sought to answer the question, if RS and BS participate differentially to the bulk subicular input to NAc. Using slice electrophysiology, under whole cell current clamp mode, a step current injection protocol is used to assess the neuronal firing patterns. Neurons are then categorized as RS or BS based upon the action potential firing pattern in response to depolarizing current injections. In addition to classifying these neurons as either RS or BS neurons, a number of properties were extracted from the electrophysiology experiments. We analyzed resting membrane properties (potential, resistance, and capacitance), access resistance and depolarization induced properties (rheobase, spike after depolarization (ADP), spike after-hyperpolarization (AHP), sag, and rebound) (Table 1).

This information was acquired to determine cell health and to investigate potential electrophysiological properties, other than AP, that may distinguish the two populations. In addition, following recording slices are fixed, mounted and imaged. The recorded cell is filled with AlexaFluor™ 594 during electrophysiology experiments which allows for visualization of the localization of the neuron from which the recording was obtained (Figure 6A). As is seen in figure 6A the neuron labeled and recorded from resided in the proximal-central area of the vSub.
We used WT uninfected animals as controls for these experiments to determine if vSub-NAcS output contained similar proportions of RS:BS neurons or if there was clear output specificity. Uninfected animals were harvested at p28 and were subjected to a rigorous electrophysiology protocol. The neurons sampled were unlabeled and represented a random population of vSub RS and BS neurons in proximal, center, and distal locations within the vSub. In control experiments, the intrinsic membrane properties and depolarization induced properties were not significantly different and are not shown. Through these experiments, it was determined that the population of vSub neurons projecting to all outputs is 40% RS, 60% BS (Figure 6B).

However, when we selectively isolated neurons that project to NAcS, we found that the population of vSub-NAcS projection neurons is made up of 30% BS neurons and 70% RS neurons, indicating a distinct pattern of participation of BS and RS neurons in the projection circuit (Figure 6C). In contrast, arbitrary sampling of neurons in the vSub projecting to all outputs revealed a distribution of 40% RS and 60% BS which was different than previous literature that showed a 70% BS and 30% RS. As shown in Table 1, in these populations, resting membrane potential was -65.6 ± 0.6 mV for RS (n=47) and -61.4 ± 1.1 mV for BS (n=23, p<0.001). The significance of this difference is not clear and requires further investigation. With all other electrophysiology properties, there was no significant difference between the two populations. Resting membrane capacitance was 79.6 ± 4.8 pF for RS and 87.3 ± 6.8 pF for BS (p=0.36) and resting membrane resistance was 94.5 ± 9.9 MΩ for RS and 80.3 ± 10 MΩ (p=0.37), access resistance was 16.94 ± 1.24 MΩ for RS and 17.45 ± 1.05 MΩ for BS (p=0.79). For depolarization induced properties: rheobase was 90.2 ± 6.9 pA for RS and 90.9 ± 9.7 pA for BS (p=0.98), 81 ± 6% of RS and 84 ± 8% of BS neurons exhibited ADP (p=0.79), 93 ± 4% of RS and 100% of BS neurons exhibited AHP (p=0.23), and 85 ± 5% of RS neurons and 91 ± 6% of BS neurons exhibited adaptation (p=0.53). RS neurons had an average sag of 1.78 ± 0.20mV while BS neurons had an average of 1.97 ± 0.27mV (p=0.61). For rebound, RS neurons averaged 1.98 ± 0.23 mV while BS neurons averaged 2.01 ± 0.26mV (p=0.92).
Unfortunately, we did not find any defining electrophysiological properties, other than AP dynamics and resting membrane potential, between the two populations, but this was not unexpected. The finding that there is a disproportionate number of RS neurons participating in the vSub-NAcS circuit was surprising. It suggests that the activation of this circuit might not be as dependent on novel contextual stimuli which is encoded by BS neurons instead, activity might be driven by non-novel (familiar) contextual stimuli which is encoded by RS neurons. The unexpected finding that vSub projections to NAcS consists of 70% RS neurons and 30% BS neurons raises the question whether this distribution is preserved independent of the postsynaptic target or if there is further projection specificity that is dependent on the postsynaptic target being a D1R or D2R expressing MSN. Insight into the fundamental cell type specific wiring of this circuit is essential to strengthen our understanding of this disease relevant circuit.

