PROGRESSIVE NEUROADAPTATIONS IN VENTRAL HIPPOCAMPUS SYNAPTIC TRANSMISSION DURING COCAINE WITHDRAWAL

by

COLLIN PRESTON

(Under the Direction of John J. Wagner)

ABSTRACT

The hippocampus is a brain region that has long been known to be critical in learning and memory processes. Over the past several years, there has been increased recognition of the hippocampus' role in addiction and addiction as a disease of learning and memory. Functionally and anatomically the hippocampal formation can be divided along its septotemporal axis into two main sectors: the dorsal hippocampus and the ventral hippocampus (vH). The vH plays an important role in responses to stress and motivational factors through its connection with the brain's mesolimbic reward circuitry. One of the most prevalently used models of addiction, the conditioned place preference assay, relies heavily on hippocampal-dependent learning. This method, however, also relies on researcher administration of drugs which fails to mimic the voluntary intake seen in both the self-administration model of addiction and human usage. To better study the effects of cocaine addiction, we developed an escalating conditioning protocol that resulted in changes of vH synaptic plasticity observable on withdrawal day (WD) 28. We found on WD28, cocaine-treated animals exhibited impaired long-term potentiation (LTP) and increased basal synaptic plasticity. Mice treated with this cocaine conditioning

also exhibited impairments in working memory during withdrawal, a behavioral consequence of these alterations to vH synaptic plasticity.

Furthermore, we investigated the temporal nature of the changes in vH synaptic transmission and found progressive changes throughout cocaine withdrawal. We recorded excitatory postsynaptic currents on either WD2, WD9, or WD28 following cocaine conditioning. Our results showed that there is increased excitatory transmission throughout withdrawal in the vH, but the increase is mediated by different types and, consequently, ratios of AMPA receptors. Early in withdrawal, excitatory transmission is increased in the vH due to an increase in canonical, calcium-impermeable AMPARs. As withdrawal progresses, calcium-permeable AMPARs are inserted into the synapse and by WD28 they account for a significant portion excitatory transmission in the vH. The presence of these calcium-permeable AMPARs after cocaine conditioning may increase responsiveness to contexts associated with cocaine use and drive cocaine craving. Additionally, they may create neurons that do not respond to normal plasticity events resulting in impaired memory ability.

INDEX WORDS: Addiction, Learning and Memory, Hippocampus, Long-Term Potentiation, Synaptic Plasticity, Cocaine, Calcium-Permeable AMPA Receptors

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by

COLLIN JAY PRESTON

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SYNAPTIC TRANSMISSION DURING COCAINE WITHDRAWAL

by

COLLIN JAY PRESTON

Major Professor: John J. Wagner
Committee: Brian S. Cummings
Nikolay M. Filipov
Jesse R. Schank

Electronic Version Approved:

Ron Walcott
Dean of the Graduate School
The University of Georgia
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 History of the Hippocampus

The hippocampus is one of the most thoroughly studied areas of the mammalian central nervous system. Early anatomists were able to easily distinguish the hippocampus as one of the first subcortical regions due to its large and distinguishable shape. The Venetian anatomist Julius Caesar Aranzi first referred to the hippocampus as such in 1587 due to similarities between the unusual, curved shape of the structure and the shape of a sea horse (in Greek, *hippo* means horse and *kampos* means sea monster). During the same period, the hippocampus was likened to another animal, the ram, with the name *Cornu Ammonis* used for the hippocampus by the Danish anatomist Jacob Winslow in 1732. This terminology is derived from the resemblance of the hippocampus to a curved Rams horn and the hippocampus itself may have been named after the Egyptian deity Amun as early as 300BC by members of the Alexandrian School of Medicine [1]. While the term hippocampus has prevailed as the designation for this region of the brain, the eponym of *Cornu Ammonis* is still retained in its abbreviated form "CA" for distinctions of hippocampal subregions which are further detailed in section 1.2. The hippocampus became a region of particular interest to neuroscientists after the invention of microscopes due to the beautifully organized and distinctly laminar structure they were easily able to observe. After pioneering a new staining method that would later be named after him, Camillo Golgi was able to observe the remarkable neuronal assemblies of the

hippocampus. This contributed to his support of the “reticular theory” that proposed the nervous system consisted of a single continuous network connected by cellular structures such as thin fibrils. This theory was later refuted by another pioneering scientist, Santiago Ramón y Cajal, using Golgi’s staining method in the hippocampus to postulate the “neuron doctrine”: a concept that the nervous system is made up of discrete individual cells. Though the two theories contrasted starkly, Golgi and Santiago Ramón y Cajal were jointly awarded the Nobel Prize in Physiology or Medicine in 1906 and their still valid work is often used in neuroscience lectures across the world. It was not until the 1950s, with the invention of electron microscopy, that the neuron doctrine was fully supported when scientists found that each neuron in the brain is indeed an individual entity.

The 1950s were also the time period that the hippocampus was recognized to play a fundamental role in some forms of learning and memory. A landmark paper by Scoville and Milner in 1957 [2] reported that the famous patient ‘H.M.’, experienced a permanent loss of the ability to encode new information into long-term memory after bilateral hippocampal removal for the treatment of epilepsy. Shortly after the report of H.M.’s memory loss it was observed in 1971 that certain hippocampal cells, known as place cells, fired in the rat hippocampus when the animal interacted with a familiar environment [3]. Two years later, the discovery of long-term potentiation (LTP) in the hippocampus was reported [4] and posited to be one of the physiological mechanisms underlying memory formation. In 1986, Morris and colleagues demonstrated the role of LTP in spatial learning at a behavioral, physiological, and receptor level by selectively impairing spatial learning via blockade of NMDA receptors in the hippocampus [5].

Further confirmation of the hippocampus's fundamental role in learning and memory came the same year when the anterograde amnesia experienced by H.M., was also reported in other patients with bilateral damage to the hippocampus in 1986 [6]. The hippocampus gained appreciation for not only its role in learning and memory, but also as a model of synaptic plasticity as evidenced by the review paper published in 1993 from Collingridge and Bliss entitled "A synaptic model of memory: long-term potentiation in the hippocampus" [7]. This review highlights both the role of NMDA receptors in Hebbian plasticity and the pivotal role of the hippocampus in neuroscience research, as it is one of the most highly cited neuroscience papers with over 2,500 citations at the time of this writing.

1.2 Hippocampal Anatomy & Neurotransmission

Neuroscientists have relied on the anatomical and functional organization of the hippocampus for decades to study synaptic transmission and as a model of other cortical regions. What most scientists refer to as the hippocampus is actually part of a functional brain system called the hippocampal formation, a bilateral structure within the limbic system composed of the hippocampus (proper), dentate gyrus (DG), subiculum, pre- & para-subiculum, and the entorhinal cortex. The hippocampus and associated cortical regions form the base of the temporal horn of the lateral ventricle in the human brain and forms the posterior wall of the lateral ventricle in the mouse brain. The long axis that lines the ventricles is referred to as the septotemporal axis while the orthogonal axis is referred to as the transverse axis. The hippocampus can be subdivided along its septotemporal axis into two major sections [8] which, due to differential positioning of the human and mouse hippocampus, have different nomenclature between the two

species; the human anterior hippocampus corresponds to the mouse ventral hippocampus and the human posterior hippocampus correlates to the mouse dorsal hippocampus (Figure 1A&B). In both species, the fimbria-fornix pathway serves as a major conduit of subcortical afferent and efferent connections and is easily visible from a gross anatomical view. For the purposes of this dissertation, I will focus on the anatomy and functionality of the rodent hippocampus since it has been the subject of many decades of research, though it is important to appreciate the anatomical distinctions of the hippocampus between rodents and humans in research.

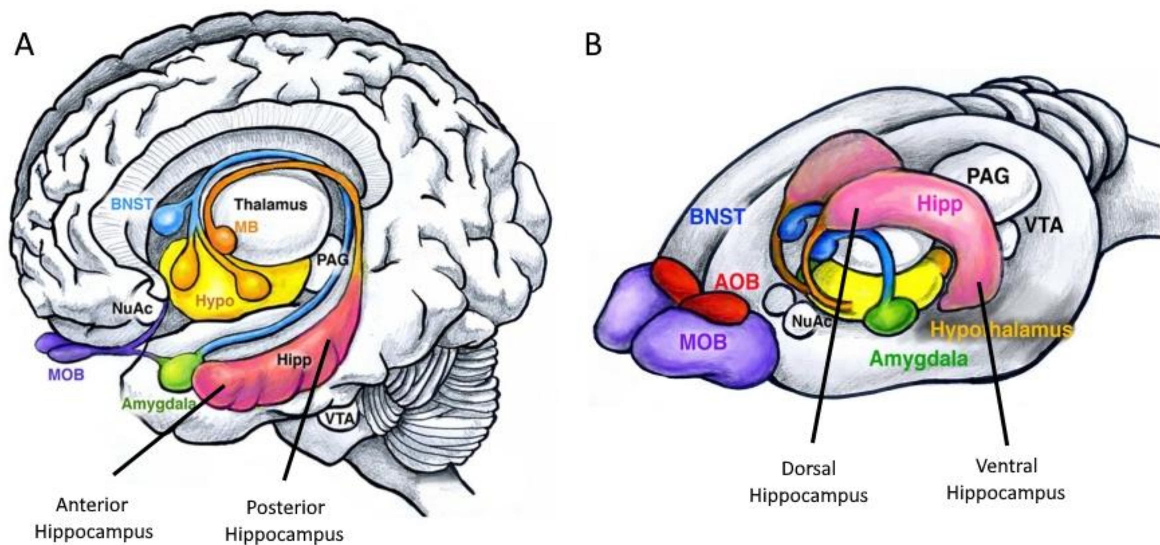

Figure 1.1. Diagram depicting the anatomical locations of the human hippocampus (A) and the rodent hippocampus (B). [9]

The rodent hippocampus is a popular model system of other cortical regions because it shares a similar laminar structure with large principal neurons and small interneurons. The principal neurons in the dentate gyrus are the granule cells, and in the hippocampus, they are the pyramidal neurons. The pyramidal cells of the hippocampus

and can be separated into three main regions, CA1, CA2, and CA3 with CA standing for *Cornu Ammonis* as a tribute to the hippocampus' earlier name. The regions are categorized by cell size and appearance with further categorization intro *stratum* (*s.*) based on the laminar structure of each region [10].The cell bodies of the dentate gyrus reside in the *s. Granulosa* and are considered to be monopolar with dendrites only extending into *s. moleculare*. The multipolar pyramidal cells in the CA1-CA3 regions have apical dendrites that traverse *s. lucidum, s. radiatum,* and *s. lacunosum moleculare* while the basal dendrites extend into *s. oriens*. Neurons in all regions contain axonal projections that form major excitatory pathways that facilitate a largely unidirectional flow of information [11], unlike other cortical regions.

There are many afferent and efferent pathways of the hippocampus that are beyond the scope of this review so only the three major excitatory pathways will be discussed: the perforant pathway, the mossy fiber pathway, and the Schaffer collateral pathway (Figure 2). Collectively, these pathways form a trisynaptic circuit [11] with the perforant pathway as the main conduit of information coming into the hippocampus. This pathway originates within the entorhinal cortex and traverses through the subiculum where it synapses at the granule cells in the DG or the pyramidal cells in CA3. The second part of the circuit is formed from granule cell axons that project to and synapse on the dendrites of CA3 pyramidal cells to form the mossy fiber pathway. The circuit is then completed by the Schaffer collateral pathway consisting of projections from CA3 pyramidal cells to the apical dendrites of CA1 pyramidal cells. After the flow of information through the trisynaptic pathway, axons from CA1 pyramidal cell neurons synapse with subiculum pyramidal neurons that project to numerous brain regions. The

targets of these afferents serve various functions including stress response by feedback regulation of the hypothalamic-pituitary-adrenocortical axis, emotional response via cross-talk with the amygdala, and episodic memory via direct projection to the prefrontal cortex.

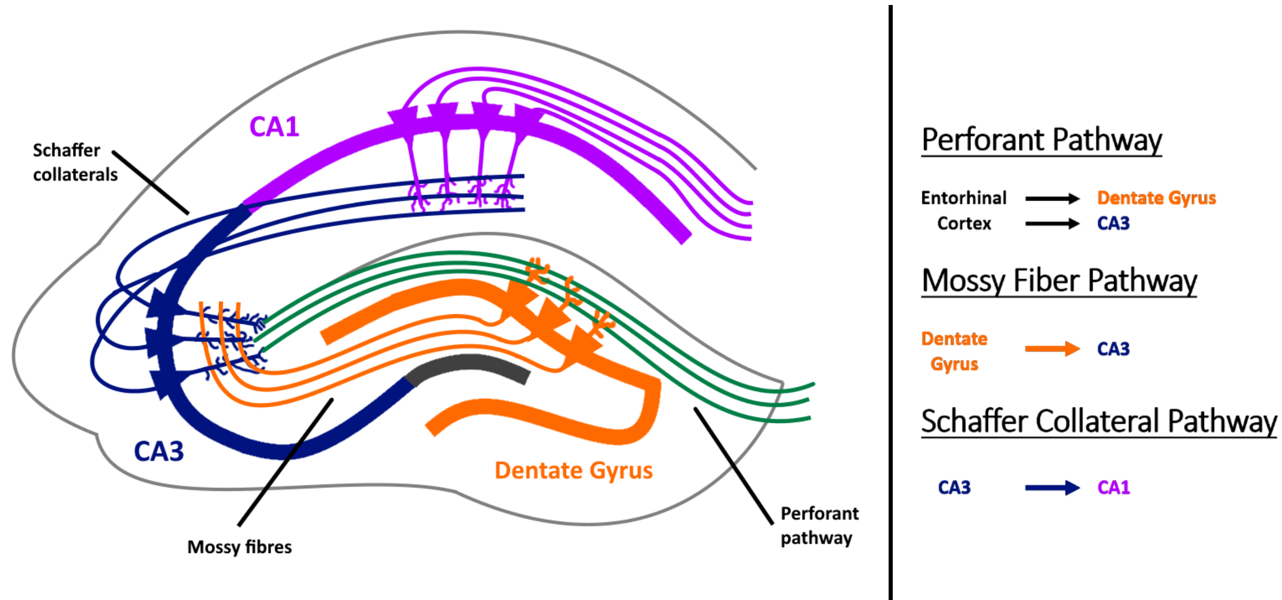

Figure 1.2. A schematic representation of hippocampal circuitry

All synapses in the trisynaptic circuit are excitatory glutamatergic synapses with the activity of the circuit regulated by a complex integration of inhibitory interneurons. It is estimated that a typical CA1 pyramidal neuron may have as many as 30,000 excitatory inputs and 1,700 inhibitory inputs [12]. Named after the first ligand found to bind to them, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are the main fast excitatory postsynaptic receptors in the hippocampus [13, 14] while the slow excitatory component is contributed by the N-methyl-D-aspartate (NMDA) receptor [15, 16]. Inhibitory neurotransmission is mediated by the γ-aminobutyric acid (GABA) receptor with two subtypes mediating fast inhibitory neurotransmission ($GABA_A$) and

slow inhibitory neurotransmission ($GABA_B$). Additionally, metabotropic receptors are present in the hippocampal synapses and can contribute to the slow components of excitatory or inhibitory neurotransmission depending on the receptor. These excitatory receptors will be focused upon in this review and are outlined below.

AMPA receptors (AMPARs) are glutamate-gated ion channels in the hippocampus that are tetrameric assemblies of four possible subunits, GluA1-4. These subunits are first assembled in the endoplasmic reticulum as dimers then come together to form a dimer of dimers to make a tetramer [17, 18]. In adult rat hippocampal pyramidal neurons, AMPARs are composed mainly of GluA1/GluA2 or GluA2/GluA3 dimers [19]. The GluA2 subunit plays a critical role in determining the conductance of the AMPAR since it contains the electrically charged amino acid, arginine, in the pore-lining domain that allows only the flow of monovalent cations (Na^+ & K^+) through the pore. AMPARs lacking the GluA2 subunit, and therefore the arginine residue in the pore-lining domain, are capable of conducting monovalent cations as well as divalent cations such as calcium. These GluA2-lacking AMPARs are referred to as calcium-permeable AMPARs (CP-AMPARs) while the GluA2-containing AMPARs that are normally found in rodent hippocampal pyramidal cells are referred to as calcium-impermeable AMPARs (CI-AMPARs). Aside from the conductance of calcium, CP-AMPARs have unique distinguishing features from CI-AMPARs such as an inwardly rectifying current-voltage relation due to voltage-dependent intracellular polyamine block of the channel and faster kinetics [20].

NMDA receptors (NMDARs) are also glutamate-gated ion receptors but differ from AMPA receptors in structural composition, permeability to cations, kinetics, and

voltage-dependence. NMDARs are tetramers, similar to AMPARs, composed of two obligatory GluN1 subunits along with either two GluN2 or GluN3 subunits. The GluN2 subunit, which contains the glutamate binding site, has 4 subtypes (GluN2A-D) while the GluN3 subunit has 2 subtypes (GluN3A&B) that determine the functionality of the NMDAR [21, 22]. When open, NMDARs conduct cations non-selectively and can have widely varying kinetics based on their subunit composition. In contrast to AMPARs, NMDARS do not conduct at resting membrane potentials due to a block in the channel pore created by extracellular Mg^{2+} ions. When the cell reaches the reversal potential of NMDARS (~0mV), Mg^{2+} is expelled from the pore allowing ions to flow freely through the NMDAR. As a result, the NMDAR acts as a molecular coincidence detector [23]: efficient activation and ion permeation through the NMDAR requires both a synaptic release of glutamate and a sufficiently strong depolarization of the membrane surrounding the NMDAR. In the hippocampus, this NMDAR activation provides the main source for extracellular calcium entry into pyramidal neurons to play a pivotal role in synaptic plasticity and memory [24].

1.3 Synaptic Plasticity & Memory

During the 1890s one of the most universally well-known studies of learning and memory was performed when Russian physiologist, Ivan Pavlov, first discovered classical conditioning by conditioning dogs to salivate upon hearing a metronome associated with food. With this discovery, Pavlov provided an objective approach to study memory through the use of conditioned reflexes which generated many theories of the underlying physiological processes behind memory formation. It wasn't until 1949 that these numerous theories were summarily described in a neuropsychological theory

by Donald Hebb who wrote “if the inputs to a system cause the same pattern of activity to occur repeatedly, the set of active elements constituting that pattern will become increasingly strongly interassociated” [25]; a statement is often paraphrased “cells that fire together, wire together”. The experience or activity dependent changes of the neuronal properties at the synaptic, function, and structural levels proposed by Hebb are cumulatively referred to as synaptic plasticity. This theory of Hebbian plasticity, also referred to as associative learning, has been the predominate way of thinking about the mechanisms and consequences of synaptic plasticity.

Soon after Hebb proposed his theory, the famous patient H.M. had bilateral removal of his hippocampus, suffered severe anterograde amnesia with limited retrograde amnesia, and thrust the hippocampus into the spotlight of learning and memory research. It was inferred from this that the hippocampus is critical for the formation of new memories whereas memory retrieval was not entirely hippocampal-dependent. Since that time the role of the hippocampus in learning and memory has been further elucidated and our general understanding of memory formation is as follows; Information about things such as an environment or an object is routed through the hippocampus as a short-term memory trace. The short-term memory capacity of the hippocampus is finite, and the trace is only retained for a matter of minutes before it is lost. If the short-term trace is deemed important enough to be stored as a long-term memory by extrahippocampal input and modification, the trace is then consolidated and permanently stored in the cerebral cortex. These consolidated memories, referred to as engrams, can be recalled by the hippocampus when necessary and are continuously modified as they are recalled [26]. This neural basis of memory formation and consolidation occurs at the cellular level in

the hippocampus via Hebbian plasticity and phenomena such as long-term potentiation (LTP). It has also been recognized that a counterbalance to Hebbian plasticity called synaptic scaling occurs via phenomena such as long-term depression (LTD) to prevent saturation of a synapse. Together, LTP and LTD are used to explain the neural basis of learning and memory.

Long-term potentiation (LTP) was first described in 1966 with Terje Lomo's abstract stating that "This represents an example of a plastic change in a neuronal chain, expressing itself as a long-lasting increase of synaptic efficacy. The effect, which may last for hours, is dependent on repeated use of the system." [27]. This work showed that subjecting hippocampal excitatory synapses to "repeated use of the system" with brief, high-frequency stimulation produced a rapid and long-lasting increase in the strength of the synapses that can persist for many days. Since this work over 50 years ago, LTP is still believed to be one of the primary mechanisms giving rise to synaptic plasticity in the brain and remains the foremost cellular model of learning and memory. Although LTP occurs at excitatory synapses throughout the brain, LTP occurring at the Schaffer collateral-CA1 synapses of the hippocampus represents the most robust and widely studied form of this phenomenon. For many years after the discovery of LTP there was debate whether the potentiation was the result of changes in the presynaptic neuron to release more glutamate or if the potentiation was the result of increased postsynaptic responsiveness to glutamate. The discovery of the ability of postsynaptic silent synapses to be unsilenced during LTP [28] along with the more recent observation that glutamate uncaging on single spines is sufficient to induce LTP [29], has largely solidified the LTP

mechanism as postsynaptic. LTP can be broken down into three phases: induction, expression, and maintenance.

Induction

Induction of LTP primarily occurs from a transient facilitation of presynaptic glutamate release, typically from a high-frequency tetanus event, and a transient increase in postsynaptic intracellular Ca^{2+} concentration [23, 30]. This transient increase in postsynaptic Ca^{2+} concentration during the induction phase is both a necessary and sufficient mechanism of LTP in hippocampal CA1 pyramidal cells as demonstrated by precluded LTP in the presence of Ca^{2+} chelators [30-33]. When sufficient increases in postsynaptic intracellular Ca^{2+} concentration occur, Ca^{2+} binds to the protein, calmodulin (Ca^{2+}/CAM), which in turn binds and activates Calcium/Calmodulin-dependent Protein Kinase type II (CAMKII), a serine/threonine protein kinase [34, 35] critical for phosphorylation of AMPARs in the expression phase of LTP [36]. Injection of Ca^{2+}/CaM into hippocampal CA1 pyramidal cells induces potentiated EPSPs that are identical to potentiated EPSPs from tetanus-induced LTP [37]. These potentiated EPSPs cannot be further potentiated with tetanus and are also observed in hippocampal CA1 pyramidal cells after injection or viral transduction of CAMKII [37, 38]. These experiments highlight the necessary and sufficient role of Ca^{2+} and its immediate downstream targets, CAM & CAMKII, on induction of LTP. Whether induced by tetanus or exogenous CAMKII activation, LTP is input specific in that once induced, LTP follows Hebbian plasticity strengthening the connection of one synapse that does not spread to other synapses [29].

