FACTORS AFFECTING SYNAPTIC PLASTICITY IN THE HIPPOCAMPUS: FOCUS ON STRESS AND ADDICTION

by

MADHUSUDHANAN MATHILAKATH KERALAPURATH

(Under the Direction of John J Wagner)

ABSTRACT

Involvement of the hippocampus in learning and memory is well known. Despite maintaining a uniform intrinsic circuitry throughout the structure, there are evidences for significant functional differences between the septal (dorsal) and temporal (ventral) sectors of hippocampus. Appreciation for the role of ventral hippocampus in the emotive and reward associated behaviors is on the rise. Behavioral and lesion studies in rodents have suggested that hippocampus plays an important role in drug reinstatement behaviors, although confirmatory molecular and electrophysiological evidences are lacking in this aspect. To study the effects of cocaine, we employed either the locomotor sensitization protocol where the drug was non-contingently administered by the experimenter, or we utilized the self-administration protocol where the rats were allowed to voluntarily administer drugs. Additionally, hippocampal feedback regulation of the hypothalamus-pituitary-adrenal axis is critical in the body's response to stress. We investigated the effects of minor stressors such as novelty, handling and i.p. injections associated with a locomotor sensitization protocol on the hippocampal synaptic plasticity.

We found that the minor, intermittent stressors associated with commonly employed behavioral protocols were sufficient to induce persistent stress-like effects. Cocaine, either

experimenter administered or self-administered by the rats, specifically altered synaptic properties in the ventral hippocampus, albeit in different ways. Non-contingent cocaine exposures acted as metaplastic triggers and persistently enhanced the artificially induced LTP in the ventral hippocampus for at least 2 weeks after the last injection. In contrast, cocaine self-administration resulted in persistent enhancement of basal synaptic transmission in the ventral hippocampus, with both increases in excitatory glutamatergic activity and suppression of GABAergic inhibition being observed. Unlike in the non-contingently exposed groups, cocaine self-administration resulted in the suppression of LTP in the ventral hippocampus, suggesting a causal link between impaired learning and addicted states observed in the human subjects. Increased sensitivity of the ventral sector of the hippocampus to stress and drugs of abuse indicate its importance in the neuronal circuitry as a key intersection point where these co-morbidities of stress and drugs of abuse overlap.

INDEX WORDS: hippocampus; dorsal; ventral; stress; cocaine

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MADHUSUDHANAN MATHILAKATH KERALAPURATH

MS, Mississippi State University, 2009

BVSc & AH, India, College of Veterinary & Animal Sciences, Pookot, 2005

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MADHUSUDHANAN MATHILAKATH KERALAPURATH

Major Professor:	John J. Wagner
Committee:	Gaylen L. Edwards
	Xiaoqin Ye

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Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

To my family back home

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Hippocampus: Historical land marks

The hippocampus was one of the earliest subcortical structures to be discovered. Due to its very prominent bulging structure, it was easily identifiable to most ancient anatomists. It was Giulio Cesare Aranzi, in the mid-16th century, who named this structure 'hippocampus' due to its similarity with a type of fish of the same name. Much before that, because it appeared like a curvy ram's horn, anatomists of the Alexandria school of medicine (300 BC – 300 AD) had called it by the name 'Cornu Ammonis'. This terminology is still retained in its abbreviated form – 'CA', as in the sub regions of hippocampus with the names CA1, CA2, CA3 and CA4. The detailed histological studies of many brain regions including hippocampus were made possible by the advent and use of microscopes. With the combination of powerful staining procedures with the microscopy-aided histological examinations, came the stunning details of the neuronal arrangements in the hippocampus. Two pioneers whose works are still valid were Camillo Golgi and Ramon Y Cajal, proponents of the 'reticular theory' and the 'neuron doctrine' respectively (later part of 1890s). Reticular theory proposed that cells of the brain – neurons – were interconnected intra-cellularly with certain cellular structures such as thin fibrils; while the 'neuron doctrine' supported the view that each neuron was individual cells without forming a syncytium. Not until the advent of electron microscopy that the answer to this conundrum was obtained when they found that each neuron in the brain are indeed individual entities, supporting the 'neuron doctrine'(Shepherd 1972).

Several prominent theories on the functional aspects of hippocampus have been proposed. Until the early part of 1930s hippocampus was considered as a part of the olfactory cortical system. This belief was proven wrong by the works of Alf Brodal (1947) which tempted others in the field to find the real functional roles of hippocampus (Andersen 2007). Meanwhile, Papez, in his still valid 'Papez circuit' (Papez 1995) described hippocampus as an assimilator and transformer of sensory information into emotional traces prior to its transfer onto anterior cingulate gyrus via the mammillary nucleus- anterior thalamic nuclei pathway. The cognitive appreciation of these emotional traces was suggested to occur in the anterior cingulate gyrus, a concept which is still not repudiated. The functional association of anterior cingulate gyrus with hippocampus was also found important in the generation of theta wave activity occurring during many types of learning and attention control episodes (Grastyan et al 1959; Green & Adey 1956).

An important role endorsed to hippocampus is its involvement in learning processes and memory encoding. Some of the early, but largely unknown, evidence for hippocampal involvement in learning came from the works of Brown & Schafer in1888 (Andersen 2007). They observed that a rhesus monkey with significant temporal lobe lesion failed miserably in a simple variant of the currently employed 'object recognition task'. In the same decade, Theodule-Armand Ribot came up with a revolutionary hypothesis in the field of memory in which, the then amorphous concept of memory was ascribed as having physiological and structural form when he posited that formation of memory traces is happening in the nerve cells (Andersen 2007). One of the first structural localization of memory attributes in human hippocampus came during 1890s when Vladmir Bekhterev observed softening of hippocampal tissues during autopsy of two patients with severe memory loss (Andersen 2007). However, the

land mark clinical case which confirmed the hippocampal involvement in memory was that of the famous patient H.M. Scoville and Milner (Scoville & Milner 1957) observed that the temporal lobe- epileptic patients like H.M, after the surgical removal of most of the temporal lobe portions including hippocampal formation, suffered from anterograde and limited retrograde amnesia. Furthermore, O'Keefe and Dostrovsky (O'Keefe & Dostrovsky 1971) observed that in rats certain hippocampal cells fired when the animal reaches familiar environment, a mechanism in turn thought to help the animal navigate in familiar environments. These cells, known as place cells, in hippocampus form a cognitive map of the environment helping the rats in spatial navigation. With the advent of imaging techniques such as MRI, and *in vivo* unit recordings of electrical activity in brain with EEG, multi-electrodes, tetrodes etc., helped to identify many further roles for hippocampus.

Structure

The hippocampus is a part of closely associated structures located deep in the medial temporal lobe of brain. An accurate nomenclature for this group of structures would be 'hippocampal formation', which consists of hippocampus proper, dentate gyrus, subiculum, presubiculum, parasubiculum and entorhinal cortex. The hippocampus proper consists of 3 regions – CA1, CA2 and CA3 (CA: Cornu Ammonis). The subregions (DG, CA3, CA2, CA1 and subiculum) of hippocampus have a well-organized cytoarchitectonic laminar structure. In the dentate gyrus, the soma of the granule cells are arranged within the granule cell layer (stratum granulosum), while the dentritic branches of the granule cells are present in the molecular layer (stratum moleculare). The cellular layer in between the U- or V- shaped stratum granulosum is the hilus, which contains mossy cells and other polymorphic cells of the dentate gyrus. In the hippocampus proper, the soma of the pyramidal cells of CA3 and CA1 regions are arranged in a

dense manner in the pyramidal cell layer (stratum pyramidale). The basal dendrites of pyramidal neurons are present in the stratum oriens, while the apical dendritic branches traverse across the stratum radiatum and stratum lacunosum-moleculare. The axonal fibers of the CA1 pryamidal cells traverse through the alveus above the stratum oriens. (Figure 1.1).

The intrinsic network within in the hippocampal formation majorly retains its unidirectionality while processing the information in three synapses, duly named as the

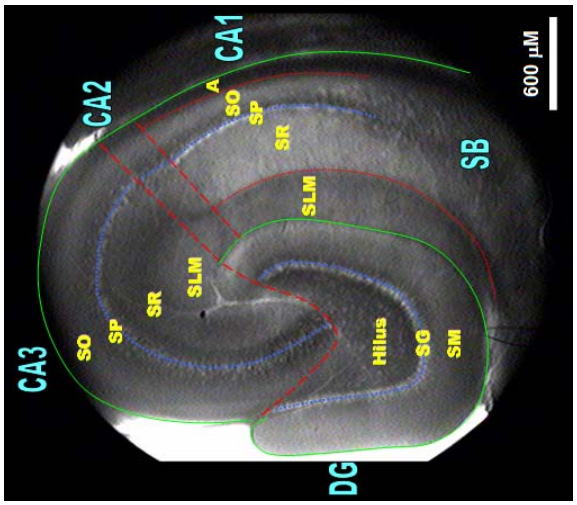

Figure 1.1. Subregions and laminar organization of hippocampus: Subregions: Cornu Ammonis (CA) regions – CA3, CA2, CA1; SB-subiculum; DG- dentate gyrus. Laminae: A-alveus, SO – stratum oriens, SP- s.pyramidale, SR- s.radiatum, SLM – s.lacunosum moleculare , SG – s.granulosum, SM- s.moleculare .Courtesy: painesnotebook.net

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trisynaptic circuit (Andersen 2007). Sensory information from the neocortical circuit reaches the hippocampus via the entorhinal cortex. In the DG, majority of the perforant path fibers originating from the entorhinal cortex synapses with the dendritic branches of the granule cells of dentate gyrus in the s.moleculare. The axonal projections from the granule cells, called mossy

fibers, project to synapse with the apical dendritic branches of CA3 pyramidal cells, forming the 2nd synapse with in the hippocampal formation. The Schaffer collaterals axons of the CA3 pyramidal cells synapse with the apical dendrites of CA1 pyramidal neurons in the s.radiatum forming the 3rd synapse of the region. A majority of the efferent axons from the CA1 neurons project to the entorhinal cortical areas, completing a full loop of information passage (Figure 1.2). A smaller proportion of CA1 pyramidal neurons synapse with the subicular neurons making an exception to the general norm of trisynaptic circuitry.

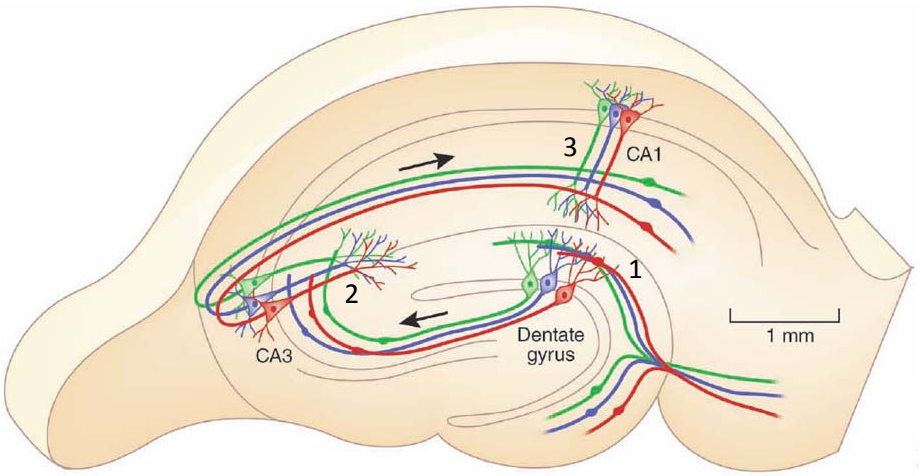

Figure 1.2. Unidirectional trisynaptic circuit in the hippocampal slice. The three synapses are numbered 1, 2 & 3 in the orthodromic order. Modified from Moser EI, Multi-laned hippocampus. Nat.Neurosci. (2011).

Septo-temporal segregation in hippocampus

Evidence for the anatomical connectivity differences in hippocampus along its septotemporal axis has been identified as early as 1970s (Siegel & Tassoni 1971). Concurrent evidences along the similar lines helped to gain wide consensus for the idea of dissociating the

hippocampus functionally into dorsal (septal) and ventral (temporal) sectors (Moser et al 1995). While the intrinsic connectivity in the hippocampus maintains its uniformity, the extrinsic afferent and efferent connectivity show gradients along the septotemporal axis. The caudo-lateral entorhinal fibers bringing mainly the visuo-spatial information from the corresponding association cortices, project to the dorsal sector, while the rostro-medial entorhinal projections receiving olfactory, visceral and gustatory information projects more into the ventral hippocampus (Burwell 2000; Dolorfo & Amaral 1998; Insausti et al 1997). Afferents from dorsal sector project to the retrosplenial and anterior cingulate cortices which are important for the visuo-spatial information processing, and to the mammillary nucleus and anterior thalamus which are important regions for aiding in the spatial navigation (Ishizuka 2001; Kishi et al 2000; Swanson & Cowan 1975). The afferents from the ventral hippocampus projects to the olfactory cortical areas (Cenquizca & Swanson 2007), amygdala, the infra- & pre- limbic and the insular cortices, implying its role in the modulation of anxiety related emotions. Hypothalamus, which is the neuroendocrine center for emotions and stress response, is one of the afferent targets primarily of the ventral hippocampus, via direct or indirect (involving BNST, lateral septum or amygdalar-body) pathways (Risold & Swanson 1996). This clearly suggests a role of hippocampus in the modulation of stress responses. Furthermore, as compared to the dorsal sector, the ventral hippocampus was shown to send more glutamatergic afferents to the shell region of nucleus accumbens (Groenewegen et al 1996; Naber & Witter 1998), indicating a significant role of hippocampus in the processing of reward related information. In general, the dorsal sector of hippocampus is mainly involved in the cognition, spatial navigation and exploration, while the ventral sector is associated with emotion, anxiety and reward related memory processes. The research presented in this document has investigated the effects of stress

and of the psychostimulant cocaine on the hippocampal synaptic plasticity along its septotemporal axis.