**Cell Type Specific Connectivity between Regular Spiking and Burst Spiking Neurons of the Ventral Subiculum and the D1R and D2R Expressing Medium Spiny Neurons of the Nucleus Accumbens Shell**

We showed that the vSub-NAcS projection circuit is made up of 70% RS and 30% BS neurons, but how these neurons selectively innervate the two different populations of NAcS medium spiny neurons (MSNs) is not understood and has never been investigated. The NAc is made up of 95% GABAergic medium spiny neurons (Scofield et al., 2016). There exists two main types of MSNs that are identified by their expression of either dopamine 1 receptor (D1R) or the dopamine 2 receptor (D2R). It has been shown that D1R MSNs positively regulate psychostimulant-induced behaviors while D2R MSNs negatively regulate these behaviors. In addition, cocaine induced plasticity occurs primarily at D1R MSNs (Pascoli et al., 2014). This all suggests that the selectivity of vSub inputs to either MSN population may serve important functions governing behavioral responses associated with
drug abuse and drug addiction. Understanding this connectivity pattern is essential because changes in the synaptic signaling may very well be contributing to the pathology associated with drug addiction.

Unfortunately, we could not use the AAV2-retro in cell-type specific experiments for a number of reasons. To restrict expression of retrograde migration and labeling from the NAcS we will utilize transgenic mice expressing Cre in either D1R or D2R MSNs. Because the AAV2retro is directly uptaken by presynaptic neurons it cannot be used to deliver Cre dependent expression constructs to the NAcS. Therefore, we investigated other AAV circuit tracing methods in order to analyze the cell-type specific innervation pattern.

To do this, we again made use of AAV carrying tracer proteins fused to fluorescent reporters similar to the anterograde circuit tracing described previously. These fusion products’ expression and subsequent retrograde migration can be placed under the control of a recombinase which allows for selective expression within either population of MSNs based on the transgenic mouse used. Wheat germ agglutinin and tetanus toxin C-fragment are two retrograde tracer proteins that have been well characterized. Here, we examined their ability to retrogradely label vSub neurons projecting to the NAcS.

We first tested the ability of AAV carrying the WGA protein to evaluate the potential for use in the cell-type specific investigations. WGA is a useful for retrograde tracing based on its low toxicity and mono-synaptic retrograde tracing (Gonatas et al., 1979; Levy et al., 2015). WGA will enter the synapse from the postsynaptic cell and be uptaken by the presynaptic cell through an undefined mechanism. This provides a strong visualization of the presynaptic cells projecting to a particular population of postsynaptic contacts. We performed retrograde tracing using stereotaxic injections of 1 µL of AAV-hSYN-mCherry-IRES-WGA-eGFP into the NAcS (Figure 7A). The internal ribosome entry site (IRES) is an enhancer sequence that allows the translational machinery to start protein synthesis by internal initiation (Fussenegger et al. 1998; Renaud-Garbados et al. 2015). This allows for translation of two different peptides: hSYN-mCherry and WGA-eGFP. hSYN-mCherry expression
is used as a marker of the injection site while the eGFP signal visualizes the WGA retrograde migration (Figure 7B). Animals were injected at p21, but were not harvested until p49. This was based upon previous studies that indicated 3-4 weeks is required for efficient WGA expression and retrograde migration. A coronal slice of the NAc shows the injection site labeled by mCherry. The expression is localized to the injection site which is shown by the white and black circles. There was some spread into the dorsal striatum; however, this is not indicative of a faulty injection. The mCherry will diffuse into local neurons following the long expression times. The eGFP fluorescence seen within this coronal slice shows the beginning of the WGA retrograde migration. A coronal slice posterior of the NAc image, displays the vSub with a representative image on the right (Figure 7C). In Allen Brain Atlas™ image, the vSub is outlined in yellow. In the tracing image, it is clear that WGA migrated from the NAcS and successfully labeled vSub neurons. Unfortunately, using the WGA presents a slight problem in that there is no trans-neuronal amplification, therefore for these studies we used increasing amounts of injected virus (0.5, 0.7, 1 µL) to obtain stronger labeling. This could present a problem due to the spread of virus from the injection site. For these experiments, it was not as important because we were just determining its ability to robustly label vSub neurons. No electrophysiology experiments were performed on these WGA-eGFP labeled vSub neurons.