Expression

Following the induction of the major form of LTP found in the brain, NMDAR-dependent-LTP, LTP is expressed as the result of increased AMPAR density in the postsynaptic membrane and/or increases single channel conductance of the AMPAR [39]. The expression of LTP has typically been broken into three stages: (1) Short-term potentiation (STP) which lasts approximately 15 minutes, (2) early-phase LTP (e-LTP) which typically lasts up to an hour, and (3) late-stage (l-LTP) that can last several hours [40]. This review will focus on the two later stages of LTP expression which have been defined by e-LTP not requiring protein synthesis whereas l-LTP requires protein synthesis and is more of a maintenance mechanism.

The expression of e-LTP is caused by an increase in AMPAR expression in the postsynaptic density (PSD) that is supplied immediately by non-synaptic pools of diffusing AMPARs, usually located in perisynaptic sites of the plasma membrane. This lateral diffusion of AMPARs to the PSD is supplemented in by exocytosis of intracellular pools of AMPARs that are anchored to the PSD via a wide variety transmembrane AMPA receptor regulatory proteins (TARPS). Trafficking of AMPARs to the PSD is highly regulated by intracellular Ca^{2+} and target kinases such as CAMKII, mitogen-activated protein kinase (MAPK), protein kinase A (PKA), and some isoforms of protein kinase C (PKC). The activation of these kinases leads to direct phosphorylation of AMPAR subunits to alter conductance and/or receptor trafficking to the postsynaptic membrane to increase postsynaptic strength. Of particular importance to synaptic plasticity is the phosphorylation of two sites of the GluA1 subunit, S831 and S845 [39, 41-44], which under basal conditions display little phosphorylation (~15%) [45]. It has been shown using gene knock-in mice that either S831 or S845 alone may support LTP in

the hippocampus, but phosphorylation of both sites is required for expression of maximal LTP suggesting a synergistic role of these two phosphorylation sites [42, 46]. Phosphorylation of GluA1 Ser831 occurs during e-LTP through CAMKII/PKC, via an increase in intracellular Ca^{2+}, to increase to increase AMPAR conductance and lateral diffusion of AMPARs to the postsynaptic density (PSD)[47-49]. Phosphorylation of GluA1 Ser845 occurs primarily by PKA phosphorylation contributing to LTP by promoting targeting of intracellular AMPARs to perisynaptic sites and retention of the AMPARs on the PSD [46, 50]. Since S845 phosphorylation can increase perisynaptic AMPAR density, PKA-mediated S845 phosphorylation can prime a synapse for LTP. By creating a larger pool of readily-available perisynaptic AMPARS, the threshold for LTP induction via CAMKII/PKC is lowered [41, 46].

AMPARS trafficked during LTP are generally assumed to be GluA1/GluA2 or GluA2/GluA3 CI-AMPARs, however there exists evidence, as well as conflicting reports, that CP-AMPARs are involved in the expression of all forms of LTP. The first evidence of the role of CP-AMPARs in hippocampal LTP was published in 2006 by Plant and colleagues who observed increased rectification during STP up to 10 minutes after tetanus [51]. This finding was followed by a report in 2007 by Gray and colleagues entitled “Long-Term Potentiation in the Hippocampal CA1 Region Does Not Require Insertion and Activation of GluR2-Lacking AMPA Receptors” [52]. While the evidence of CP-AMPARs during STP has been conflicting, the role of CP-AMPARs during e-LTP and l-LTP have been more well defined. The critical factor that determines whether the potentiation comprises e-LTP alone or a combination of e-LTP and l-LTP is the timing and strength of the induction trigger. CP-AMPARs can be inserted into the PSD by a

single high frequency stimulation and when subsequently activated promote protein synthesis to drive the expression of l-LTP[53, 54]. This initial insertion of CP-AMPARs is driven by PKA and thought to contribute to synaptic tag and capturing. Activation of PKA can occur as a result of G_s-coupled GPCR activation such as the D1-type dopamine receptor (D1R) and the β-adrenergic receptor which act as neuromodulators of hippocampal memory and LTP [55]. Importantly, drugs of abuse increase dopamine levels in the brain which can lead to increased D1R activation and consequently PKA activation. As outlined above, PKA activation can lower the threshold for LTP induction via increased perisynaptic AMPARs and promoting synaptic tag and capture.

1.4 Cocaine Addiction as a Disease of Learning and Memory

Indigenous people of the Amazon Rainforest and Andes Mountains have chewed on the leaves of the coca plant for energy for thousands of years until scientists isolated cocaine (benzoylmethylecgonine) in the 1850s. The molecule was touted as a miracle drug through the early 1900s with Sigmund Freud publishing a book entitled “Uber Coca” claiming that cocaine could help cure heroin addiction. It was so popular that it was even included in the original formula of the soft drink Coca Cola. Cocaine was made illegal in the U.S. with the passing of the Harrison Narcotics Act of 1914 and as of 2018 is the second most commonly used drug of abuse, behind only marijuana, with over 5 million users per year [56]. Cocaine acts as a dopamine transporter (DAT) inhibitor to raise synaptic levels of dopamine (DA) and produce a euphoric and energetic feeling for its users. The drug also inhibits other monoaminergic reuptake transporters such as the serotonin transporter (SERT), as well as the norepinephrine transporter (NET), further

contributing to the euphoric and energetic feelings [57]. These euphoric, or rewarding, properties are a hallmark of addictive substances that are commonly abused.

Addiction is a complex brain disease that is characterized by compulsive drug seeking and use despite harmful consequences. These changes are often persistent, which is why drug addiction is considered a “relapsing disease” – people in recovery from substance use disorder are at increased risk of returning to drug use even after years of abstinence. Repeated drug use results in strong associations between the rewarding properties of drugs of abuse and the context that the drug was taken, driving this relapse feature of addiction [58]. These maladaptive associations and resulting behavioral changes that drive drug-seeking are a consequence of persistent, underlying changes in neural plasticity [59-61]. As a consequence, individuals who suffer from cocaine addiction exhibit learning deficits and cognitive inflexibility [62]. There has been increasing appreciation for the role of the brain’s learning and memory systems in addiction over the past several years due to the high degree of overlap between the neurobiology these systems and those primarily responsible for addiction [63, 64]. As emphasized prior, the hippocampus is a brain region critical to learning and memory with a high degree of plasticity. Given the role of this region in forming contextual memories, drugs of abuse likely produce alterations in hippocampal function that have a profound, negative effect on behavior [65].

Researchers have developed animal behavioral paradigms in an attempt to elucidate the neural mechanisms of drug addiction: the two most common paradigms of cocaine addiction are conditioned place preference (CPP) and self-administration (S.A.).

Both models require hippocampal learning and memory systems and are briefly detailed at the end of this section.

Conditioned Place Preference (CPP)

Conditioned Place Preference is a commonly used addiction behavioral models across due to the ease with which it can be performed [66]. The typical set up comprises of two compartments that utilize different floor, lighting, walls, etc. to create two contextually distinct environments. These chambers should represent a neutral environment to an animal and when the animal is given the option to freely move between both, should not favor one side over the other. Researchers can use a drug or non-drug treatment to serve as an unconditioned stimulus (UCS) that is repeatedly paired with one of the two chambers. If the UCS has rewarding properties, as is the case with cocaine, the animal will prefer the side of the chamber in which it received the drug and thus display conditioned place preference. Conversely, if the UCS is a noxious compound, the animal will avoid the side of the chamber in which it was given the noxious UCS and display conditioned place aversion. Since the UCS is given to the animal by a researcher, CPP is considered a non-contingent model of addiction.

Self – Administration

Self-administration is a form of operant conditioning with drug as a reward. The drug is administered remotely through an I.V. catheter, for immediate effect, when the animal performs a task such as pressing on a lever. This creates an associative learning process through which the behavior is positively reinforced by the drug. Self-administration is a contingent paradigm since the animal has control of the drug administration, but the researcher can still modify the experiment in many ways; this

includes the use of cues, different schedules of administration, and the amount of time the animal has access to the drug. Using these variations of the S.A. paradigm researchers can observe individual differences in drug-taking behavior which may be helpful to parallel individual susceptibility to drug use in humans.

Self-administration is thought to most closely mimic human use since the drug intake is voluntary and the animal usually escalates its intake under its own will. Since CPP requires researcher administration of the drug, it has drawn some criticism for not being as relevant to drug addiction studies as self-administration [59]. However, both behavioral paradigms engage learning and memory processes relevant to addictive behavior such as acquisition, extinction, and reinstatement of drug-induced conditioned responses. Due to the ease with which CPP can be performed compared to S.A., especially in smaller rodents such as mice, it is important to identify and reconcile the differences between the two in future addiction research studies. Much of the work presented in this dissertation aims to demonstrate that CPP is a valid model of addiction that can produce persisting and pertinent neurophysiological changes. It is here that I would like to highlight the previous studies which influenced the trajectory of my graduate career and the research that will be presented.

The traditional model of CPP is performed over several days with animals receiving a fixed dose of cocaine (usually 5-20 mg/kg i.p.). Yossef Itzhak's lab at the University of Miami postulated that this fixed dosing schedule did not mimic the escalation of intake seen in both self-administration and human use. His lab demonstrated that an escalating dosing schedule resulted in a larger CPP shift that persisted through extinction training, even though the total amount of cocaine administered was the same as

the fixed dosing schedule group [67, 68]. These papers provided the inspiration for the escalating doses of cocaine that I used throughout my experiments. Additionally, I need to recognize the work of a previous graduate student in our lab, Madhu Keralapurath, who recognized the effects of stress from the CPP paradigm on hippocampal plasticity [69]. By identifying the role of the kappa opioid system during CPP, I was able to easily replicate his findings (see Chapter 2) and ameliorate the confounding stress variable from my experiments; a variable that may account for some of the discrepancies in results from CPP and S.A. studies.

1.5 The Circuit Model of Addiction

To understand the role of the hippocampus in the learning and memory of addiction it is necessary to first understand its position within the brain's mesolimbic, or reward, circuitry. At its most basic level, the mesolimbic system consists of the ventral tegmental area (VTA) and the nucleus accumbens (NAC). The VTA serves as the major source of dopamine for the mesolimbic system projecting to the GABAergic neurons of the NAC, which in turn project back to the VTA. Increased release of dopamine (DA) in this mesolimbic pathway linking the VTA to the NAC plays a major role in the rewarding properties of both drugs of abuse as well as natural rewards and has been aptly named the "Reward Pathway" [70]. While the foundation of the mesolimbic system is built around this dopamine pathway, the VTA and NAC also project to, and receive modulatory input from, numerous brain regions. These connections are highlighted in Figure 3 and contribute to the "Circuit Model of Addiction" that has been developed as the role of glutamate in addiction has gained increased appreciation [61].

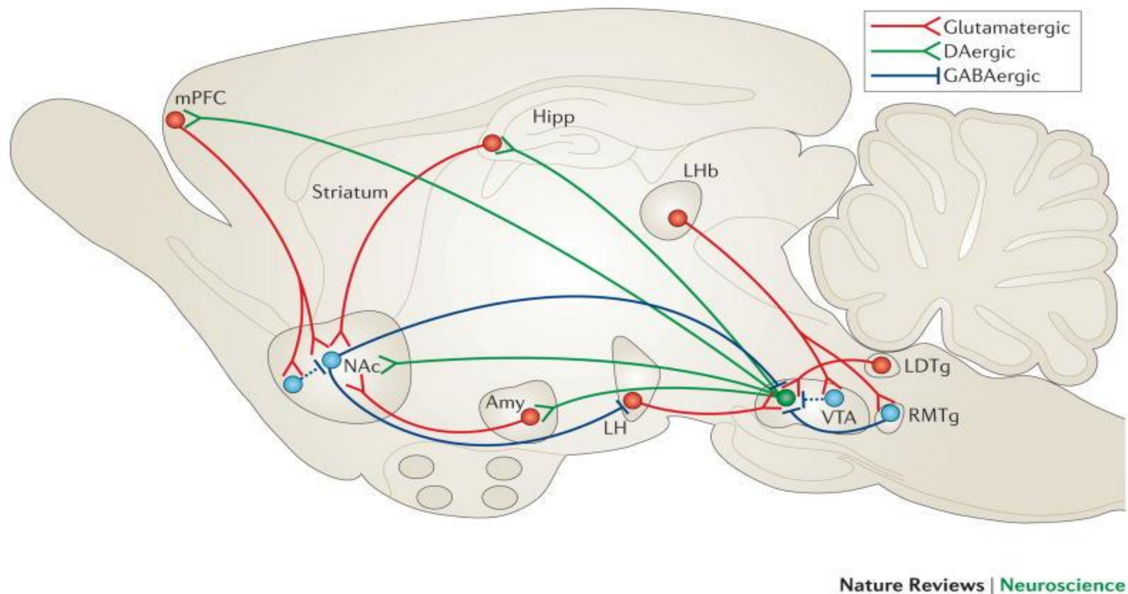

Figure 1.3. Is a diagram depicting the major brain regions involved in the circuit model of addiction [71]

The mesolimbic system is responsible for encoding an individual's responses to natural rewards, such as food and sex, and provide motivational drive towards these evolutionarily advantageous objectives. When a rewarding stimulus is encountered, the mesolimbic system can encode contextual information via increased dopamine release from the NAC to the VTA. The next time the individual encounters this context, the mesolimbic system is primed to expect a rewarding stimulus and releases dopamine. If there is no subsequent reward, then a decrease in dopamine levels occurs shortly after to update that information that this context should no longer be associated with rewarding stimuli. This process is known as the reward prediction error hypothesis and is thought to incentivize and drive evolutionarily beneficial goals [72, 73]. Cocaine, like naturally rewarding stimuli, increases tonic dopamine levels at VTA afferents by inhibiting the

dopamine transporter to prevent reuptake [74]. However, the dopamine increase in the VTA is larger from cocaine than from natural stimuli leading to a euphoric feeling that rivals or surpasses a food or sex reward. Since cocaine acts throughout the entire brain, it increases dopamine at all VTA afferents such as the medial prefrontal cortex (mPFC), hippocampus, and the amygdala. It is the long-lasting changes of these brain regions, along with their subsequent changed inputs into the NAC and VTA, that are thought to underly the behaviors that drive drug seeking and relapse [61].

Of particular interest is the hippocampus in this circuit model of addiction. The hippocampal formation can be separated both functionally and anatomically along its septotemporal axis into two main sectors: the dorsal hippocampus (dH) and ventral hippocampus (vH). The dH is primarily involved in mediating cognitive functions while the vH plays an important role in stress responses and motivational factors [8, 75]. With regards to cocaine addiction, the vH is anatomically positioned to play a modulatory role in the addiction circuit since it receives dopaminergic innervation from the VTA [76-78] and projects glutamatergic fibers to the NAC [79]. This dopaminergic innervation of the hippocampus has been shown to potentially play an important role in memory formation and encoding [55, 80] (also section 1.3).

This circuit model of addiction is proposed to contribute to the incubation of cocaine craving, a phenomenon where the onset of drug craving in rodents progressively increases throughout withdrawal. Lasting changes of the inputs into the nucleus accumbens, as well as changes in VTA synaptic plasticity, are thought to drive this incubation of cocaine craving via the insertion of CP-AMPARs [59, 61, 81]. The VTA undergoes immediate (within 2 hours) changes in synaptic transmission in response to a

single cocaine injection that lasts around 5 days [82]. Similarly, a single 15 mg/kg i.p. injection is sufficient for CP-AMPAR insertion in the VTA within 48 hours [83]. This information, combined with the fact that a single 5 mg/kg injection of cocaine is sufficient to produce CPP in C57Bl/6J mice [84], displays the potent ability of cocaine to alter VTA synapses and behavior in a persisting manner. These changes in VTA synaptic transmission are thought to drive the changes seen throughout withdrawal in the NAC [85]. The NAC however displays an increase in excitatory transmission within a week of cocaine withdrawal [86] with CP-AMPARs not being observed until withdrawal day 25+[85, 87]. Given the temporal segregation of CP-AMPAR insertion in these two brain regions, it is reasonable to postulate that an intermediate brain region, such as the vH, may play an important role in driving the changes in the NAC associated with the incubation of cocaine craving. Consistent with this theory, synaptic alterations are observed within the vH CA1 region [69, 88]as well as in vH-NAc synapses after cocaine withdrawal [89]. These changes in vH plasticity during withdrawal appear to be dynamic and progressive; enhanced long-term potentiation (LTP) is observed one week after cessation of self-administration [90, 91] and decreased LTP is observed after longer withdrawal periods from cocaine self-administration or conditioned place preference (CPP) [88, 92].

1.6 Introductory Summary

Given the important role of the hippocampus in both learning & memory and the reward system of the brain highlighted above, the studies here have focused on the changes that occur in the ventral hippocampus (VH) following cocaine administration. In the following chapter, I will discuss the development of our escalating conditioning

protocol as well as the effects of long-term (>28 days) cocaine withdrawal on VH synaptic plasticity. In the third chapter I build on the findings of the second chapter with whole-cell electrophysiology data that allowed us to assess the changes in VH receptor composition at three different withdrawal time points. I hope that, taken together, these data help outline the progressive changes in VH synaptic transmission during withdrawal and can be used to further study both the mechanisms of addiction and potential therapeutics for this disease.

1.7 References

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CHAPTER 2

COCAINE CONDITIONING INDUCES PERSISTING CHANGES IN VENTRAL HIPPOCAMPUS SYNAPTIC TRANSMISSION, LONG-TERM POTENTIATION, AND RADIAL ARM MAZE PERFORMANCE IN THE MOUSE [1]

1. Preston CJ, Brown KA, Wagner JJ. 2019. Neuropharmacology. 150:27-37. Reprinted here with permission of the publisher.

Abstract

The effects of drugs of abuse, such as cocaine, on learning and memory processes are thought to contribute to drug craving and relapse susceptibility. Using two different escalating cocaine i.p. dosing schedules (Esc and 2x Esc) with the conditioned place preference (CPP) model we investigated the persisting effects of cocaine conditioning on long-term potentiation (LTP) in the CA1 region of the ventral hippocampus (vH), and spatial working memory in a radial arm maze (RAM) task. Interestingly, vH LTP was increased in animals that received only saline vehicle injections up to 4 weeks after the last injection day. A single pre-treatment with the kappa-opioid receptor antagonist, norbinaltorphimine, blocks this stress-like effect of the conditioning protocol on vH LTP without altering the behavioral responses of the animals to cocaine. In animals that received the 2x Esc/norBNI cocaine conditioning, vH LTP was significantly decreased compared to those that received saline vehicle 4 weeks after the last dose. These 2x Esc/norBNI treated animals also exhibited a significant leftward shift in the stimulus-response curve of the baseline fEPSP measurements. A separate group of 2x Esc/norBNI displayed an impaired ability to learn a spatial working memory RAM task compared to saline-conditioned mice following a similar 4-week abstinence period. Together, these results demonstrate that cocaine-induced alterations in synaptic transmission and LTP in the vH are associated with persisting drug-induced impairments in learning and memory performance.

2.1 Introduction

Learning and memory processes modified following drug use contribute to the formation of contextual associations with the drug-taking environment that are maintained through abstinence and may contribute to relapse. These drug associations, as well as cognitive performance in behavioral assays such as the radial arm maze (RAM), have been shown to rely on a hippocampal-dependent forms of learning and memory [1]. Functionally and anatomically the hippocampal formation can be divided along its septotemporal axis into at least two sectors: the dorsal hippocampus (dH) and the ventral hippocampus (vH). The dH mediates cognitive functions such as spatial memory while the vH plays an important role in responses to stress and motivational factors [2,3]. The CA1 region of the vH receives dopaminergic innervation from the Ventral Tegmental Area (VTA) [4,5] and projects glutamatergic fibers to the Nucleus Accumbens (NAc) [6,7]; two regions involved in cocaine craving and addiction[8,9]. The vH displays enhanced long-term potentiation (LTP) in the CA1 region one week after cessation of cocaine self-administration [10], followed by a decrease in LTP after longer periods of withdrawal as a result of increased basal transmission [11]. This drug-induced metaplasticity persists despite weeks of abstinence, indicating a possible contribution to reinstatement of drug-seeking behaviors long after short-term withdrawal effects have subsided [8,12].

Over the past five years there have been over 1,300 publications using the conditioned place preference (CPP) model of drug motivated behavior (PubMed, Embase). Despite the prevalence of this model, there is no consensus regarding the ability of CPP to produce the persisting changes in neural transmission and plasticity that

have been observed using operant models of cocaine self-administration. For example, some groups have obtained similar results after abstinence when comparing non-contingent and contingent administration in regions such as the NAc [13,14] whereas other groups have not observed comparable cocaine-induced changes in plasticity following non-contingent administration [15,16]. Nevertheless, non-contingent drug exposure protocols (e.g. CPP) can provide considerable advantages such as lack of surgical intervention and maintenance of catheter patency, adaptability to species not readily amenable to catheterization, and uniform drug exposure among treatment groups [17]. Additionally, cocaine conditioning has been shown to be hippocampal-dependent [49,50] and can strengthen the connections between the hippocampus and the nucleus accumbens [51]. Given such practicalities and the established use of the CPP approach, it is crucial to further refine the use of such non-contingent administration models and establish their validity.

Building from findings that cocaine-induced CPP was increased in mice that received an escalating conditioning dose compared to those that received a “standard” fixed dose [18,19], we used two non-contingent escalating dosing schedules (Esc and 2x Esc) to recapitulate the long-lasting changes in synaptic function in the vH that we have previously observed following cocaine self-administration [11]. As cocaine use disorder has been shown to produce cognitive deficits in humans [20,21], we assessed the impact of escalating cocaine conditioning in our rodent model on both neurophysiological and behavioral performance. We recorded field excitatory postsynaptic potentials (fEPSPs) from the CA1 region of the mouse vH to assess changes in synaptic transmission and LTP that persist after prolonged cocaine abstinence. Behavioral performance in a

modified Win-Shift task using the RAM to test spatial working memory was also measured after a similar abstinence period. Our results indicate that persisting alterations in synaptic transmission and plasticity in the vH correspond with drug-induced impairments of hippocampal-dependent learning.