Hippocampal neurotransmission

Glutamatergic- and th GABA-ergic neurotransmission are the respective excitatory and inhibitory components responsible for the majority of hippocampal synaptic activity. The neurons forming the trisynaptic circuit in hippocampus proper are the glutamate releasing excitatory pyramidal cells. In addition to these principal cells, there are a variety of GABAergic inhibitory interneurons present in all sub-regions of hippocampus. The fast paced excitatory transmission is mainly carried out by the activity of a type of ligand gated glutamate receptor named as α-amino-3-hydroxy-5-methyl-4-isoxazolepriopionate (AMPA) receptor, and by the kainite receptors to a lesser extent. The slow excitatory component is contributed by the N-methyl-D-aspartate (NMDA) receptor. The fast inhibitory GABAergic neurotransmission is mediated by the $GABA_A$ receptors, while the slow inhibitory component is contributed by $GABA_B$ receptors. In addition, certain types of metabotropic glutamate receptors are also responsible for the slow components of both excitatory and inhibitory neurotransmission in the region. A brief description of the major ionotropic neurotransmitter receptors such as AMPA, NMDA and GABA, which determine the hippocampal activity, is given below.

The AMPA- glutamatergic receptor is a hetero-tetramer composed of $GluA_{1-4}$ subunits. Subunit composition of AMPA receptors varies depending on the type of neurons where they are present. For example, hippocampal pyramidal neurons mainly express $GluA_1$ and $GluA_2$ subunits, while the interneurons in the region mainly express $GluA_1$ and $GluA_4$ subunits (Andersen 2007). The channel conductance is mainly determined by the $GluA_2$ subunit of the tetramer. Unique property of $GluA_2$ subunit is due to the presence of amino acid arginine at the

M2 pore-loop segment, whereas other subunits have glutamine in this locus. When $GluA_2$ subunit is present in the receptor complex, it only allows monovalent cations (Na^+, K^+) to pass through the ion pore of the receptor, while the $GluA_2$- lacking AMPA allows divalent Ca^{2+} ions to pass through it. This has greater significance in the context of activity-dependent synaptic plasticity changes happening in the region.

NMDA- glutamatergic receptors are different from the AMPA receptors not only in the structural composition, but also in the channel permeability to cations, voltage dependence, kinetics etc. The subunit types of NMDA receptors are NR_1, NR_2 (NR_2A, NR_2B, NR_2C, NR_2D) and NR_3 (NR_3A and NR_3B), wherein the NR_1 subunit is the essential component of a functional NMDA receptor complex (Andersen 2007). The NR_1 and NR_3 subunits bind glycine, while glutamate binds with the NR_2 subunit. At the resting membrane potentials, NMDA receptors at the synapse do not conduct ions due to a block in the channel pore created by the extracellular Mg^{2+} ions. However, when the membrane is sufficiently depolarized, Mg^{2+} will be expelled and the block removed, the channel conducts both monovalent and divalent cations into the cell. Subsequent increase in the Ca^{2+} permeability activates variety of specific intracellular signaling cascades resulting in persistent changes in the neuronal structure and properties.

The excitatory neurotransmission in the hippocampus is regulated mainly by the inhibitory activity of the GABAergic interneurons. GABA receptors have pentameric composition, with main subunit types such as α_1, α_2, α_4, α_5, $\beta_{1\text{-}3}$, γ_2 and δ present in hippocampus (Andersen 2007). They conduct Cl^- ions through the channels whose entry into the cells hyperpolarize the neurons. Tonic inhibitory activity of the region is imparted by the activity of the extrasynaptic $GABA_A$ receptors while the phasic inhibition is carried out by synaptically located $GABA_A$ receptors. The metabotropic $GABA_B$ receptors also contribute to the tonic

inhibition, but not usually activated at the basal GABA levels. GABAergic interneurons in the region are classified based on the structure and type of receptors expressed (Kullmann 2011). Depending upon the type of GABAergic interneurons synapsing with the pyramidal cells, it can modulate the firing rate and spike timing of the pyramidal cell activity.

Synaptic plasticity

Experience or activity dependent changes in the neuronal properties at the synaptic, functional and structural levels are termed as synaptic plasticity (Ho et al 2011). The well-studied structure and organization of the hippocampal neuronal network made it a good model to study the mechanisms of synaptic plasticity. Any change in the release of neurotransmitters from the presynaptic terminals can alter the neurotransmission in the circuit. Presynaptic proteins which determine the vesicular mobilization, docking, priming and exocytosis all regulate this process. One such important protein required for vesicular mobilization and tethering of readily releasable pool of neurotransmitter vesicles is the synapsin protein (Cesca et al 2010). Proteins such as SNARE (soluble-NSF attachment protein receptor) complex, RIM (Rab3-interacting molecule), and Munc-13 are some of the presynaptic molecules which are essential for the docking, priming and exocytosis of the presynaptic vesicles (Ho et al 2011). Post synaptic mechanisms which contribute to synaptic plasticity involves changes in the receptor expression and conductance, and increase in the Ca^{2+} permeability.

Short term plasticity: Various forms of short term plasticity (which lasts for a few milliseconds – tens of seconds) such as augmentation, facilitation, depression, post-tetanic potentiation etc. haven been described. The phenomenon of facilitation and depression depends upon the release probability of the presynaptic vesicles which in turn is dependent upon the amount of Ca^{2+} at the release sites (Schulz et al 1994). When the release probability of

neurotransmitter at a particular synapse is higher, then when two successive stimuli separated by few milliseconds are given, the second response evoked at the postsynaptic neuron will be smaller than the first response. This phenomenon is called the paired pulse depression. The alternate phenomenon which is usually expressed in the hippocampal CA3- Schaffer collateral synapses is called paired pulse facilitation.

Long term plasticity: Two major types of long term plasticity described are long term potentiation (LTP) and long term depression (LTD). LTP occurs when the presynaptic fibers are stimulated by high frequency stimulation, while LTD happens when the pathway is stimulated by low frequency pulses for a longer time (Bear & Malenka 1994). LTP was initially demonstrated at the perforant path- granule cell synapses in anesthetized rabbit by Bliss and Lømo (Bliss & Lomo 1973). LTP and LTD observed in the hippocampus and other memory regions in the brain are thought to be the cellular substrates for memory since they share some common attributes such as input specificity, cooperativity and associativity (Bear & Malenka 1994). Postsynaptic NMDA receptors contribute to the unique feature of associativity of the LTP phenomenon. NMDA receptors act as 'coincidence detectors' at these synapses, where they will become active only when they bind to the glutamate released in concurrence with a sufficient depolarization of the postsynaptic neuron (Collingridge et al 1983). Calcium entry into the neurons activates kinases such as the calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC), as well as phosphatases such as calcineurin (Collingridge et al 1983). Calcium-entry initiated postsynaptic cellular signaling cascades also result in the changes in the expression and conductance of the NMDA- and AMPA- receptors affecting the excitatory neurotransmission of the region. Activity dependent changes in the AMPA receptor expression, density and subunit composition are the critical determinants of synaptic plasticity in in several regions of brain

including hippocampus (Malinow & Malenka 2002). Of the several factors affecting synaptic plasticity in brain, the ones which are of interest in the current dissertation are exposure to stress and drugs of abuse, specifically cocaine.

Stress

Stress or stress response is a homeostatic mechanism occurring in a living organism towards an external stimulus. Initial responses towards a stressor is meant to be adaptive, but when sustained may result in pathological consequences (McEwen 2001). Stress response occurs mainly in two steps – first is an acute response initiated by the sympathetic- adrenal medullary system, which releases adrenaline hormones preparing the subject for the so called 'fright, fight and flight' response (McGaugh 1973). The second set of responses is comparatively slower and involves the activation of hypothalamus-pituitary-adrenal cortex (HPA) system. After the somatosensory information presented during the stressed environment are perceived by the brain, hypothalamus secretes corticotrophin-releasing factor (CRF) which activates adrenocorticotropic hormone (ACTH) release from the anterior pituitary further inducing adrenal cortex to release the corticosterone (cortisol in humans) or the 'stress' hormone into the peripheral circulation (McEwen 2001). Corticosterone in turn reaches various brain regions including hippocampus and activates corticosteroid receptors, and in turn initiates feedback action on the HPA axis (Andersen 2007).

Two types of corticosteroid receptors have been described – mineralocorticoid (MR) and glucocorticoid (GR) receptors. MRs have a 10 fold affinity to corticosterone over the GRs and hence most of MRs would be in the bound state even during the basal conditions (Joels 2001). Hippocampus contains the highest density of corticosteroid receptors (especially MR) among the brain regions (McEwen et al 1980), a finding which indicated the importance of this otherwise

conventional memory structure in the homeostatic response towards stress. Corticosteroid receptor activation induces both acute non-genomic responses as well as delayed, but long lasting genomic/transcriptional changes in the neurons. In hippocampus, when these receptors are activated, it initiates an allostatic negative feedback regulation on the paraventricular nucleus of hypothalamus (McEwen 2001). In addition, other experimental evidence points to the fact that structures such as hippocampus which are important in learning & memory, also help the animal 'anticipate' the occurrence of stressful stimuli (Herman et al 1998). In such cases, it is posited that hippocampal regulation of the PVN helps the release of corticosterone, thus setting forth the stress responses in the body.

Stress & hippocampal plasticity

When the activity of corticosterone sustains, it results in the pathological modification of hippocampal synaptic plasticity, structural alterations and even atrophy of the region, ultimately resulting in impaired feedback regulation (McEwen 1999). Stress induced changes in the synaptic plasticity in hippocampus has been a subject of investigation for many years. Effects of stress on hippocampus depends upon the duration (acute vs. chronic) or severity (minor vs. intense) of the stressors. Most acute effects are non-genomic and mediated by membrane-bound or cytoplasmic MRs. Altered neurotransmission affecting glutamatergic and GABAergic systems in the CA1 pyramidal cells of hippocampus have been observed within few minutes of acute stress in rats which were found to be mediated by MRs.(Karst et al 2005). Most of the delayed effects of stress are genomic in origin mediated by the GRs. Activation of transcription by GRs also can modify the general excitability of the hippocampal circuits by altering the Ca^{2+} currents and associated events such as the Ca^{2+} -dependent K^{+} currents, thereby influencing the hippocampal dependent tasks (Joels 2001). A bidirectional effect of stress on hippocampal tasks

and synaptic plasticity is in concurrence with the inverted U-relationship between stress & memory. It was found that minor stress or low levels of corticosterone in the circulation could enhance the plasticity events such as LTP (Pavlides et al 1994), while intense stressors or high levels of corticosterone impaired the expression of LTP or enhanced LTD in the CA1 region and dentate gyrus (Diamond et al 1992; Kim et al 1996; Xu et al 1997). Parallel studies have demonstrated that mild levels of stress enhanced memory and hippocampal dependent tasks (Sandi et al 1997), while moderate to high levels of stress impaired such functions (Diamond et al 1999). Furthermore, moderate to high levels of stress were shown to induce dendritic atrophy in the in dentate gyrus, CA3 (Magarinos & McEwen 1995) and CA1(Sapolsky et al 1985) regions, suppress neurogenesis in the dentate gyrus (Gould et al 1997), all of these might contribute the overall atrophy of the hippocampus (Starkman et al 1992).

Addiction

Addiction is characterized by the compulsive binging of drugs despite negative consequences, and an extreme tendency to relapse to drug seeking and use even after long periods of abstinence (Hyman & Malenka 2001). According to the recent (2012) survey conducted by the National Survey on Drug Use and Health (NSDUH), illicit drug use in America has increased from 8.3 % to 9.2% of population 12 years and older, owing to an increase in the marijuana users with either a no change or decrease in the use of other illicit drugs such as cocaine, tobacco, alcohol etc. Estimated economic burden to the American society considering the treatment, crime prevention and loss of productivity accounts for more than $600 billion annually (source: www.drugabuse.gov). Transformation from the stage of occasional use of illicit drugs to dependence and addiction takes the subjects through stages of impulsive intake & binging, withdrawal symptoms and craving during abstinence, and compulsive and habit forming

binging. Regions involved in the development and expression of these stages in addiction is briefly described below.

