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The Effect of Acute Cocaine Exposure on NMDA Receptor Subunits in Pedunculopontine Nucleus to Substantia Nigra Pars Compacta Synapses

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Abstract

The substantia nigra pars compacta (SNc) is implicated in cocaine addiction due to increased NMDA receptor-mediated current found after acute cocaine exposure. The present study seeks to determine if this increase is driven by a subunit change that decreases magnesium blockage of the receptor and thus increases current. To test this, NMDA receptor-mediated current was isolated and recorded at holding voltages from -80 mV to +40 mV. It was found that at negative holding voltages, NMDA receptors conduct more current, potentially indicating that a subunit change occurs. However, more data collection will be needed to affirm these results. Continued support of a subunit change hypothesis would give researchers more knowledge on the cellular mechanisms of early-stage cocaine addiction, and provide guidance when investigating potential treatment options.

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Literature Review

Substance abuse disorder, including cocaine addiction, is a debilitating and increasingly common condition that leads to serious health complications and even death. The National Institute on Drug Abuse found that the number of deaths by cocaine overdose has been rising annually for the last decade, with 15,883 deaths in 2019 (National Institute on Drug Abuse, 2021). The opioid crisis has exacerbated existing problems with cocaine addiction, with overdose due to cocaine mixed with opioids, namely fentanyl, sharply increasing annually since 2014 (National Institute on Drug Abuse, 2021). In addition to overdose, cocaine use increases risk to a variety of health conditions such as Parkinson's disease, HIV, hepatitis, and drug-induced psychosis (National Institute on Drug Abuse, 2021). These health concerns are particularly alarming, given that there are currently no drug treatments for cocaine addiction or cocaine overdose (National Institute on Drug Abuse, 2020). However, several behavioral interventions are currently in practice. Many programs use contingency management (CM), where prizes that promote healthful lifestyles (ex: a gym membership) are offered as prizes for cocaine abstinence (National Institute on Drug Abuse, 2016). Other common methods include cognitive-behavioral therapy (CBT), which helps individuals better understand their relationship with cocaine improve coping skills (National Institute on Drug Abuse, 2021). Despite these methods' success, the continued high overdose and relapse rate demonstrates the need for novel treatment options.

The lack of adequate treatment emphasizes the need for further research into the mechanisms behind cocaine addiction. For several decades, the “dopamine hypothesis” has been the predominant hypothesis behind the cellular mechanism for cocaine addiction (Kuhar et al., 1991). It is believed that cocaine binds to dopamine transporters responsible for dopaminergic reuptake. This increases synaptic levels of dopamine and thus increases neurotransmission, causing a pleasurable, rewarding feeling (Kuhar et al., 1991). While less researched, norepinephrine and serotonin have also been shown to undergo similar reuptake blockage by cocaine (Einhorn et al., 1988) (Figure 2). However, research finding that Dopamine antagonists reduced cocaine reinstatement led to dopamine being considered the main driver of addiction (Woolverton & Virus, 1989).

Research has suggested that cocaine’s blockage of dopamine reuptake deregulates dopamine currents even after reuptake functionality returns. Despite the short-term increase in dopamine binding, continued cocaine exposure leads to dopamine depletion, marked by lowered endogenous dopamine levels and reduced dopamine binding affinity to postsynaptic targets (Martinez et al., 2009). This has led some researchers to subscribe to the “dopamine depletion hypothesis”, which states that just as increased dopamine transmission gives cocaine its euphoric effect, subsequent dopamine depletion causes feelings of withdrawal (Dackis & Gold, 1984). Such a neurotransmitter-driven model supports cocaine to be a physical addiction more than a psychological one (Dackis & Gold, 1984). This makes a study of cocaine’s cellular effects particularly important, with understanding of cocaine’s effects potentially leading to effective treatments for cocaine addiction.

Cocaine has also been shown to become a learned reward after repeat cocaine use, providing insight into the mechanisms of cocaine addiction. Learning also often leads to associated reward cues, which leads to cue-induced relapse further complicating treatment (Ito et al., 2000). Dopaminergic currents have a baseline level of firing that increases after administration of cocaine (Morikawa & Paladini, 2011). However, classical conditioning can attach this increase in dopaminergic firing to a neutral stimulus, with a drop off in firing if the presumed cocaine reward is not subsequently delivered (Morikawa & Paladini). This alteration in dopaminergic firing independent of a physiological reaction to cocaine is an example of long-term changes in plasticity from cocaine, and provides insight towards a model of cocaine addiction.

A growing list of brain regions have been implicated in the plasticity and reward learning that occurs after cocaine exposure. The most well-researched regions are those in the mesolimbic dopamine system, including the ventral tegmental area, substantia nigra, and amygdala (Thomas et al., 2008). Several of these midbrain structures are implicated in the "spiral to addiction", a hypothesis on what leads to the induction of cocaine-seeking behavior (Lüscher & Bellone, 2008). The "spiral" describes the output of dopaminergic current from the VTA travelling to the NAc shell, with the NAc shell subsequently projecting GABAergic current back to the VTA. After carrying current through a GABAergic interneuron, the VTA then projects more dopaminergic current, this time to the NAc core. The NAc core then recruits the SNc into the spiral with a GABAergic projection. The spiral ends when the SNc subsequently recruits the dorsal striatum with dopaminergic current (Lüscher & Bellone, 2008). The recruitment of the dorsal striatum is believed to mark the onset of cocaine-seeking behavior in mammals, including

humans (Lüscher & Bellone, 2008). This hypothesis makes the midbrain regions in question of extreme interest for cocaine research. Other regions, including the pedunculopontine nucleus and subthalamic nucleus, also became focuses of cocaine research due to their glutamatergic currents towards structures associated with these "spiral" regions (Morikawa & Paladini, 2011), (Beaudoin et al., 2018). With the growing complexity of scientific understanding on how each region of the brain is affected by cocaine exposure, as well as the relationships between regions, it is helpful to review each region individually.

Amygdala

The amygdala is a limbic system structure most often associated with emotional processing (Nestler. 2001). The amygdala has long been hypothesized to have some connection to addiction, but researchers have only very recently begun uncovering the details of the relationship. Previous research showed that individuals who use cocaine had significantly lower amygdala volume when compared to the general population (Makris et al., 2004). While the exact cause of the volume difference is unclear, researchers hypothesized that it was due to natural differences in amygdala size that caused predispositions for cocaine addiction (Makris et al., 2004). However, it is not clear whether amygdala size predicts propensity to try cocaine or propensity to begin habitual use. The amygdala has also received attention in addiction research due to its projections to the NAc, a region frequently associated with addiction and reward (Nestler, 2001).

Over a decade later, the amygdala-NAc pathway would become the focus of promising addiction research. Researchers found that optogenetically activating amygdala-NAc currents

could reduce learned and unconditioned alcohol consumption (Millan et al., 2017). This suggests the amygdala potentially exhibiting a moderating effect on learned rewarding behavior. While our current understanding restricts this phenomenon to alcohol use, future research could determine if this effect is also observed with cocaine and other drugs of misuse. If this is the case, we would also have a better understanding of the relationship between amygdala size and propensity for cocaine addiction.

Dorsal Striatum

The dorsal striatum is a subsection of the striatum containing the caudate nucleus and the putamen (Vanderschuren et al., 2005). The dorsal striatum has been heavily implicated in reward learning, particularly in cases of cocaine addiction. In particular, the dorsal striatum has been heavily implicated in later stages of addiction, including relapse. Dopamine release occurs in the dorsal striatum in response to drug-associated cues and subsequent cocaine-seeking behavior, implicating the region in cue-induced relapse (Ito et al., 2002). Researchers believe that the recruitment of the dorsal striatum from intertwined cocaine-sensitive midbrain structures marks the onset of cocaine-seeking behavior in animals (Lüscher & Bellone, 2008). These regions consist of the VTA, NAc core and shell, and SNc, with ultimate recruitment occurring from dopaminergic SNc current (Lüscher & Bellone, 2008). Additionally, research showing that inhibition in the region induced by GABAergic currents leads to reduced drug relapse of cocaine-seeking behavior (Fuchs et al., 2006). This is potentially through GABAergic mediation of dopaminergic currents in the dorsal striatum, which is supported by the finding that direct inhibition of dopamine receptors in the dorsal striatum also leads to reduced

cocaine-seeking behavior (Vanderschuren et al., 2005). Overall, current understanding of the dorsal striatum depicts the region as an important factor in the late stages of cocaine addiction, largely mediated by dopaminergic current. Due to the complicated nature of well-established cocaine addiction, including high likelihood of relapse, understanding of the midbrain dopaminergic structural changes that lead to recruitment of the dorsal striatum are particularly promising topics of future research.