2.2 Methods

Animal Maintenance

C57Bl/6J (Jackson Laboratory, Bar Harbor, ME) mice aged 8-10 weeks were housed in groups of 5 in clear Micro-Isolator cages. Animals were maintained on a 12 h light/dark cycle (0700/1900 h) and provided with food and water *ad libitum*. Animals were allowed at least 1 week to acclimate to their home cages after arrival and received 3 days of habituation to handling prior to the start of behavioral experiments. For each cage of 5 mice used in this experiment, 1 mouse was identically handled but not subjected to behavioral protocols to establish a naïve group to assess effects of the protocols. All experiments were performed in compliance with the University of Georgia Animal Care and Use Committee guidelines.

Apparatus

Behavioral tests requiring an open-field setup were performed in a 43.2 cm x 43.2 cm chamber with a smooth, solid floor and clear plastic walls (Med Associates Inc., St. Albans, VT). These chambers were located in sound-attenuating boxes in a room separate from the home cage location and equipped with two lights (~20 lux) and a ventilation fan. Two pairs of 16 beam infrared arrays were used to record horizontal activity with

Activity Monitor software (Med Associates Inc.) to count beam breaks. Conditioned place preference (CPP) was performed in the same boxes as open-field testing with the addition of a two-compartment insert (Med Associates Inc.) specifically designed for use with mice. Each compartment was identical in size but differed in both flooring and luminosity as previously described [22].

Locomotor Sensitization

Open-field locomotor activity was recorded on day 1 of behavioral testing and again on the challenge day one week after the final conditioning day (Figure 1A). Data for locomotor sensitization (LMS) was obtained by recording ambulatory and stereotypic counts after placing the animal in the center of the open field chamber for 30 minutes followed by an intraperitoneal injection of either 0.9% saline or cocaine (10 mg/kg). The animal was subsequently returned to the open field chamber and recorded for 60 minutes. Animals, chosen at random, to receive cocaine injections for LMS also received cocaine during CPP training while those that received saline were never exposed to cocaine. Animals pretreated with norbinaltorphimine (norBNI; 10 mg/kg i.p.) received injections 24 hours before the activity day 1 (on the last handling day). LMS was determined by increased total locomotor activity on the challenge day in response to drug or saline compared to the initial activity day response.

Conditioned Place Preference

For the place preference assay, mice were allowed free-access to both chambers on the pretest day (day 2) to assess the most- and least-preferred compartments as determined by

percent of time spent in each compartment over a 15 minute period. Each conditioning day consisted of two sessions with mice restricted to one compartment for 15 minutes in each session. We utilized a bias design with mice in all groups received saline on their most preferred side in the morning (~10:00) and then either cocaine or saline on their least preferred side in the afternoon (~14:00). Mice in the cocaine-paired groups received escalating doses of cocaine from one of the two following schedules:

Escalating (Esc): 4 Sessions over days 3-6 with mice receiving cocaine at 4,8,16,24 mg/kg i.p.; one dose per day.

Double-Escalating (2x Esc): 8 Sessions over days 3-6 and 9-12 with mice receiving cocaine at 4,8,16,24 mg/kg i.p. and 16,24,32,32 mg/kg i.p., respectively; one dose per day.

A 15-minute post-test was performed 24 hours after the final conditioning session where mice were again placed in the place-preference chamber with access to both compartments. CPP results are reported as the change in time the mice spent in the least preferred side on the post-test day compared to the pre-test day.

Extracellular field recording

Mice were sacrificed either 1 or 4 weeks after the last exposure day using halothane to deeply anesthetize the mice prior to decapitation. Brains were removed from the mice and submerged in oxygenated (95% O_2 / 5% CO_2), ice-cold dissection artificial cerebrospinal fluid (dACSF; 120 mM NaCl, 3 mM KCl, 4 mM $MgCl_2$, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose). The brain was mounted on its dorsal pole and sectioned along the horizontal plane with a vibratome to obtain 400 µm thick sections containing

the ventral hippocampus (vH). The vH was isolated from the rest of the slice and the CA3 region was removed. Theses slices were then transferred to a submersion-type chamber and perfused with oxygenated (95% O_2 / 5% CO_2) ACSF (120 mM NaCl, 3 mM KCl, 1.5 mM $MgCl_2$, 1 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10mM glucose) and allowed to recover for 45 minutes at room temperature followed by 45 minutes at the recording temperature of 30°C. Following the 90 minute recovery, a bipolar stimulating electrode (Kopf Instruments, Tujunga, CA) was placed within the stratum radiatum of CA1 and a 1.0 MΩ tungsten recording microelectrode (World Precision Instruments, Sarasota, FL) was placed in the same layer of CA1. A stimulus pulse was applied once every minute consisting of a single square wave of 270 µs duration to elicit field excitatory postsynaptic potentials (fEPSPs) at Schaffer collateral-CA1 synapses. Data was digitized at 10 kHz, low-pass filtered at 1 kHz, and analyzed with pCLAMP 10.2 software (Axon Instruments, Sunyvale, CA).

A stimulus response curve was obtained at the beginning of each experiment by incrementally delivering stimulus pulses ranging from 40-180 µA in intensity and measuring fEPSP slope (mV/ms) in a 1ms window immediately following the fiber volley. The stimulus intensity was adjusted to elicit stable baseline responses of ~35% of the maximal fEPSP slope. Paired-pulse responses were performed at an inter pulse interval of 50 ms between pulses. Baseline responses were recorded each minute for 30 minutes prior to a high-frequency stimulation protocol (3 x 100 Hz / 1 s trains at 20 s intervals) used to induce long-term potentiation (LTP). Responses were recorded 60 minutes following HFS. fEPSP slopes were normalized to baseline using the average value of the five responses immediately prior to HFS. LTP values were determined by

averaging the normalized fEPSP slope values at 56-60 min after high-frequency stimulation. Reported n-values (x(y)) indicate the number of slices (x) and the number of mice (y) assessed.

Radial arm maze apparatus and animal preparation

A standard 8-arm radial arm maze (RAM) (Med Associates, St. Albans, VT) was used to assess learning and memory function [23] in mice after undergoing the 2x Esc/norBNI or 2x Sal/norBNI conditioning protocols. The maze consisted of an octagonal central chamber with 8 equal length arms extending from each side of the central chamber. At the distal end of each arm was a food trough containing photosensors that dispense a 20mg sucrose pellet when activated. Between the central chamber and the trough in each arm were photosensors to track mouse movement. The top and sides of each arm were composed of clear plastic to allow mice to use external visual cues placed around the room as a basis for spatial navigation. MED-PC software 4.0 (Med Associates, St. Albans, VT) was used to control and record photosensor, food, and door signals from a computer in an adjacent room.

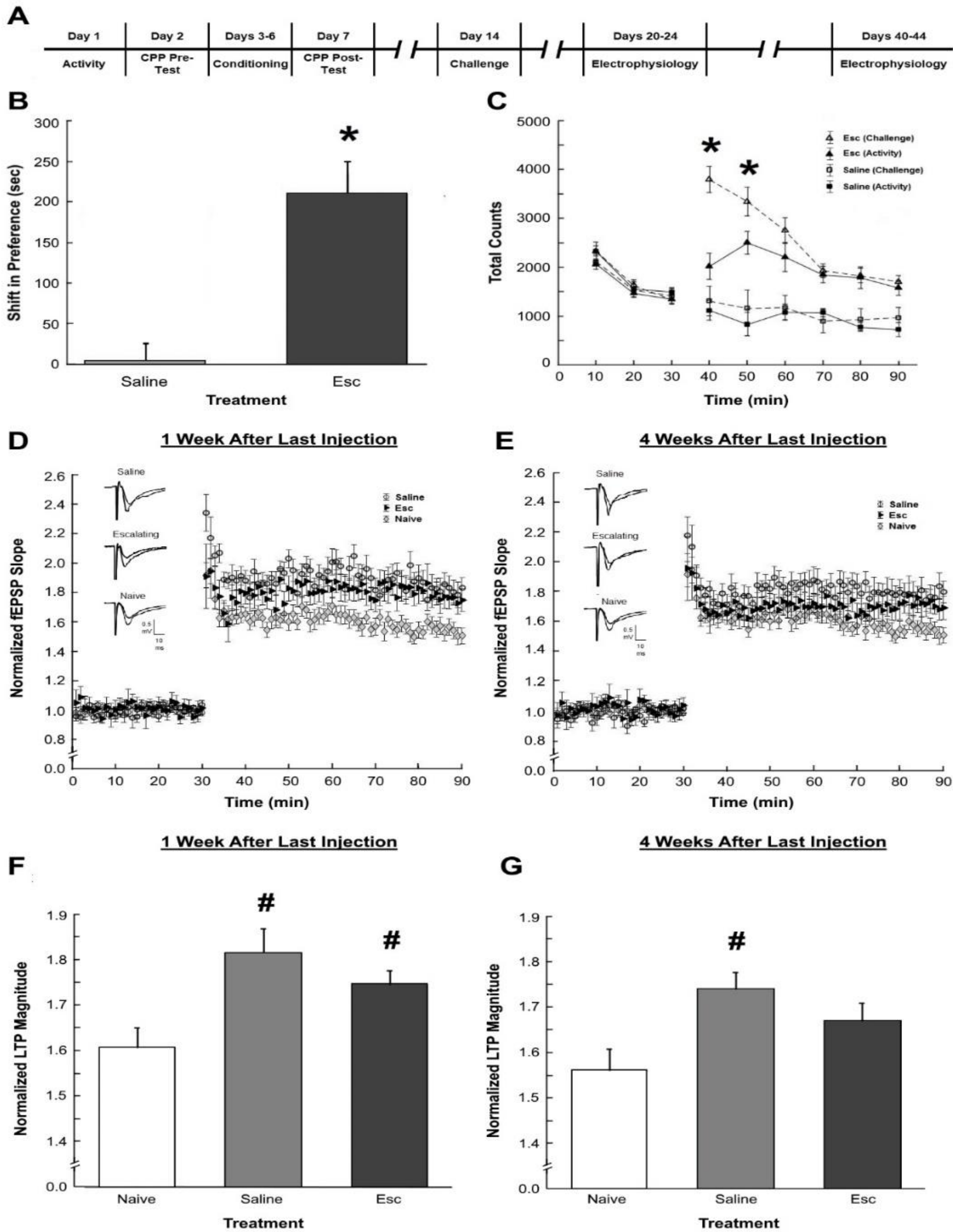
Behavioral assessments in the radial arm maze were performed 14-34 days after the challenge session (Figure 5A). Immediately following the challenge day, mice were individually housed and given norBNI (10 mg/kg i.p.) to mitigate the stress effects of single housing. The following day, mice began a food restricted diet to maintain a body weight at 85% of their average free-fed body weight. Mice were behaviorally acclimated, and the maze was cleaned between sessions as previously described in Clark *et al,* 2015 [24].

Radial arm maze testing to assess spatial working memory

Spatial short-term working memory was assessed in a standard 8-arm RAM uninterrupted task once a day, at the same time of day, over 10 consecutive days. Each mouse was placed in the central chamber for a 1 minute acclimation period after which all 8 doors opened allowing free access to all arms. In this task, each arm is "baited" so that one food reward is dispensed upon the first head poke into the food trough of each arm (Figure 5B1). If an animal revisited a trough after the initial visit, this was considered an error in spatial short-term working memory; the response variable was recorded as the number of errors in the first 8 choices.

Following the final day of the spatial short-term working memory task, spatial long-term working memory was tested over 10 consecutive days using a modified delayed spatial Win-Shift assay (see [24]). This assay utilizes a 2-phase procedure with an interposed delay (Figure 5C1). During the initial study phase, 4 randomly chosen doors of the 8 opened allowing the mouse access to 4 baited arms. After collecting the last food reward, the mouse was returned to the home cage and subjected to a 4 minute retention interval while the maze was cleaned. The mouse was then returned to the central chamber, allowed a 1 minute acclimation period (total retention interval of 5 minutes), and began the test phase. During the test phase, all 8 doors opened allowing free access to 8 arms where only head pokes in the previously unvisited 4 arms resulted in a food reward. A visit to an arm that was previously baited in the study phase was considered an error in spatial long-term working memory; the response variable was recorded as the number of errors in the first 4 choices in the test phase.

2.3 Results



<u>Figure 2.1</u> A. Timeline of the schedule for the Esc dosing protocol. **B.** Results from conditioned place preference assay are shown as shifts in time (sec) spent on the least preferred / cocaine-paired side for Esc (213 +/- 40, n=10) and Sal (7 +/- 21, n=7) mice. **C.** Effects of Saline or Esc conditioning on total locomotor counts from activity and challenge days are shown. Summary plots of normalized fEPSP values recorded either 1 (**D**) or 4 (**E**) weeks after final injection day. Each plot compares saline-treated mice, cocaine-treated, and behaviorally-naïve mice. Insets show representative sweeps composed of average sweeps from 5 minutes pre-tetanus and 56-60 minutes post-tetanus. **F.** Summary quantification of LTP values recorded 1 week after challenge day from Saline (1.82 +/- 0.06, n=10(3)), Esc (1.75 +/- 0.03, n=13(5)), and naïve (1.61 +/- 0.04, n=30(11)) animals. **G.** Summary quantification of LTP values recorded 4 weeks after challenge day from Saline (1.74 +/- 0.03, n=15(5)), Esc (1.67 +/- 0.04, n=19(6)), and naïve (1.57 +/- 0.03, n=30(11)) animals. * $p<0.05$ compared to treatment group; # $p<0.05$ compared to behaviorally naïve mice; Error bars +/- SEM.

2.3.1 Saline i.p. injections and associated behavioral protocol experience enhance vH LTP for at least 4 weeks

Mice that received the Esc dosing protocol displayed a significant shift in place preference (Figure 1B) for the cocaine-paired side as compared to the Sal-treated mice ($p<0.001$). Figure 1C shows the locomotor activity assessed on the activity/challenge days from Esc and Sal conditioned animals. A two-way repeated measures ANOVA revealed a significant effect of day (activity or challenge) on the total locomotor counts ($F=6.89$, $P<0.001$) for mice in the Esc group. Subsequent SNK post-hoc analysis revealed

a significant ($p<0.05$) increase in the total locomotor activity counts 10 and 20 minutes post-injection between the activity and challenge days of mice in the Esc group. LTP values obtained from the CA1 region of vH slices 1 week after the challenge day (Figures 1D,F), indicated significant treatment effect ($F=5.12$, $p<0.01$) between Sal, Esc, and Naïve animals. Post-hoc analysis revealed that both Sal and Esc conditioned mice had significantly elevated LTP ($p<0.05$) when compared to behaviorally naïve mice that received neither i.p. injections nor behavioral conditioning. No significant difference was observed in post-hoc analysis between Esc and Sal groups. LTP values obtained from vH slices 4 weeks after last injection (Figures 1E,G) again indicated significant treatment effect between Sal, Esc, and Naïve animals ($F=3.07$, $p<0.05$). Post-hoc analysis revealed a significant increase in vH LTP of the Sal group when compared to the behaviorally naïve group ($p<0.05$).

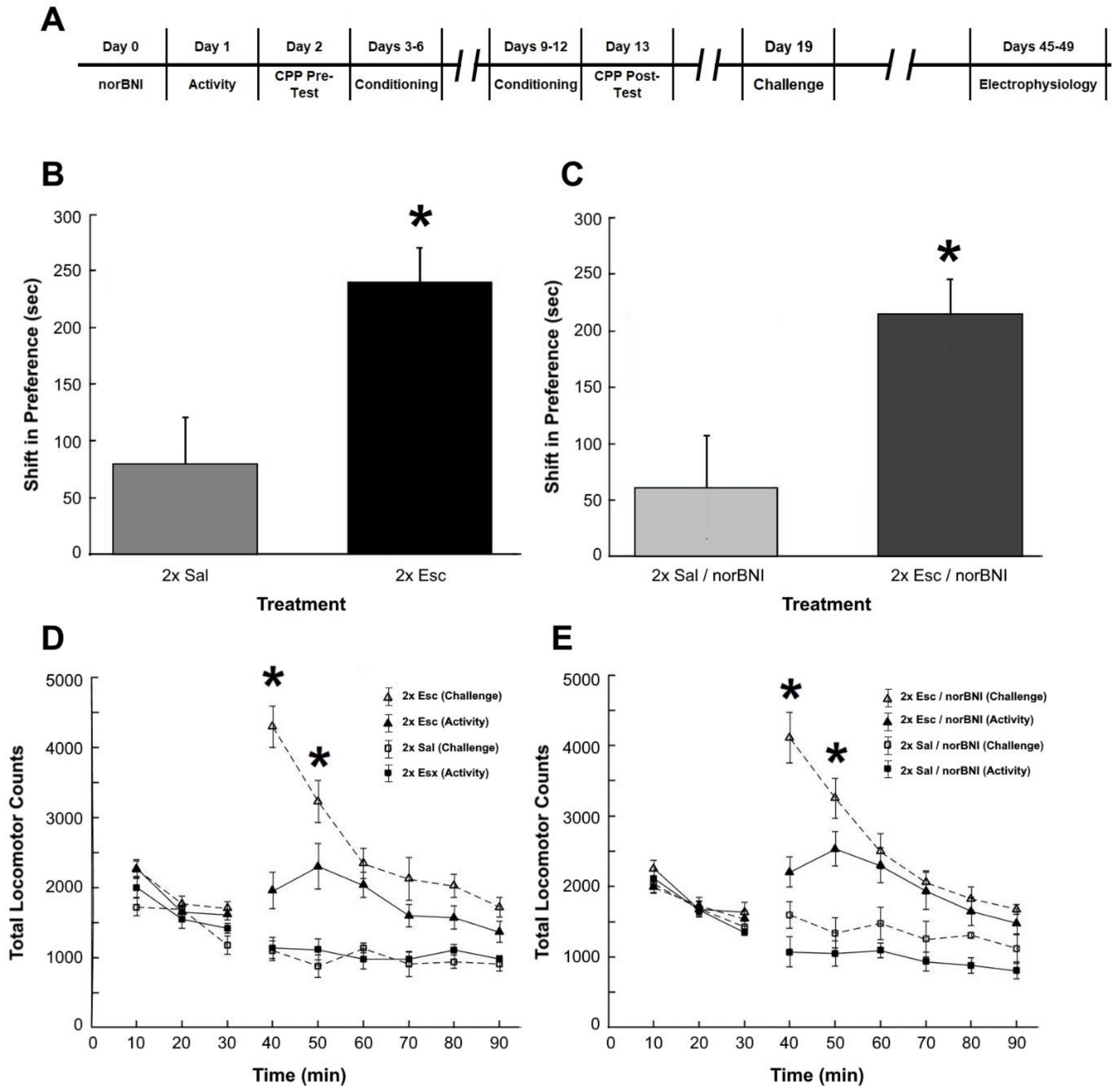


Figure 2.2 **A.** Timeline of the schedule for the conditioning schedule for mice that received the 2x Esc protocol. **B** Results from conditioned place preference assay are shown as shifts in time (sec) spent on the least preferred / cocaine-paired side for 2x Esc (240 +/- 30, n=9) and 2x Sal (80 +/- 41, n=4) animals. **C.** Results from conditioned place preference assay are shown as shifts in time (sec) spent on the least preferred / cocaine-

paired side for 2x Esc/norBNI (215 +/- 31, n-10) and 2x Sal/norBNI (61 +/- 46, n =5).Effects of 2x Sal or 2x Esc conditioning with (**E**) or without (**D**) norBNI on total locomotor counts from activity and challenge days are shown. * $p<0.05$ compared to treatment group. Error bars +/- SEM.

2.3.2 The 2x Esc dosing schedule produced increased CPP and LMS that were not altered by norBNI pretreatment.

In order to identify and potentially isolate the effects of stress on vH LTP from those of cocaine, CPP and LMS were assessed in mice that received either the 2x Esc or 2x Sal conditioning protocol (Figure 2A) with separate groups of mice receiving norBNI pretreatment (10mg/kg). Additionally, the increase in number of conditioning days (and associated dosages) was done in an effort to mimic the escalation of drug intake observed in long-access cocaine self-administration protocols. In a two-way ANCOVA, we observed a significant ($p<0.001$) increase in place preference of 2x Esc mice compared to 2x Sal mice. A significant ($p<0.001$) increase in place preference was also observed in 2x Esc/norBNI mice compared to 2x Sal/norBNI. There was no significant treatment effect of norBNI ($F=0.28$, $p=0.60$) or interaction effect ($F=2.56$, $p=0.12$) on shift in preference.

Figures 2D and 2E show the total locomotor activity counts assessed on the activity and challenge days of mice receiving the 2x Esc or 2x Sal protocols with (Figure 2E) or without (Figure 2D) norBNI pretreatment. A two-way repeated measures ANOVA showed a significant effect of day (activity or challenge) on total locomotor counts in 2x Esc mice ($F=7.18$, $p<0.001$) and 2x Esc/norBNI mice ($F=6.77$, $p<0.001$). Subsequent

SNK post-hoc analysis revealed a significant difference in the total locomotor activity counts 10 and 20 minutes post injection between the activity and challenge days of both 2x Esc ($p<0.05$) and 2x Esc/norBNI groups ($p<0.05$). There was no significant treatment effect of norBNI ($F=2.02$, $p=0.18$) or interaction effect on locomotor counts ($F=0.69$, $p=0.42$). These CPP & LMS results demonstrate that norBNI pretreatment does not impact these behavioral measures of drug conditioning, allowing for the determination of cocaine-induced effects on vH function to be made in the absence of overlying stress-induced effects.