Neurocircuitry of addiction

Increased release of dopamine (DA) in the mesolimbic DA-ergic pathway (or so called 'reward pathway' linking ventral tegmental area to nucleus accumbens, Figure 1.3) is thought to be the reason for the rewarding and reinforcing properties of drugs of abuse (Spanagel & Weiss 1999). However, the functional details of DA-ergic pathway alone are not sufficient enough to explain various behavioral manifestations in addiction and stages through which it is progressed.

Three simple observations vouch for the above argument: a) even a non-addictive substance such as sucrose could create DA-triggered feelings of euphoria or satiety in subjects; b) potential addictive drugs produces the same DA-induced feeling of euphoria or 'high' in most subjects, but the tendency to get addicted is restricted only to a few among those who are exposed; c) It has been shown that in cocaine-addicts, cocaine intake alone no longer induced heightened DA levels in the reward pathway in the advanced stages of addiction; yet they compulsively seek, crave and use drugs (Volkow et al 2009). In fact, in addition to the reward pathway, other closely associated regions implicated in decision making (orbito-frontal cortex (OFC), anterior cingulate cortex (ACC)), habit formation (dorsal striatum), emotion & salience (amygdala, lateral habenula, lateral hypothalamus), learning & memory (hippocampus), craving (insula) etc., are all thought to play vital roles in formation and progression of addictive behaviors (for review, see (Koob & Le Moal 2008). Besides the role of DA, significance of other neurotransmitter systems such as glutamatergic, GABAergic and cholinergic pathways in modulating the drugs of abuse induced pathological modifications in the synaptic plasticity have also been demonstrated (Kalivas & O'Brien 2008).

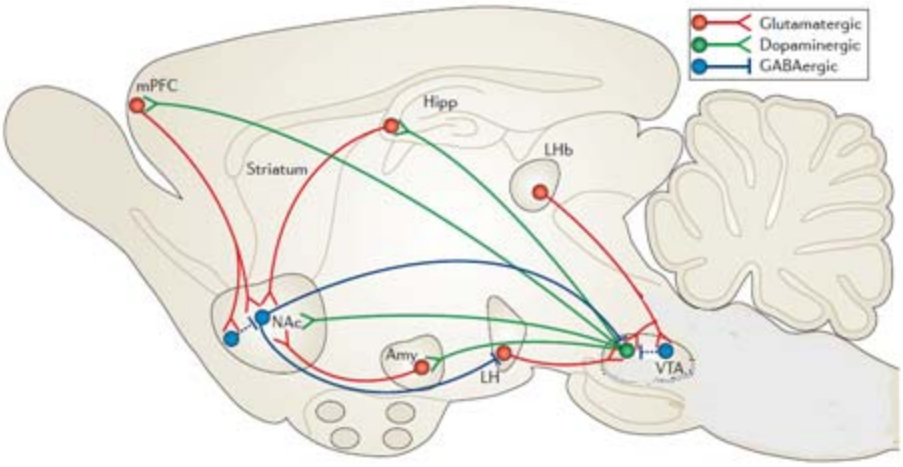

Figure 1.3. Circuitry of reward and addiction: VTA- ventral tegmental area, LH- lateral hypothalamus, Amy- amygdala, NAc- nucleus accumbens, LHb- lateral habenula, Hipp- hippocampus, mPFC- medial prefrontal cortex. Modified from Russo SJ & Nestler EJ, Nat Rev Neurosci. (2013)

Molecular targets of common drugs of abuse

Illicit drugs come under different chemical classes. Many of these drugs have one or more molecular targets at the synapse. Nevertheless, the acute euphoria after their administration occurs subsequent to the release of dopamine in the system. Depending upon the molecular mechanism of action, drugs can be classified into 3 broad groups (Luscher & Ungless 2006). First, those which bind to the G protein-coupled receptors such as $G_{i/o}$,which have direct inhibition effect on the postsynaptic neuronal activity as well as on the presynaptic neurotransmitter release. The acute effects of those drugs occur by the disinhibition (inhibition of GABAergic interneurons) in the VTA dopamine neurons. Drugs such as morphine and heroine fall under this category. Second group of drugs acts on the ionotropic receptors such as $GABA_A$

and nicotinic receptors and can directly or indirectly (via glutamatergic and dopaminergic circuits) mediate the dopamine cell activity. Ethanol, stimulants such as nicotine, depressants such as barbiturates etc. comes under this class. The third group acts on monoamine transporter proteins thereby affecting the synaptic and extrasynaptic levels of dopamine in the circuit. Psychostimulants such as cocaine and amphetamine come under this class

Table 1.1. Molecular targets of common classes of drugs. DAT- Dopamine transporter, SERT- serotonin transporter, NET- norepinephrine tranporter, VMAT- vesicular monoamine transporter, nAChRs- nicotinic acetylcholine receptors, CB_1Rs – Cannabinoid receptor subtype 1, 5-HT3- serotonergic receptor type 3.

Category	**Drugs**	**Molecular Targets**
Stimulants	Cocaine Amphetamine, ecstasy Nicotine	DAT, SERT, NET DAT, SERT, NET, VMAT nAChRs
Opioids	Heroin Opium	mu-opioid Rs
Depressants	Barbiturates (pentobarbital) Benzodiazepines (Alprazolam)	$GABA_A$ Rs
Cannabinoids	Marijuana Hashish	CB_1Rs
Alcohol	Alcohol (ethyl alcohol)	$GABA_A$Rs, $5\text{-}HT_3$, nAChRs

Amphetamines and ecstasy function by reversing the actions of respective transporter proteins (DAT and SERT respectively) thereby resulting in the depolarization and activation of dopamine cells. Cocaine, another psychostimulant drug, acts by blocking the DAT resulting in the increased dopamine concentration at the synapses. Table 1.1. describes some common classes of drugs and their molecular targets.

Drugs of abuse & synaptic plasticity

In addition to the acute dopamine increasing effects of drugs of abuse, other more complex mechanisms by which synaptic plasticity in the reward- related regions are modified, also occur. For example, disrupting the glutamate homoeostasis in the ventral striatum and afferent targets is one way that many drugs of abuse induce persisting changes in the neuronal synaptic transmission (Kalivas et al 2009). The effect on the glutamatergic receptors such as AMPA and NMDA receptors varies depending upon the region and stage of addiction (Wolf & Tseng 2012). Following an acute injection of drugs such as cocaine, amphetamine, nicotine, alcohol, morphine etc., increased trafficking and conductance of GluR1 subunit of AMPARs were observed in dopaminergic neurons in VTA which lasted for not more than five days (Saal et al 2003; Ungless et al 2001), whereas contingent intake of cocaine for several days resulted in increased AMPAR expression which stayed for longer duration in VTA (Chen et al 2008) and nucleus accumbens (Conrad et al 2008).

However, a re-exposure to cocaine during the abstinence reversed this increased expression of AMPAR, due to the decreased membrane expression of GluR2 subunit-lacking AMPARs (Boudreau et al 2007; Conrad et al 2008; Kourrich et al 2007). An acute injection of cocaine also resulted in an enhanced NMDA-mediated excitatory current due to insertion of NR2A subunits onto the postsynaptic membrane (Borgland et al 2006). Chronic administration of ethanol increased synaptic expression of NMDAR and thereby influenced the AMPA/NMDA ratio in the mesencephalic dopaminergic neurons (Carpenter-Hyland et al 2004). These changes in the expression of ionotropic glutamate receptors are believed to be essential for the drugs of abuse- induced synaptic plasticity.

Addiction & hippocampus

Declarative memory processing is involved in the contextual conditioning occurring in the addicts, in which the subjects learn to link the drug usage with the situational context/cues (for review,(Koob & Volkow 2010). In fact, dorsal hippocampus (dH) and ventral hippocampus (vH) were shown to be important in the context- and cue-induced relapse to drug usage respectively, which are evidences for the role of hippocampus in the contextual conditioning (Rogers & See 2007). Hippocampus interacts closely with the dopaminergic reward circuitry (Nestler 2005), and hence can influence the firing rate of meso-striatal dopaminergic pathway containing ventral tegmental area (VTA) and nucleus accumbens (NAc) neurons (Lisman & Grace 2005). It has been demonstrated that the theta burst stimulation of this putative memory storage center resulted in the reinstatement of cocaine usage in rats (Vorel et al 2001). These properties make hippocampus an ideal candidate to study the mechanism of action of drugs of abuse in changing the properties & functioning of neural circuits leading to the pathology of addiction. A major focus of this document is to investigate the changes occurring in the glutamatergic and GABAergic neurotransmitter systems in the hippocampus in response to exposure to cocaine.

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CHAPTER 2

EFFECTS OF INTERMITTENT, MINOR STRESSORS ON THE HIPPOCAMPAL SYNAPTIC PLASTICITY

Introduction

Stress can be considered as the response by the organisms' homeostatic mechanism to a physical or psychological challenge. Such responses initiate favorable compensatory mechanisms towards the challenge; however, the persistence of stress may result in pathological consequences. Hippocampal feedback regulation of hypothalamus-pituitary-adrenal axis is critical in mediating the neuroendocrine changes associated with various stress responses in the body. Located in the medial temporal lobe of the brain, hippocampus plays a major role in learning and in the processing of different forms of memory. According to the current consensus, it is involved in the various stages of memory formation such as acquisition, processing, consolidation and storage of both explicit and implicit memory traces (Preston & Eichenbaum 2013). These basic roles performed by hippocampus endorse its importance in other related facets such as cognition, spatial navigation, emotion, fear conditioning, addiction and stress.

Substantial evidence exists for a functional dissociation within the hippocampus along its septotemporal axis in various physiological and pathological (such as stress and addiction) conditions (Fuchs et al 2005; Rogers & See 2007). With respect to cellular mechanisms of learning and memory, long-term potentiation (LTP) has been reported to exhibit different characteristics between the septal (dorsal) and temporal (ventral) sectors of the hippocampus. For example, LTP induced in the CA1 region in ventral hippocampus (vH) was found to be of lesser

magnitude than the LTP in the dorsal hippocampus (dH) in unstressed normal rats (Maggio & Segal 2007b; Papatheodoropoulos & Kostopoulos 2000b). Importantly, the functional distinction between dH and vH was further evidenced by the differential response to an acute stress protocol in which the magnitude of vH-LTP was significantly increased, whereas dH-LTP was decreased (Maggio & Segal 2007a). Thus the consequences of a stressful experience on hippocampal function can vary according to the specific sector of the hippocampus being assessed, a finding that contrasts with the prior description of a general stress-induced impairment of LTP in this brain region (Diamond & Rose 1994; Foy et al 1987; Kim et al 1996; Xu et al 1997).

With respect to the impact of stress on the function of the nervous system, the hippocampus has been found to be affected by various forms of stressful stimuli (Diamond & Rose 1994; Foy et al 1987; Kim et al 1996; Xu et al 1997). Such studies have typically involved in evaluating the effects of intense and often chronic stressful experience on measures of hippocampal function such as learning and memory performance (Diamond & Rose 1994; Foy et al 1987; Kim et al 1996; Xu et al 1997). In contrast, the consequences of repeated exposure to minor, intermittent stressors (which may approximate more typical real-life experiences), are seldom explored (Holm et al 2011; Parihar et al 2011; Rothwell et al 2011). The current study investigates the effects of minor, intermittent stressors associated with a typical conditioning protocol employed to test the locomotor sensitization in rats, on the hippocampal synaptic plasticity along its septotemporal axis.

Interestingly, we found that experiencing the conditioning protocol itself resulted in persistent, stress-induced changes in the hippocampus of saline-conditioned rats. The two potential stressors - novelty exposure & i.p. injections- were then tested to determine the major contributor to the stress associated with the behavioral protocol. Our results demonstrate that a

combinatorial effect of open-field novelty exposure and repeated i.p. injections together was necessary to induce stress-like metaplasticity in the expression of LTP of both the dorsal and ventral sectors of the hippocampus. Stress induced metaplasticity in vH was prevented by antagonizing the corticosteroid- or dynorphin-kappa opioid- receptors. It could be argued that such differential metaplasticity along the septotemporal axis could dramatically alter the hippocampal influence on the activity of associated neuronal circuitry, as shifting the efficacy of efferent output from predominantly dH (cortical circuits) to predominantly vH (limbic circuits) would occur (Segal et al 2010).

Materials and methods

Animal maintenance: 8-10 week old male Sprague-Dawley rats (Harlan, IN, USA) were housed in pairs in clear plastic cages and maintained on a 12 h light/dark cycle (0700/1900 h). Food and water were available *ad libitum* except during the behavioral sessions. Behavioral sessions were conducted daily between 0900 and 1200 h. Experiments involving rats were performed in compliance with the University of Georgia Institutional Animal Care and Use Committee guidelines.

Apparatus: A description of the apparatus used and measurements of activity have been described in detail elsewhere (Gosnell 2005). Briefly, activity was measured in four 43.2 × 43.2 cm chambers with clear plastic walls and a solid smooth floor (Med Associates, St. Albans, VT, USA). The chambers were individually housed in sound-attenuating cubicles equipped with two lights (approximately 20 lux) and a ventilation fan. Two banks of 16 infrared photo beams and detectors, mounted at right angles 3.5cm above the floor were used to detect horizontal activity. Activity Monitor software (Med Associates) was used to count photo beam- breaks. This

software subdivides total counts into stereotypic and ambulatory counts according to the repetitive (stereotypic) vs. sequential (ambulatory) patterning of the beam-breaks.