Hippocampus

The hippocampus, like the amygdala, is a limbic system structure associated with emotional processing and memory (Nestler, 2001). The hippocampus has particularly strong associations with learning and demonstrates high synaptic plasticity, making its relevance to addiction intuitive (Kutlu & Gould, 2016). However, unlike the amygdala, hippocampal volume does not vary significantly between individuals who use cocaine and the general population (Makris et al., 2004). Interestingly, researchers have noted that use of cocaine and other stimulants leads to increased memory-formation related activity (Kutlu & Gould, 2016). This reduces after use stops, which researchers have noted as evidence of a self-medication model for addiction. It is believed that, after tolerance builds and the initial euphoric effects of cocaine fade, individuals who use cocaine continue to avoid the negative symptoms when neural activity reduces due to lack of cocaine (Kutlu & Gould, 2016).

Chronic cocaine exposure, or exposure in high doses, leads to deficits in working memory caused by hippocampal cell death and decreased neurogenesis (Domínguez-Escribà et al., 2016), (Sudai et al., 2010). This cell death and reduced neurogenesis is largely specific to the

dentate gyrus, a subregion of the hippocampus (Domínguez-Escribà et al., 2016), (Sudai et al., 2010). It has not been explicitly explained how this apparent cell death and decrease in neurogenesis fits with the apparent lack of change in hippocampal volume after cocaine exposure.

Nucleus Accumbens

The NAc is an input structure of the basal ganglia, known to receive inputs from a plethora of regions, including but not limited to the amygdala, Prefrontal Cortex (PFC), VTA, substantia nigra, and hippocampus (Scofield et al., 2016). The NAc is considered the center to reward circuitry and addiction, including cocaine addiction (Koob & Bloom, 1988). The region is divided into the NAc core and NAc shell, both of which are integral to the "spiral to addiction" do to their reception of dopaminergic inputs from the VTA and transmission of GABAergic currents to the VTA and SNc (Lüscher & Bellone, 2008). Dopaminergic transmission to the NAc has long been thought to be the primary cause of cocaine addiction and relapse (Caine & Koob, 1994). However, dopamine is no longer believed to be the only relevant neurotransmitter. As previously mentioned, GABAergic inputs appear to play a role by connecting key regions affected by cocaine exposure (Lüscher & Bellone, 2008). Glutamate transmission has also been shown to mediate cocaine addiction and relapse (Cornish & Kalivas, 2000). Overall, it is arguably the most important region for addiction, with most other relevant regions contributing to addiction at least in part through the NAc.

The NAc also seems to undergo synaptic plasticity in response to cocaine exposure. Chronic exposure to cocaine has been shown to increase the number of dendritic branches in

NAc neurons (Robinson & Kolb, 1999). These dendritic changes also appear to be persistent, remaining at least one month after discontinuation of cocaine treatment (Robinson & Kolb, 1999). There is also evidence of acute cocaine exposure altering NAc pathways, particularly through alterations to NMDA receptors. NMDA receptors generally contain four subunits, two obligatory NR1 subunits, and then two other subunits, most commonly matching NR2 subunits (Tong et al., 2008). NR2 subunits can be further divided into subtypes, known as NR2A and NR2B. In the NAc shell, the outer region of the NAc, the NR2A/NR2B ratio changes due to an increase in relative NR2B levels, while NR2A levels remain constant (Huang et al., 2009). Total levels of NR1 also do not change, but excess NR1 travels to the synapse alongside novel NR2B subunits to create novel NMDA receptors (Huang et al., 2009). Researchers. proposed that this may potentially be the mechanism resulting in the creation of silent synapses after exposure to cocaine (Huang et al., 2009). Further research is needed to determine if similar processes take place in other brain regions.

Pedunculopontine Nucleus

The pedunculopontine nucleus (PPN) is a mesencephalic locomotor region (MLR) structure in the upper brainstem (Geula et al., 1993). The PPN is oftentimes associated with addiction due to its glutamatergic projections to the SNc and VTA (Morikawa & Paladini, 2011). The PPN is also the sole cholinergic input to the midbrain, which has similarly granted it attention in addiction research (Dautan et al., 2016). However, cholinergic inputs were not found to be activated during reward learning (Lanca et al., 2006), causing a shift in focus to GABA-ergic and glutamatergic inputs (Corrigall et al., 2001).

The PPN is not the sole glutamatergic input to the SNc, with other notable inputs including the dorsal raphe (DR) and subthalamic nucleus (STN) (Morikawa & Paladini, 2011). However, only The PPN's glutamatergic inputs to the SNc have demonstrated synaptic plasticity after cocaine exposure (Beaudoin et al., 2018). It was found that NMDA receptor-mediated current increases in PPN-SNc synapses after acute cocaine exposure, along with property changes to the current (Beaudoin et al., 2018). These property changes included a reduced rise time and decay time of current (Beaudoin et al., 2018). These property changes are particularly interesting, as they suggest a change in NMDA receptors that is not limited to quantity of receptors or availability of binding. Instead, changes in property of current suggests possible changes to the properties of the receptor. Further research is needed to investigate the nature of such potential property changes.

Prefrontal Cortex

Like the NAc, the PFC is a forebrain structure that is linked to addiction due to its reception of major dopaminergic currents from regions associated with addiction (Scofield et al., 2016). As with the NAc, the PFC undergoes large increases in dopamine levels after cocaine exposure, and is believed to contribute to the euphoric state that cocaine produces (Scofield et al., 2016). Furthermore, the layer V pyramidal cells in the medial PFC undergo an increase in the number of dendritic spines and branches after cocaine exposure (Robinson & Kolb, 1999). This change is applicable to both apical and basilar dendrites (Robinson & Kolb, 1999).

The PFC also appears to respond to cocaine through changes to the NMDA receptors. However, unlike the NAc, the exact nature of these changes have appeared to be inconsistent.

Researchers have independently found both increases and decreases in NMDA receptor subunit expression and NMDA receptor ligand binding (Ortinski, 2015). The one constant, however, is that these changes to NMDA receptor activity in the PFC return to baseline shortly after cocaine withdrawal (Ortinski, 2015). Overall, more research is needed to bring understanding of the PFC's contribution to addiction to the level of other regions.

Ventral Tegmental Area

The ventral tegmental area (VTA) is a midbrain structure that is most commonly implicated in addiction due to its dopaminergic inputs to both the PFC and the NAc (Nestler, 2001). Furthermore, the VTA receives glutamatergic and GABAergic inputs back from the PFC and NAc, respectively (Morikawa and Paladini, 2011). Other notable projections to the VTA include glutamatergic inputs from the pedunculopontine tegmental nucleus and laterodorsal tegmental nucleus, GABAergic inputs from the rostromedial tegmental nucleus and ventral pallidum, and norepinephrine inputs from the locus coeruleus (for a full review, see Morikawa and Paladini, 2011 & Uchida et al., 2012). Such an extensive network of connections, as well as its strong dopaminergic currents to regions associated with reward, have made the VTA a focus of extensive addiction research (Beaudoin et al., 2018).

The VTA's dopaminergic currents have also been shown to be influenced by cocaine. After a single cocaine exposure, the ratio of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor to N-methyl-D-aspartate (NMDA) receptor mediated current from the VTA's glutamatergic inputs increased significantly (Ungless et al., 2001). This alteration was shown to not occur in the hippocampus or with GABAergic neurons in the VTA, suggesting that

the effect may be unique to dopaminergic neurons in the VTA (Ungless et al., 2001). Furthermore, inhibiting NMDA receptor-mediated current suppressed the cocaine-induced potentiation of AMPA receptor-mediated current, suggesting that NMDA receptors, while apparently unaffected, played an important role in the process (Ungless et al., 2001). Further research revealed that AMPA/NMDA receptor-mediated current ratio changes were not constant in all DA VTA neurons, but instead varied by cell terminal location (Lammel et al., 2011). Cell terminal location also was found to determine AMPA/NMDA receptor-mediated current ratio sensitivity to cocaine, with the increased AMPA/NMDA receptor-mediated current ratio reported by Ungless et. al only occurring in cells projecting to the NAc (Lammel et al., 2011).