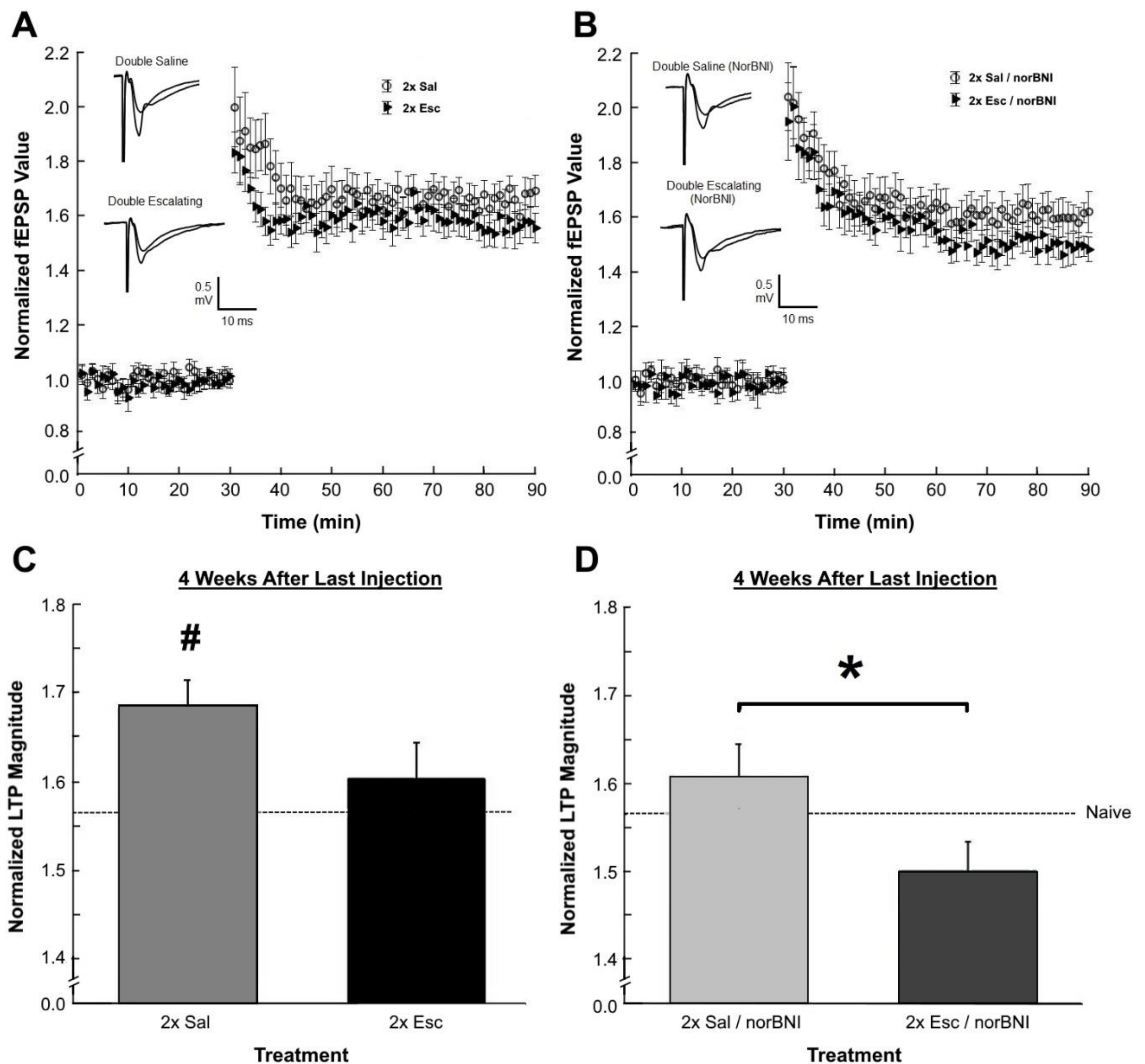
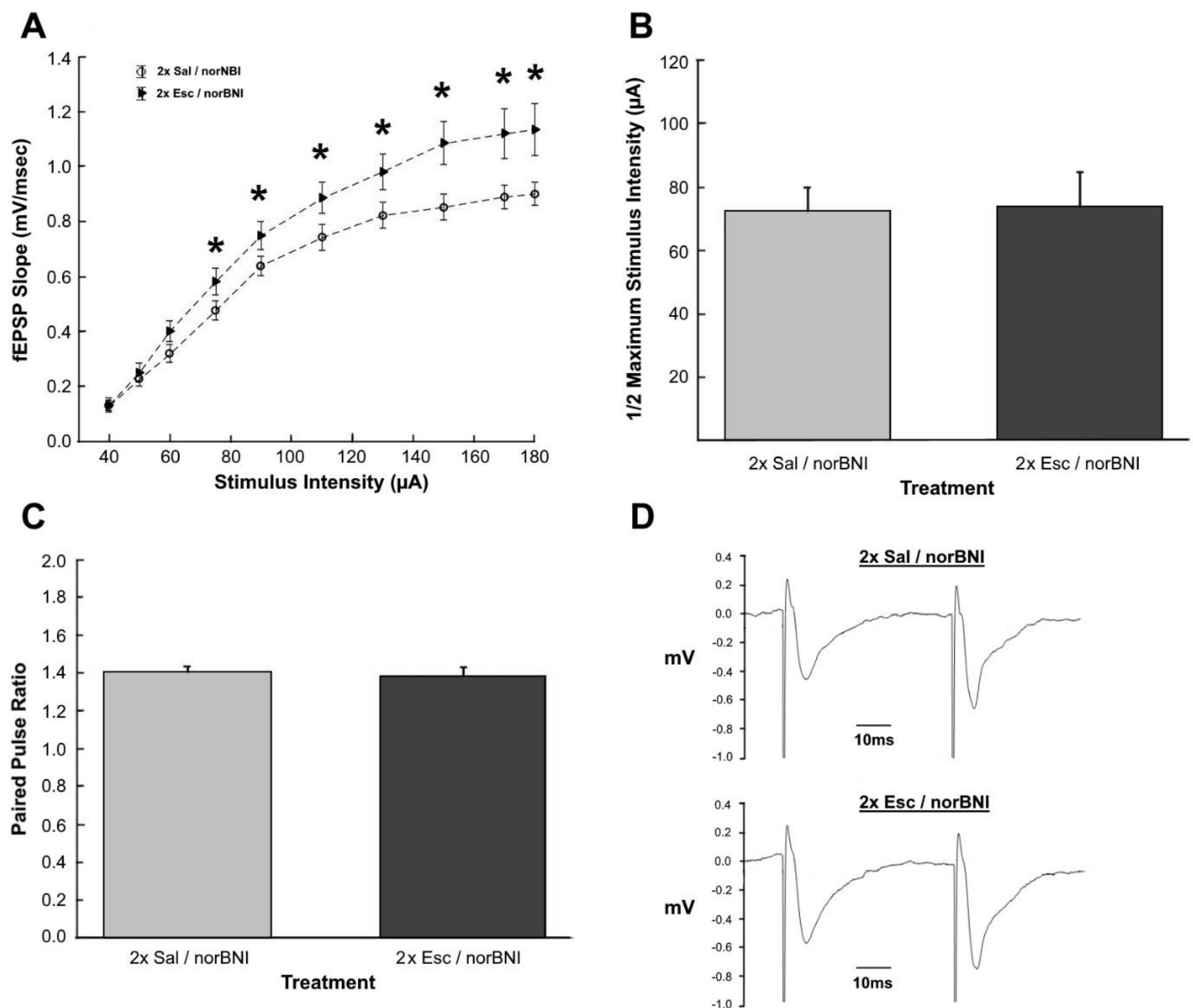


Figure 2.3 Summary plots of normalized fEPSP slopes in cocaine and saline treated mice that did (**B**) or did not (**A**) receive norBNI pretreatment. **C.** Summary quantification of LTP values recorded 4 weeks after mice received 2x Sal (1.69 +/- 0.03, n=23(5)) or 2x Esc (1.61 +/- 0.04, n=21(7)) animals. **D.** Summary quantification of LTP values recorded 4 weeks after mice received 2x Sal/norBNI (1.61 +/- 0.03, n-23(6)) or 2x Esc/norBNI (1.51 +/- 0.03, n=32(8)). # p<0.05 compared to Naïve LTP values (1.57; dashed line). * p<0.05 compared to treatment group. Error Bars +/- SEM.

2.3.3 LTP is decreased in the ventral hippocampus 4 weeks following 2x Esc/norBNI conditioning

Following the experimental protocols outlined in Figure 2A, vH LTP was assessed following a 4-week abstinence period (Figure 3). The previously observed increase in vH LTP from saline conditioning (Figure 1G) is also seen after the 2x Sal protocol as shown by a significant treatment effect in a one-way ANOVA between Naïve, 2x Sal, and 2x Esc animals (Figure 3C; F=3.27, p=0.0387). Using a 2-way ANOVA with LTP as the response variable from conditioned animals (Figure 3C&D; main effects: Cocaine Treatment and norBNI Pretreatment) revealed a significant effect of both Cocaine (F=8.35, p=0.0048) and norBNI (F=4.74, p=0.0322) on LTP in the vH with no Cocaine x norBNI interaction effect on vH LTP (F=0.05, p=0.8211). Although neither the 2x Sal/norBNI nor 2x Esc/norBNI were significantly different from the naïve group (Figure 3D), a planned t-test revealed that the 2x Esc/norBNI group had significantly decreased (p<0.05) LTP compared to the 2x Sal/norBNI group.

Importantly, post-hoc analysis of a one-way ANOVA between Naïve, 2x Sal, and 2x Sal/norBNI (F=3.61, p=0.0321) revealed that LTP from the vH of animals that received the 2x Sal protocol was significantly elevated (p<0.05) compared to behaviorally Naïve mice (Figure 3C) whereas 2x Sal/norBNI mice exhibited LTP that was not significantly different from those of behaviorally-naïve mice. Coupled with the lack of interaction noted above between cocaine and norBNI on CPP and LMS results, this indicates that norBNI pretreatment allows for the actions of cocaine to be observed in isolation from the concomitant effects of stress associated with the behavioral protocol.

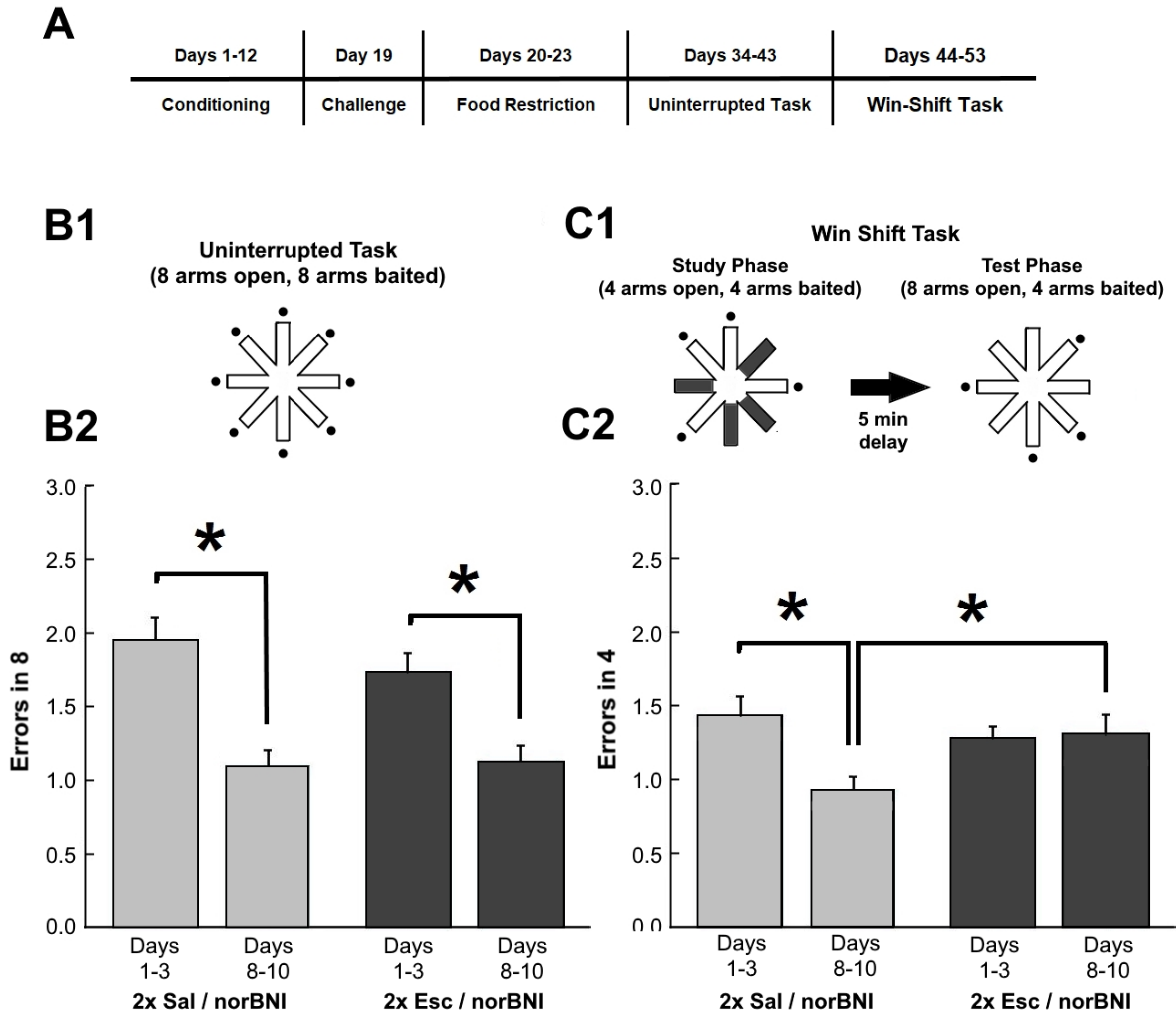


<u>Figure 2.4</u> A. Stimulus-response relationship of the average fEPSP slopes elicited by increasing stimulus intensities is illustrated from animals that received either 2x Sal/norBNI or 2x Esc/norBNI conditioning. **B.** The average value for the stimulus intensity that elicited ½ maximal fEPSP slope is displayed for 2x Sal/norBNI (73 +/- 11 μA) and 2x Esc/norBNI (72 +/- 7 μA) treated animals. **C.** The paired-pulse ratio for 2x Sal/norBNI (1.38 +/- 0.05) and 2x Exc/norBNI (1.40 +/- 0.03) is shown where pulses were elicited a 50 ms interval; average traces of the paired pulse facilitation are shown in (**D**). * $p<0.05$ compared to treatment group; Error Bars +/- SEM.

2.3.4 The reduction in LTP observed in 2x Esc/norBNI mice is associated with an increase in basal synaptic activity

Prior to each LTP experiment, fEPSPs were recorded from vH stratum radiatum of the CA1 region at increasing intensities to generate a stimulus-response curve for each slice. Figure 4A illustrates the stimulus response relationship in slices from mice that received norBNI pretreatment. A significant ($p<0.05$) increase in basal synaptic transmission in the 2x Esc/norBNI mice compared to the 2x Sal/norBNI mice was observed at 7 of 10 stimulus intensities (75,90,110,130,150,170, and 180 μA). The average value at which ½ maximal fEPSP slope was obtained was not significantly different in 2x Esc/norBNI mice compared to 2x Sal/norBNI mice (Figure 4B). Rather, the apparent leftward shift of the stimulus response curve in these mice resulted from a concurrent increase in maximal fEPSP slope elicited**.** To assess whether presynaptic mechanisms contributed to increased basal synaptic transmission, paired-pulse ratio was recorded at a 50ms interpulse interval. There was no significant difference between 2x Esc/norBNI and 2x Sal/norBNI animals

(Figure 4C), suggesting that presynaptic mechanisms are not involved in the persisting effects of the 2x Esc/norBNI conditioning protocol.



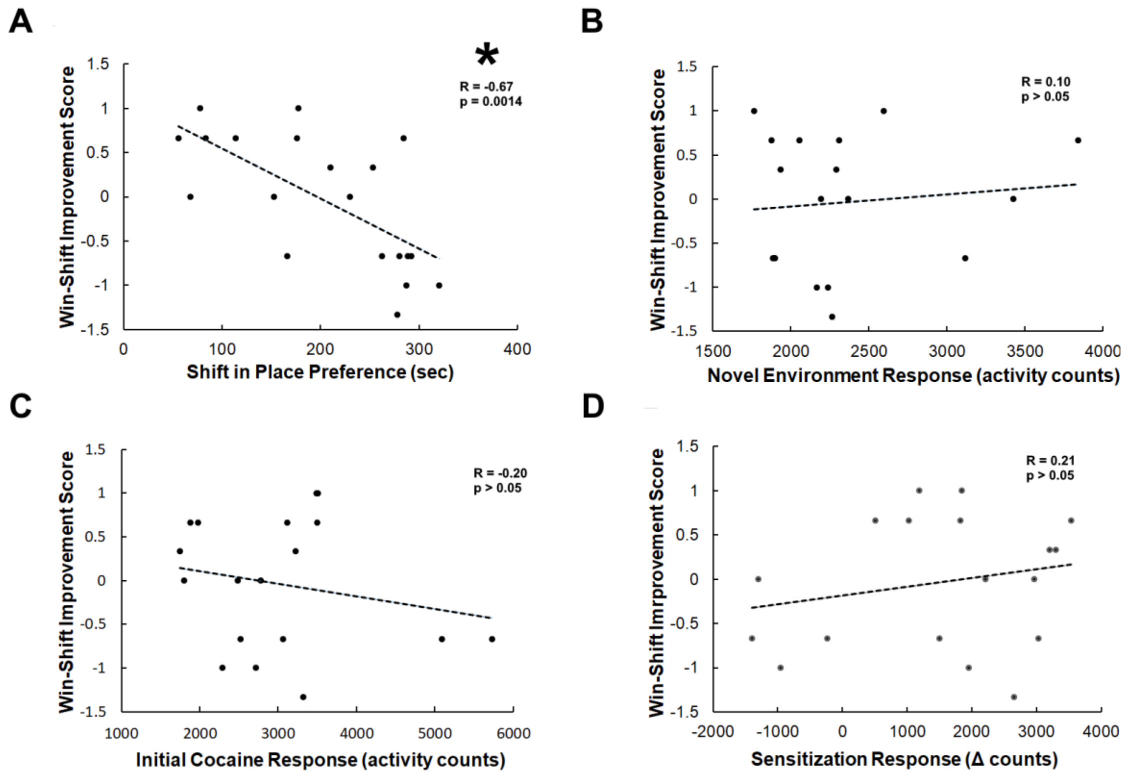
<u>Figure 2.5</u> A. A timeline of the schedule for conditioning and subsequent behavioral maze testing. **B1** displays a schematic representation of the uninterrupted task (• represents sugar pellet baited arm). **B2** displays the average Errors in 8 made by Non-Cocaine (n=20) and Cocaine-treated (n=20) animals on days 1&2 and days 9&10 of testing for the uninterrupted task. **C1** displays a schematic representation of the Win-Shift task (• represent sugar pellet baited arm). **C2.** Displays the average Errors in 4 made by

Non-Cocaine (n=20) and Cocaine-treated (n=20) animals during the test phase on days 1&2 and days 9&10 of the Win-Shift task. * $p<0.05$ compared to treatment group.

2.3.5 2x Esc/norBNI mice exhibit impaired ability to perform a spatial working memory task

Given the involvement of the vH in goal-directed behavior [25] and the association between impaired vH LTP and long-term spatial working memory [24,26], we assessed if cocaine exposure resulted in persisting impairment of spatial working memory. Three groups of mice that received either 2x Esc/norBNI, 2x Sal/norBNI, or norBNI without conditioning (i.e. behaviorally naïve) were tested in a RAM 3-5 weeks after completion of the treatment protocol (see Figure 2.5A). Behaviorally naïve mice (n=6) and 2x Sal/norBNI mice (n=14) showed no difference in their RAM performance and were subsequently grouped together as a "non-cocaine" (n=20) group for statistical analyses. An uninterrupted 8-arm protocol (Figure 2.5B) was used over ten consecutive days to assess short-term spatial working memory and demonstrated that mice from both non-cocaine (n=20) and cocaine-treated (n=20) groups showed significant ($p<0.001$) improvement in their performance after training (Figure 2.5B2). Neither group displayed significant differences on the initial or final day errors made in the first 8 choices. Thus, this short-term form of spatial working memory was unaffected by prior cocaine conditioning**.** A modified Win-Shift task (Figure 2.5C1), requiring a greater working memory load than the uninterrupted task, was used over ten consecutive days to assess long-term spatial working memory. This task demonstrated that the non-cocaine mice showed a significant improvement in their performance ($p<0.01$) whereas the cocaine-

treated mice did not show a significant improvement after training (Figure 2.5C2). This impairment in long-term spatial working memory occurs after 4-5 weeks of abstinence from the 2x Esc/norBNI conditioning; the same abstinence period in which we observed a reduction in the magnitude of vH LTP (Figure 2.3D).



<u>Figure 2.6</u> Win-Shift Improvement Scores were obtained by subtracting the average errors in 4 made on days 8–10 from the average errors in 4 made on days 1–3 for each animal. **A.** A significant inverse correlation between the shift in preference (sec) from the CPP assay and Win-Shift Improvement Scores is displayed ($R = -0.67$, $p = 0.0014$). **B.** No relationship was evident between Novel Environment Response and Win-Shift Improvement Scores. **C.** No relationship was evident between Initial Cocaine Response and Win-Shift Improvement Scores. **D.** No relationship was evident between

Sensitization response and Win-Shift Improvement Scores. All plots in Figure 6 display only 2x Esc/norBNI treated animals (n = 20).

3.6 Cocaine-induced CPP in 2x Esc/norBNI mice was negatively correlated with performance in the Win-Shift task

When comparing individual maze performance of cocaine-treated mice with their cocaine-induced CPP shift, a negative correlation is observed between increases in time spent on the cocaine-paired side and their improvement in the modified Win-Shift performance (Figure 2.6). This negative correlation is significant when analyzed using the Pearson product-moment correlation coefficient ($p < 0.05$), indicating mice that exhibit a greater CPP response to 2x Esc/norBNI conditioning also demonstrate a greater impairment in their ability to perform a long-term spatial working memory task.

2.4 Discussion

In this study, we have shown that a non-contingent conditioning protocol using a double-escalating dose of cocaine produced persisting changes in synaptic function in the CA1 region of the ventral hippocampus that include an enhancement of basal transmission and a coinciding reduction of LTP magnitude. In addition to effects mediated by cocaine conditioning, the stress associated with the behavioral protocol also had a significant impact on synaptic plasticity of the vH as the magnitude of LTP was increased in saline-conditioned animals. Importantly, the kappa opioid receptor antagonist norBNI was effective in attenuating this confounding stress effect, thereby allowing the persisting actions of cocaine conditioning to be differentiated. Finally, we have demonstrated an impairment in long-term spatial working memory following 4

weeks of abstinence from this cocaine conditioning protocol analogous to impairments seen in humans with cocaine use disorder.