We next investigated the effectiveness of the tetanus toxin-C fragment (TTC) to label neurons in the vSub that project to the NAcS. The TTC is a portion of the full length, toxic, tetanus toxin (TeNT) (Fishman & Savitt, 1989; Roux et al., 2005; Toivonen et al., 2010). The fragment retains the TeNT affinity for neurons and the rapid uptake by these neurons. Once the TTC enters the brain it is uptaken by the presynaptic neuron through interactions with the membrane and subsequent clathrin mediated endocytosis. The TTC is then transported to the soma and from there it can continue up the dendrites of the presynaptic neuron. We made injections of 1 µL AAV-CAG-Nrxn1βSP-eGFP-TTC into the mouse NAcS (Figure 8A). The Nrxn1β signal peptide drives the expression of this construct specifically within excitatory neurons. This construct did not contain a fluorescent marker for the site
of infection. For these experiments, animals were injected at p21 and harvested at p35, p42, and p49 to determine the expression time required for robust labeling. A coronal slice of the NAc displays the injection site (Figure 8B). There is strong eGFP expression seen around the injection site which is demarked by the white and black circles. The spread of eGFP is not indicative of a poor injection, but rather the beginning of the retrograde migration. A coronal section posterior of the injection site is displayed (Figure 8C). The green labeling in the representative image shows the two sites that displayed some eGFP expression. The dHipp was marked as well as the anteroventral periventricular nucleus (AVPV) of the hypothalamus. This was labeled due to known connections between this structure and the NAcS (Hsu & Price, 2009). This helped demonstrate that we were, in fact, getting retrograde migration of the eGFP-TTC, but this migration did not make it to the vSub. Unfortunately, this was as far as the virus spread even with increased volume injections and longer expression times. Again, the issue with these retrograde tracers is the lack of trans-neuronal spread following infection. Therefore, for cell-type specific investigations, we sought a way by which we could amplify this signal providing to produce more robust labeling of cell-type specific connections within the vSub-NAcS circuit.

Our experiments showed WGA as an effective retrograde tracer; however, the labeling of vSub neurons was not as pronounced as the AAV2-retro. In an effort to amplify this WGA labeling for subsequent electrophysiology experiments, we will use a dual injection protocol. We generated two AAVs for use in these experiments (Figure 9). First, we produced a Cre-recombinase dependent expression vector: AAV-DIO-CAG-WGA-Flpe-mRuby which will be injected into the NAcS (Figure 9A). This vector is constructed in such a way that it is flanked by LoxP sites in the double inverted orientation (Van Duyne, 2001; Tronche et al., 2002; Saunders et al., 2012). Injecting this virus into the NAcS of either the D1R-Cre or A2A-Cre expressing transgenic mice allows for expression of the WGA retrograde tracer fusion product in a cell-type specific manner. From the particular NAcS MSN, the WGA-Flpe construct travels in the retrograde direction to the presynaptic vSub neurons where it will
interact with our second AAV, FLEX/FRT-eGFP (Figure 9B). FLEX/FRT-eGFP will be injected into the vSub, but will only express in the presence of Flpe which will travel in a retrograde direction to the vSub via WGA (McLeod et al., 1986; Branda & Dymecki, 2004). This will produce dual labeling of vSub neurons confirming cell-type specific connections. With this labeling strategy, we will subject the labeled neurons to the same rigorous electrophysiology protocol. This will allow us to dissect the cell-type specific innervation pattern between RS and BS neurons of the vSub and D1R or D2R MSNs of the NAcS. Determining this cell-type specific innervation pattern is essential in furthering our knowledge into the mechanisms driving context induced drug relapse.
Figure 1: Identification of stereotaxic coordinates for ventral subiculum injections. Left, sagittal view of the mouse brain showing the injection protocol. Animals were injected with 0.7 µL of AAV-hSYN-mRuby at p21 and brains were harvested at p28. Right, horizontal section of the mouse brain showing the ventral hippocampus. mRuby expression labels the sight of infection. Coordinates used for injection: -3.15 A/P, +/-3.05 M/L, -3.15 D/V (cornu ammonis 1, CA1; cornu ammonis 3, CA3; dentate gyrus, DG; entorhinal cortex, EC; ventral hippocampus, vHipp; ventral subiculum, vSub).
Figure 2: Anterograde tracing from ventral subiculum to the nucleus accumbens shell. (A) Sagittal view of the mouse brain showing the injection protocol. Animals were injected at p21 with 0.7µL AAV-hSYN-eGFP-P2A-synaptophysin-mRuby at the defined vSub stereotaxic coordinates. (B) Horizontal section of the mouse brain. eGFP labels the injection site within the vSub. (C) Left, coronal slice showing mRuby labeling within the NAcS. Right, representative image from the Allen Brain Atlas™ outlining the NAc in red with NAcC and NAcS locations labeled.
Figure 3: Representative traces showing the action potentials fired in response to depolarizing current. Right, burst spiking cell displays a bursting pattern during the first spike. Left, regular spiking cell displays regularly spaced action potentials.