Research has also been conducted to determine the cellular mechanisms causing the altered AMPA/NMDA receptor-mediated current ratio. Researchers identified a subunit change in NMDA receptors, where a subunit composition of two NR1 subunits, one NR2 subunit, and one NR3 subunit is found at higher frequencies (Yuan et al., 2013). NMDA receptors are most commonly composed of two NR1 subunits and two NR2 subunits (Yuan et al., 2013).

Overall, the VTA and its related regions have been shown to play an important role in addiction. The acute changes that occur to the region's AMPA receptor-mediated current suggest that the VTA may be particularly important for early stages of addiction. Continued research has supported the initial findings of an increased AMPA receptor-mediated current, while adding more nuance by identifying specific pathways where it occurs and providing some evidence of the cellular mechanisms behind it.

Substantia Nigra

The substantia nigra (SN) is a midbrain structure composed of two distinct subregions, the substantia nigra pars reticulata (SNR) and substantia nigra pars compacta (SNc), with GABAergic inputs communicating between the two subregions (Morikawa & Paladini, 2011). The SNc carries dopaminergic current to the striatum, meditated by inputs from several regions, many of which, such as its glutamatergic input from the pedunculopontine nucleus (PPN), are shared with the VTA. (Morikawa & Paladini, 2011). The SNc is also implicated in addiction research due to its role in the "spiral to addiction", receiving GABAergic inputs from the NAc core (Lüscher & Bellone, 2008). Perhaps more importantly, the SNc is responsible for recruiting the dorsal striatum, which is believed to mark the onset of habitual cocaine-seeking behavior in animals (Lüscher & Bellone, 2008).

Historically, the SNc has not received the same focus as the VTA in addiction research (Wise & Koob, 2013). This is in part because early experiments testing for similarly altered AMPA/NMDA receptor-mediated current ratio changes after cocaine exposure found negative results (Ungless et al., 2001 & Lammell et al., 2011). However, the methodology used was a relatively crude form of electrical stimulation that could not specifically stimulate individual currents (Beaudoin et al., 2018). Due to optogenetic activation, researchers are now able to individually activate currents. Using this technology, this lab found that the SNc dopaminergic cells receiving glutamatergic input from the PPN experienced a decreased AMPA/NMDA receptor-mediated current ratio after cocaine exposure (Beaudoin et al., 2018). Cells receiving glutamatergic inputs from the dorsal raphe and subthalamic nucleus were unaffected (Beaudoin et al., 2018). Notably, the changes in PPN-SNc synapses contrasted changes found in the VTA,

where an increased AMPA/NMDA receptor-mediated current ratio was observed (Ungless et al., 2001).

Like with the changes found in VTA, researchers found that the ratio change in the SNc were caused by changes in NMDA receptors (Beaudoin et al., 2018). However, the cause of the increased NMDA receptor-mediated current is not clear. There are several potential hypotheses, including an increase in NMDA receptors, a centralization of NMDA receptors to increase ligand blocking, an increase in terminals interacting at the synapse, or a change in NMDA receptors' subunit composition (Figure 1). It is worth noting that, in addition to increased NMDA receptor-mediated current, there were changes to NMDA receptor-mediated current properties, namely a decrease in current rise time and decay time (Beaudoin et al., 2018). A change in receptor properties suggests that cocaine exposure leads to alterations in the receptor itself, not merely an increase in ligand binding. This lowers interests in hypotheses of increased or localized NMDA receptors and suggests a heightened possibility of a subunit change.

It benefits to expand on the hypothesis of a subunit change, as it may not be immediately apparent why changing a NMDA receptor subunit would increase NMDA receptor-mediated current. NMDA receptors contain four subunits, two obligatory NR1 subunits along with two other subunits, most commonly a pair of NR2 subunits (Tong et al., 2008). However, researchers have recently identified an additional subunit, NR3, which occurs naturally in low quantities during early development (Wong et al., 2002). Interestingly, researchers found that NR3 levels significantly increase after cocaine exposure in the VTA (Yuan et al., 2013). This introduces the possibility that cocaine exposure causes NR3 subunits to

replace some NR2 subunits in the SNc as well. If this subunit change occurs, it would result in an alteration of NMDA receptor shape, which could in turn result in reduced magnesium ion affinity to the NMDA receptor (Figure 3). With reduced magnesium affinity, the NMDA receptor would be less likely to be inactivated, allowing for current flow when glutamate and glycine bind and thus increasing NMDA receptor-mediated current.

The possibility of a NR3 subunit replacing an NR2 subunit raises the question of whether NR3 selectively replaces a specific NR2 subtype. In the NAc, the NR2B/NR2A ratio increases after cocaine exposure due to a relative increase in NR2B levels (Huang et al., 2009). Researchers are yet to determine whether a similar change also occurs after cocaine exposure in the SNc. Because this change could potentially be a side effect of an NR3 subunit change, it is unclear whether a change in NR2B/NR2A ratios would alter magnesium ion affinity and thus contribute to changes in NMDA receptor-mediated current levels.

Optogenetics

Optogenetic activation of PPN neurons is necessary to specifically activate the PPN-SNc glutamatergic current (Beaudoin et al., 2018). This is done using a virus containing coding instructions for channelrhodopsin-2 (ChR2), a light-activated ion channel (Berndt et al., 2011) (Boyden et al., 2008). Neurons infected with the virus produce ChR2 and become sensitive to light, allowing researchers to then specifically depolarize them (Berndt et al., 2011). The virus also contains coding instructions for yellow fluorescent protein (YFP) (Beaudoin et al., 2018). When viewing sections of tissue, the infected area glows bright green due to YFP production,

allowing researchers to easily determine if the target area was infected (Berndt et al., 2011) (Boyden et al., 2008).

In the present study, optogenetics allowed us to selectively activate glutamatergic PPN neurons while responses of dopaminergic SNc neurons are measured. This prevents interference from other regions, and allows us to more accurately investigate the observed increase in NMDA receptor-mediated current previously identified in the region (Beaudoin et al., 2018).

Summary

Cocaine addiction is a difficult condition to live with that has high overdose and relapse rates due to its manipulation of neural reward pathways. A variety of factors contribute to these high rates, including gaps in current treatment options and cocaine's strong ability to elicit cue-induced cravings. Regions implicated in the "spiral to addiction", namely the VTA, SNc, NAc, and dorsal striatum are the main sites that undergo plasticity as a result of cocaine exposure and are thus the most common focus of research (Lüscher & Bellone, 2008). However, considerable contributions to addiction appear to stem from neighboring regions of the brain that project non-dopaminergic axons to these regions, especially the VTA and SNc. Overall, the effects of cocaine in various brain regions are complexly interwoven, with many potential research questions still left to be explored. Fortunately, optogenetics and other developing lab techniques allow researchers to probe regions and currents with much greater specificity than researchers from decades past. These continually evolving techniques may lead to rapid growth of our understanding of cocaine-induced plasticity in the next few years.

Introduction

Drug addiction is a highly complex condition marked by initial pleasure upon drug administration causing continued drug use, even after severe consequences (Camí & Farré, 2003). Even after a period of drug abstinence, underlying addiction remains, making cases of relapse highly common, further complicating treatment of people struggling with addiction. Several midbrain regions, such as the substantia nigra pars compacta (SNc), have been found to be important regions for this so-called "spiral to addiction," (Lüscher & Bellone, 2008).

Given the seemingly irreversible effects of long-term addiction, early intervention may be the best strategy for treatment. This makes understanding of the mechanisms of synaptic plasticity after acute cocaine exposure particularly important to understand. Early research found that the VTA underwent changes to its α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor to N-methyl-D-aspartic (NMDA) receptor-mediated current ratio in glutamatergic synapses after cocaine exposure, suggesting importance for early reward learning in cocaine addiction (Ungless et al., 2001). AMPA and NMDA receptors are glutamatergic receptors found on postsynaptic sites in neurons. NMDA receptors are distinct from AMPA receptors due to extracellular Mg^{2+} blocking current when the cell is hyperpolarized. This has also implicated the receptors in synaptic plasticity.