Studies employing conditioning protocols using escalating dose cocaine regimen have been argued to be more effective at creating drug-associated memories than standard fixed dosing protocols, possibly due to the unexpected nature of drug reward [18]. An escalating dose also recapitulates some aspects of the increase in drug consumption observed in long-access self-administration studies as well as duration of exposure [11,27]. However, non-contingent cocaine administration has not been shown to produce equivalent results to self-administration in terms of persisting electrophysiological changes in some instances [16,28]. Here we have that non-contingent cocaine administration using conditioning with the 2x Esc/norBNI protocol produced long-lasting electrophysiological changes in the vH of mice similar to those previously reported following 3-5 weeks of abstinence from self-administration in rats [11]. These changes in LTP and basal synaptic administration were not observed in animals that received the Esc/norBNI conditioning protocol (Figure 2.7) highlighting the potential importance of dosage and duration on the ability of non-contingent administration of cocaine to produce persisting changes in the vH. Furthermore, pairing of cocaine with a context such as during CPP appears to be necessary to induce these changes as home cage injections of the same dosing schedule produced opposite results in LTP elicited from cocaine-treated animals (Figure 2.7D&E). Crucially however, this result was only apparent when the stress effects of experiencing the behavioral protocol itself are accounted for and negated by administering a KOR antagonist.

The physiological changes observed from injections of saline [29], introduction to novel environment [30], and various behavioral protocols [31,32] can introduce confounding stress-effects that alter LTP in the vH when compared to naïve animals. In the current report, saline-conditioned mice exhibited significantly elevated LTP compared to behaviorally naïve mice; an effect that persists at least 4 weeks (Figures 1&3). This stress effect involves the KOR system since pre-treatment of saline-treated animals with the KOR antagonist norBNI resulted in LTP comparable to naïve mice (Figure 2.3, see also [31]). Given the prevalent use of the CPP model, the relatively minor, yet repetitive, stress experienced during conditioning is a potentially confounding variable that investigators using CPP protocols should consider. In the current study, despite the role of KORs in the mesolimbic reward system [33-35], pretreatment with norBNI did not change the behavioral responses of the mice to cocaine conditioning (Figure 2.2). Neither CPP scores nor LMS values were significantly altered between cocaine treated mice with or without norBNI, consistent with previous results [31,37]. Pre-treatment with norBNI resulted in saline treated-animals with LTP magnitudes similar to behaviorally-naïve; mice which then serve as appropriate controls for evaluating effects of cocaine without the associated stress effects. Only in these norBNI pretreatment groups were significant cocaine-induced differences in synaptic plasticity and transmission observed (Figures 2.3D & 2.4A).

The significant differences we have observed here in vH synaptic transmission and plasticity observed after 4 weeks of cocaine abstinence resemble changes in other areas of the brain following long abstinence periods, such as in the VTA [38]. Increased basal synaptic transmission has been linked to the phenomenon of incubation of drug

craving that occurs following cocaine abstinence and has been attributed to persisting changes in glutamatergic signaling such as insertion of calcium-permeable AMPA receptors in the NAc [15]. In addition to these studies, an escalating morphine protocol produced increased basal synaptic transmission in the dorsal hippocampus in mice [39,40] that resemble changes seen in the vH of rats following cocaine self-administration [11]. A postsynaptic mechanism would seem to underlie our current findings as paired-pulse ratios were not significantly different between saline- and cocaine-treated animals (Figures 2.4C&D), suggesting presynaptic mechanisms were unchanged. The insertion of additional AMPARs into the CA1 Schaffer collateral synapse could explain our observed increases in vH basal synaptic transmission and associated reduction of LTP and is also consistent with previous reports of cocaine-enhanced hippocampal output [6,41,42]. Regardless of the specific mechanism(s) underlying the cocaine-induced persisting alterations in vH synaptic transmission and plasticity, the significance of such changes for hippocampal function has not been previously determined.

In order to further characterize the impact of the observed modulations in synaptic transmission following abstinence from cocaine conditioning, we used the 8-arm radial arm maze and a Win-Shift task to assess hippocampal-dependent learning and memory. It has been reported that shortly after cessation of cocaine self-administration hippocampal LTP is elevated [10,43] and Morris Water Maze performance is either unaffected [44] or possibly enhanced [45]. In contrast, after prolonged abstinence from self-administration LTP is decreased [10,11], which we also observed here in the 2x Esc/norBNI treated animals (Figure 2.3D). However, the behavioral significance of this diminished LTP on

hippocampal-dependent learning and memory has not previously been assessed. We have previously shown that impairment of vH LTP results in decreased long-term working memory function [24] and the importance of vH-mPFC connectivity for working memory is well established [25,26,46]. In mice tested in the Win-Shift task we observed a profound impairment of spatial working memory in the cocaine conditioned animals (Figure 2.5C2) not seen in saline-conditioned or their control counterparts (Figure 2.8B). Persistent impairments in working memory have also been reported in human cocaine users [47,48]. Interestingly, we also observed a negative correlation between the magnitudes of CPP shift and maze performance, implying that the strong associative learning that occurred during cocaine conditioning diminishes the capacity for subsequent hippocampal-dependent learning. This further highlights the possible impact of cocaine context-associated learning and memory on persisting synaptic changes in the vH and related cognitive impairments.

In summary, we have employed a cocaine conditioning protocol in mice that involves the formation of drug-context associations in order to characterize the subsequent effects on hippocampal function in abstinent mice. The hippocampus is involved with the acquisition and expression of cocaine-induced CPP, where persisting memories of the drug experience are maintained. Effects of the double-escalating protocol included decreased vH LTP and increased basal synaptic transmission, consistent with our previous findings in the rat vH following long-access self-administration of cocaine (Keralapurath at al. 2017). Importantly, persisting working memory deficits have been reported in human drug abusers and a similar deficit was observed here, suggesting this model may recapitulate clinically relevant aspects of

cocaine use and abstinence. Altogether, the results are consistent with a primary role of the ventral hippocampus as a key participant in the neurobiological circuitry underlying drug craving and relapse to seeking/taking.

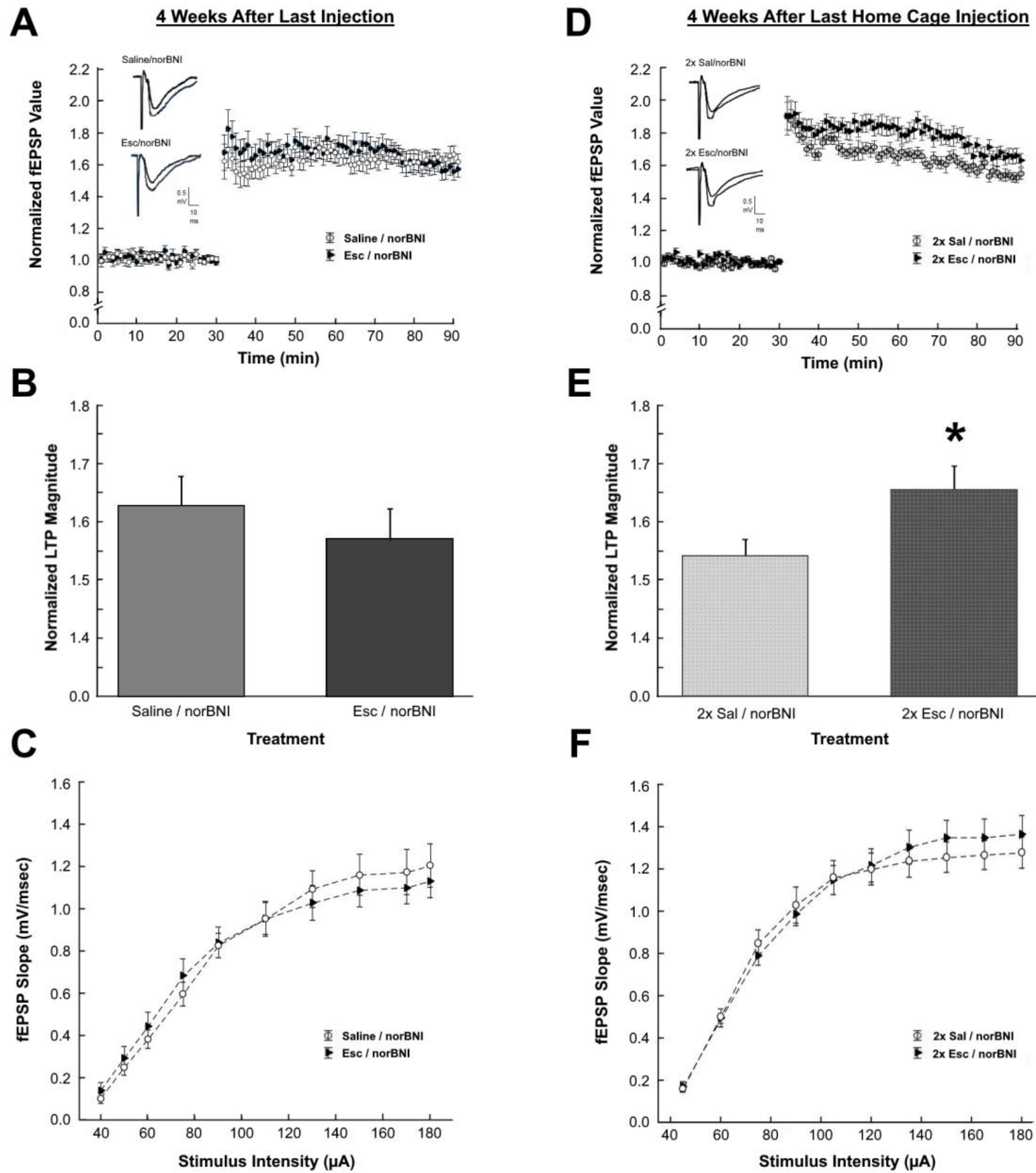


Figure 2.7 Summary plot of normalized fEPSP values from animals that received either the escalating conditioning protocol or equivalent saline protocol with norBNI pretreatment (Esc/norBNI or Saline/norBNI). Insets show representative sweeps

composed of average sweeps from 5 min pre-tetanus and 56–60 min post-tetanus. **B.** Summary quantification of LTP values recorded from Saline/norBNI (1.63 ± 0.05, n = 20(8)) and Esc/norBNI (1.57 ± 0.05, n = 19(8)) treated animals. **C** Stimulus-response relationship of the average fEPSP slopes elicited by increasing stimulus intensities is illustrated from animals that received either Saline/norBNI or Esc/norBNI conditioning. **D.** Summary plot of normalized fEPSP values from animals that received either the 2x Esc/norBNI or 2x Sal/norBNI dosing protocols *in their home cages.* Insets show representative sweeps composed of average sweeps from 5 min pre-tetanus and 56–60 min post-tetanus. **E.** Summary quantification of LTP values recorded from animals that received 2x Sal/norBNI (1.54 ± 0.03, n = 22(4)) or 2x Esc/norBNI (1.65 ± 0.04, n = 26(5)) in their home cages. **F.** Stimulus-response relationship of the average fEPSP slopes elicited by increasing stimulus intensities is illustrated from animals that received either 2x Sal/norBNI or 2x Esc/norBNI dosing in their home cages. ***** $p < 0.05$ compared to treatment group; Error bars±SEM

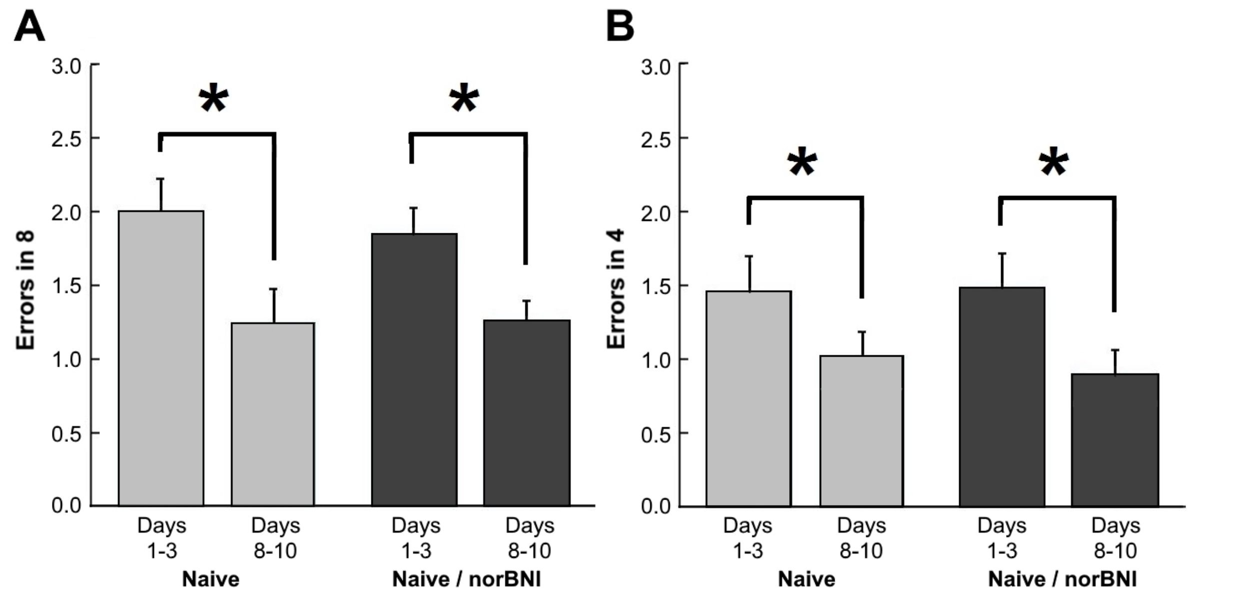


Figure 2.8 displays the average Errors in 8 made by behaviorally naïve (n = 5) and behaviorally naïve animals pretreated with norBNI (Naïve / norBNI; n = 6) during days 1–3 and days 8–10 of the uninterrupted task. **B.** displays the average Errors in 8 made by behaviorally naïve (n = 5) and behaviorally naïve animals pretreated with norBNI (Naïve / norBNI; n = 6) during days 1–3 and 8–10 of the Win-Shift task. ***** $p < 0.05$ using paired *t*-test

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CHAPTER 3

COCAINE CONDITIONING PROGRESSIVELY ALTERS VENTRAL HIPPOCAMPUS AMPA RECEPTOR TRANSMISSION DURING COCAINE WITHDRAWAL [1]

1. Preston, CJ & Wagner JJ. To be submitted to Addiction Biology

Abstract

Drugs of abuse, such as cocaine, produce aberrant changes in synaptic transmission and plasticity that emerge throughout withdrawal. One region of the brain that displays a high degree of synaptic plasticity, as well as connectivity with mesolimbic structures such as the nucleus accumbens, is the ventral hippocampus (vH). Here, we investigated the effects of an escalating cocaine dosing schedule on vH CA1 excitatory transmission by measuring place preference and recording EPSCs at 3 different withdrawal time points: withdrawal day (WD) 2, 9, or 28. Behaviorally, this escalating cocaine conditioning protocol was capable of producing conditioned place preference that persisted through WD28. Physiologically, cocaine conditioning produced an increase in vH excitatory transmission on WD2 that appeared to be the result of an increase in calcium-impermeable (CI) - AMPA receptor density. Excitatory transmission was still enhanced in cocaine-treated animals on WD9, however we detected a significant increase in the contribution of calcium-permeable (CP) - AMPA receptors to EPSCs compared to WD2. By WD28, these CP-AMPA receptors appeared to be major contributors to vH CA1 excitatory transmission producing synaptic responses distinct from WD2 and WD9. These results highlight progressive changes in vH synaptic transmission throughout withdrawal that may contribute to relapse by driving cocaine seeking & craving.

3.1 Introduction

Approximately 1 million Americans meet the DSM-5 criteria for cocaine addiction [1]; a mental illness characterized by addictive behaviors such as compulsive drug seeking and drug taking. Imbalances in the brain's natural reward circuitry drives these behaviors by perpetuating drug cravings throughout abstinence leading to an

intractable feature of addiction, relapse. Interestingly, drug cravings tend to increase over the duration of abstinence which represents a phenomenon known as the “incubation of drug craving” [2]. Classic reward pathways such as the Ventral Tegmental Area (VTA) and Nucleus Accumbens (NAc) exhibit synaptic changes throughout withdrawal from cocaine [3, 4] and have received the majority of attention as the drivers of incubation. However, many other brain regions are implicated in the incubation of drug craving [5, 6] and few have been studied at multiple withdrawal time points to assess their possible contribution to this phenomenon. One particular region that has been increasingly appreciated for its role in the aberrant learning and memory that drives drug seeking is the ventral hippocampus [7].

The hippocampal formation can be separated both functionally and anatomically along its septotemporal axis into two main sectors: the dorsal hippocampus (dH) and ventral hippocampus (vH). The dH is primarily involved in mediating cognitive functions while the vH plays an important role in stress responses and motivational factors [8, 9]. With regards to cocaine addiction, the vH is anatomically positioned to play a modulatory role in the mesolimbic system since it receives dopaminergic innervation from the VTA [10-12] and projects glutamatergic fibers to the NAc [13] . Synaptic alterations are observed during cocaine withdrawal within the vH CA1 region [14, 15] as well as in vH-NAc synapses [16], indicating potential long-term adaptations of the vH as a result of cocaine exposure. These changes in vH plasticity during withdrawal appear to be dynamic and progressive; enhanced long-term potentiation (LTP) is observed one week after cessation of self-administration [17] and decreased LTP is observed after longer withdrawal periods from cocaine self-administration or conditioned place preference

(CPP) [14, 15]. Similarly, dynamic changes in synaptic function have been observed in the NAc and VTA during cocaine abstinence and attributed to the presence of calcium-permeable AMPARs [18]. Therefore, we sought to explore if the synaptic changes following cocaine exposure and abstinence in the VH could also be the result of an increased physiological role of VH calcium permeable-AMPARs.

AMPA receptors are the main fast excitatory postsynaptic glutamate receptors in the hippocampus [19, 20]. These receptors are hetero-tetrameric assemblies of the subunits GluR1-4, which canonically contain two GluR2 subunits. These canonical AMPARs are calcium-impermeable AMPARs (CI-AMPARs) due to an electrically charged arginine residue in the pore-lining domain of the GluR2 subunits [21]. Variations in subunit composition can result in calcium-permeable AMPARs (CP-AMPARS) that lack the GluR2 subunit and have distinguishing features from canonical CI-AMPARS such as conductance of Ca^{2+} and Zn^{2+}, faster channel kinetics, and an inwardly rectifying current-voltage relation due to voltage-dependent intracellular polyamine block of the channel [22]. In the hippocampus, these CP-AMPARs are minimally expressed in the synapse under basal conditions [23, 24], but have been implicated to play a role in homeostatic plasticity [25, 26]. Insertion of CP-AMPARs has been observed in both the VTA after a single cocaine exposure for up to 5 days [27-29] and in the NAc after 30-45 days of withdrawal from cocaine self-administration [30, 31] and appears to be dependent on signaling from PKA and phosphorylation of S845 of the GluR1 subunit [32, 33]. Similarly, CP-AMPAR insertion has been observed in the dH after morphine conditioning and was associated with an increase in phosphorylation of GluR1 S845 [34-36]. Previous results from our lab indicated a numerical increase in the rectification

index, indicative of the presence of CP-AMPARs, following 3 weeks of withdrawal from cocaine self-administration [15]. However, these recordings were done without the intracellular polyamine, spermine, in the recording pipette which is necessary to observe the inward rectification resulting from voltage-dependent channel block of CP-AMPARs [37, 38]. Given the appearance of CP-AMPARs in reward circuitry at various time points after cocaine exposure, we investigated if, and when, CP-AMPARs are functionally present in the vH following cocaine conditioning.

The conditioned place preference (CPP) model of drug motivated behavior is a commonly used model that forms drug contextual associations by pairing drug administration with a context [39]. In our previous paper [14], we observed a decrease in vH long-term potentiation following 28 days of withdrawal from CPP similar to findings from our lab after >21 days of with withdrawal from cocaine self-administration [15]. In addition to a decrease in LTP, we also observed an increase in basal synaptic transmission and an impairment in special working memory in cocaine-treated animals after 28 days of withdrawal. After observing persisting effects of cocaine conditioning on vH plasticity, we attempt here to provide temporal resolution to the synaptic changes occurring in the vH during withdrawal. We performed an escalating conditioning protocol and utilized whole-cell voltage clamp recording on withdrawal days (WD) 2, 9, or 28 to assess the progression of synaptic changes in the vH. Our results indicate that following cocaine conditioning on WD2, there is an initial increase in CI-AMPARs and a minimal appearance of CP-AMPARs on WD2. By WD9, these CP-AMPARs appear to account for a greater proportion of vH excitatory transmission and appear to be a major contributor in vH glutamatergic excitatory transmission by WD28.

3.2 Materials & Methods

Animal Maintenance

7-week-old C57Bl/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME) and housed in clear Micro-Isolator cages in groups of 5. Animals were maintained on a 12 h light/dark cycle (0700/1900 h) and provided with food and water *ad libitum*. Animals were allowed at least 1 week to acclimate to their home cages in our facility after arrival. Each mouse was handled, tail-marked, and scruffed for 3 days prior to the start of conditioned place preference to habituate them to handling. All experiments were performed in compliance with the University of Georgia Animal Care and Use Committee guidelines.

Conditioned Place Preference

The conditioned place preference (CPP) assay was performed in sound-attenuating cubicles in a room separate from the home cage facility. Each cubicle contained a two-compartment chamber with two pairs of 16 beam infrared arrays to record horizontal activity with Activity Monitor software, all obtained from Med Associates Inc. (St. Albans, VT). To create different contexts, each compartment was identical in size but differed in both flooring and luminosity as previously described [40]. Mice were allowed free-access to both chambers on the pre-test day for 15 minutes to assess the most- and least-preferred compartments as determined by the percent of time spent in each compartment. Each conditioning day consisted of two sessions with mice restricted to one compartment for 15 minutes in each session. All mice received saline on

their most preferred side in the morning (~10:00) and then either cocaine or saline on their least preferred side in the afternoon (~14:00). Mice in the cocaine-paired groups received escalating doses of cocaine previously shown to affect hippocampal plasticity (Preston et. al, 2019). The dosing protocol consisted of 4 days with mice receiving i.p. cocaine at 4,8,16,24 mg/kg, 2 days of no conditioning, and a subsequent 4 days with mice receiving i.p. cocaine at 16,24,32,32 mg/kg i.p. (Fig. 1A). Mice were allowed free-access to both chambers on the first post-test day , 24 hours after the final conditioning day, for 15 minutes to obtain CPP scores which are reported as the change in time the mice spent in the least preferred side on the post-test day compared to the pre-test day. To assess incubation of cocaine craving in the conditioned place preference model, a second post-test day was performed on mice at either the WD8 or WD27 time point (Figure 3.1A).