Figure 4: Identification of stereotaxic coordinates for NAcS injections. Left, sagittal view of the mouse brain displaying the injection protocol. Animals were injected at p21 with 0.7 µL of AAV-hSYN-mRuby and brains were harvested at p28. Right, coronal slice showing the injection site as labeled by mRuby next to a representative image from the Allen Brain Atlas™, with the NAc outlined in red. The white and black circles within the image show the injection site at +1.7 A/P, +/-0.95 M/L, -3.85 D/V (Anterior commissure, AC).
Figure 5: AAV2-retro provides robust retrograde labeling of vSub neurons. (A) Sagittal view of the mouse brain showing the injection protocol. At p21, animals were injected with AAV2-retro-hSYN-mRuby at +1.7, +/-0.95, -3.85 and brains were harvested at p28. (B) Coronal slice of the mouse brain showing the injection site (white and black dots). mRuby signal shows local injection into the NAc. (C) Horizontal slice showing the retrograde migration of AAV2-retro-hSYN-mRuby. Within the vHipp there is robust labeling of the vSub, particularly in the proximal portion. The white circle outlines the vSub in the coronal slice which is similarly marked with a yellow outline in the representative image.
<table>
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<td>Rebound (mV)</td>
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Table 1: Properties of RS and BS neurons within the ventral subiculum-nucleus accumbens shell circuit. All values are +/- standard error. Resting membrane potential (p<0.001), resting membrane capacitance (p=0.36), resting membrane resistance (p=0.37), access resistance (0.79), rheobase (p=0.98), ADP (p=0.79), AHP (p=0.23), Adaptation (p=0.53), Sag (p=0.61), Rebound (p=0.92).
Figure 6: Electrophysiological characterization of ventral subiculum output projection to the nucleus accumbens shell. (A) Horizontal image of the hippocampus. 0.7 µL of AAV2-retro-hSYN-mRuby was injected into p21 animals at the defined NAcS stereotaxic coordinates. mRuby signal displays the retrograde labeling of vSub neurons. The AlexaFluor™ 594 labels the neuron from which the recording was obtained. (B) Non structure specific output (control) from the vSub consisted of 60% BS and 40% RS. (C) NAcS specific output from the vSub consisted of 30% BS and 70% RS (regular spiking, RS; burst spiking, BS).
Figure 7: Wheat germ agglutinin retrograde tracing labels ventral subiculum neurons connected to the nucleus accumbens shell. (A) Sagittal view of the mouse brain displaying the injection protocol. 1 µL of AAV-mCherry-IRES-WGA-eGFP was injected into the NAcS at the defined stereotaxic coordinates at p21 and brains were harvested at p49. (B) Coronal slice showing the injection site into the NAc (white and black circles). The mCherry expression marks the site of injection within the NAc. The WGA-eGFP signal is seen migrating from the site of infection. (C) Coronal slice of the hippocampus showing the vSub with a representative image from the Allen Brain Atlas™ on the right. The vSub is effectively labeled with eGFP.
Figure 8: Tetanus toxin C-fragment retrograde tracing fails to label ventral subiculum neurons projecting to the nucleus accumbens shell. (A) Sagittal view of the mouse brain with the injection protocol. 0.7 μL of AAV-Nrxn1βSP-eGFP-TTC was injected into the NAcS of p21 animals at the defined coordinates. (B) Coronal slice displaying the injection site. eGFP expression labels the initial site of infection. (C) Coronal slice anterior of the vSub showing labeling within the dorsal hippocampus and the AVPV of the hypothalamus denoted by the green labeling within the Allen Brain Atlas™ representative image on the right (anteroventral periventricular nucleus, AVPV).
Figure 9: Dual injection protocol to label cell-type specific connections between the ventral subiculum and nucleus accumbens shell. (A) DIO-WGA-Flpe-mRuby is injected into mouse expressing Cre recombinase in either D1R MSNs or D2R MSNs. Inversion and expression occurs in the presence of Cre recombinase. (B) FLEX/FRT-eGFP will be injected into the vSub. Interaction of WGA-Flpe-mRuby and FLEX/FRT-eGFP produces dual labeling of vSub neurons making up a cell-type specific projection.
CHAPTER IV
DISCUSSION