Interestingly, the changes in AMPA and NMDA receptor-mediated current in the VTA appear to be relatively short term in the absence of additional cocaine use, lasting more than five but less than ten days (Ungless et al., 2001). This was the beginning of understanding midbrain structures as the primary drive behind the early stages of cocaine addiction. However,

no such change was immediately found in other regions, implicating that the change may be specific to the VTA (Ungless et al., 2001).

Other regions, including the SNc, did not immediately produce results suggesting similar changes, as the crude mechanisms for activation meant that projections from different regions could not be measured individually. However, the development of optogenetic activation has allowed the question of individual current's sensitivity to cocaine to be revisited. This lab has found that glutamatergic currents to the SNc from Pedunculopontine nucleus (PPN) undergo increased NMDA receptor-mediated current after acute cocaine exposure (Beaudoin et al., 2018). Other glutamatergic inputs from the subthalamic nucleus (STN) and dorsal raphe (DR) demonstrated no such effect, partially explaining the negative findings from previous research (Beaudoin et al., 2018, Lammel et al., 2011). In addition to increased current, property changes to the PPN-SNc current occur after cocaine exposure (Beaudoin et al., 2018).

The present study seeks to gain understanding of the mechanism behind this increased AMPA/NMDA receptor-mediated current ratio in the PPN-SNc synapses. The ratio change is driven by an increase in NMDA receptor-mediated current (Beaudoin et al., 2018). However, there are several viable hypotheses behind this increase (Figure 1). It is possible that cocaine exposure leads to an increase in available NMDA receptors for binding, either through an increase in neurons interacting at the synapse, an increase in NMDA receptors in existing cells, and/or a recentralization of NMDA receptors so they are more available for binding. However, such changes would not account for the property changes found after cocaine exposure, such as the decrease rise time and decay time of current (Beaudoin et al., 2018). Such changes suggest not merely an increase in NMDA receptors, but also property changes to the receptors.

This study investigated whether acute cocaine exposure leads to subunit changes in SNc cells receiving glutamatergic PPN current. NMDA receptor subunits are divided into three subtypes, NR1, NR2, and NR3 (Paoletti & Neyton, 2007). From these subtypes exist eight distinct isomers of NR1, four NR2 subunits (NR2A-D) (Cull-Candy et al., 2001), and two distinct NR3 subunits (NR3A and NR3B), (Traynelis et al., 2010). A NMDA receptor consists of two obligatory NR1 subunits forming dimers with either NR2 or NR3, with NR2 being considerably more common in adult mammals (Traynelis et al., 2010). NR3 subunit types are much more recently discovered and are the least extensively categorized NMDA receptor subunit category (Yao et al., 2008). These four subunits form a central ion pore through which current can flow - however, when the cell is hyperpolarized, extracellular magnesium typically blocks current flow (Burnashev et al., 1992). However, NMDA receptors containing an NR3 subunit demonstrate a weaker fit for Magnesium pore blockage, resulting in reduced sensitivity to magnesium and increased NMDA receptor-mediated current (Tong et al., 2008). This study investigated whether the property changes associated with a NR2 to NR3 subunit change contribute to the increased NMDA receptor-mediated current observed in PPN to SNc currents after cocaine exposure (Figure 3).

This study aims to develop understanding of cocaine's effect on the midbrain at a cellular level. Understanding the cellular mechanism behind cocaine's increase of NMDA receptor-mediated current would be a helpful contribution in understanding the midbrain's role in cocaine addiction. Subsequent research could utilize this understanding to improve treatment options available for people who are affected by cocaine addiction, and hopefully curtail the continued rise in use, health complications, and fatal overdoses (NIH, 2021).

Materials and Methods

Subjects

All mice (n = 6, male = 5, female = 1) were of the Balb/C strain and housed at the Trinity University vivarium in San Antonio, TX. Mice were at least 12 weeks old when undergoing viral injection. Mice had *ad libitum* access to food and water, and underwent standard 12:12 dark-light cycles. Mice were housed in 500 cm^2 cages containing 0-3 other mice of the same sex. All were moved to individual cages after viral injection. Mice were provided cardboard tubing enrichment both before and after surgery. All mice handling and conditions were approved by the Trinity University Animal Research Committee.

Stereotaxic Surgery

The purpose of stereotaxic surgery was to inject an adeno-associated virus (AAV) encoding channelrhodopsin (ChR2) and yellow-fluorescent protein (YFP) into the PPN. The AAV was significant because it allows us to verify accurate injection via YFP expression and optogenetically activate the PPN during electrophysiology via ChR2 expression. Prior to placement on the stereotaxic surgery site, mice were anesthetized with 4% isoflurane in 2 L/min oxygen. and injected with Carprofen (Rimadyl) and Enroflox at a dosage of 10 mg/kg. Ear bars were used to secure the head during surgery. Lidocaine jelly was applied to the bars to avoid discomfort. Similarly, altalube was applied to the eyes to avoid drying. Once secured onto the stereotaxic surgery site, the mice were injected with 10mg/kg of lidocaine above the mouse skull. Both sides of skull above the PPN were drilled and the PPN was injected using coordinates

from Paxinos and Franklin's *The Mouse Brain in Stereotaxic Coordinates* (M/L = +/- 1.22 mm, A/P = -4.6 mm, D/V = -3.7 mm). The injection was carried out over a five minute period for each side.

Neural Slicing

Twenty four hours before they were sacrificed and at least three weeks after they were injected with AAV, mice were anesthetized by 4% isoflurane in 2 L/min oxygen and subsequently underwent an IP injection with 10mg/kg of either saline or 10mM cocaine in saline. Twenty four hours later, the mice were sacrificed and their brain was extracted for electrophysiology.

Once the brain was extracted, it was quickly placed in cutting artificial cerebrospinal fluid (cACSF), composed of 2.5 mM KCL, 1.25 mM NaH_2PO_4, 110mM choline chloride, 2.6 mM $NaCO_3H$, 7 mM MgCl, 0.5 mM $CaCl_2$, 10mM dextrose, 2.4mM sodium pyruvate, 1.3 mM ascorbic acid dissolved in 18 MOhm water. The brain is continuously held in cASCF while cut into 250 µm horizontal sections by a Leica VT1000 S vibratome. During cutting, physiological pH was maintained by bubbling in 95% O_2 and 5% CO_2. Slices containing SNc were extracted and placed in recording artificial cerebrospinal fluid (RACSF). RACSF is composed of 125 mM NaCl, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 4 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM NaHCO, 2.4 mM sodium pyruvate, 1.3 mM ascorbic acid, and 0.16 nM L-glutathione. Slices containing PPN were also collected and imaged to determine YFP expression. This can be used to verify that the PPN was infected with the AAV during viral injection.

Electrophysiology

Slices containing SNc were continuously held in RACSF while undergoing electrophysiology. Suspected dopaminergic SNc cells were patched onto before being tested to ensure the cell is dopaminergic and in SNc. Additionally, we tested to ensure that the cell is not infected with the AAV. Spiking data was recorded, and spiking rates under 8 Hz with action potentials lasting longer than 1.5 ms confirmed cells to be dopaminergic (Beaudoin et al., 2018). Furthermore, once we entered the cell, we used a voltage clamp to hyperpolarize the cell to -110 mV. If hyperpolarization-activated cyclic nucleotide-gated (HCN) cation channels subsequently opened, the cell was confirmed as being dopaminergic and in SNc.

To ensure that the cells we were recording from were not directly infected with the AAV, we tested the synaptic response to blue light. Cells were confirmed to be indirectly activated by blue light, as the light activated infected PPN cells that subsequently projected to the dopaminergic SNc cells. If there was not a >2 ms delay between laser firing and the return to baseline potential, or there was a sustained response to a 100 ms light pulse, we suspected that the SNc cell was infected.