Whole-Cell Electrophysiology

Mice were sacrificed at either withdrawal day 2, 9, or 28 (WD2, WD9, or WD28) to assess changes in hippocampal function during withdrawal from cocaine. Halothane was used to anesthetize the mice prior to decapitation and removal of brains. Once removed, brains were submerged in oxygenated (95% O_2 / 5% CO_2), ice-cold dissection artificial cerebrospinal fluid (dACSF; 120 mM NaCl, 3 mM KCl, 4 mM $MgCl_2$, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose). The brain was mounted on its dorsal pole and sectioned along the horizontal plane with a vibratome to obtain 400 µm thick sections containing the ventral hippocampus (vH). Slices were then sectioned to isolate the vH and remove the CA3 region. Theses slices were then transferred to a submersion-type chamber and perfused with oxygenated (95% O_2 / 5% CO_2) ACSF (120 mM NaCl, 3

mM KCl, 1.5 mM $MgCl_2$, 1 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10mM glucose) and allowed to recover for 45 minutes at room temperature followed by 45 minutes at the recording temperature of 30°C.

A bipolar stimulating electrode (Kopf Instruments, Tujunga, CA) was placed within the stratum radiatum of the CA1 region and whole-cell voltage clamp recordings were performed using 3-5 MΩ pipettes pulled from 1.5mm thin-walled borosilicate glass with a P-97 horizontal pipette puller (Sutter, Novato, CA). Pipettes were filled with an intracellular solution consisting of: 145mM $CsMeSO_3$, 5mM QX-314, 2mM $MgCl_2$, 0.2mM $CaCl_2$, 2mM EGTA, 2mM HEPES, 2mM Mg-ATP, 0.5 Mg-GTP, and 100µM Spermine at pH 7.2. A multi-clamp 700B amplifier was used to establish voltage clamps and data was digitized at 10 kHz, low-pass filtered at 1 kHz, and analyzed with pCLAMP 10.2 software (Axon Instruments, Sunyvale, CA). Cells were excluded if the input resistance drifted more than 20% from the baseline resistance.

All voltage clamp experiments were performed in the presence of the GABA antagonist picrotoxin (100µM) to isolate excitatory postsynaptic currents (EPSCs) generated by a single square-wave stimulation pulse of 270 µs duration delivered every 30 seconds. An input/output (I/O) curve was obtained at the beginning of each experiment at +40mV by incrementally delivering stimulus pulses ranging from 50-200 µA in intensity. Cells were only included if a stable baseline response of at least 400pA was acquired. To isolate AMPA-EPSCs from baseline responses, the NMDA antagonist D-APV (50µM) was bath applied to the slice for at least 20 minutes. The NMDA-EPSC was determined by subtracting the resulting AMPA-EPSC from the baseline EPSC and used to calculate the AMPA/NMDA ratio. After isolating AMPA-EPSCs, the I-V

relationship was determined using 4 different holding potentials (-80, -40, 0, and +40mV) and a rectification index (R.I.) value was obtained by dividing the V_m(+40mv) EPSC amplitude by the V_m(+40mv) EPSC amplitude. This rectification index is a useful measurement to quickly determine if inwardly rectifying ion channels are present in the membrane. Since calcium-permeable AMPA receptors (CP-AMPARs) do not conduct current well at positive holding potentials [22], another I/O curve was recorded at -60mV in the presence of d-APV(50µM). After establishing a stable baseline at -60mV, the CP-AMPAR antagonist NASPM was bath applied to the slice to assess the presence and contribution of CP-AMPARs to pyramidal cell EPSCs after cocaine- or saline-conditioning. The stimulus intensity at which the half-maximal EPSC was elicited was determined to calculate the $S_{1/2}$ value for the I/O assessments. The decay time (τ) of averaged EPSC sweeps was also calculated by fitting sweeps with a single exponential function in Clampfit 10.3. The decay constant was assessed since CP-AMPARs display faster channel decay kinetics than CI-AMPARs. Reported n-values [x(y)] indicate the number of slices (x) and the number of mice (y) assessed.

3.3 Results

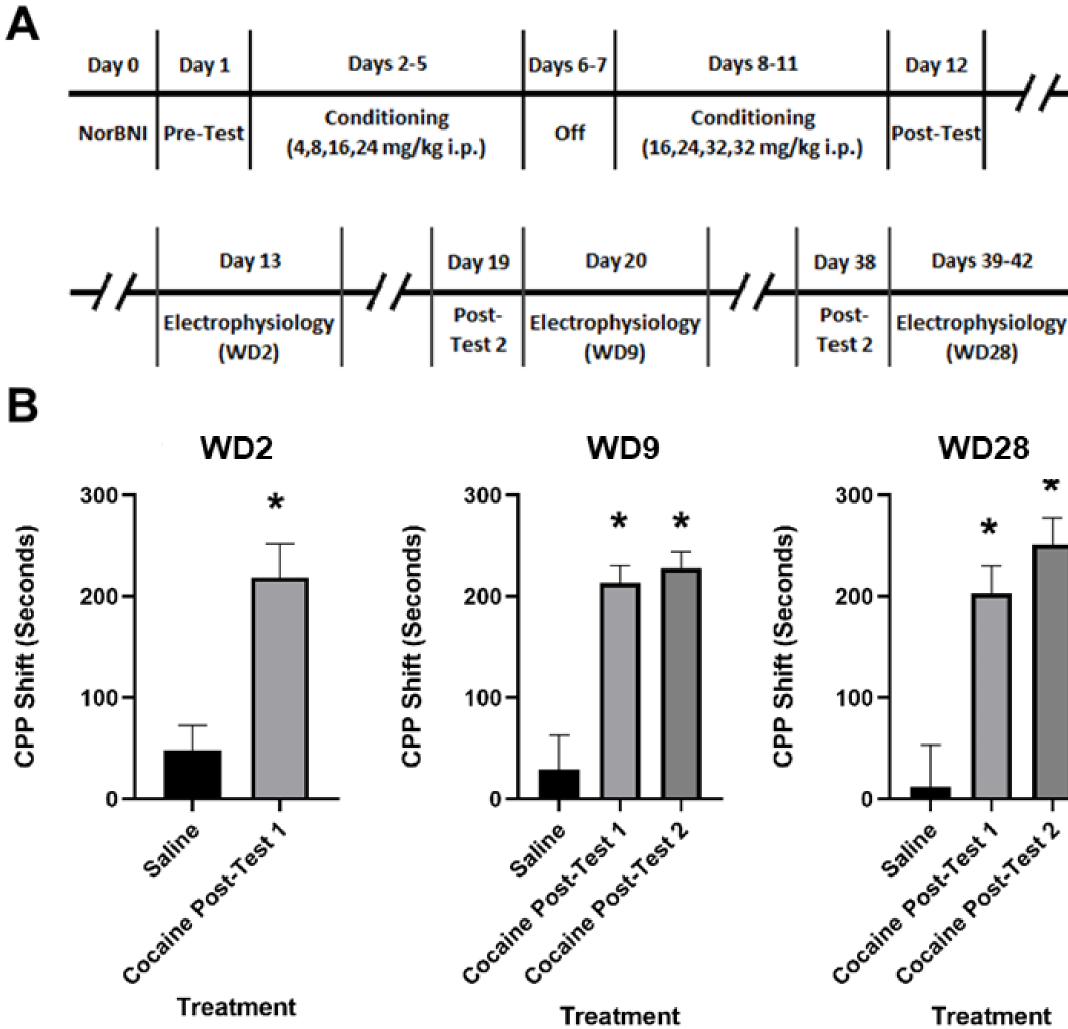


Figure 3.1 (**A**) Timeline for cocaine or saline conditioning and subsequent electrophysiology experiments. (**B**) Results from the conditioned place preference assay are shown as shifts in time spent (seconds) on the least preferred / cocaine-paired side between Pre-Test and Post-Test measurements. N-values are as follows: WD2 Saline (n=10) WD2 Cocaine (n=14) WD9 Saline (n=10) WD9 Cocaine (n=12) WD28 Saline (n=11) WD28 Cocaine (n=14). Only WD9 (n=8) and WD28 (n=8) treatment groups were assessed with a second Post-Test day. * $p < 0.01$ compared to saline control group; Error bars +/- SEM.

3.3.1 Cocaine conditioning with an escalating dosing protocol produces significant place preference that persists at least 28 days.

The conditioning protocol outlined in Figure 3.1A was used to assess the persisting effects of cocaine conditioning on WD2, 9, and 28. To assess if the incubation of cocaine craving is observable in this CPP model, some animals from the WD9 and WD28 group were selected to receive a second post-test. All three cocaine-treated groups showed significant ($p<0.01$) place preference compared to saline-treated animals (Fig 1B,C,D) on the first post-test day. Cocaine-treated animals from both the WD9 and WD28 groups continued to exhibit significant ($p<0.01$) place preference compared to saline-treated animals on their second post-test date (Fig. 1C,D). While there was a numerical increase in the shift in place preference between the second and first post-test days for the WD9 and WD28 groups, no statistical significance was achieved using a two-way ANOVA. It is possible that our results were limited by a potential “ceiling effect” on the maximum possible shift induced by cocaine conditioning.

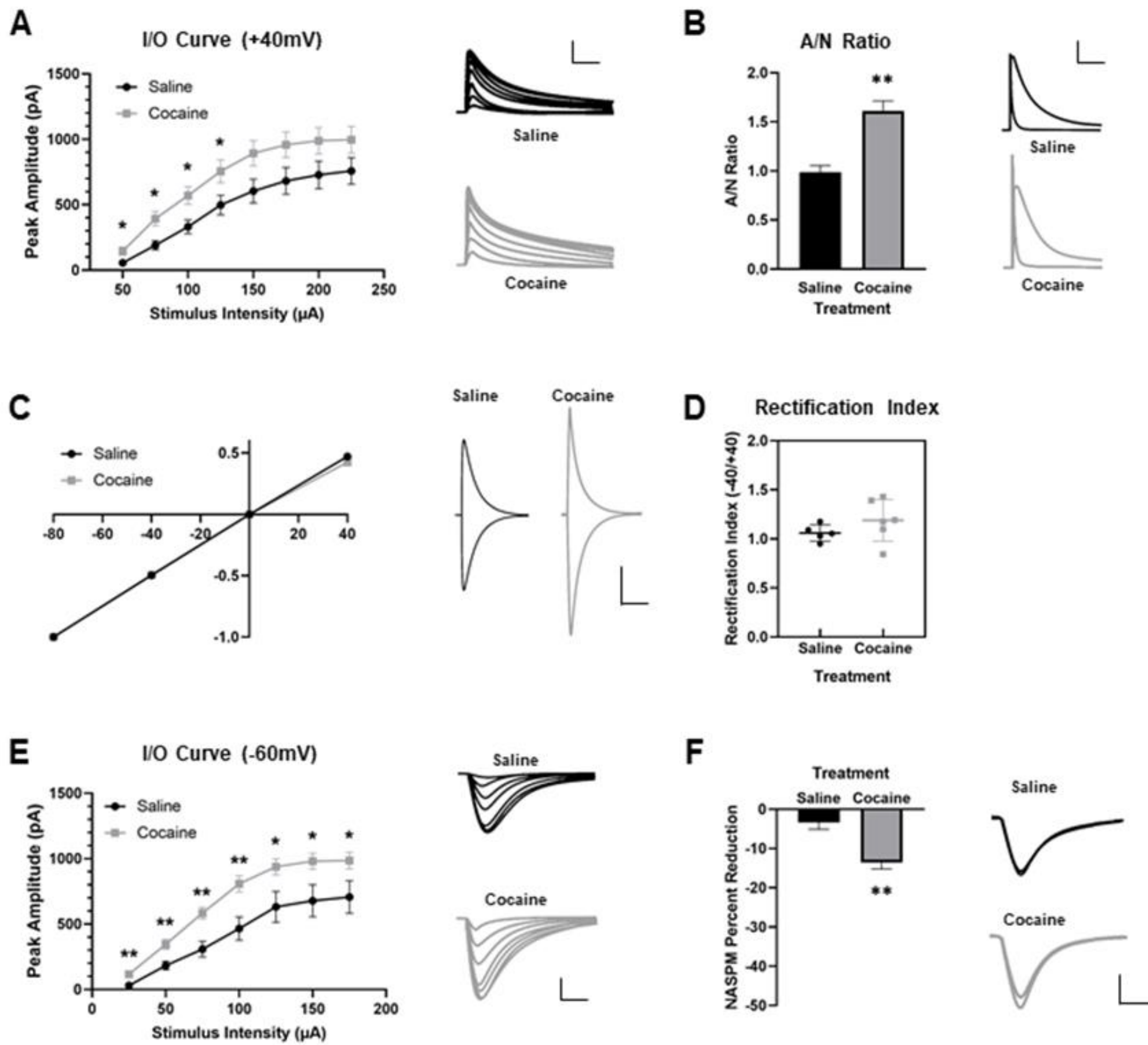


Figure 3.2 **(A)** Input / Output (I/O) curve of EPSCs recorded in vH slices from saline [n=9(8)] or cocaine [n=14(12)] treated animals recorded at a +40mV holding potential. Average traces of the EPSCs are shown to the right; vertical scale bar = 200pA and horizontal scale bar = 100ms. **(B)** The summary quantification of the AMPA/NMDA (A/N) ratio recorded at +40mV holding potential in saline [n=7(7)] and cocaine [n=9(8)] treated animals. Average traces of the AMPA-EPSCs and NMDA-EPSCs are shown to the right; vertical scale bar = 100pA and horizontal scale bar = 300ms. **(C)** The current-

voltage (IV) relationship shown with normalized AMPA-EPSC amplitudes plotted against the holding potentials at which the responses were evoked in saline [n=5(5)] and cocaine [n=6(6)] animals. Average traces of AMPA-EPSCs recorded at +40mV (top trace) and -40mV (bottom trace) holding potentials; vertical scale bar = 400pA and horizontal scale bar = 40ms. **(D)** Summary quantification of the rectification index ($EPSC_{-40mV}/EPSC_{+40mV}$) obtained from the IV relationship. **(E)** Input / Output (I/O) curve of EPSCs recorded in vH slices from saline [n=7(5)] or cocaine [n=5(5))] treated animals recorded at a -60mV holding potential. Average traces of the EPSCs are shown to the right; vertical scale bar = 300pA and horizontal scale bar = 20ms. **(F)** Summary quantification of the percent reduction in AMPA-EPSC peak amplitudes recorded at -60mV following NASPM bath application in saline [n=5(5)] and cocaine [n=6(6)] animals. Averages traces are shown to the right of AMPA EPSCs before and after NASPM bath application; vertical scale bar = 200pA and horizontal scale bar = 20ms. * = p<0.05 ** = p<0.01 compared to saline control group. Error bars +/- SEM.

3.3.2 Increased vH AMPA-EPSC transmission observed on WD2 in cocaine-treated mice can be attributed to increase in CI-AMPAR response with a modest contribution of CP-AMPARs.

Mice were sacrificed 2 days after the final injection day of the CPP protocol (WD2) and EPSCs were evoked utilizing the whole-cell voltage-clamp mode while perfusing the slices with picrotoxin (100µM). An input-output (I/O) curve was obtained at the beginning of each experiment while holding cells at +40mV, a holding potential at which CP-AMPARs conduct poorly [22]. We observed a leftward shift of the +40mV I/O

curve of cocaine-treated animals with significantly higher outputs ($p<0.05$, Fig. 2A) at 50,75,100,& 125µA compared to saline-treated animals. Slices were subsequently perfused with D-APV (50µM) to isolate AMPA-EPSCs and obtain an AMPA/NMDA (A/N) ratio. The A/N ratio was significantly higher on WD2 in cocaine-treated animals compared to saline-treated animals ($p>0.001$, Fig. 2B) suggesting an increased contribution of AMPARs to vH excitatory transmission in cocaine-treated animals. Since CP-AMPARs display an inward rectification at positive holding potentials, a current-voltage (IV) curve was obtained by holding cells at -80mV, -40mV, 0mV, and +40mV in the presence of both picrotoxin and D-APV. The rectification index (RI) was obtained by dividing the peak AMPA-EPSC amplitudes obtained at -40mV by the amplitudes obtained +40mV. On WD2, we did not observe a significant difference between cocaine- and saline-treated animals in the rectification index ($p=0.12$, Fig. 2D). After obtaining the IV curve, another I/O curve of AMPA-EPSCs was performed at a -60mV holding potential where CP-AMPARs more readily conduct current than a +40mV holding potential [21, 22]. We observed a leftward shift of the -60mV I/O curve, similar to the +40mV I/O curve (Fig. 2A), of cocaine-treated animals with significantly higher outputs at all recorded stimulus intensities ($p<0.05$, Fig. 2E). To assess possible CP-AMPAR contribution to -60mV AMPAR-EPSC, NASPM (100µM) was bath applied and reductions in AMPAR-EPSCs were quantified as a percent reduction from baseline response in Figure 3.2F. NASPM induced a significantly greater ($p<0.01$) reduction in EPSC amplitude on WD2 in cocaine-treated animals than saline-treated animals (Fig. 2F). It would appear from these results that the leftward shift of the +40mV I/O curve and increased A/N ratio of cocaine-treated animals on WD2 can largely be attributed to

additional postsynaptic CI-AMPARs as CP-AMPARs do not conduct at the +40mV holding potential at which these experiments were performed. While we did not observe an increase in the RI normally associated with the presence of CP-AMPARs, we did observe a significantly greater reduction of EPSCs in cocaine-treated animals compared to saline-treated animals when NASPM was bath applied. Taken together, these results suggest an increase in CI-AMPAR function in the vH of cocaine-treated animals on WD2 with a small, but measurable, contribution of CP-AMPARs to vH synaptic responses.

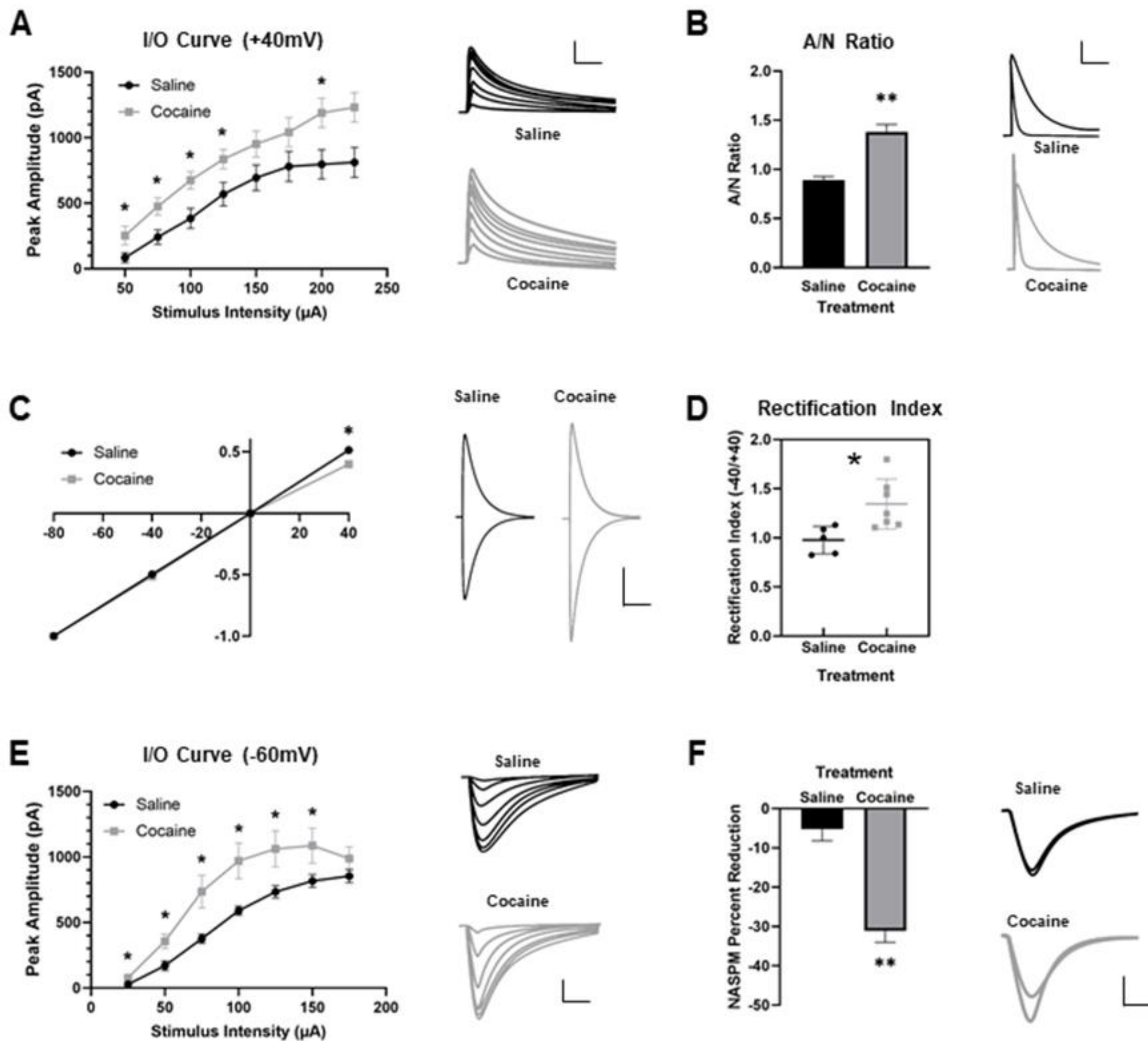
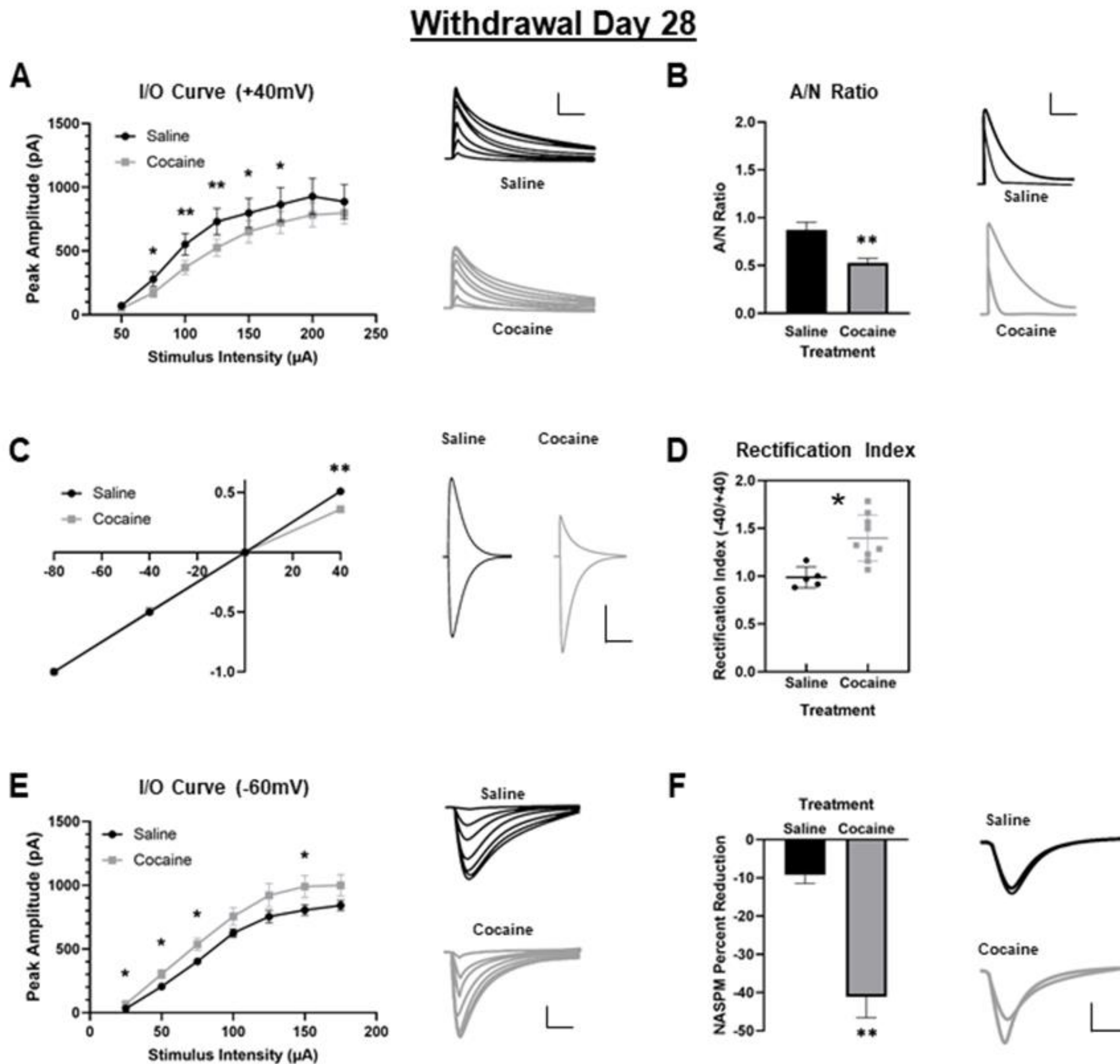