Locomotor sensitization study (LMS): Rats were habituated to handling for 3 days prior to locomotor activity testing. As depicted in the time line (Figure 2.1.A), rats were subjected to saline treatments for the next 5 days (an initial activity day and 4 subsequent conditioning days), followed by another treatment (challenge) day 1 week later. Saline conditioned rats received 0.5ml saline i.p. injections and were tested for open field locomotor activity (saline group) or maintained in the home cages (home cage injection – inj). Subgroups of saline group were administered either a single dose of kappa-opioid receptor (KOR) antagonist, nor-binaltorphimine (10mg/kg i.p.) on the last day of handling, or daily doses of one of the following: glucocorticoid receptor antagonist (mifepristone 25mg/kg) or mineralocorticoid receptor antagonist (spironolactone 20mg/kg) on all of the locomotor activity testing days. Yet another group of rats maintained as novelty exposure controls (novel environment), were tested for their locomotor activity on the testing days without receiving any injections. Locomotor activity was recorded, for 30 min prior and 60 min post injection, by the activity monitor software and was further analyzed using Sigma Stat 3.1 software (Systat software inc., CA). Two-way repeated measures ANOVA was used to detect main effects of test day (activity vs. challenge) and time (60 min post injection) on the locomotor activity of rats following conditioning. If ANOVA revealed an effect of test days in a treatment group, Student-Newman-Keuls (SNK) post hoc analysis was performed on the total locomotor counts at various post-injection time points (40^{th} to 90^{th} minute of the behavioral sessions in 10 minute bins) to reveal significant differences across time ($p < 0.05$).

Measurement of blood plasma corticosterone levels: The blood plasma corticosterone levels were measured from trunk blood samples collected on the day of sacrifice obtained from naïve and saline conditioned rats, as well as an acutely stressed group of animals for positive control. There were no significant differences between the corticosterone levels measured (Corticosterone EIA kit, Cayman Chemicals, MI) between the naïve and saline conditioned groups (50-100ng/ml, n=3-4 each) whereas the acutely stressed rats (elevated platform, 30 min) gave the expected increase (250-1000 ng/ml, n=4). This result demonstrates that a persisting elevation in blood plasma corticosterone levels did not occur in the rats experiencing the conditioning protocol when measured 1-2 weeks following the last behavioral session.

Extracellular electrophysiology: 500 μm thick hippocampal slices were prepared from either naive (no treatment, remained in the home cage) or conditioned rats within 1–2 weeks after the challenge day. All rats were anesthetized with 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane) prior to decapitation. The brain was removed and submerged in ice-cold, oxygenated (95% O2/ 5% CO2) dissection artificial cerebrospinal fluid (ACSF) containing (mM): NaCl (120), KCl (3), MgCl2 (4), NaH2PO4 (1), NaHCO3 (26), and glucose (10). Horizontal and coronal sections were prepared from either half of the brain to obtain both ventral and dorsal hippocampal slices respectively, from the same animal. We estimate that our vH and dH slices are obtained from the extreme 30% of either temporal or septal pole, respectively. The CA3 region was removed and the remaining hippocampal tissue held in a submerged slice chamber and perfused with oxygenated (95% O2/5% CO2) standard ACSF containing (mM): NaCl (120), KCl (3), MgCl2 (1.5), NaH2PO4 (1), CaCl2 (2.5), NaHCO3 (26), and glucose (10) at approximately 1 ml/min. Slices recovered in the recording chamber for 45 min at room temperature, and then another 45 min at 30°C, the recording temperature. A bipolar stimulating

electrode (Kopf Instruments) was placed on the CA3-side of the CA1 region in the stratum radiatum and a 1.0 MΩ tungsten recording microelectrode (World Precision Instruments) was then positioned in the same layer in CA1 to record the field potential changes occurring in the region. Field excitatory post synaptic potentials (fEPSPs) were elicited by stimulation of the Schaffer collateral-commissural pathway in stratum radiatum once every 60 s (0.0167 Hz) for the duration of the experiment. A stimulus-response curve was obtained at the beginning of each experiment, with stimulus pulses consisting of a single square wave of 270 μs duration delivered at 40–160 μA intensities with 20 μA increments. The stimulation intensity was then adjusted to obtain fEPSP amplitude of 1.0–1.5mV to begin baseline recording. Once a stable baseline was established, LTP was induced by a high frequency stimulation (HFS) protocol consisted of 100 pulses of 30 Hz trains, repeated three times separated by a 20 s interval. This particular HFS protocol was chosen as it yields a sub-maximal (i.e. non-saturated) LTP magnitude, which in preliminary studies we found to be sensitive to the effects of cocaine to induce metaplasticity of LTP.

Quantification of synaptic plasticity: Data were digitized at 10 kHz, low-pass filtered at 1 kHz, and analyzed with pCLAMP 10.2 software (Axon Instruments). The initial slope of the fEPSP responses was measured and normalized to the last 5 min of baseline prior to tetanization. LTP was quantified by comparing the normalized fEPSP slopes from the last 5 minutes of the pre- tetanus baseline with that of the 25-30 minutes post-tetanus. Planned comparisons were made using unpaired *t*-tests. In reporting our results, n-values indicate the number of slices and the number of animals in parenthesis.

Drugs (vehicle): Cocaine hydrochloride (saline) and nor-binaltorphimine (saline) were provided by NIDA. Spironolactone (DMSO), mifepristone (DMSO), SCH 23,390 (saline) and eticlopride (saline) were obtained from Tocris.

Results

1) Repeated saline injections did not induce locomotor sensitization towards saline challenge:

Young adult (225-275g) male rats were tested for locomotor activity following i.p. injections of saline (0.5 ml per injection) in the activity box. These open field activity sessions were conducted for the first five days (activity day followed by 4 conditioning days), and on a challenge day 1 week after. Saline conditioning via i.p. injections did not induce significant changes in locomotor responses during open field activity sessions nor did it induce locomotor sensitization towards a challenge dose (Figure 2.1.B). Following a 1-2 week abstinent period, hippocampal slices were prepared and fEPSP responses were recorded from the stratum radiatum of the CA1 region.

2) Conditioning with saline did not change the basal synaptic transmission in the ventral or dorsal hippocampus:

Input-Output relationship of the field potential responses were measured at various stimulus intensities ranging from 40 to 160 μA at increments of 20 μA **s**timulus-response curves were obtained from the ventral (Figure 2.2.A) and dorsal (Figure 2.2.B) hippocampal slices of saline-conditioned rats and compared with respective naïve controls. This naïve group of rats was maintained in their home cages throughout the conditioning protocol days without administering injections or testing for locomotor activity. The sensitivity of the fEPSPs was indexed by calculating the stimulus intensity which gave the ½ maximal response ($S_{1/2}$, μA) from the stimulus-response curves. No significant change in the $S_{1/2}$ (μA) of saline-conditioned rats

were observed when compared to the naïve controls (vH-saline: 95 ± 4, n=18(10) vs. vH-naïve: 96 ± 3, n= 13(6); or dH-saline: 93 ± 4, n=12(6) vs. dH-naïve: 95 ± 3, n=22(7)). These results suggested that our experimental protocol did not induce any change in the basal synaptic transmission in the hippocampus along its septo-temporal axis.

3) Saline conditioning induced metaplastic effects in vH & dH:

LTP was assessed in the ventral and dorsal hippocampal slices from the saline-conditioned animals 1-2 weeks following the challenge day of the behavioral protocol. (Figure 2.3). The tetanus protocol consisted of 3 trains of 30Hz/100 pulses, and LTP magnitude was calculated at 30 minutes post tetanus in the CA1 region of hippocampal slices. Experiencing behavioral protocol caused a significant increase in the vH LTP (Figure 2C, left bars) in the saline conditioned rats (1.49 ± 0.04, n=18(10)) when compared with that in the naïve ventral slices (1.28 ± 0.05; n= 13(6)). In contrast, dH LTP (Figure 2C, right bars) in the saline conditioned group (1.29 ± 0.04, n=12(6)) was significantly decreased when compared to the naïve group (1.42 ± 0.03; n=22(7)). Furthermore, using our unique tetanus protocol (30Hz), we successfully reproduced the differential expression of LTP with in the two zones of hippocampus in the control environment as reported which employed the standard tetanus protocol (100Hz). (Maggio & Segal 2007a; Papatheodoropoulos & Kostopoulos 2000a). The LTP magnitude in the vH (1.28 ± 0.05; n= 13(6)) of naïve rats was significantly lower than the LTP in the dH (1.42 ± 0.03; n=22(7)).

The changes observed in the LTP expression via electrically induced stimulation occurred without any obvious change in the basal synaptic transmission (Figure 2.2). Hence it could be rightly argued that experiencing the saline conditioning protocol was affecting as a metaplastic trigger in the hippocampal circuit.

4) Saline conditioning induced metaplasticity was reversed by glucocorticoid receptor antagonist and mineralocorticoid receptor antagonist in the dH and vH respectively:

Forced swim stress induced differential effect on the two sectors of hippocampus was observed when LTP was measured within 30 minutes of the stress protocol (Segal et al 2010). They demonstrated that the stress induced differential effects on vH- and dH- LTP were due to corticosterone mediated activation of mineralocorticoid receptors in the vH and glucocorticoid receptors in the dH. Hence we were curious to test whether the saline conditioning induced stress-like effects on the hippocampal LTP.

Daily injections of glucocorticoid receptor (GR) antagonist mifepristone (25 mg/kg, n=4), when co-administered with the saline vehicle, (Figure 2A) did not cause any change in the locomotor behavior in the saline conditioned rats. The vH LTP in the mifepristone injected rats was still significantly elevated relative to the naïve group and was similar to the magnitude of that of the saline conditioned rats (1.45 ± 0.06, n=10(4)) (Figure 2.4.D, left bars). Interestingly, the dH LTP was significantly enhanced in the mifepristone injected rats than that of the saline group, and was closer to the naïve dH LTP value (1.41 ± 0.02, n=10(4)) (Figure 2.4.D, right bars).

Mineralocorticoid receptor (MR) antagonist spironolactone (20 mg/kg, n=4) also did not cause any changes in the locomotor activity in rats (Figure 2.5.A). Moreover, spironolactone injections did not affect the saline conditioning induced LTP expression in the dH (1.36 ± 0.03, n=11(4)) (Figure 2.5.D, right bars). However, vH LTP was significantly reduced in the spironolactone injected rats (1.28 ± 0.02, n=14(4)) compared to that of the vH LTP in the saline conditioned rats, and were similar to the naïve LTP magnitude (Figure 2.5.D, left bars).

These two results were similar to the observations of Maggio & Segal (Maggio & Segal 2007a) in which they concluded that an MR-involved mechanism (spironolactone-sensitive) is causing the acute stress induced enhancement of vH LTP, while a GR-involved mechanism (mifepristone -sensitive) is behind the acute stress induced suppression of dH LTP. Hence it could be argued that experiencing the behavioral protocol involving behavioral manipulations such as handling, repeated i.p. injections, exposure to novelty etc., could induce persistent stress-like effects in the hippocampus.

5) Additive effects of novelty and i.p. injection procedures involved in the behavioral protocol caused stress-like effects in the hippocampus.

Potential stressors involved in our conditioning protocol were the exposure to novel environment (activity chambers), repeated handling and i.p. injections (Gartner et al 1980; Lapin 1995; O'Callaghan et al 2002; Shors & Wood 1995) etc. We tried to isolate the major contributing factor to the overall stress-like effects on hippocampal LTP. In the vH (Figure 2.6.A), the magnitudes of LTP in the novelty exposed (1.40 ± .05, n=18(10)) or home cage injected (1.33 ± .04, n=13(6)) rats were not significantly higher than the naïve controls (1.28 ± 0.05; n= 13(6)). Similarly, the dH (Figure 2.6.B) LTP magnitudes in the novelty exposed (1.36 ± .05, n=20(10)) or home cage injected (1.32 ± .03, n= 14(6)) rats were not significantly different than the naïve controls (1.42 ± 0.03; n=22(7)). These results show that none of the putative stress factors could alone induce stress-like effects on the hippocampal LTP. Hence it could be argued that the significant stress induced by the behavioral protocol is a combinatorial effect of all the putative stress factors involved.

6) Kappa opioid receptor antagonist prevented the stress-induced metaplasticity in vH, but not in dH:

Repeated daily injections of corticosteroid receptor antagonists prevented saline-conditioning induced stress effects on the hippocampal LTP (Figures 2.4 & 2.5). These suggest that corticosterone released during the stress episodes was contributing to the induction of stress-like effects on hippocampus. However, analysis of plasma corticosterone in rats on the day of sacrifice did not show elevated levels of circulating corticosterone (see Materials and methods), indicating the possible role of other factors in the maintenance/persistence of stress-induced metaplasticity phenomenon in hippocampus.