Imaging

At the conclusion of electrophysiology, slices containing SNc were fixed in paraformaldehyde for later analysis and imaging. All cells (*n* total = 7) that were recorded from were dialyzed with biocytin. We then stained slices in streptavidin, which binds to biocytin. Because only cells we record from are dialyzed with biocytin, streptavidin staining allowed us to specifically locate our recording cells. Cells were also stained with anti-tyrosine hydroxylase (TH) antibodies. The anti-TH antibodies were used to locate cells containing TH, the rate limiting

enzyme for synthesizing dopamine (Daubner & Wang, 2010). Thus, anti-TH stained cells were confirmed to be dopaminergic.

For staining, slices were first incubated with 1% Triton X-100 in phosphate buffered saline (PBS) with 40 rpm shaking overnight at 4°C. Slices were then washed in a 1:800 dilution of streptavidin Alexa 568 in 1% Triton in PBS overnight before being washed three times with 0.5% Triton X-100 in PBS for 5 minutes at 40 prm shaking at room temperature (RT). After the third wash, slices were blocked and permeabilized in 5% normal goat serum (NGS) and 0.5% triton X-100 at room temperature for 4 hours at 40 rpm shaking. Slices were subsequently stained overnight with chicken anti-tyrosine hydroxylase antibody (ABCAM ab76442, 1:500) in 1% NGS and 0.1% Triton in PBS. Excess anti-TH antibody was washed away using three washes in 0.1% Triton in PBS. Slices were then held overnight with the secondary antibody, 2mg/mL goat anti-chicken antibody Alexa 405 (ABCAM ab175675, 1:1000). At the conclusion of the final stage of staining, slices were placed on coverslips for imaging.

Data Analysis

Data were standardized by dividing current at each holding voltage by their current at +40 mV. Due to the limited data collection, particularly in the cocaine condition (*n* saline = 5, *n* cocaine = 2), statistical analysis has not yet been conducted. However, with additional data, a pooled-t test can be used to determine whether mean NMDA receptor-mediated current at each holding voltage is significantly different between saline-exposed and cocaine-exposed mice.

Results

Several steps were utilized to confirm that a given cell was dopaminergic, in SNc, not infected by viral injection, and receiving current from a properly infected PPN region. YFP was expressed alongside channelrhodopsin so slices could be imaged to confirm that the PPN was properly infected (Figure 4).

Cells are confirmed to be dopaminergic during electrophysiology by observing their firing properties. Dopaminergic cells show a firing rate of approximately 3 Hz, an action potential width of 1.5 ms, and an I_H current of -110 mV. Thus, observing similar properties in our cell is a potential means to confirm that the cell is dopaminergic (Kimm et al., 2015). Cells are also stained for biocytin when patched, allowing us to later locate cells used for recording. Anti-tyrosine hydroxylase staining of biocytin-stained cells additionally confirmed cells to be dopaminergic (Figure 5).

An IV plot was made to conglomerate the NMDA receptor current recorded at holding voltages from -80 mV to +40 mV, in 20 mV increments (Figure 6). Samples were individually normalized by their +40 mV current. -80 mV holding levels had standardized currents of *M* = -0.217, *SEM* = 0.0635 in the saline condition (*n* =5) and standardized currents of *M* = -0.178, *SEM* = 0.0436 in the cocaine condition (*n* = 2). In -60 mV holding levels, *M* standardized current = -0.216, *SEM* = 0.0436 for saline and *M* standardized current = -0.382, *SEM* = 0.0298 for cocaine. When holding levels were changed to -40 mV, *M* standardized current = -0.367, *SEM* = 0.0769 for the saline condition and *M* standardized current = -0.719, *SEM* = 0.298 for the cocaine condition. In our last negative holding voltage, -20 mV, the standardized current was *M* = -0.578, *SEM* = 0.160 for saline and *M* = -0.578, *SEM* = 0.160 for cocaine. At 0 mV holding

voltage, the saline condition had a standardized current of *M* = -0.162, *SEM* = 0.0745 while the cocaine condition had a standardized current of *M* = -0.204, *SEM* = 0.0930. Finally, the +20 mV holding voltage demonstrated saline standardized current of *M* = 0.326, *SEM* = 0.041 and a cocaine standardized current of *M* = 0.338, *SEM* = 0.037. Note that, because currents were measured by their +40 mV holding voltage recording, the standardized current of the +40 mV holding voltage is 1 for both conditions. Due to the current sample size for conditions (saline *n* = 5, cocaine *n* = 2), data analysis has not been conducted, and will not be until more data is collected.

Discussion

YFP expression was visible in the PPN of all slices used for recording (Figure 4), demonstrating that slices contained properly AAV-infected PPN. Furthermore, recorded cells, identified by biocytin staining, were shown to also be stained by anti-TH (Figure 5). Because TH is the rate-limiting enzyme of dopamine synthesis, these results demonstrate that the cells recorded from were dopaminergic.

Slices from cocaine-injected mice demonstrated stronger standardized current at negative holding voltages compared to saline-injected mice (Figure 6). The exception to this is holding voltage at -80 mV, which demonstrated slightly more current in saline-treated mice (Figure 6). However, from -60 mV to -20 mV, with the largest difference occurring at a -40 mV holding voltage (Figure 6). These differences potentially indicate a NR2 to NR3 subunit change in NMDA receptors. However, more data is needed to further support current trends, as our cocaine condition in particular has a notably low sample size (*n* = 2).

Further data collection may also resolve some discrepancies between our current data trends and those expected for receptors containing NR3. Namely, previous research comparing NMDA receptors with and without NR3 subunits demonstrated that NMDA receptors with NR3 have increased current at -80 mV (Tong et al., 2008). However, the -80mV currents of our saline and cocaine conditions appear similar (Figure 4). This may be in part due to anomalies in some of our saline condition data, which demonstrates current at -80mV that is uncharacteristic of native NMDA receptor-mediated current. This leads to suspicion that the current may be from another source, most likely uninhibited AMPA receptors. However, if this trend continues with further data collection, it would weaken the hypothesis of a NR3 subunit change.

The primary limitation of the present study is the low *n* collected for both conditions, particularly for the cocaine condition ($n = 2$). A goal for future research should be to continue data collection to a more reasonable milestone, such as the original *n* proposal of 10 for each condition. Only at that point can conclusions be drawn with any reasonable level of confidence from our results.

It is always important to note that the mouse model for cocaine addiction, like all animal models, is not a perfect analogue for human cocaine addiction. Some midbrain effects of cocaine found in mice may not translate. Similarly, some apparent effects found in humans, such as dopaminergic cell death, do not apparently occur in mice (Little et al., 2009). Unfortunately, the research questions of this paper are too invasive to investigate in any meaningful way with participants, so direct verification of results is not possible. However, it does provide a mechanism, the subunit change of NMDA receptors, for researchers to attempt to manipulate in a search for treatment options. If a treatment option were to become ready for

human testing, we would then be able to better understand the applicability of our model's findings to humans.

The immediate future direction for this research project is to continue data collection of isolated NMDA receptor-mediated current to increase confidence in our current observed trends. This would allow us to overcome the current projects' central limitation. Depending on the results of this continued data collection, other proposed methods of supporting results may not prove necessary. However, it is helpful to consider future directions for research, should the data for the current project continue to support the hypothesis of an NR3 subunit change.

There are several promising avenues to help further determine whether or not a subunit change is occuring in the SNc to PPN current. Previous research has been able to measure NMDA receptor-mediated current with and without the presence of extracellular magnesium. In such circumstances, NMDA receptors containing NR3 are similar to standard NMDA receptors in the absence of magnesium, but undergo less blockage in the presence of magnesium (Tong et al., 2008). Such an experiment could be replicated in SNc to help further demonstrate that the observed increase in NMDA receptor-mediated current is due to a subunit change.

Additionally, measuring current both with and without extracellular magnesium could help indicate whether other potential causes for increased NMDA receptor-mediated current play a role. Recall that there were several potential mechanisms for increased NMDA receptor-mediated current, including an increase in cells interacting at the synapse, and increase in NMDA receptors, and a centralization of NMDA receptors for increased glutamate binding (Figure 1). Unlike a subunit change, each of these mechanisms is not anticipated to be sensitive to magnesium blockage. Thus, if cocaine-injected mice continue to demonstrate

increased NMDA receptor-mediated current compared to saline-injected mice, even in the absence of magnesium, it would suggest that an additional factor is playing a role.