Prior investigations into the ventral subiculum-nucleus accumbens shell circuit relied on electrophysiology experiments, behavioral analysis, and/or pharmacological approaches to reveal communication between these two structures and that the interaction was mediated through glutamaterigic input, but these studies still lacked the tools to fully characterize this specific circuit (Yang and Morgenson, 1985; Mulder et al., 1998; Brudzynski and Gibson, 1997; Britt et al., 2012; Kim & Spruston, 2012). More recent advances in behavioral manipulations has identified this circuit as a major contributor to context induced drug relapse (Taepavarapruk et al., 2014; Cruz et al., 2014; Bossert et al., 2016). This suggests understanding this circuit is key to furthering our knowledge of the underlying synaptic and molecular mechanisms that drive this context induced drug relapse.

This study took advantage of novel circuit tracing methods to characterize the vSub output projection to the NAcS. We defined stereotaxic coordinates for precise injections into the vSub and adapted known coordinates for injections of virus into the NAcS. This allowed for reproducible infections with circuit tracing virus. With our new coordinates we were able to selectively target the vSub for anterograde tracing experiments and the NAcS for retrograde tracing experiments. Through the use of the synaptophysin-mRuby fusion construct, we showed the vSub as providing robust input that terminates and forms active presynaptic boutons within the NAcS. We used the novel AAV variant, AAV2-retro to effectively label vSub neurons with minimal toxicity. With this robust labeling by the AAV2-retro retrograde tracer virus we were able to perform electrophysiology experiments to analyze the proportions of RS and BS neurons. Here, we showed the vSub bulk output to all structures is 40% RS and 60% BS neurons while NAcS specific output was made up of 70% RS and 30% BS. The information acquired provides the basis for future investigations into the mechanisms associated with context-induced drug relapse.
Advances in methods by which to visualize circuit connections has provided more insight into the circuit. Britt et al. (2012) performed injections of AAV expressing EYFP into the vHipp. Due to the nature of this AAV, EYFP filled the neurons without marking any particular structure of the neuron. This showed the vHipp sent a dense projection of fibers to the NAcS, but lacked the ability to label and identify terminal projections. In addition, methods of retrograde circuit tracing have provided information that the NAc receives input from the vSub, yet failed to identify which substructure within the NAc that is involved in this circuit. With this information it was not clear as to which substructure within the vHipp provided the dense projection specifically to the NAcS. And with the retrograde experiments it was unclear as to where the vSub projections were going within the NAc. Through the use of our anterograde tracing method, we were able to clarify decades of research in a relatively short amount of time. The robust projections from the vSub to the NAcS suggests the vSub can exert a strong influence over the NAcS which may ultimately contribute to context induced relapse.