One important neuromodulatory factor is the dynorphin-kappa opioid receptor system which is shown to cause various stress-associated behavioral changes in rodents (McLaughlin et al 2003; Nabeshima et al 1986; Shirayama et al 2004). A long-acting kappa opioid receptor antagonist, nor-binaltorphimine (nBNI), was employed in order to investigate the role of dynorphin-kappa opioid system in conditioning protocol- induced stress. To this end, a single dose of nBNI (10mg/kg i.p.; n=7) was i.p. injected in rats one day prior to the behavioral protocol, and further locomotor protocol was conducted. nBNI administration did not cause any significant effect on the locomotor activity in rats (Figure 2.7.A). However, the stress-induced enhancement of LTP in vH was significantly reduced by the nBNI administration (1.35 ± 0.04, n=18(7)) (Figure 2.7.D, left bars), but no change was observed in dH (1.32 ± 0.02, n=18(7)) (Figure 2.7.D, right bars). These results show that dynorphin-kappa opioid receptor system is involved directly or indirectly in the pathways/mechanisms by which stress alters the plasticity in the ventral zone of hippocampus. Moreover, for both the induction and the persistence of the

effects of stress, ventral and dorsal sectors of hippocampus actively engages different molecular candidates or possibly non overlapping neuronal circuits.

Discussion

The major findings of the current study are summarized here. Experiencing a commonly employed locomotor sensitization protocol differentially altered the LTP expression in the ventral and dorsal sectors of the hippocampus, and these changes were persistent at least until 2 weeks of the final behavioral manipulation. Changes in the electrically induced LTP expression occurred without any concurrent changes in the basal synaptic transmission. This altered metaplasticity observed in the hippocampus was contributed by a combinatorial effect of potential stressors such as novelty exposure, i.p. injections and handling involved in the conditioning protocol. Involvement of stress was further confirmed when GR and MR antagonists successfully reversed the induction of metaplasticity in dH and vH respectively. Additionally, we showed the specific involvement of the dynorphin-kappa opioid receptor system in the stress-induced changes in vH, and not in dH.

The behavioral protocol that we employed in the current study to test locomotor sensitization towards pharmacological agents, involved many potential stressors such as novelty exploration, handling, repeated noxious stimuli such as i.p. injections. Handling, which is meant to habituate the lab animals to the new environment and reduce anxiety, was shown to induce acute stressful behaviors in rats (Gartner et al 1980; Korz & Frey 2003). Similarly, repeated noxious stimuli such as i.p. injections also create episodes of stress in lab animals (Lapin 1995; O'Callaghan et al 2002; Saal et al 2003). However, the consequences of such events are usually neglected by the experimenters. Additionally, the forceful exposure to strange environments (novelty exposure) was shown to induce acute changes in the synaptic plasticity in regions such

as hippocampus (Shors et al 1989; Xu et al 1998) and nucleus accumbens (Rothwell et al 2011).However, all these studies demonstrated the acute (within minutes to few hours of stressful experience) effects of various types of stressors. In the current study, we found that the locomotor sensitization protocol that involved the above mentioned stressors, itself was sufficient to cause a persistent (at least until 2 weeks after the last manipulation) alteration in the hippocampal LTP expression. Moreover, the LTP magnitudes in the dH (decreased) and vH (increased) was differentially affected by the conditioning protocol (Figure 2.3). Since there was not any obvious change in the basal synaptic transmission (Figure 2.2), these potential stress-like effects were inducing metaplastic changes in the hippocampal circuit. It turned out that a combinatorial effect of all the potential stressors such as novelty and i.p. injections in the protocol was necessary to induce sufficient stress to cause persistent changes in hippocampal LTP (Figure 2.6).

There has been a growing appreciation of the functional and anatomical segregation of various zones of hippocampus along its septotemporal axis. (Fanselow & Dong 2010; Segal et al 2010). Because of its bilateral association with higher order cortical centers of the brain, dH is considered important in cognition and spatial navigation (Fanselow & Dong 2010). vH is integrated to the reward pathway and other limbic regions, hence thought to play major roles in the reward associated behaviors such as addiction and craving. Electrophysiological studies have reported differences in the intrinsic excitability, density distribution of voltage (Dougherty et al 2012), and ligand gated (Pandis et al 2006) ion channels etc., between the dH and vH. Furthermore, electrically induced LTP in the CA1 region of dH was found to be significantly greater than that in the vH in naïve control rats (Papatheodoropoulos & Kostopoulos 2000a). In agreement to this finding, in the current report we successfully reproduced the greater expression

of LTP magnitude in dH when compared to the vH in our naïve group of rats, although employing a different stimulus protocol.

The differential metaplasticity observed in the hippocampal sectors in the saline conditioned rats was similar to the effects of acute stress on hippocampal LTP reported earlier (Maggio & Segal 2007a). In this study, they demonstrated that stress induced release of corticosteroid hormones differentially activates its receptors in the two sectors of hippocampus, resulting in reduced LTP in dH (due to the activation of GRs) and enhanced LTP in vH (due to MR activation). We extended this observation by showing that even minor stressors when repeated for a few days were sufficient enough to induce persistent stress-like effects on the hippocampus. Involvement of stress was further confirmed by co-administering MR or GR antagonists in the saline conditioned rats which successfully blocked the metaplasticity observed in vH and dH respectively. Interestingly, a blood plasma corticosterone level in the saline conditioned rats on the day of sacrifice was not elevated to the levels of the acutely stressed rats (see, Materials and methods for the corticostene result). This indicates that corticosterone was not essential for the persistence of the metaplasticity induced by stress in the hippocampus. It is possible that the initial corticosterone activity engages other neurotransmitter systems causing the persistent maintenance of metaplasticity. An important candidate in this regard is the dynorphin-kappa opioid receptor system, which has been previously shown to be involved in the stress-associated behavioral changes (McLaughlin et al 2003; Shirayama et al 2004).

Dynorphin is an endogenous opioid peptide that is released during stress which acts on the kappa opioid receptor system in various brain regions including hippocampus (Drake et al 2007). For example, stress-induced place aversion was found to involve dynorphin and subsequent activation of kappa opioid receptors in brain regions such as basolateral amygdala,

hippocampus, dorsal raphe nucleus, etc. (Land et al 2008). The long lasting, selective kappa opioid receptor antagonist, nor-binaltorphimine (nBNI) has been successfully used to prevent stress-induced analgesia and immobility (McLaughlin et al 2003; Nabeshima et al 1986; Shirayama et al 2004) as well as stress-induced potentiation of conditioned place preference and self-administration of ethanol (Sperling et al 2010) in rodents. Therefore we investigated the effectiveness of nBNI to prevent changes in hippocampal LTP following saline conditioning. Interestingly, administration of nBNI one day prior to saline conditioning selectively blocked the stress- induced enhancement of LTP in vH, but did not affect the reduction of LTP in the dH.

The reason for this differential effect of nBNI in these sectors of the hippocampus is unclear, but could involve the previously mentioned distinct anatomical connectedness of the vH and the dH. Dynorphin mediated direct (Graziane et al 2013) or indirect (via BNST circuit) (Li et al 2012) disinhibition of dopaminergic neurons in VTA, could result in the activation of dopaminergic efferents (Jalabert et al 2009) synapsing with other regions of brain, including hippocampus . Additionally, anatomical evidence has been shown for the vH receiving more prominent dopaminergic afferents than the dH from the midbrain dopamine cells (Gasbarri et al 1996; Verney et al 1985). Hence it is expected that dynorphin-induced activation of dopamine system would have more effect on the vH than on the dH. Regardless of the specific mechanism at work, the selective role for the kappa opioid system in modulating stress-induced metaplasticity in the vH could explain disparate findings among previous studies assessing the effects of stress on hippocampal dependent tasks, where performance is influenced by stress either positively (Luine et al 1993; Nijholt et al 2004) in the vH (where LTP is increased) or negatively (Beylin & Shors 1998; Diamond et al 1996) in the dH (where LTP is decreased).

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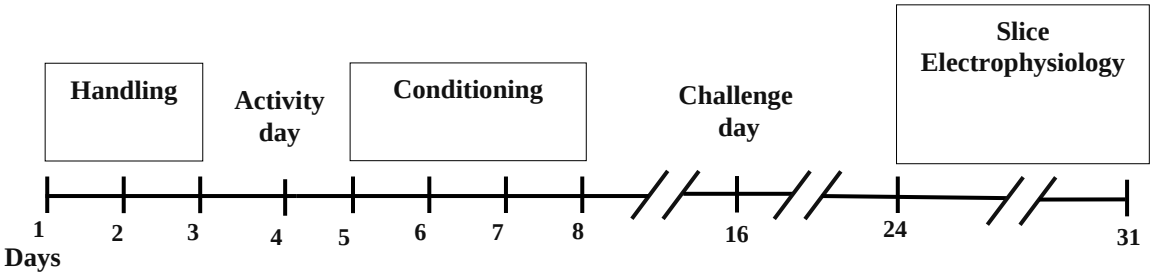
A



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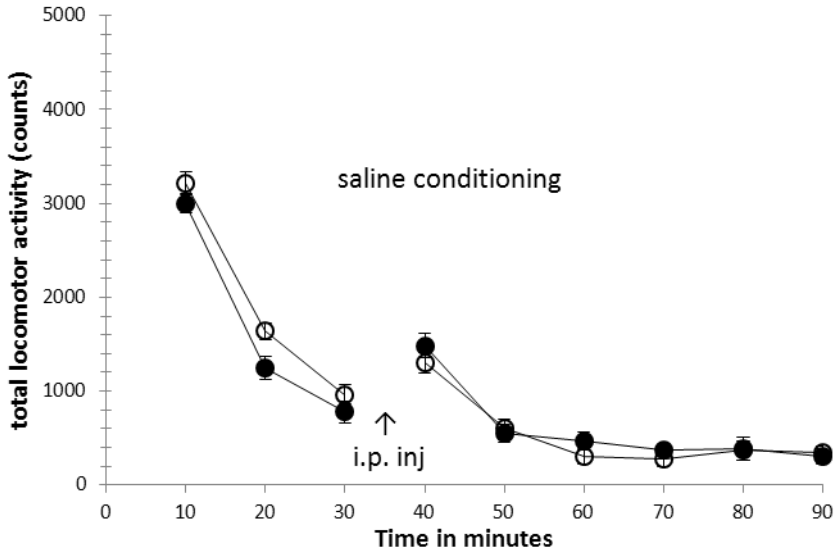


Figure 2.1. Locomotor sensitization was not induced in rats in response to repeated injections of saline. **(A)** Experimental time line for the locomotor sensitization behavioral protocol and subsequent electrophysiological recordings from ex vivo hippocampal slices. **(B)** Effect of saline conditioning on the locomotor activity of rats during activity (○) and challenge (●) days. The x-axis shows time (in minutes) for rats tested for locomotor activity during pre-injection (first 30 minutes) and post-injection (last 60 minutes). The y-axis shows the locomotor activity (total photo-beam breaks) in the open field chamber. Error bars ± SEM.

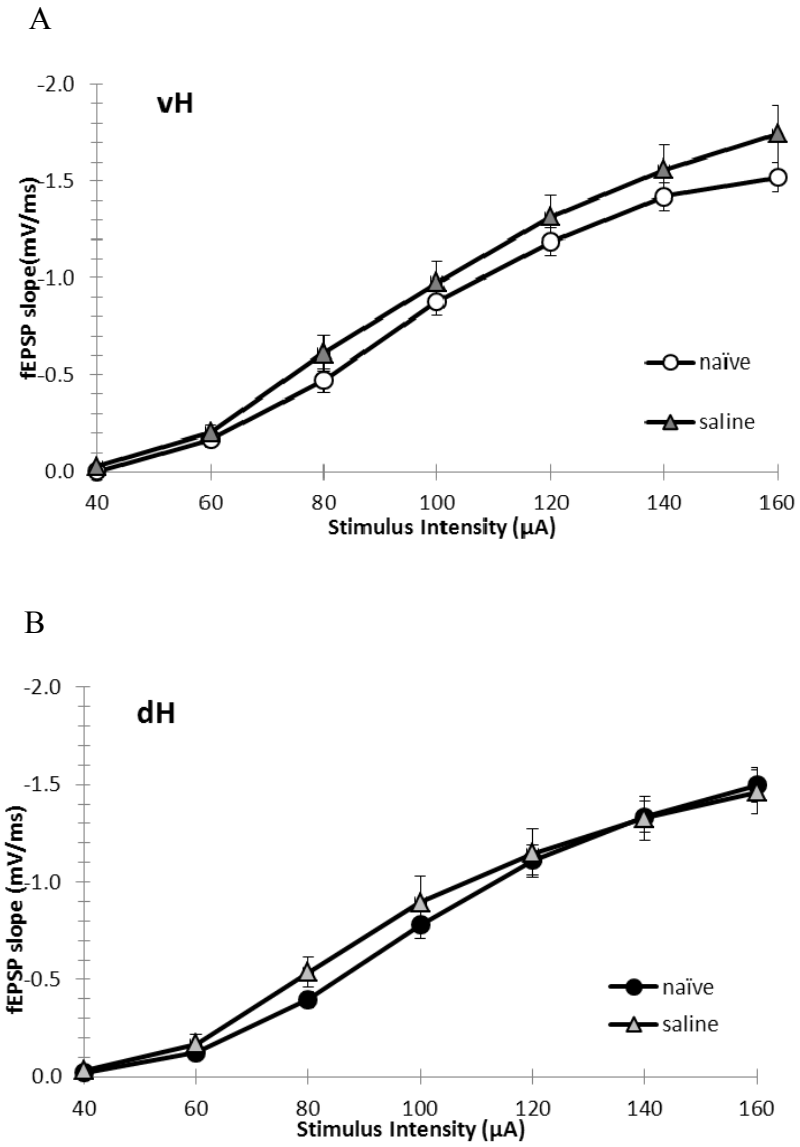


Figure 2.2. Saline conditioning did not affect the basal synaptic transmission in hippocampus. **(A)** Stimulus response curves obtained from the vH of naive (○) and saline (▲) groups of rats. **(B)** Stimulus response curves obtained from the dH of naive (●) and saline (▲) groups of rats. The x- axis represent stimulus intensities (µA) at which hippocampal slices are stimulated at the baseline frequency. The y-axis show the normalized slopes (normalized to the slope of maximal fEPSP response) of fEPSPs obtained at each stimulus intensities. Error bars ± SEM.