There are additional known properties of NR3-containing NMDA receptors that could be measured to test for a subunit change. Namely, NR3-containing NMDA receptors exhibit reduced Ca^{2+} permeability compared to wild type NMDA receptors (Tong et al., 2008). Ca^{2+} permeability can be measured by recording current at varying concentrations of extracellular Ca^{2+} and finding the reversal potential (Jahr & Stevens, 1993). Thus, researchers can determine whether there is a significant difference in Ca^{2+} permeability in SNc NMDA receptors between cocaine-exposed and cocaine naive mice. A significant increase in permeability after cocaine exposure would be additional evidence of a subunit change.

Observed changes could be further supported by immunostaining of NR3 subunits. Rabbit anti-NR3 antibodies are available for both NR3A and NR3B (Wee et al., 2008). This could not only provide further evidence for the heightened presence of NR3 after cocaine exposure, but also help determine which forms of NR3 are present.

It would also be interesting to investigate the longevity of the observed NMDA receptor changes. Previous studies of midbrain structures have shown that acute cocaine exposure can cause rather long-lasting changes to the receptors. For example, an increase in the AMPA/NMDA receptor current ratio found in the VTA was demonstrated to remain five, but not ten, days after acute exposure to cocaine (Ungless et al., 2001). Further research on the increased NMDA receptor-mediated current in SNc would help us understand how its longevity compares to other AMPA receptor and NMDA receptor changes in related midbrain structures.

Once the question of a NR3 subunit change is settled, there will still be the question of what subunit type the NR3 is replacing. The two NR1 subunits would not be replaced, as they are obligatory for NMDA receptors (Traynelis et al., 2010). However, there are multiple subtypes of NR2 subunits (Traynelis et al., 2010), one of which may be a selective site for NR3 replacement. A selective replacement would alter relative NR2 subtype levels in SNc by decreasing levels of the replaced subunit. Figure 7 depicts one hypothetical replacement, in which NR3 selectively replaces NR2A, leading to a relative increase in other NR2 subtypes, such as NR2B. This partially mirrors the relative increase in NR2B subunit subtypes that occurs after cocaine exposure in the NAc (Huang et al., 2009).

Overall, the hypothesis of a subunit change in NMDA receptors after cocaine exposure in SNc shows promise, but will need further investigation. Researchers can undergo this testing by continuing data collection on the current project, as well as pursuing more novel methods that have shown to be reliable in measurements of other midbrain structures. Such methods would also help confirm whether results noted are due to a subunit change or other potential hypotheses, such as an increase in postsynaptic NMDA receptors, centralization of NMDA receptors, or increased postsynaptic cells interacting at the synapse (Figure 1). It is also important to remember that these hypotheses are not mutually exclusive, and continued findings supporting a subunit change should not discourage researchers from investigating alternative hypotheses.

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Tables and Figures

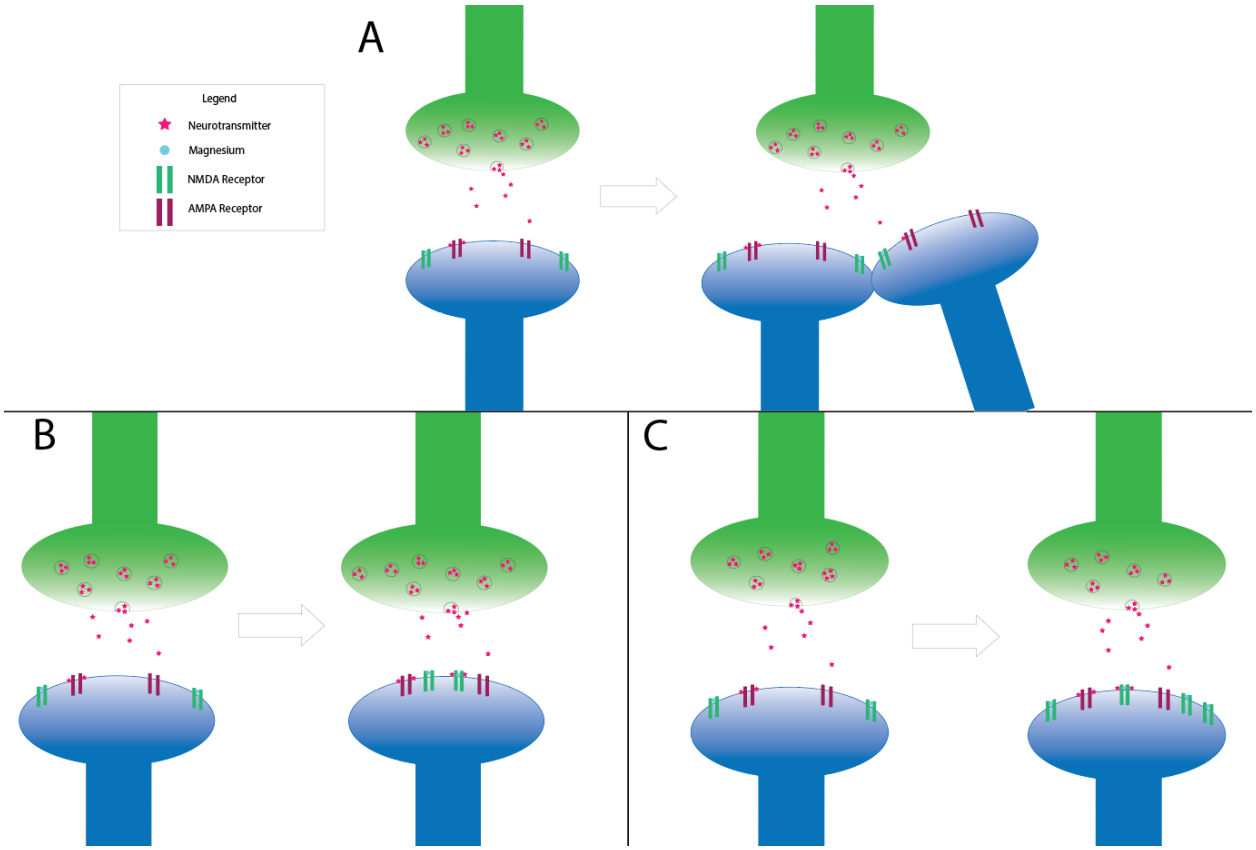

Figure 1. A depiction of potential alternate hypotheses for the observed increase in NMDA receptor-mediated current in SNc after acute cocaine exposure. **A.** Hypothesis that an increase in postsynaptic cells interacting at the synapses leads to an increased NMDA receptor-mediated current. **B.** Hypothesis that a centralization of NMDA receptors leads to increased binding availability for glutamate and thus increase NMDA receptor-mediated current. **C.** Hypothesis that an increase in NMDA receptors is the cause of the observed increase in NMDA receptor-mediated current.