Figure 3.3 **(A)** Input / Output (I/O) curve of EPSCs recorded in vH slices from saline [n=8(7)] or cocaine [n=10(9)] treated animals recorded at a +40mV holding potential. Average traces of the EPSCs are shown to the right; vertical scale bar = 200pA and horizontal scale bar = 100ms. **(B)** The summary quantification of the AMPA/NMDA (A/N) ratio recorded at +40mV holding potential in saline [n=6(6)] and cocaine [n=7(7)] treated animals. Average traces of the AMPA-EPSCs and NMDA-EPSCs are shown to the right; vertical scale bar = 100pA and horizontal scale bar = 300ms. **(C)** The current-voltage (IV) relationship shown with normalized AMPA-EPSC amplitudes plotted against the holding potentials at which the responses were evoked in saline [n=5(5)] and cocaine [n=7(7)] animals. Average traces of AMPA-EPSCs recorded at +40mV (top trace) and -40mV (bottom trace) holding potentials; vertical scale bar = 400pA and horizontal scale bar = 40ms. **(D)** Summary quantification of the rectification index ($EPSC_{-40mV}/EPSC_{+40mV}$) obtained from the IV relationship. **(E)** Input / Output (I/O) curve of EPSCs recorded in vH slices from saline [n=6(6)] or cocaine [n=8(7)] treated animals recorded at a -60mV holding potential. Average traces of the EPSCs are shown to the right; vertical scale bar = 300pA and horizontal scale bar = 20ms. **(F)** Summary quantification of the percent reduction in AMPA-EPSC peak amplitudes recorded at -60mV following NASPM bath application in saline [n=4(4))] and cocaine [n=5(5)] animals. Averages traces are shown to the right of AMPA EPSCs before and after NASPM bath application; vertical scale bar = 200pA and horizontal scale bar = 20ms. * = $p<0.05$ ** = $p<0.01$ compared to saline control group. Error bars +/- SEM.

3.3.3 Increased vH AMPA-EPSC transmission observed on WD9 in cocaine-treated mice can be attributed to increase in both CI-AMPARs and CP-AMPARs

Similar to WD2, we observed a significant leftward shift in the +40mV I/O curve on WD9 of cocaine-treated animals with significantly greater ($p<0.05$, Fig 3A) EPSC amplitudes observed at µA input intensities of 50,75,100,125,&175. A significant ($p<.01$, Fig. 3B) increase in the A/N ratio of cocaine-treated animals compared to saline-treated animals was also observed on WD9 similar to WD2. In contrast to WD2, on WD9 we observed a significant decrease ($p<0.01$) in the +40mV peak EPSC amplitude of cells from cocaine-treated animals (Fig. 3C) with a concurrent significant ($p<0.01$) increase in the rectification index (Fig. 3D). This indicates that CP-AMPARs are expressed postsynaptically at a high enough density in the vH of cocaine-treated animals on WD9 in relation to CI-AMPARs for us to observe an inward rectification at positive membrane potentials. We further observed a leftward shift in the -60mV I/O curve of cocaine-treated animals on WD9 with significantly higher outputs at all input stimulation intensities except for 175µA. Bath application of NASPM induced a significantly greater decrease ($p<0.01$) in AMPAR-mediated EPSC amplitude in vH cells from cocaine-treated animals compared to saline treated animals. Similar to WD2 (Fig. 2A&B), the leftward shift of the +40mV I/O curve and increased A/N ratio of cocaine-treated animals on WD9 would indicate an increase in CI-AMPARs that persist from WD2 to WD9. However, vH slices from these animals also displayed a significant increase in the RI (Fig. 3D) and a significant decrease in EPSC amplitude after NASPM application (Fig 3F). It would appear on WD9 that there is an increase in both CI-AMPARs and CP-AMPARs in the vH

of cocaine-treated animals compared to saline-treated animals based on the data presented in Figure 3.3.



<u>Figure 3.4</u> (A) Input / Output (I/O) curve of EPSCs recorded in vH slices from saline [n=9(8)] or cocaine [n=13(11)] treated animals recorded at a +40mV holding potential. Average traces of the EPSCs are shown to the right; vertical scale bar = 200pA and horizontal scale bar = 100ms. **(B)** The summary quantification of the AMPA/NMDA

(A/N) ratio recorded at +40mV holding potential in saline [n=7(7)] and cocaine [n=10(9)] treated animals. Average traces of the AMPA-EPSCs and NMDA-EPSCs are shown to the right; vertical scale bar = 100pA and horizontal scale bar = 300ms. **(C)** The current-voltage (IV) relationship shown with normalized AMPA-EPSC amplitudes plotted against the holding potentials at which the responses were evoked in saline [n=5(5)] and cocaine [n=9(8)] animals. Average traces of AMPA-EPSCs recorded at +40mV (top trace) and -40mV (bottom trace) holding potentials; vertical scale bar = 400pA and horizontal scale bar = 40ms. **(D)** Summary quantification of the rectification index ($EPSC_{-40mV}/EPSC_{+40mV}$) obtained from the IV relationship. **(E)** Input / Output (I/O) curve of EPSCs recorded in vH slices from saline [n=7(6)] or cocaine [n=8(8)] treated animals recorded at a -60mV holding potential. Average traces of the EPSCs are shown to the right; vertical scale bar = 300pA and horizontal scale bar = 20ms. **(F)** Summary quantification of the percent reduction in AMPA-EPSC peak amplitudes recorded at -60mV following NASPM bath application in saline [n=5(5)] and cocaine [n=7(7)] animals. Averages traces are shown to the right of AMPA EPSCs before and after NASPM bath application; vertical scale bar = 200pA and horizontal scale bar = 20ms. * = $p<0.05$ ** = $p<0.01$ compared to saline control group. Error bars +/- SEM.

3.3.4 Increased in vH AMPA-EPSC transmission observed on WD28 only at negative holding potentials due to a significant contribution of CP-AMPARs to AMPA-EPSCs

In contrast to WD2 and WD9, on WD28 we observed a significant rightward shift in the +40mV I/O curve with significantly decreased peak EPSC amplitudes at stimulus intensities of 75,100,125,150,&175µA ($p<0.05$, Fig. 4A). We also observed a significant

decrease in the A/N ratio ($p<0.01$, Fig. 4B) in vH slices from cocaine-treated animals. This stark contrast on WD28 compared to WD2 and WD9 may be attributed to increased CP-AMPAR contribution in vH synapses since CP-AMPARs conduct poorly when cells are held at +40mV. We observed a significant decrease ($p<0.01$) in the +40mV IV response of cells from cocaine-treated animals (Fig. 4C) with a concurrent significant ($p<0.01$) increase in the rectification index (Fig. 4D) that would be expected if CP-AMPARs are present. A leftward shift of the -60mV I/O curve, similar to WD2 and WD9, was observed on WD28 of cocaine-treated mice compared to saline-treated mice with significantly higher output amplitudes from cocaine-treated animals at stimulus intensities of 25,50,75,&150µA ($p<0.05$, Fig 4E). The rightward shift in the +40mV I/O curve (Fig. 4A). The leftward shift of the -60mV I/O curve (Fig. E) of cocaine-treated animals on WD28 could possibly be due to a high proportion of postsynaptic CP-AMPARs which do not conduct at the +40mV holding potential but conduct readily at a -60mV holding potential. Indeed, after bath application of NASPM, vH EPSCs were significantly reduced ($p<0.01$, Fig. 4F) by 41% compared to a 9% reduction in saline-treated animals. Cumulatively, these indicate that CP-AMPARs are present, similar to WD9, but account for a much larger proportion of postsynaptic AMPARs in the vH of cocaine-treated animals on WD28.

		Saline	Withdrawal Day 2	Withdrawal Day 9	Withdrawal Day 28
Depolarized (+40mV)	A/N Ratio	0.94 ± 0.05	1.67 ± 0.10 (↑)	1.48 ± 0.07 (↑)	0.5 ± 0.05 (↓↓↓)
	NMDA τ (decay)	243 ± 7 ms	255 ± 7 ms	257 ± 13 ms	253 ± 9 ms
	AMPA τ (decay)	25.8 ± 1.0 ms	24.4 ± 0.8 ms	27.4 ± 2.1 ms	26.1 ± 1.5 ms
	Rectification Index	1.01 ± 0.03	1.15 ± 0.08	1.28 ± 0.10 (↑)	1.36 ± 0.07 (↑)
Hyperpolarized (-60mV)	S1/2	93 ± 5 µA	73 ± 8 µA (↓)	68 ± 9 µA (↓)	78 ± 7 µA (↓)
	I/O Max	782 ± 58 pA	985 ± 105 pA (↑)	1087 ± 115 pA (↑)	999 ± 95 pA (↑)
	NASPM Reduction	5.1 ± 2.1 %	12.3 ± 1.8 % (↑)	31.1 ± 2.9 % (↑↑)	41.1 ± 5.5 % (↑↑)
	τ - AMPA before NASPM	28.6 ± 2.0 ms	27.8 ± 1.8 ms	20.8 ± 1.0 ms (↓↓)	18.7 ± 1.6 ms (↓↓)
	τ - AMPA after NASPM	27.9 ± 2.2 ms	28.0 ± 1.4 ms	27.5 ± 2.0 ms	26.7 ± 1.5 ms

Table 3.1 Displays values recorded from ventral hippocampal CA1 Schaffer-collateral synapses following saline- or cocaine-conditioning at either a 2, 9, or 28 day withdrawal period. ↑/↓ $p < 0.05$ increase / decrease compared to saline. ↑↑/ ↓↓ $p < 0.05$ compared to saline and compared to WD2. ↑↑↑/↓↓↓ $p < 0.05$ compared to saline, WD2, and WD9 treatment groups.

3.3.5 Comparison of EPSC responses between WD2, WD9, and WD28.

When comparing all three withdrawal time points using a two way ANOVA (main effects: treatment and time) and LSD post-hoc test, we observed that the A/N ratio was significantly lower on WD28 in cocaine-treated mice than in saline-treated mice ($p<0.05$) or cocaine-treated mice on WD2 ($p<0.01$) or WD9 ($p<0.01$). Additionally, in cocaine-treated animals on WD9 the A/N ratio was only numerically lower ($p=0.058$) than in cocaine-treated animals on WD2. Further, no change in AMPA-EPSC or NMDA-EPSC kinetics was observed, as the decay constant τ was not significantly different between any of the treatment groups. While the rectification index of cocaine-treated animals was higher on WD9 and WD28 to their saline counterparts, no statistically

significant difference was detected among the cocaine-treated animals across withdrawal timepoints. When the stimulus intensity at which half-maximal EPSCs were elicited, the $S_{1/2}$, was calculated for the -60mV holding potential I/O curves, vH slices from cocaine-treated animals exhibited significant decreases in $S_{1/2}$ compared to time-matched saline-treated animals. Similarly, the max peak EPSC amplitude recorded during the -60mV I/O curve was significantly higher for cocaine-treated animals compared to time matched saline-treated animals at all 3 withdrawal timepoints. This indicates a significant leftward shift of the I/O curve that persists from WD2 to WD28, but is not reflected in the +40mV I/O curve, and may reflect a proportional change in contribution of CP-AMPARs to vH synaptic function throughout withdrawal. The presence of CP-AMPARs in the vH of cocaine-treated animals is evidenced by the significant increase AMPA-EPSC NASPM sensitivity at all 3 withdrawal time points. When comparing all three withdrawal time points using a two way ANOVA (main effects: treatment and time) and LSD post-hoc test, we observed a significantly greater NASPM sensitivity of EPSCs on WD9 and WD28 compared to WD2 in cocaine-treated animals with only a numerical increase ($p=0.08$) in NASPM sensitivity on WD28 compared to WD9.

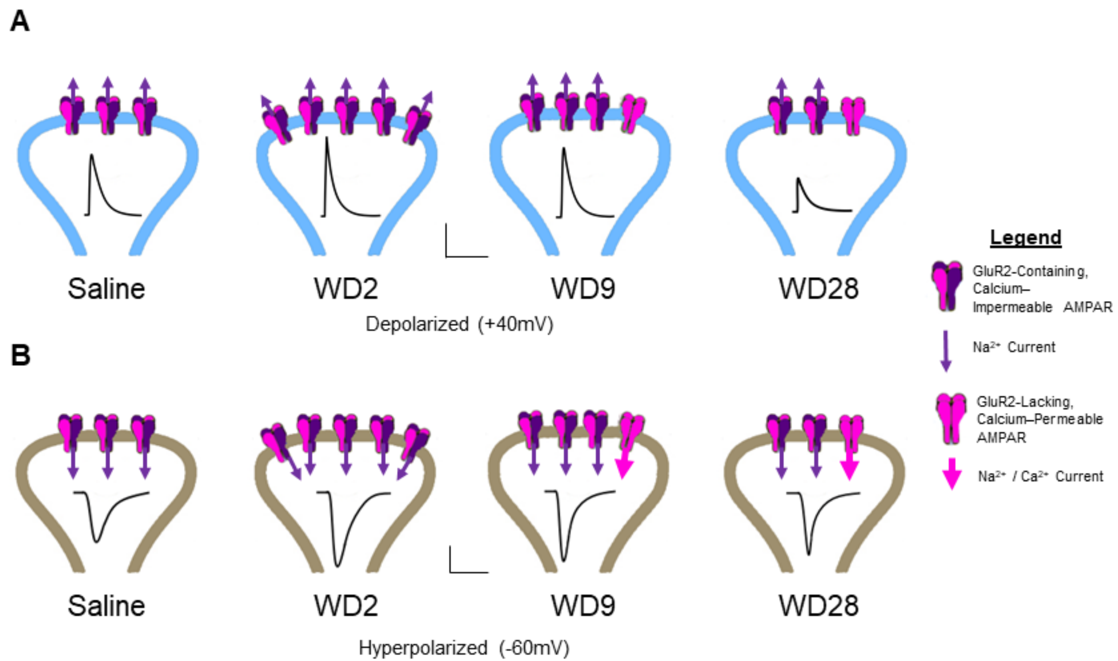


Figure 3.5 (A) Schematic representation of AMPA-EPSC currents in vH CA1 neurons held at a depolarizing holding potential (+40mV). Vertical scale bar = 200pA, Horizontal scale bar = 40ms. **(B)** Schematic representation of AMPA-EPSC currents in vH CA1 neurons held at a resting membrane holding potential (-60mV). Vertical scale bar = 100pA, Horizontal scale bar = 20ms.

3.4 Discussion

In this study, we have shown that ventral hippocampus (vH) CA1 Schaffer-collateral synapses undergoes dynamic changes between withdrawal day (WD) 2 and 28 following cocaine conditioning. We found evidence of increased AMPA receptor function on WD2, followed by changes in AMPA receptor composition on WD9, with

significant changes in AMPA receptor composition ensuing on WD28. This significant reshaping of the synapse can be attributed to increased CI-AMPARs early in withdrawal and subsequent removal and replacement with CP-AMPARs. In adult mice, these CP-AMPARs are minimally expressed in the hippocampus but have been previously linked to the incubation of cocaine craving through their appearance in the NAc during cocaine withdrawal[6]. The presence of CP-AMPARs in the Schaffer collateral synapses in the CA1 region of the vH following cocaine conditioning has not been previously studied and their insertion appears to be temporally dependent.

On the earliest withdrawal time point, WD2, we observed an increase in vH synaptic transmission that we attribute largely to an increase in the number of CI-AMPARs in the postsynaptic density. The increase in vH synaptic transmission of cocaine-treated animals on WD2 is evidenced by the leftward shift of the I/O curves at both +40mV and -60mV holding potentials, as well as an increase in the AMPA/NMDA ratio. CI-AMPARs are distinguishable from CP-AMPARs due to the unique channel properties of CP-AMPARs such as poor conductance at positive holding potentials[21, 22] resulting in an inward rectification that can be quantified as a rectification index. This increase in transmission on WD2 can be mainly contributed to an increase in CI-AMPARs as evidenced by a lack of increase in the rectification index. We did, however, observe a modest, significant increase in NASPM (a CP-AMPAR selective antagonist) sensitivity in slices from cocaine-treated animals compared to saline-treated animals on WD2. This would suggest that while there is a significant increase in CI-AMPAR density, there is also a small, but observable, increase in CP-AMPAR density in vH synapses as early as WD2.

As the withdrawal duration increased, we observed an increase in synaptic CP-AMPARs in the vH CA1 Schaffer-collateral synapse. On WD9 there is a significant increase in CP-AMPARs in vH slices from cocaine-treated animals as illustrated by the increased rectification index and increased NASPM sensitivity compared to both saline-treated animals and cocaine-treated animals on WD2 (Table 1). Although CP-AMPARs conduct poorly at positive holding potentials, we still observe a leftward shift of the I/O curve on WD9 at a +40mV holding potential and an increase in the A/N ratio in cocaine-treated animals. This would suggest that while there is a significant presence of CP-AMPARs in vH Schaffer-collateral synapses on WD9, there is also a persistent, concurrent increase in CI-AMPARs. From these data, we can infer that early in withdrawal (WD2) there is an initial increase in CI-AMPARs, of which some are replaced by CP-AMPARs as withdrawal progresses (Figure 3.5). These results are of interest since they are similar to the changes in glutamatergic transmission seen in the VTA on WD2 after a single i.p. injection of cocaine[29] and those seen in the NAc following extended withdrawal (WD30+) from cocaine self-administration[4] (for a more in depth review of these changes see Wolf & Tseng, 2012[18]).

By withdrawal day 28, the functionality of the vH CA1 Schaffer-collateral synapses exhibit characteristics unique from both saline-treated animals and cocaine-treated animals on WD2 and WD9. In contrast to WD2 and WD9, we observed a rightward shift of the +40mV I/O curve and reduction in the A/N ratio, which is also recorded at +40mV, on WD28 from cocaine-treated animals. While the rectification index and NASPM sensitivity on WD28 is not significantly different from those values on WD9, there is a numerical increase in both numbers suggesting an even greater

contribution of CP-AMPARs to vH synaptic function on WD28 than on WD9. Indeed, given that CP-AMPARs do not conduct well at +40mV, the rightward shift of the +40mV I/O curve and decreased A/N of cocaine-treated animals is likely the result of CP-AMPARs accounting for a greater proportion of AMPARs in the synapse on WD28 in cocaine-treated animals than on WD9. This is supported by results in vH slices from cocaine-treated animals still exhibiting a leftward shift of the -60mV I/O curve, a holding potential where CP-AMPARs conduct easily, on WD28. Additionally, we calculated the EPSC decay kinetics (τ) at the +40mV holding potential for both AMPA-EPSCs and NMDA-EPSCs as well for AMPA-EPSCs at the -60mV holding potential both before and after NASPM application. At the positive holding potential, we did not observe any difference in the decay kinetics of AMPA- or NMDA-EPSCs in cocaine-treated animals at any withdrawal timepoint compared to saline-treated animals. This further supports the idea that the increase in the A/N ratio seen on WD2 and WD9 can be attributed to an increase in number, rather than composition, of CI-AMPARs while decrease in the A/N ratio on WD28 can be attributed to a decrease in number of CI-AMPARs compared to saline-treated animals. Since CP-AMPARs display faster channel kinetics than CI-AMPARs [22, 41, 42], we are able to show further evidence of their contribution to vH synaptic transmission by observing faster channel kinetics at the -60mV holding on both WD9 and WD28 that is reversed by NASPM bath application. We have previously shown in vH field potentials that 28 days after cocaine conditioning there is an increase in basal synaptic transmission with a concomitant decrease in LTP[14]. This increase in basal synaptic transmission may be the result of higher conductance CP-AMPARs in the vH synapses. As a result of the increase in synaptic strength, LTP may be blunted due to

an occlusion of further synaptic strengthening [15, 27, 43]. Alternatively, the reduction in LTP observed on WD28 may be due to the inward rectification of these CP-AMPARs, which would decrease conductance during intense periods of depolarization[44].