To identify the types of vSub neurons that project to the NAcS, we had to design an effective method of retrograde circuit tracing. Recent advances in technology now permit robust retrograde circuit tracing. Apart from the AAV2 method described here, there are two other commonly used viral tracing approaches: monosynaptic rabies virus and canine adenovirus-2 (CAV-2) (Kuypers & Ugolini, 1990; Kremer et al., 2000; Inoue et al., 2004; Hnasko et al., 2006; Callaway, 2008; Salinas et al. 2009; Ohara et al., 2013; Zussy & Salinas, 2014). While each approach effectively provides retrograde tracing, both have significant limitations. Monosynaptic rabies virus provides effective labeling of presynaptic neurons following infection; however, this virus is incredibly toxic to neurons. Infected neurons die within two weeks and preceding cell death are changes in the transcriptional profiles of infected neurons. This was a major impediment two reasons. 1, it is unclear if cell toxicity due to rabies virus would alter the intrinsic membrane properties of vSub neurons, thereby altering spiking properties and 2, because as a future direction, we plan on performing single-cell RNA-sequencing of vSub neurons that project to either D1R or D2R MSNs to test the hypothesis that there is a distinct molecular
code that defines the connectivity and/or synaptic transmission properties at each type of synapse. CAV-2 is another robust retrograde viral tracer that is much less toxic than the monosynaptic rabies virus; however, this virus is distributed by a single laboratory at the Institut Genetique Moleculaire Montellier. Acquiring this virus takes a significant amount of time and ordering custom CAV-2 is nearly impossible meaning that we would be restricted to a select variety of already made CAV-2. Given the alternatives for retrograde tracing, the generation of the AAV2-retro variant was rather important to these studies. The robustness of labeling combined with the lack of neural toxicity allowed us to perform electrophysiological analysis of the vSub projection without altering cell health or transcriptional profile.

A previous study by Kim and Spruston (2012) also focused on characterizing the vSub cell types involved in the vSub-NAc circuit; however, there were significant methodological problems that made interpreting their findings difficult. First, Kim and Spruston used Retrobeads injected into the NAc (NAcC and NAcS) whereas in our study we specifically targeted the NAcS. Second, and most importantly, the health of their cells was a major concern. In order to fire vSub neurons, the authors had to inject 800-1000 pA of current. By contrast, in our study we successfully fired APs with ~90 pA of current. The fact that their required current injection to fire the cells was an order of magnitude higher is indicative of unhealthy neurons. Our methods used here provide a more accurate description of the NAcS specific vSub output projection. Surprisingly, we identified a significant difference in resting membrane potential between RS and BS neurons projecting to the NAcS. This is likely due to differences in channel dynamics and ion flux at rest. Because these two types of vSub neurons propagate information the distinct proportion of RS and BS neurons suggests selective roles in the modification of NAcS mediated behaviors.

The differential effects of the RS and BS neurons may change based upon the postsynaptic landscape of the MSNs. Changes in the postsynaptic MSNs have been shown to occur during drug abuse and subsequent withdrawal. These changes produce an increase in AMPAR/NMDAR ratio at
vHipp inputs to the NAc and cause more GluA2 containing AMPAR to be inserted at the surface specifically at D1R MSNs (Pascoli et al., 2014). Changes in the postsynaptic landscape can ultimately enhance the effects of a particular input. These synaptic changes suggest that the NAc MSNs become poised to respond to stimulation via the vSub and that contextual information associated with drug use may be more likely to fire the MSNs.

In the future, we will attempt to further describe the innervation pattern between RS and BS neurons within the vSub and the D1R or D2R MSNs of the NAcS. There is no current literature that addresses the question of how the RS and BS neurons of the vSub innervate the D1R or D2R MSNs of the NAcS. Significant literature exists describing the different functions of the two populations of MSNs (Le Moine & Bloch, 1995; Lobo et al., 2006; Beaulieu & Gainetdinov, 2011; Scofield et al., 2016). And research has shown that selective plasticity occurs at D1R MSNs. This suggests that the input from the vSub to the NAcS may be exerting different effects through selective innervation of either D1R MSNs or D2R MSNs and this differential impact on postsynaptic signaling may be more pronounced in connections to D1R MSNs. Further characterization of this circuit would provide more detailed information on how the vSub is exerting influence over the mesolimbic DA system. And this information can be used for further analysis of the pathophysiology associated with drug addiction. To accomplish this, we will utilize an intersectional retrograde virus approach. The in vivo stereotaxic injection of AAVs expressing cre-dependent retrograde Flpe recombinase into the NAc shell of cre-expressing D1R or D2R MSNs and Flpe-dependent expressing mRuby into vSub will identify the proportion of RS and BS presynaptic subicular neurons that innervate D1R or D2R MSNs. The results from this experiment will address whether the disproportionate distribution of vSub projecting RS and BS neurons is preserved uniformly, independent of postsynaptic cell type, or if there is an additional degree of projection specificity that is dependent on postsynaptic cellular identity. The anticipated results will provide the first cell-type-specific insight into the wiring of this circuit and will expand our understanding about how this circuit is altered in addiction and schizophrenia.
The functional circuit tracing will reveal the identities, distribution and proportion of vSub neurons that synapse onto D1R or D2R MSNs, however, tracing experiments do not tell us about the strength or other functional properties of these synapses. Therefore, it is essential to gain a more complete understanding of this circuit by interrogating the cell-type-specific synaptic transmission properties of the vSub-NAc shell circuit. To measure vSub-mediated synaptic transmission onto D1R or D2R MSNs, we will infect mice that selectively express red fluorescent protein in D1R MSNs (D1R-tdTomato) with AAV expressing channelrhodopsin-2(ChR2) in the ventral subiculum. We will assess cell-type-specific basal synaptic transmission properties (AMPA-receptor and NMDA-receptor mediated synaptic responses) and synaptic plasticity. By dissecting the basal and activity-dependent properties of synaptic transmission, we will be able to address the question: “is synaptic transmission from vSub uniform between D1R and D2R MSNs or is there an inherent cell-type bias in synaptic strength?” By addressing this fundamental question, we will gain an understanding of which neurons and synapses in NAc might be more robustly affected by disease.