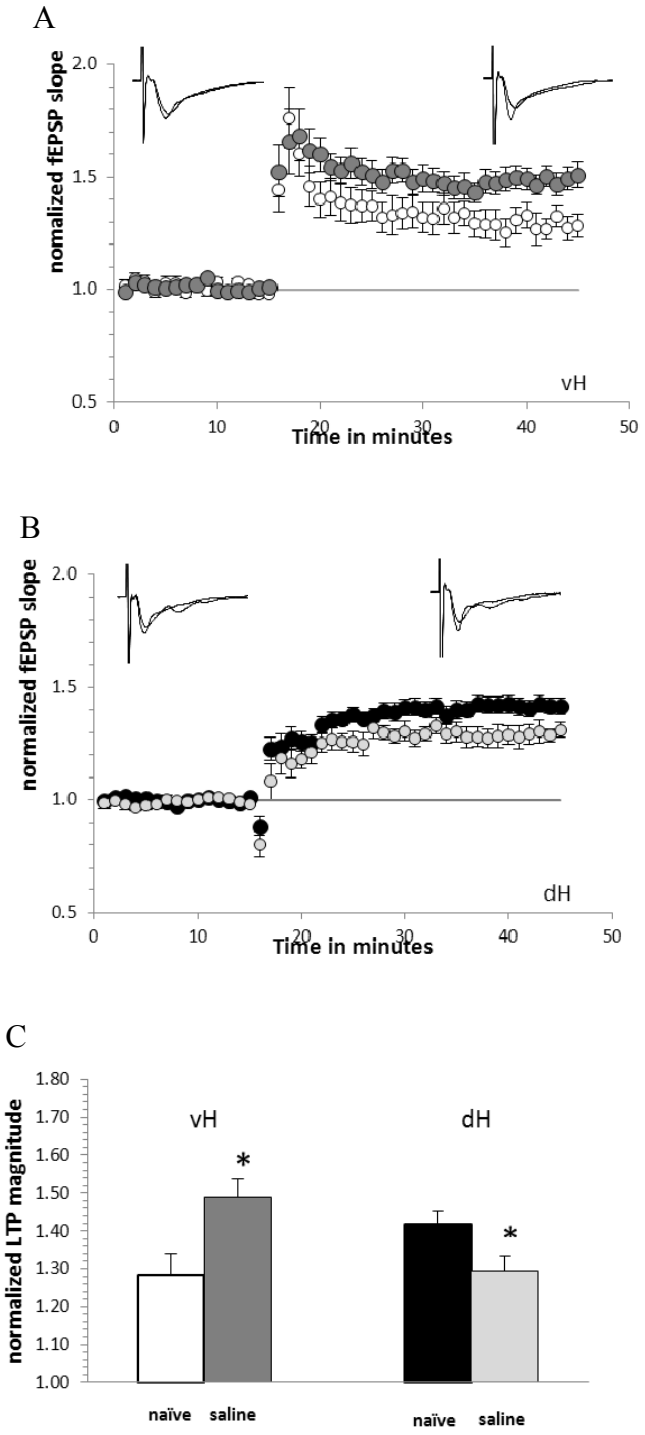
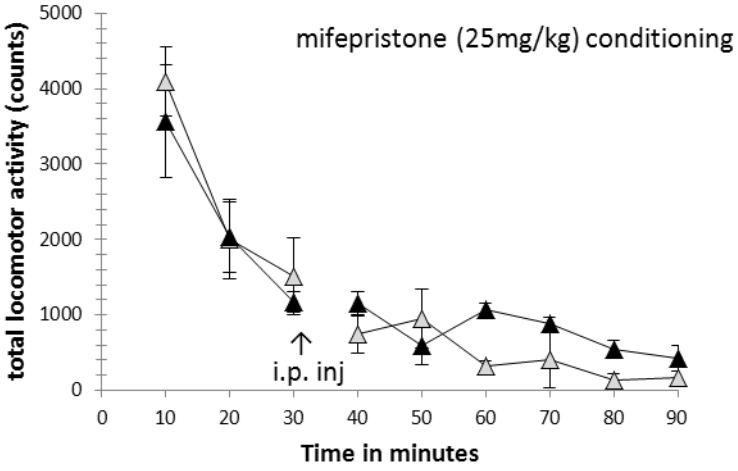


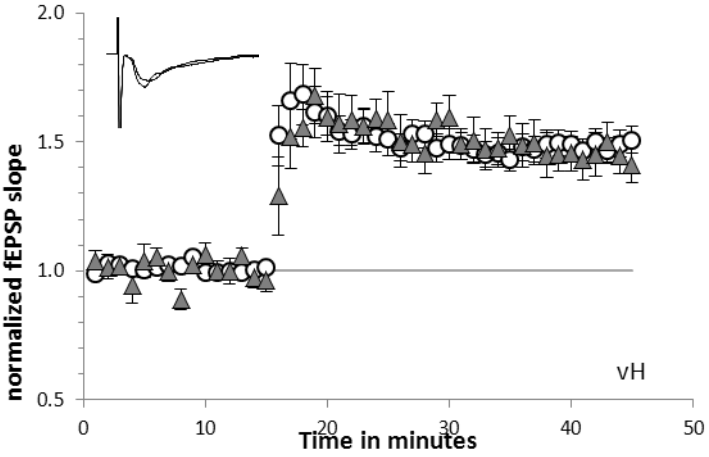
Figure 2.3. Saline conditioning induced differential metaplastic effects on the vH & dH. **(A)** Summary plot of normalized fEPSP slope measurements in the vH (●) of saline conditioned rats in comparison with respective naive controls (○). **(B)** Summary plot of normalized fEPSP slope

measurements in the dH (○) of saline conditioned rats in comparison with respective naive controls (●). The x- axis show the time (in minutes) for which the fEPSP slopes was recorded before and after tetanus. Inset shows the superimposed 50 ms representative fEPSP sweeps from the last 5 minutes of the pre tetanus baseline and 25-30 minutes post-tetanus. **(C)** Summary quantification of LTP magnitudes in the vH (left bars) and dH (right bars) comparing naive and saline conditioned groups of rats. * $p < 0.05$; Error bars ± SEM.

A


mifepristone (25mg/kg) conditioning
total locomotor activity (counts)
5000
4000
3000
2000
1000
0
i.p. inj
0
10
20
30
40
50
60
70
80
90
Time in minutes

B


normalized fEPSP slope
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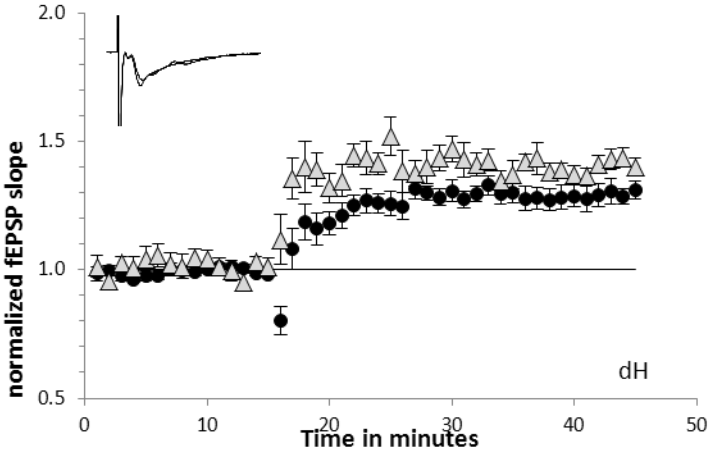
1.5
1.0
0.5
vH
0
10
20
30
40
50
Time in minutes

C


normalized fEPSP slope
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1.5
1.0
0.5
dH
0
10
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50
Time in minutes

D

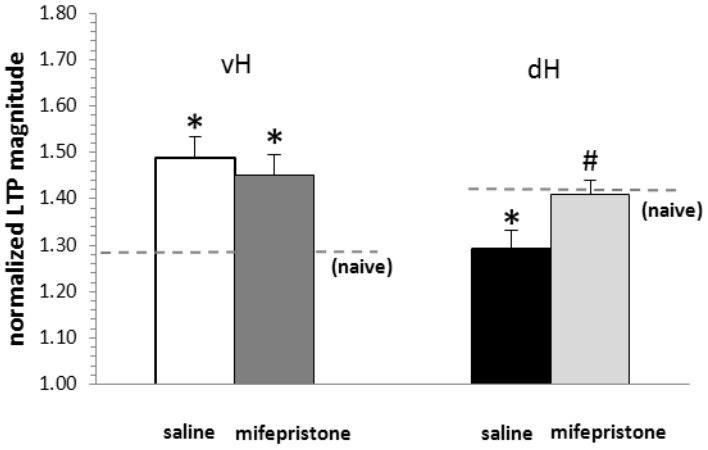
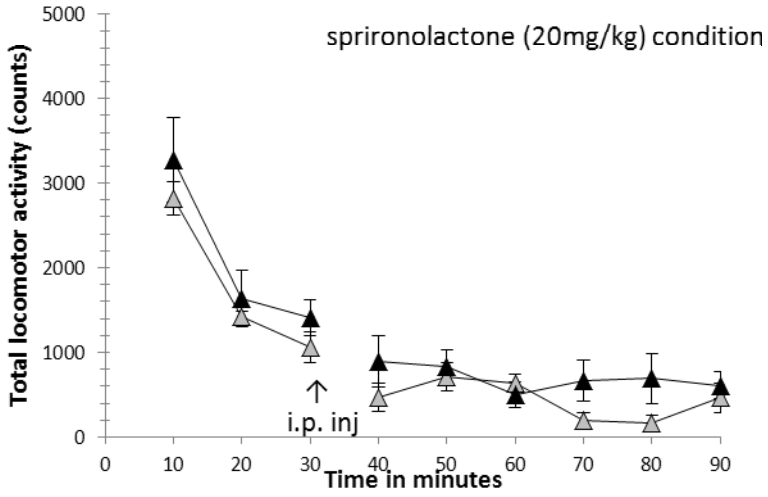


Figure 2.4. Glucocorticoid receptor antagonist prevented the conditioning- induced metaplasticity in dH. **(A)** Effect of mifepristone (glucocorticoid receptor antagonist) conditioning on the locomotor activity of rats during activity (△) and challenge (▲) days. The x- axis shows time (in minutes) for which rats were tested for locomotor activity during pre-injection (first 30 minutes) and post-injection (last 60 minutes). The y- axis shows the locomotor activity (total photo-beam breaks) in the open field chamber. **(B)** & **(C)** Summary plot of normalized fEPSP slope measurements in the vH (**B**;▲) and dH (**C**; △) of mifepristone conditioned group of rats in comparison with saline conditioned group (saline-vH ○; saline-dH ●). The x- axis show the time (in minutes) for which the fEPSP slopes was recorded before and after tetanus. Inset shows the superimposed 50 ms representative fEPSP sweeps from the last 5 minutes of the pre tetanus baseline and 25-30 minutes post-tetanus. **(D)** Summary quantification of LTP magnitudes in the vH (left bars) and dH (right bars) of mifepristone conditioned group compared with saline conditioned and naive (dashed line) groups. * significant difference between the treatment groups and the naïve group; # significant difference between treatment groups; $p < 0.05$; Error bars ± SEM.