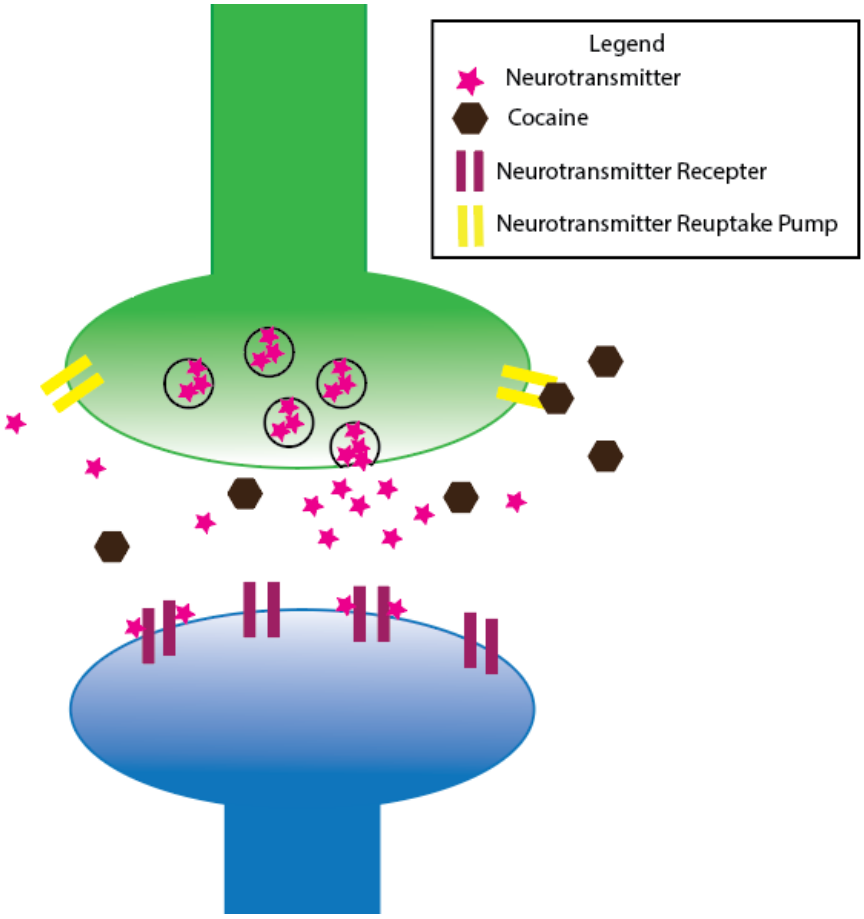

Figure 2. Depiction of cocaine's blockage of neurotransmitter reuptake, increasing binding to postsynaptic sites. Model is known to be applicable to dopamine, norepinephrine, and serotonin.

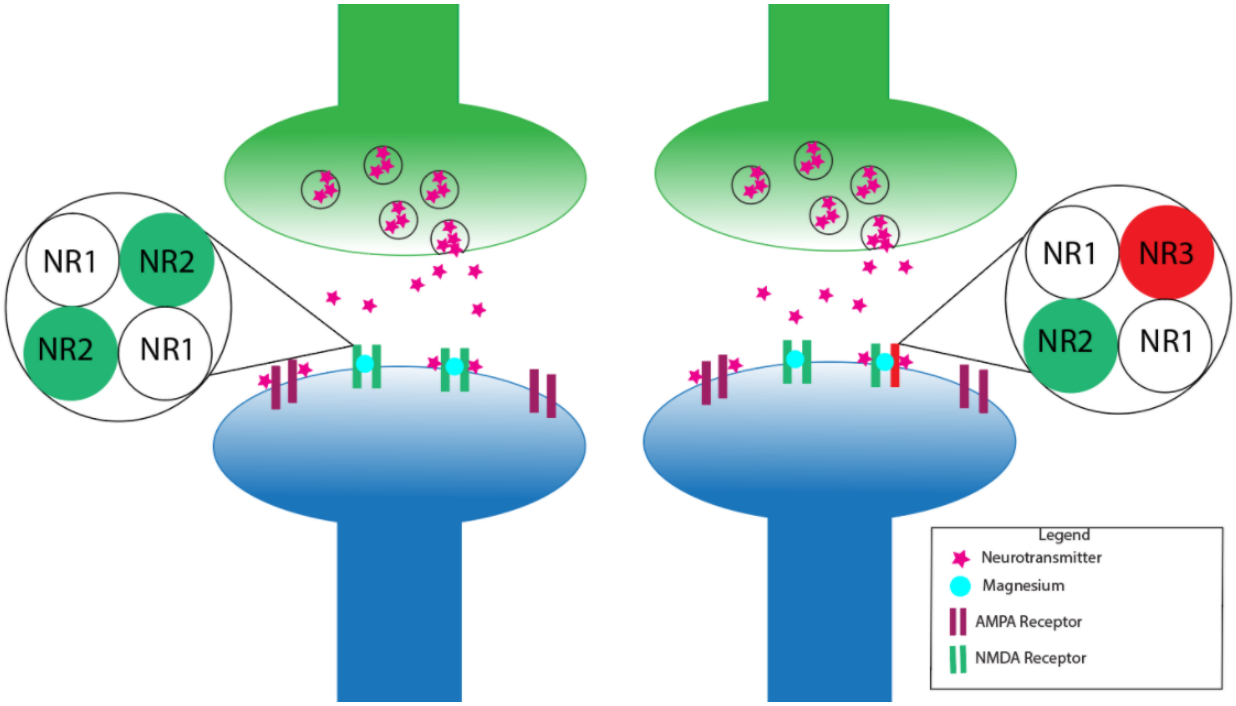

Figure 3. A depiction of cocaine exposure altering NMDA receptor subunit composition by facilitating NR3 replacement for NR2. This would increase relative NR2 levels and weaken magnesium ions' fit into NMDA receptor's central cavity, resulting in increased NMDA receptor-mediated current.

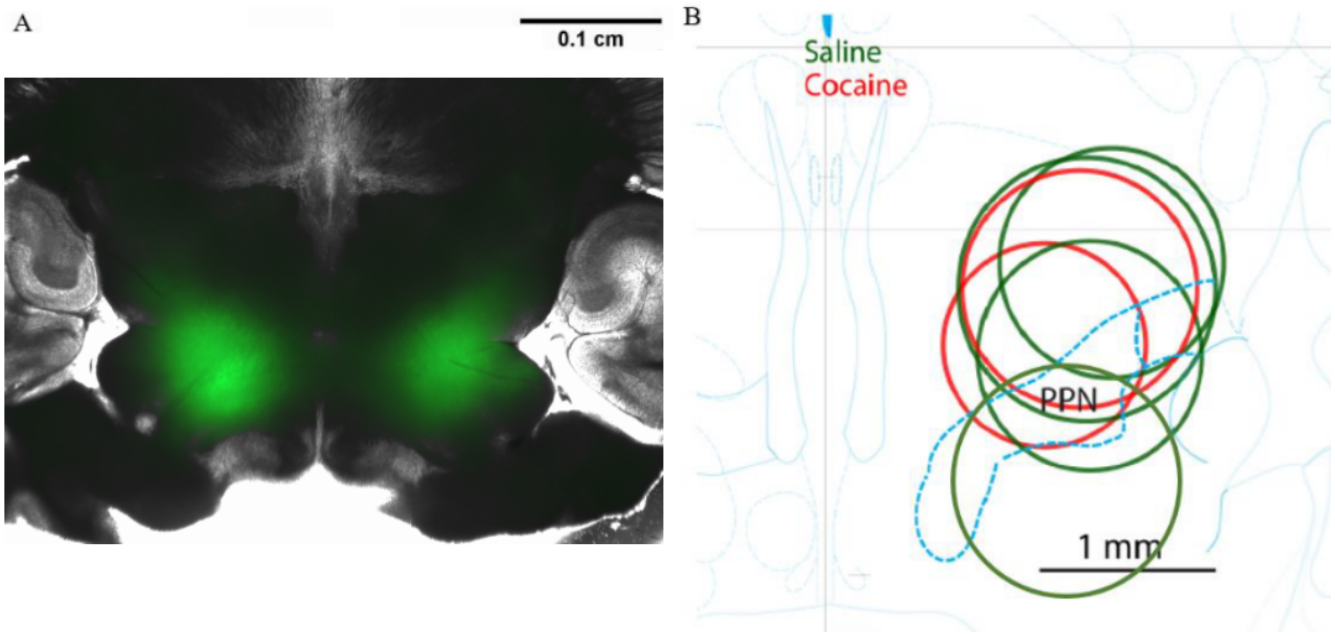

Figure 4. YFP expression is tracked to ensure accurate injections into the PPN. **A.** A bilateral PPN injection is shown to accurately infect the PPN through YFP expression. **B.** A mapping of injection sites for all brains that contained data used in this study. Notably, all infection sites include the PPN.