In summary, we assessed the temporal changes of vH excitatory transmission by utilizing an escalating cocaine conditioning protocol; this protocol has previously been shown to produce changes in vH synaptic plasticity on WD28 as well as impairment in a vH-dependent learning and memory task[14]. The timing of these changes is of importance due to the apparent disconnect between the immediate insertion of CP-AMPARs in the VTA[43] and the delayed insertion of CP-AMPARs in the NAc during cocaine withdrawal, despite the consistent increase in incubation of cocaine craving through WD60[2]. We have shown here increased excitatory transmission in the vH on WD2 with subsequent insertion and contribution of CP-AMPARs to synaptic transmission as early as WD9. By WD28 we observed a significant remodeling of the vH excitatory synapse with CP-AMPARs acting as a major contributor to neurotransmission. Considering the strong influence of the vH on the NAc**[45],** these changes in vH synaptic transmission early in cocaine withdrawal may drive the incubation of cocaine craving by contributing to the previously observed increases in vH-NAc synaptic activity late in withdrawal[16].

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CHAPTER 4

SUMMARY

The hippocampus has been known to play a role in learning and memory ever since the report of the famous patient, H.M., in 1957. Since that time, the hippocampus has gained appreciation as a bilateral structure with the ventral hippocampus (vH) playing an important role in stress and mood-related responses and the dorsal hippocampus (dH) playing an important role in spatial navigation and declarative memory. The vH has also gained appreciation for its role in addiction due to its excitatory projections to the nucleus accumbens (NAC) and the change in strength of these synapses observed during cocaine withdrawal [1]. These vH-NAC projections have been shown to be important in both the acquisition [2] and reinstatement [3] of conditioned place preference (CPP). The role of the hippocampus in CPP is of particular importance since addiction is a context-dependent phenomenon whereby previously drug-paired environments trigger drug craving, leading to drug seeking and relapse. Despite the known importance of the vH-NAC synapse in addiction, the synaptic changes within the vH itself following cocaine conditioning have not been extensively studied. Here we showed electrophysiological evidence for changes in both synaptic transmission and plasticity during cocaine withdrawal as well as relevant behavioral phenotypes.

4.1 Developing an adequate assay for studying addiction

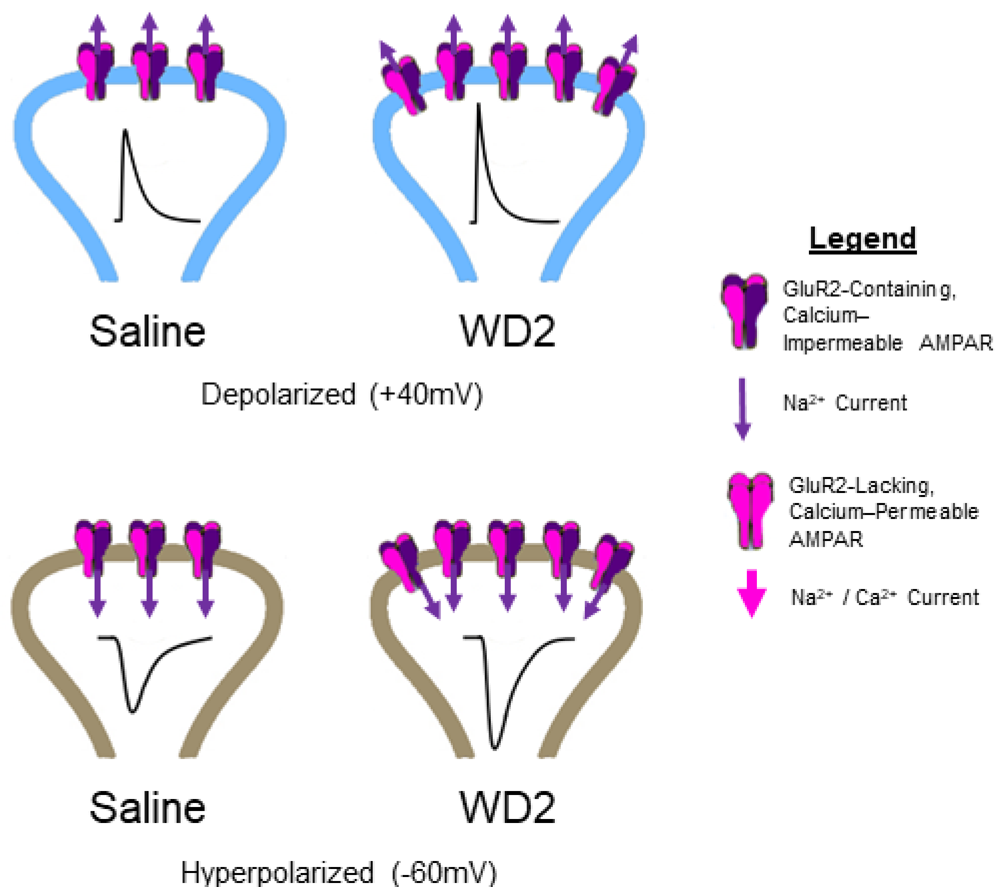
Within the field of addiction biology there is debate as to whether or not the CPP model of addiction is sufficient to mimic human behavior and produce equivalent results

to the self-administration model of addiction [4-6]. Given the ease with which the conditioned place preference (CPP) can be performed compared to self-administration, one goal of this research was to create a CPP model in mice that produced results similar to those seen in rats after cocaine self-administration [7]. We first attempted this by using a single escalating dose of cocaine but failed to observe significant changes in vH LTP on withdrawal day (WD) 28 (Figure 2.3). By utilizing a double escalating dose, we increased both the total amount of cocaine received by the mice as well as the duration of the CPP assay. This dosing schedule, which became our standard for future CPP assays, was sufficient to produce changes vH synaptic plasticity that could be observed on WD28 as outlined in Chapter 2. It is important to note that this dosing protocol, when paired with a context in the CPP model, resulted in decreased LTP on WD28 compared to home cage injections which resulted in an increase in LTP on WD28. It would appear that this dosing protocol creates strong contextual memories if paired with an environment that impairs future LTP ability, while the more pharmacological effects observed after home cage injections seem to prime the synapse for LTP and production of these contextual memories.

This initial research also highlighted the importance of adequate controls, not just for CPP or addiction studies, but for all scientific experiments. We observed increased LTP in saline-treated animals, the typical control for the CPP assay, on WD28 when compared to behaviorally naïve animals, a result also previously observed by our lab [8]. Since an i.p. injection of isotonic saline should not produce a pharmacologic response, it is reasonable to assume that the handling and injection of mice during saline-conditioning was sufficient to create a stress-induced increase in LTP that lasts at least 28 days. Had

we not compared our saline-treated animals to behaviorally naïve animals we would not have noticed this confounding stress variable from an assay that would not typically be regarded as stressful for rodents. In most experiments, saline is assumed to be a sufficient control, but we have shown here the importance of comparing saline- or control- treated animals to naïve animals to ensure the control treatment is not having its own effect. Luckily, we were able to attenuate this confounding factor with a single pre-treatment of the kappa-opioid antagonist NorBNI and gain confidence in our model to elucidate the effects with cocaine conditioning with adequate controls and without a confounding stress factor.

4.2 Progression of ventral hippocampus synaptic changes throughout withdrawal



<u>Figure 4.1</u> Withdrawal Day 2 Summary Diagram

After developing a dosing protocol sufficient to produce changes in vH synaptic plasticity at WD28, we sought to explore the time course of when and how these in plasticity changes occur. Utilizing whole-cell voltage clamp recordings allowed us to observe the progressive and dynamic changes in vH CA1 Schaffer-collateral synaptic transmission between WD2, WD9, and WD28. At the earliest withdrawal timepoint, WD2, we observed an increase in EPSCs resulting from an increase in the number of synaptic CI-AMPARs (Figure 4.1). We also observed a small contribution of CP-AMPARS as reflected by the increased NASPM sensitivity of cocaine-treated mice at WD2. This increase in CP-AMPARs, however was not sufficient to produce a significant change in the rectification index, consistent with the idea that the main increase in excitatory transmission on WD2 is from CI-AMPARs. It is important to note that at this time point, changes in VTA synaptic transmission are detectable [9-12] while changes in the NAC synaptic transmission are not yet seen [13-15]. Interestingly, the aforementioned changes in VTA synaptic plasticity are detectable within hours of cocaine injection [9]. While we did not record that soon after the final injection, it would appear that vH synaptic changes are occurring either in conjunction with VTA changes or shortly after.

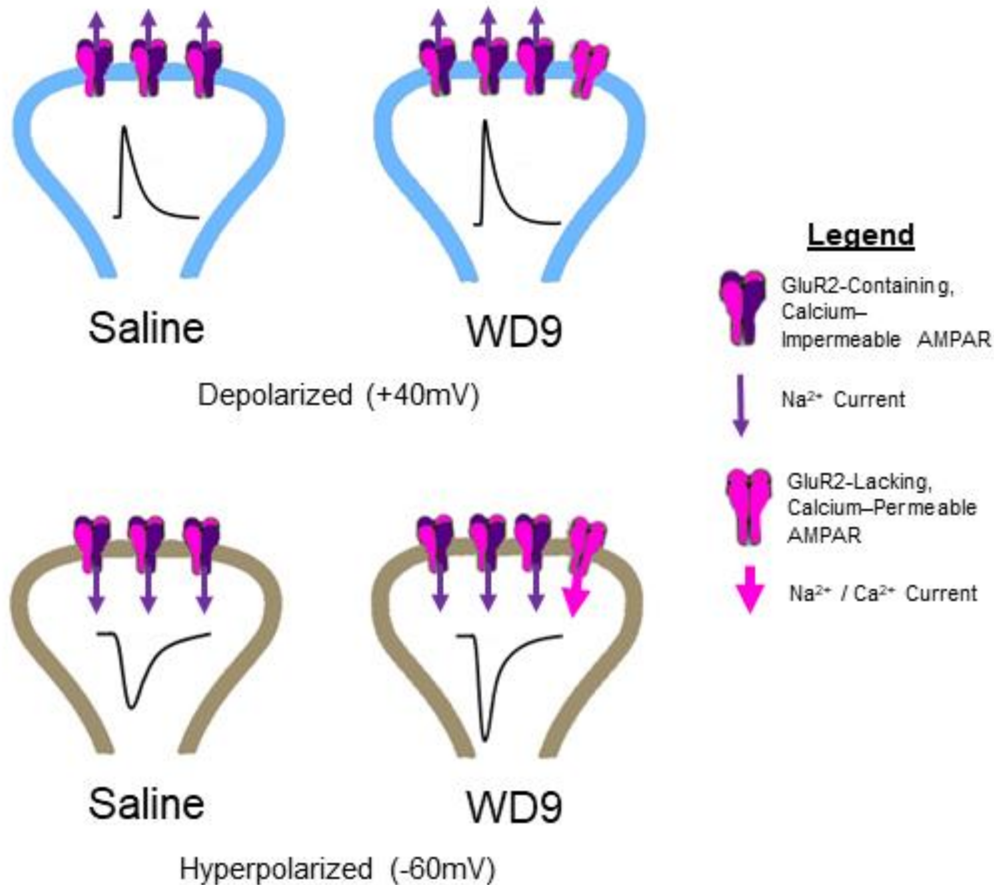


Figure 4.2 Withdrawal Day 9 Summary Diagram

On WD9 we still observed an increase in excitatory synaptic transmission of vH CA1 Schaffer-collateral synapses. The increase in CI-AMPAR synaptic density appears to maintain from WD2 to WD9 as evidenced by the increased A/N ratio on both WD2 and WD9 in cocaine-treated animals compared to saline-treated animals. However, we also observed an increased contribution of CP-AMPARs to the excitatory transmission similar to the changes seen on WD2 in the VTA [16] as evidenced by an increase in the rectification index as well as increased sensitivity of AMPA-EPSCs to NASPM (Figure 4.2). This shift from increased CI-AMPAR density on WD2 to an increase in both CI-AMPARs and CP-AMPARs may possibly result from the insertion of these CP-AMPARs

into the synapse as a form of homeostatic scaling that is inadvertently triggered by cocaine use.

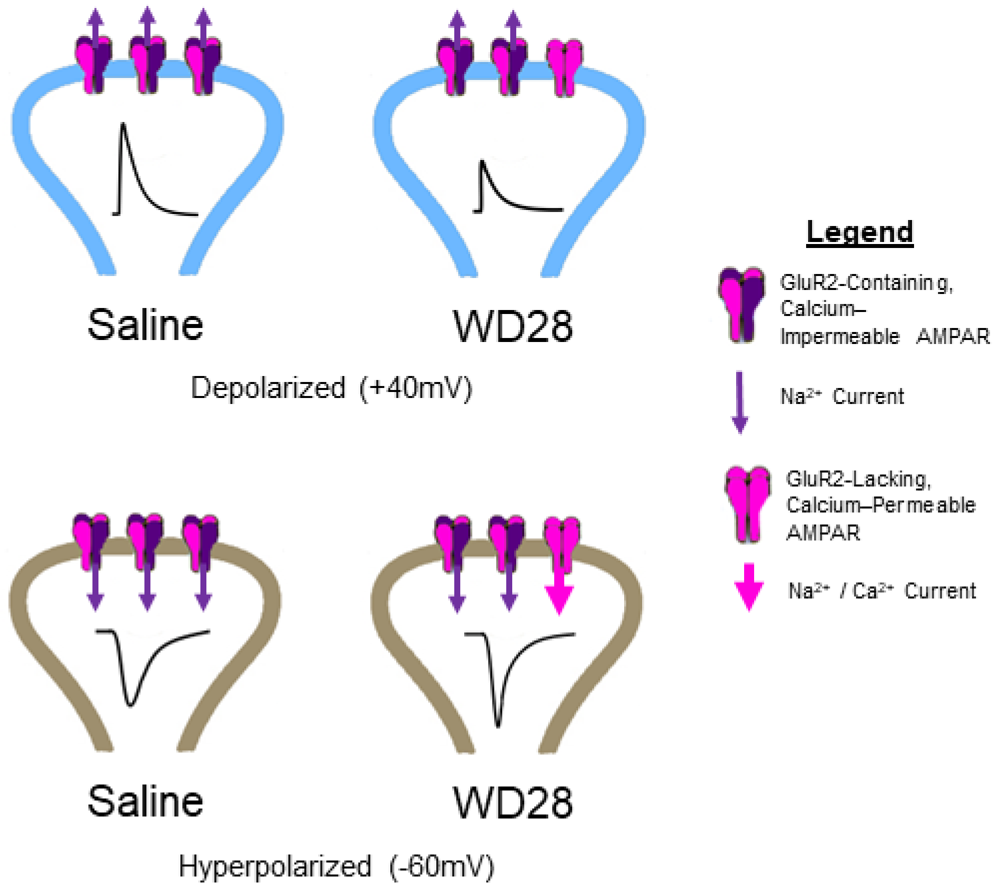


Figure 4.3 Withdrawal Day 28 Summary Diagram

On WD28 we saw changes in vH excitatory synaptic transmission that were markedly different from WD2 and WD9. At this withdrawal timepoint, we observed that CP-AMPARs contributed to a major portion of vH transmission. This was apparent by reduced EPSC amplitude and A/N ratio at positive holding potentials, where CP-AMPARs do not conduct well. However, at a negative holding potential of -60mV we observed an increase in EPSC amplitude compared to saline-treated animals. Despite this duality of increased AMPA conductance near the resting membrane potential and

decreased AMPA conductance in the depolarized state, the postsynaptic neuron of cocaine-treated animals would still be more easily excitable than saline-treated counterparts. This is due to the fact that threshold for propagation of an action potential through the postsynaptic neuron is around -55mV and these CP-AMPARs, that conduct both Ca^{2+} and Na^{+}, would have a greater contribution to reaching this threshold potential than CI-AMPARs. Additionally, we have shown utilizing field potentials that the fEPSPs of cocaine-treated animals are larger than saline-treated animals suggesting an increase in conductance of the postsynaptic neuron.

The increase in vH excitatory transmission at all time points may play an important physiological role in cocaine seeking during withdrawal. Since these neurons are more easily excitable it is possible that when the same context in which cocaine was given, is encountered again, then there may be an aberrantly strong response of the hippocampus to the context. This increased response may result in increased excitatory output to NAC driving the craving when a drug-paired context is re-encountered. Furthermore ,this increased signaling to the NAC may drive some of the changes seen in the NAC later in withdrawal that further contribute to the incubation of cocaine craving.

The significant presence of the CP-AMPARs will, however, change the rules of plasticity for the vH synapses and potentially not allow for Hebbian plasticity to occur. This typical, Hebbian plasticity in the vH is largely NMDA-dependent with the NMDA receptor requiring a positive membrane potential to remove a magnesium block to allow calcium into the cell. If these CP-AMPARs stop conducting at positive holding potentials, it is possible that during a tetanus event there is inadequate depolarization of the postsynaptic synapse to allow calcium entry through the NMDA receptor, thus

blunting the ability to potentiate. This anti-Hebbian plasticity has been seen in the VTA early in withdrawal [16] and would explain our results of decreased LTP on WD28. It is important to note that we NMDA-EPSCs, following cocaine-conditioning, were unchanged in the kinetics or peak throughout withdrawal.

The decrease in the ability of vH CA1 Schaffer-collateral neurons to potentiate at WD28 may have significant impacts on the drug users. As shown here, we observed a decrease in working memory in mice following cocaine conditioning, a feature also seen in human cocaine users. This could have a detrimental impact on the daily life and work of former cocaine addicts. Furthermore, since these neurons do not respond to Hebbian plasticity and exhibit decreased LTP, it is possible that the cocaine contextual association is essentially hard-wired into the brain's circuitry. This means that despite re-exposure to the context in which cocaine is used, the memory of cocaine use there will persist since the memory cannot be updated. This means that cocaine seeking, and craving will always be driven in this context regardless of the length of abstinence from cocaine.

4.3 Future Directions and Limitations

By utilizing electrophysiological techniques, such as whole-cell patch-clamp electrophysiology, these studies have shown evidence of these CP-AMPARs being functionally present in vH CA1 Schaffer-collateral synapses during cocaine withdrawal. These studies, however, could have been stronger with additional data from biochemical assays such as western blots. One such method is BS3 cross-linking which is able to cross-link only surface-expressed receptors to surrounding proteins. This cross-linking increases the molecular weight of the surface-expressed receptors which would allow us to distinguish between surface and intracellular receptor concentrations in a Western Blot

experiment. We attempted to perform this cross-linking on both hippocampal and nucleus accumbens tissue from all three withdrawal time points to further confirm an increase in GluA1 receptor subunits on WD9 and WD28. This experiment did not work but would have helped shed additional light on where these CP-AMPARs come from during withdrawal (i.e. intracellular pools or perisynaptic sites). While this cross-linking assay is quite useful for comparing surface vs intracellular receptor levels, the phospho-specific antibodies relevant to CP-AMPARs cannot be used with it.

Trafficking to and stabilization in the synapse of both CP-AMPARs and CI-AMPARs occurs via phosphorylation of the GluA1 subunit. However, the stabilization of CP-AMPARs in the synapse is dependent on GluA1 Ser845 [17] while the trafficking and stabilization of CI-AMPARs is dependent on GluA1 Ser831. Interestingly, the GluA1 Ser845 is phosphorylated by PKA which is activated via dopamine D1/5 receptors. This means that an increase in synaptic dopamine via cocaine blockade of the dopamine transporter could increase D1/5 activation, PKA activity, and PKA phosphorylation of CP-AMPARs for their trafficking to the synapse. It is possible to enhance LTP in hippocampal slices simply by washing on cocaine and this effect can be blacked by a D1/5 antagonist [18]. It would be interesting to explore this effect further to see if PKA inhibitors could also prevent the cocaine-induced LTP. If so, a CP-AMPAR antagonist such as NASPM or philanthotoxin could be bath applied to assess the contribution of CP-AMPARs to both the induction and expression of LTP following bath application of cocaine.

4.4 Final Thoughts

In conclusion, cocaine conditioning produces progressive changes in ventral hippocampus physiology throughout withdrawal that can have profound behavioral effects. These progressive changes can be attributed to the insertion of calcium-permeable AMPA receptors (CP-AMPARs) into the CA1 Schaffer-collateral synapses of the ventral hippocampus (Figure 3.5) and may contribute to the impaired working memory observed in mice (Figure 2.5) that is also seen human cocaine users [19].

Since cocaine produces aberrantly strong memories of the context in which cocaine was used, it should not come as a surprise that drug rehabilitation and / or imprisonment do little to treat addiction. It is important to investigate ways to ameliorate these memories in order to develop a therapeutic aid for those people who wish to abstain from drug use. One interesting facet of the CP-AMPARs that appear to produce these strong memories that can't be updated is that they may be able to be selectively removed via mGlur1 activation [20]. This provides an avenue for future experimentation where it may be possible for the strong CPP memory, that persists at least 28 days, to be selectively removed, or at least reduced by an mGlur1 agonist. Additionally, if deep brain stimulation were able to recapitulate the effects of optogenetic stimulation in rodents, it may provide a useful therapeutic to alter synapses in a frequency-specific fashion. For example, Pascoli and colleagues were able to selectively remove CP-AMPARs from medial prefrontal cortex (mPFC) to NAC synapses following cocaine self-administration using a 13 Hz optogenetic stimulation [1]. This group was also able to reverse the increase in CI-AMPARs from vH to NAC synapses following self-administration using a 1 Hz optogenetic stimulation. Both of these optogenetic manipulations abolished cocaine

seeking in rats following self-administration indicating a potential therapeutic avenue for cocaine addiction.

Given the fact that CP-AMPARs are minimally expressed in non-disease states, there is additional therapeutic potential for traditional oral drugs that may target these receptors. Since the CP-AMPARs are minimally expressed, the likelihood of off-target interactions and negative side effects is reduced. Drugs that are able to target these CP-AMPARs may be able to be given in conjunction with a rehabilitation program to help improve outcomes or may be used on an as-needed basis when the user is experiencing drug cravings. By targeting these CP-AMPARs either via traditional drug routes or less traditional DBS or transcranial stimulation, researchers may be able to provide assistance to former drug users in preventing relapse.

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