A


sprironolactone (20mg/kg) condition
Total locomotor activity (counts)
5000
4000
3000
2000
1000
0
i.p. inj
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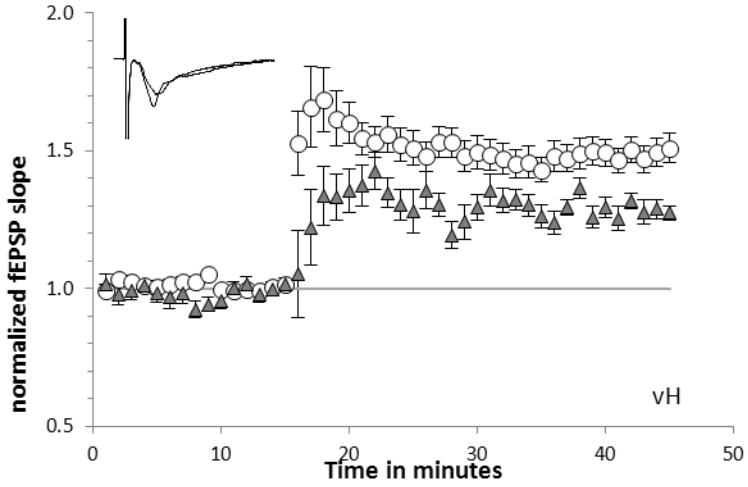
60
70
80
90
Time in minutes

B


normalized fEPSP slope
2.0
1.5
1.0
0.5
vH
0
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40
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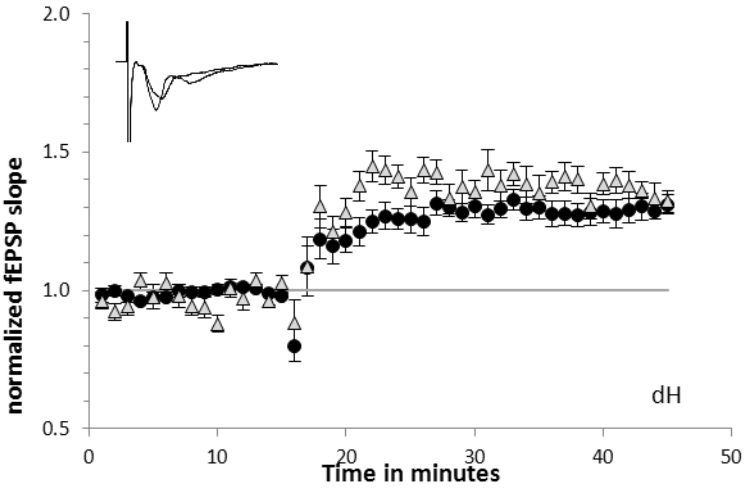
Time in minutes

C


normalized fEPSP slope
2.0
1.5
1.0
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dH
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Time in minutes

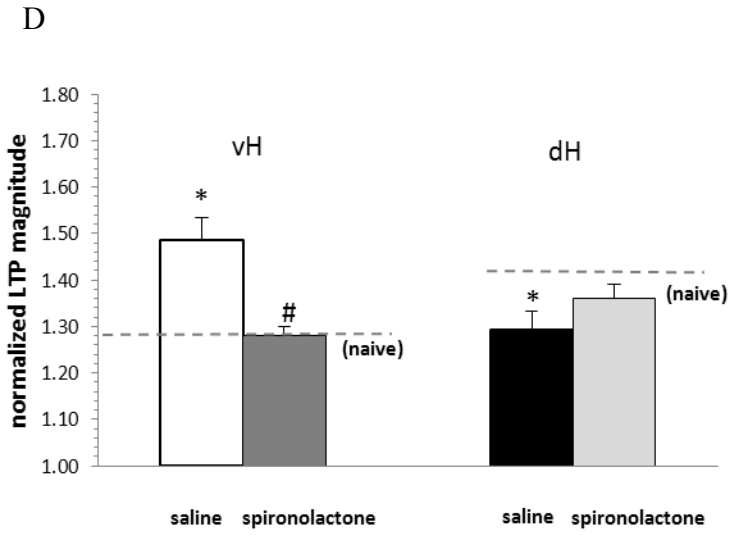
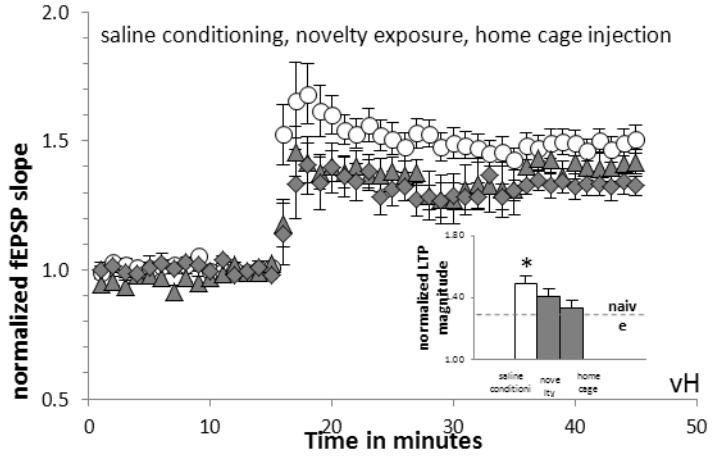


Figure 2.5. Mineralocorticoid receptor antagonist prevented the conditioning- induced metaplasticity in vH. **(A)** Effect of spironolactone (mineralocorticoid receptor antagonist) conditioning on the locomotor activity of rats during activity (△) and challenge (▲) days. The x-axis shows time (in minutes) for which rats were tested for locomotor activity during pre-injection (first 30 minutes) and post-injection (last 60 minutes). The y- axis shows the locomotor activity (total photo-beam breaks) in the open field chamber. **(B)** & **(C)** Summary plot of normalized fEPSP slope measurements in the vH (**B**; ▲) and dH (**C**; △) of spironolactone conditioned group of rats in comparison with saline conditioned group (saline-vH ○; saline- dH ●). The x- axis show the time (in minutes) for which the fEPSP slopes was recorded before and after tetanus. Inset shows the superimposed 50 ms representative fEPSP sweeps from the last 5 minutes of the pre tetanus baseline and 25-30 minutes post-tetanus. **(D)** Summary quantification of LTP magnitudes in the vH (left bars) and dH (right bars) of spironolactone conditioned group compared with saline conditioned and naive (dashed line) groups. * significant difference between the treatment groups and the naïve group; # significant difference between treatment groups; $p < 0.05$; Error bars ± SEM.

A



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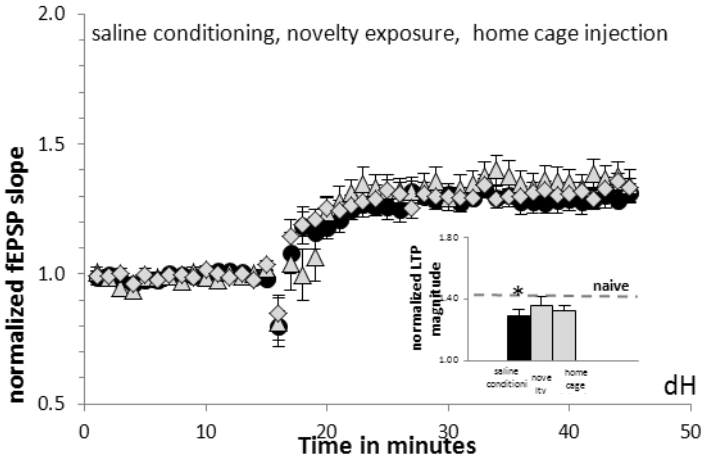
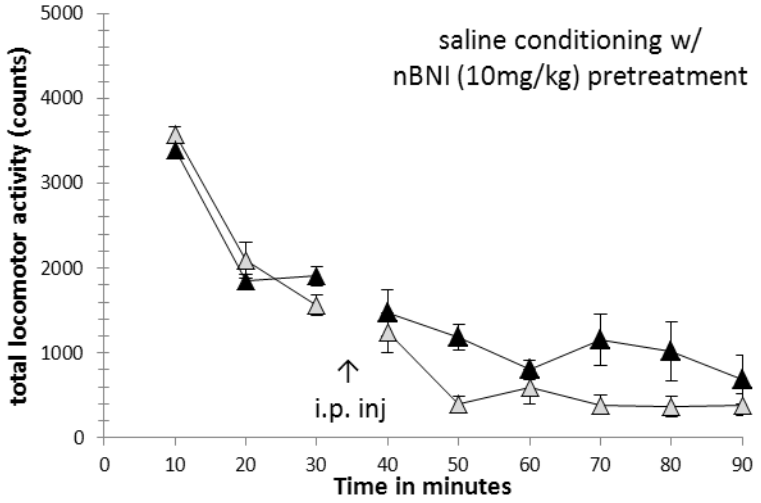


Figure 2.6. Exposure to novelty and i.p. injections caused additive stress-like effects in the hippocampal LTP. **(A)** Summary plot of normalized fEPSP slope measurements recorded in the stratum radiatum layer of the CA1 region of vH-slices in the novelty exposed (△) and home cage injected (◇) rats in comparison with saline conditioning group (○). **(B)** Summary plot of normalized fEPSP slope measurements recorded in the stratum radiatum layer of the CA1 region of dH-slices in the novelty exposed (△) and home cage injected (◇) rats in comparison with saline conditioning group (●). The abscissa show the time (in minutes) for which the fEPSP slopes was recorded during the pre- (first 15 minutes) and post- (last 30 minutes) tetanus. The

ordinate show the normalized fEPSP slopes (normalized to the average of last 5 sweeps prior to HFS). Inset bar graphs show LTP magnitudes of saline conditioning, novelty exposed and home cage injected groups, in the order mentioned, in comparison with naïve (dashed lines) groups. * shows significant difference between the treatment groups and the naive; $p < 0.05$; Error bars show ± SEM.

A


saline conditioning w/
nBNI (10mg/kg) pretreatment
total locomotor activity (counts)
5000
4000
3000
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i.p. inj
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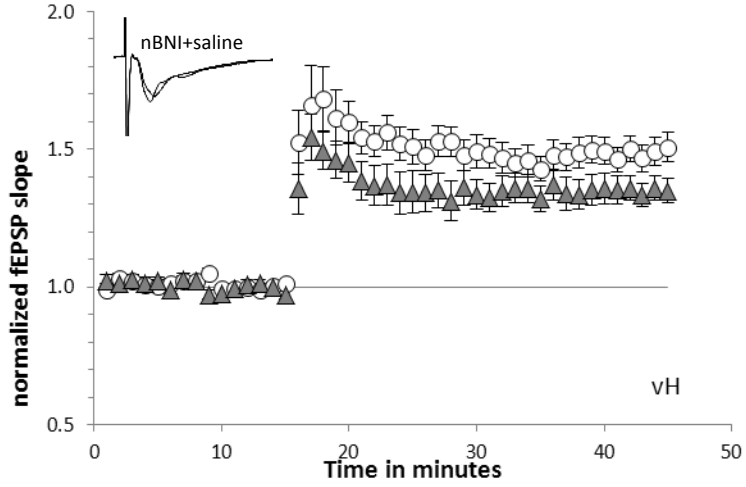
80
90
Time in minutes

B


nBNI+saline
normalized fEPSP slope
2.0
1.5
1.0
0.5
vH
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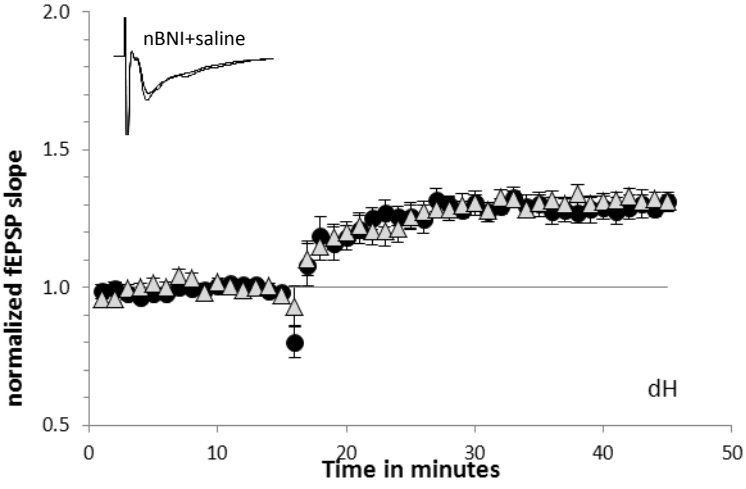
Time in minutes

C


nBNI+saline
normalized fEPSP slope
2.0
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1.0
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dH
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40
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Time in minutes

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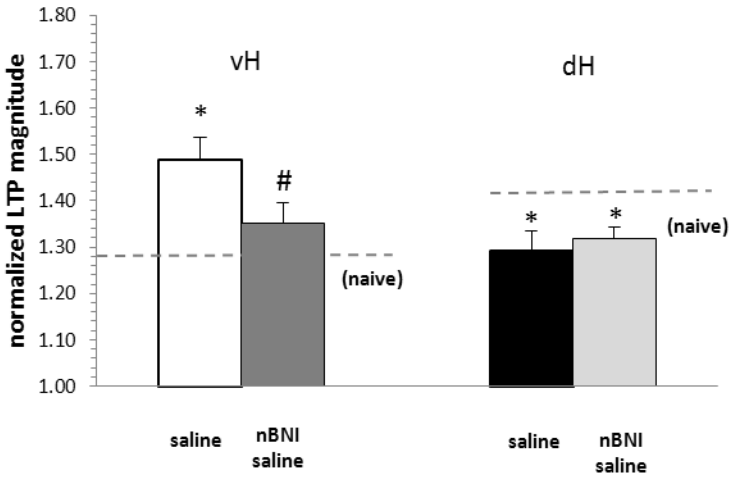