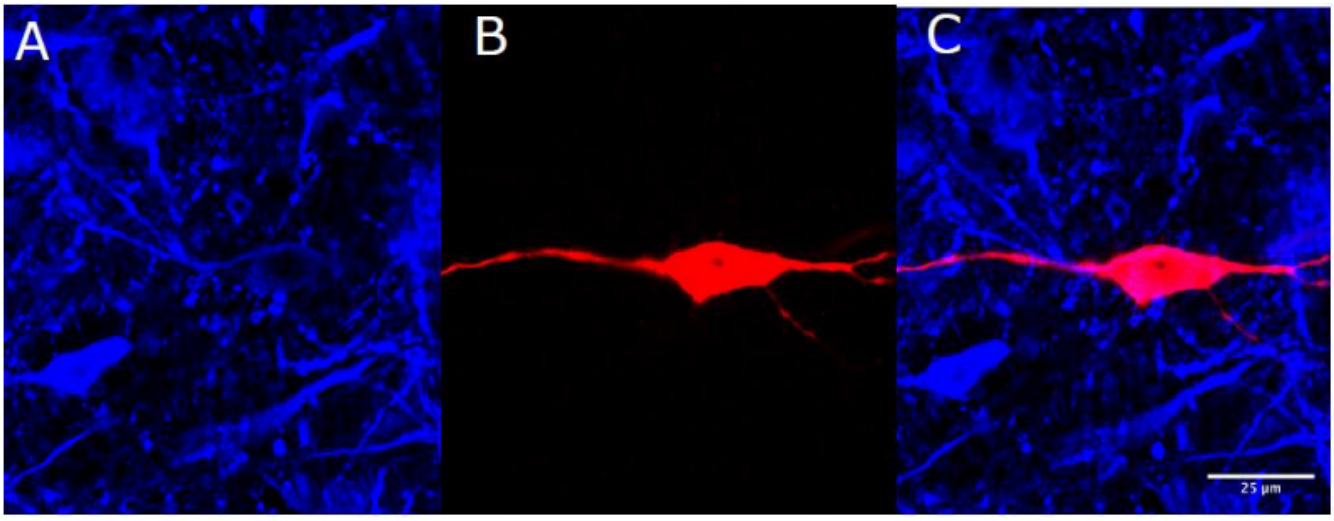

Figure 5. Anti-TH staining and biocytin staining are used to ensure that the recorded cells are dopaminergic. The Anti-TH staining (**A**) and biocytin staining (**B**) can be combined (**C**) to demonstrate that the recorded cell was also affected by anti-TH staining and is thus dopaminergic.

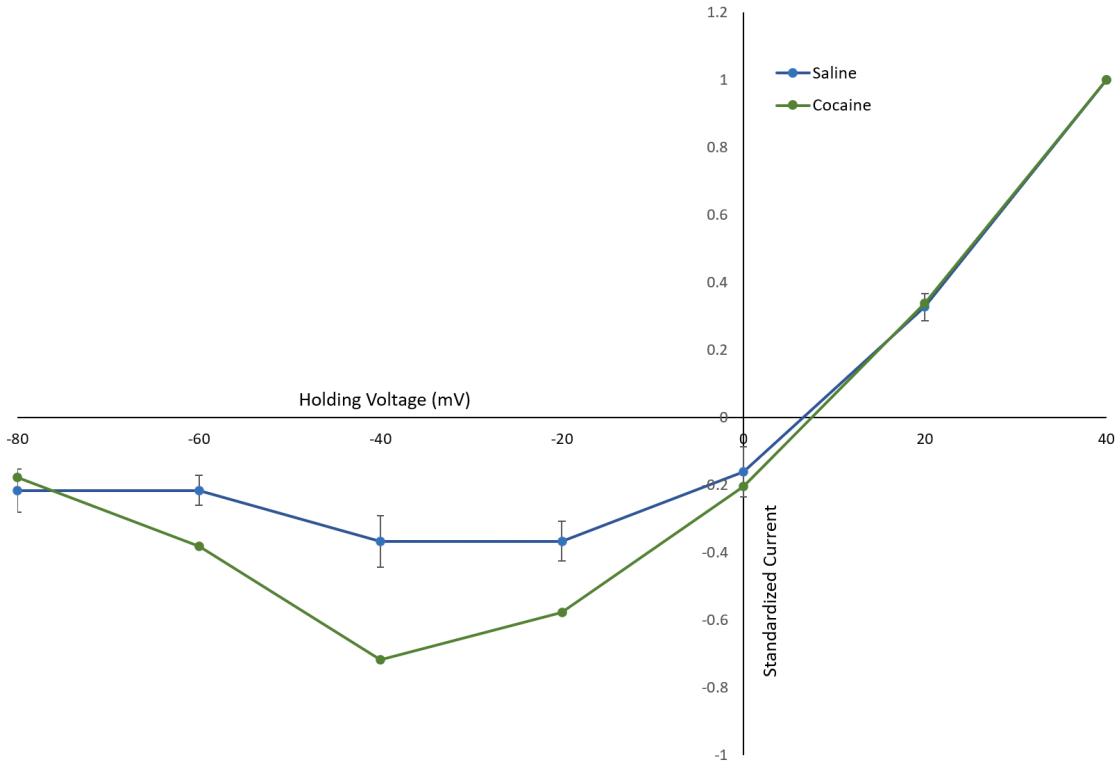

Figure 6. An IV Plot of standardized NMDA receptor-mediated current of cocaine and saline-injected mice. Blue represents saline (*N* = 5) and green represents cocaine (*N* = 2). Errors bars are +/- 1 SEM.

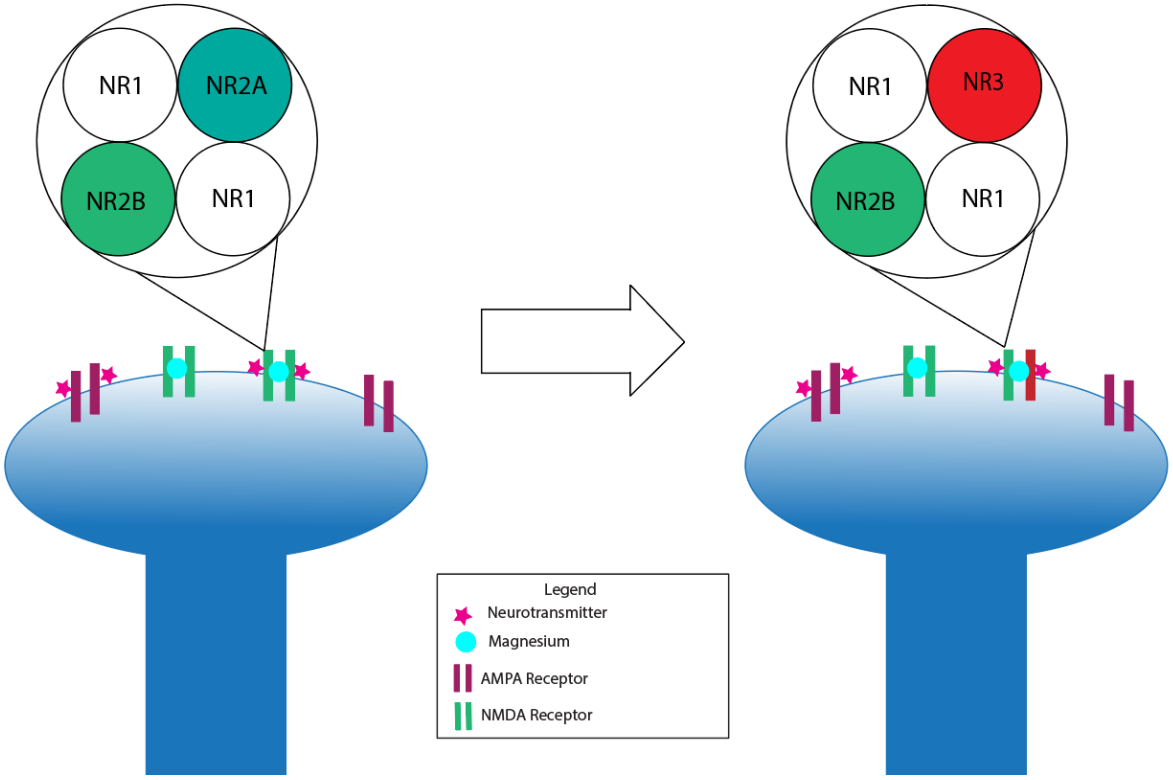

Figure 7. A depiction of selective NR3 replacement of NR2A. This is one of several possible selective replacements that could occur during NR3 replacements. Such selective replacement would lead to consistent subunit compositions for NMDA receptors containing NR3 and alter relative subunit levels of NR2 subtypes.