In addition to functionally characterizing synaptic transmission at vSub-D1R and vSub-D2R MSN synapses, a long-standing interest of the laboratory is to understand how disease-relevant molecules control transmission properties at these synapses. One molecule that is frequently associated with both drug addiction and schizophrenia is Neurexin-3 (Herd-Costa et al., 2009; Vaags et al., 2012). Moreover, dysfunction of vSub-NAcS circuit has been commonly implicated in both diseases and is thought to underlie the increased dopamine levels found in patients suffering from schizophrenia and addiction. Thus, we hypothesize that NRXN-3 may play a dominant role at vSub-NAcS synapses. As a presynaptic cell adhesion molecule, NRXN-3 has been shown to have circuit and cell type specific functions (Aoto et al., 2013; Aoto et al., 2015; Aoto et al., 2016). In the hippocampus, NRXN-3 controls AMPAR surface expression and NMDAR dependent synaptic plasticity. By contrast, in the olfactory bulb, NRXN-3 controls presynaptic release at inhibitory synapses without altering glutamateergic transmission. It is unclear how NRXN-3 affects the synaptic landscape at vSub-D1R
and vSub-D2R MSN synapses and whether these effects are similar or distinct among the two different population of MSNs.

To evaluate the role of NRXN-3 within this circuit, the lab has generated a transgenic mouse cross carrying a conditional knockout (cKO) of NRXN-3 and D1R-tdTomato labeling. We will make injections of an AAV expressing ChR2 and active or inactive (control) cre. We will assess electrophysiological properties listed above on either D1R expressing MSNs (tdTomato+) or D2R expressing MSNs (tdTomato-) in response to the NRXN-3 KO. This will allow us to elucidate a possible role of NRXN-3 within the circuit and how the loss of NRXN-3 contributes to the pathology. It will also provide insight in relation to the GWAS studies that connected NRXN-3 to DA related illnesses such as schizophrenia and drug addiction.

To conclude, the data presented here provides an initial characterization of this circuit which will allow for further investigations into the mechanisms driving drug addiction and context induced relapse. Through anterograde tracing we showed the vSub as providing robust input to the NAcS which suggests contextual information can exert a strong influence over NAcS and subsequent downstream signaling within the mesolimbic DA system. Utilizing AAV2-retro allowed us to effectively label vSub neurons without compromising cell health. With this labeling we were able to classify the NAcS specific output projection from the vSub consists of 70% RS and 30% BS neurons while bulk subicular output was shown to be 40% RS and 60% BS. Through the use of these novel techniques we were able clarify decades of research in a relatively short amount of time and provided the ground work for further investigations into this circuit. In addition, future experiments will clarify the cell-type specific innervation between RS and BS neurons and D1R or D2R MSNs allowing for subsequent analysis of how these synaptic connections are altered in response to drug addiction and how they contribute to context induced relapse. Lastly, identifying a possible molecular mechanism contributing to the pathophysiology within this circuit would provide immense insight into the circuit and possible explain the synaptic changes that occur that ultimately drive context induced relapse.
REFERENCES


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