Figure 2.7. Kappa opioid antagonist prevented stress induced metaplasticity in vH but not in dH. **(A)** Effect of nBNI (kappa opioid receptor antagonist) administration 1 day prior to the saline conditioning on the locomotor activity of rats during activity (△) and challenge (▲) days. The x-axis shows time (in minutes) for which rats were tested for locomotor activity during pre-injection (first 30 minutes) and post-injection (last 60 minutes). The y- axis shows the locomotor activity (total photo-beam breaks) in the open field chamber. **(B)** & **(C)** Summary plot of normalized fEPSP slope measurements in the vH (**B**; ▲) and dH (**C**; △) of saline conditioned rats which were pretreated with nBNI in comparison with saline conditioned group (saline-vH ○; saline- dH ●). The x- axis show the time (in minutes) for which the fEPSP slopes was recorded before and after tetanus. Inset shows the superimposed 50 ms representative fEPSP sweeps from the last 5 minutes of the pre tetanus baseline and 25-30 minutes post-tetanus. **(D)** Summary quantification of LTP magnitudes in the vH (left bars) and dH (right bars) of naïve rats (dashed line) and saline conditioned rats with/without nBNI pretreatment. * significant difference between the treatment groups and the naïve group; # significant difference between treatment groups; $p < 0.05$; Error bars ± SEM

CHAPTER 3

EFFECTS OF REPEATED NON-CONTINGENT EXPOSURES OF COCAINE ON THE HIPPOCAMPAL SYNAPTIC PLASTICITY

Introduction

Hippocampal dependent learning and memory encoding is thought to occur via pattern separation processing of contextually associated information within in the intrinsic circuits of hippocampus (Lledo et al 2006; Smith & Bulkin 2014). Substantial evidence exists for the functional dissociation within the hippocampus along its septotemporal axis, with regards to the processing and encoding of different types of memory. Septal (dorsal) sector/zone of hippocampus (dH) is mainly involved in the context dependent cognitive and spatial memory processing, while the temporal (ventral) sector of hippocampus (vH) is mainly involved in the processing of contextual memory traces with emotional valence (Fanselow & Dong 2010). Persistence of drug- context associated & reward memories is thought to contribute many of the behavioral attributes of addiction, such as the reinstatement of drug seeking in addicts (Lee et al 2006). Hence the role of hippocampus in the processing of memories relevant to addiction has been widely appreciated and is a subject ongoing investigation.

Studies to find the molecular and electrophysiological changes induced by various drugs of abuse have mainly centered around the brain regions such as ventral tegmental area, nucleus accumbens, medial prefrontal cortex etc., which are parts of the so called ‘dopaminergic reward pathway’. Impaired glutamate homeostasis in the nucleus accumbens is posited to contribute for withdrawal symptoms and subsequent relapse associated with abused psychostimulants such as

cocaine (Kalivas 2009). Hippocampal glutamatergic afferents synapsing with the medium spiny neurons in the nucleus accumbens is a key modulator of glutamate homeostasis and dopaminergic neurotransmission within the reward pathway (Britt et al 2012; Floresco et al 2001). Indeed, earlier evidences from behavioral and lesion studies have shown that hippocampus is involved in the reinstatement of drug use in rodents (Lasseter et al 2010; Rogers & See 2007; Sun & Rebec 2003). Moreover, an electrical stimulation of the ventral subiculum of hippocampus was shown to reinstate the cocaine seeking behavior in rats (Vorel et al 2001).

Despite all these evidences, only very few investigations have been conducted to study the effects of cocaine on hippocampal synaptic plasticity. Additionally, differences in the expression of synaptic plasticity between dH and vH were also identified recently. For example, long-term potentiation (LTP) induced in the CA1 region in the vH was found to be of lesser magnitude than that of the dH in naïve, unstressed rats (Maggio & Segal 2007a; Papatheodoropoulos & Kostopoulos 2000a). Furthermore, an external stimuli, such as an acute stressful experience was found to induce differential responses in the dH and vH, where the magnitude of vH LTP was significantly increased, whereas dH LTP was decreased (Maggio & Segal 2007a). Given such accumulating evidence for both the functional dissociation between the vH and dH and the potential impact on resulting hippocampal connectivity, our current report investigated the effects of cocaine conditioning on LTP in these two sectors of the hippocampus using a locomotor sensitization behavioral protocol.

We found that repeated i.p. injections (conditioning) of cocaine induced locomotor sensitization in the rats. Importantly, cocaine conditioning resulted in sector specific effects with in the hippocampus, as it enhanced LTP in the vH which persisted 1-2 weeks following the last exposure to drug. Change in the expression of LTP occurred with no concurrent changes in the

basal synaptic transmission, and hence we believe that cocaine conditioning acted as a metaplastic trigger on the hippocampal neuronal circuit. The cocaine induced enhancement of LTP in vH was prevented by co-administration of dopamine D2- receptor antagonist eticlopride, but not D1/5 receptor antagonist SCH 23390. Furthermore, we demonstrated that phenomenon of locomotor sensitization towards cocaine is independent of the changes occurring in the expression of LTP in the hippocampus. In the context of addiction, metaplasticity of LTP in the CA1 may be a candidate cellular mechanism mediating behavioral responses such as the reinstatement of drug seeking in addictive subjects.

Materials and methods

Animal maintenance: 8-10 week old male Sprague-Dawley rats (Harlan, IN, USA) were housed in pairs in clear plastic cages and maintained on a 12 h light/dark cycle (0700/1900 h). Food and water were available *ad libitum* except during the behavioral sessions. Behavioral sessions were conducted daily between 0900 and 1200 h. Experiments involving rats were performed in compliance with the University of Georgia Institutional Animal Care and Use Committee guidelines.

Apparatus: A description of the apparatus used and measurements of activity have been described in detail elsewhere (Gosnell 2005). Briefly, activity was measured in four 43.2 × 43.2 cm chambers with clear plastic walls and a solid smooth floor (Med Associates, St. Albans, VT, USA). The chambers were individually housed in sound-attenuating cubicles equipped with two lights (approximately 20 lux) and a ventilation fan. Two banks of 16 infrared photobeams and detectors, mounted at right angles 3.5cm above the floor were used to detect horizontal activity. Activity Monitor software (Med Associates) was used to count photobeam- breaks. This

software subdivides total counts into stereotypic and ambulatory counts according to the repetitive (stereotypic) vs. sequential (ambulatory) patterning of the beam-breaks.

Locomotor sensitization study (LMS): Rats were habituated to handling for 3 days prior to locomotor activity testing. As depicted in the time line (Figure 3.1.A), rats were subjected to cocaine treatments for the next 5 days (an initial activity day and 4 subsequent conditioning days), followed by another treatment (challenge) day 1 week later. All the rats in the cocaine group received cocaine i.p. injections (10mg/kg on the activity & challenge days, and 15mg/kg during the conditioning days) and tested for locomotor activity. Subgroups of cocaine rats were administered either a single dose of kappa-opioid receptor (KOR) antagonist, nor-binaltorphimine (10mg/kg i.p.) on the last day of handling, or daily doses of one of the following: glucocorticoid receptor antagonist (mifepristone 25mg/kg), mineralocorticoid receptor antagonist (spironolactone 20mg/kg), dopamine (DA) $D_{1,5}$ receptor (SCH 23390 0.5mg/kg) antagonist or DA $D_{2\text{-like}}$ antagonist (eticlopride 0.1mg/kg) on all of the locomotor activity testing days. Locomotor activity was recorded, for 30 min prior and 60 min post injection, by the activity monitor software and was further analyzed using SigmaStat 3.1 software (Systat software inc., CA). Two-way repeated measures ANOVA was used to detect main effects of test day (activity vs. challenge) and time (60 min post injection) on the locomotor activity of rats following conditioning. If ANOVA revealed an effect of test days in a treatment group, Student-Newman-Keuls (SNK) post hoc analysis was performed on the total locomotor counts at various post-injection time points (40^{th} to 90^{th} minute of the behavioral sessions in 10 minute bins) to reveal significant differences across time ($p < 0.05$).

Extracellular electrophysiology: 500 μm thick hippocampal slices were prepared from either naive (no treatment, remained in the home cage) or conditioned rats within 1–2 weeks

after the challenge day. All rats were anesthetized with 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane) prior to decapitation. The brain was removed and submerged in ice-cold, oxygenated (95% O2/ 5% CO2) dissection artificial cerebrospinal fluid (ACSF) containing (mM): NaCl (120), KCl (3), MgCl2 (4), NaH2PO4 (1), NaHCO3 (26), and glucose (10). Horizontal and coronal sections were prepared from either half of the brain to obtain both ventral and dorsal hippocampal slices respectively, from the same animal. We estimate that our vH and dH slices are obtained from the extreme 30% of either temporal and septal pole, respectively. The CA3 region was removed and the remaining hippocampal tissue held in a submerged slice chamber and perfused with oxygenated (95% O2/5% CO2) standard ACSF containing (mM): NaCl (120), KCl (3), MgCl2 (1.5), NaH2PO4 (1), CaCl2 (2.5), NaHCO3 (26), and glucose (10) at approximately 1 ml/min. Slices recovered in the recording chamber for 45 min at room temperature, and then another 45 min at 30°C, the recording temperature. A bipolar stimulating electrode (Kopf Instruments) was placed on the CA3-side of the CA1 region in the stratum radiatum and a 1.0 MΩ tungsten recording microelectrode (World Precision Instruments) was then positioned in the same layer in CA1 to record the field potential changes occurring in the region. Field excitatory post synaptic potentials (fEPSPs) were elicited by stimulation of the Schaffer collateral-commissural pathway in stratum radiatum once every 60 s (0.0167 Hz) for the duration of the experiment. A stimulus-response curve was obtained at the beginning of each experiment, with stimulus pulses consisting of a single square wave of 270 μs duration delivered at 40–160 μA intensities with 20 μA increments. The stimulation intensity was then adjusted to obtain fEPSP amplitude of 1.0–1.5mV to begin baseline recording. Once a stable baseline was established, LTP was induced by a high frequency stimulation (HFS) protocol consisted of 100 pulses of 30 Hz trains, repeated three times separated by a 20 s interval. This particular HFS

protocol was chosen as it yields a sub-maximal (i.e. non-saturated) LTP magnitude, which in preliminary studies we found to be sensitive to the effects of cocaine to induce metaplasticity of LTP.

Quantification of synaptic plasticity: Data was digitized at 10 kHz, low-pass filtered at 1 kHz, and analyzed with pCLAMP 10.2 software (Axon Instruments). The initial slope of the fEPSP responses was measured and normalized to the last 5 min of baseline prior to tetanization. LTP was quantified by comparing the normalized fEPSP slopes from the last 5 minutes of the pre- tetanus baseline and 25-30 minutes post-tetanus. Planned comparisons were made using unpaired *t*-tests. In reporting our results, n-values indicate the number of slices and the number of animals in parenthesis.

Drugs (vehicle): Cocaine hydrochloride (saline) and nor-binaltorphimine (saline) were provided by NIDA. Spironolactone (DMSO), mifepristone (DMSO), SCH 23,390 (saline) and eticlopride (saline) were obtained from Tocris.

Results

1) Rats exhibited locomotor sensitization towards cocaine challenge

Locomotor activity recorded on the activity and challenge days prior to- and post-cocaine injections were compared to assess the sensitization towards cocaine dose in rats (15 mg/kg, i.p.; n=9). Significant effect of day (activity vs. challenge) was revealed on a two-way repeated measures ANOVA ($F = 5.59$, $p < 0.001$), confirming that rats exhibited locomotor sensitization to cocaine challenge (Figure 3.1.B). Significant differences in the locomotor activity were present up to first 40 minutes between activity and challenge days (SNK post hoc analysis, $p < 0.001$).

2) Conditioning with cocaine did not change the basal synaptic transmission in the ventral or dorsal hippocampus

Input-Output relationship of the field potential responses were measured at various stimulus intensities ranging from 40 to 160 μA at increments of 20 μA. **S**timulus-response curves were obtained from the ventral (Figure 3.2.A) and dorsal (Figure 3.2.B) hippocampal slices of cocaine-conditioned rats and compared with respective naïve controls. This naïve group of rats was maintained in their home cages throughout the experimental days. The sensitivity of the fEPSPs was indexed by calculating the stimulus intensity at ½ maximal response ($S_{1/2}$, μA) from the stimulus-response curves. No significant change in the $S_{1/2}$ (μA) of cocaine-conditioned rats were observed when compared to the naïve controls (vH-cocaine: 91± 3, n=18(10) vs. vH-naïve: 96 ± 3, n= 13(6); or dH-cocaine: 93 ± 4, n=12(6) vs. dH-naïve: 95 ± 3, n=22(7)). These results suggested that cocaine conditioning did not induce any change in the basal synaptic transmission in the hippocampus along its septo-temporal axis.

3) Cocaine conditioning-induced metaplastic effects in vH, but not in dH

vH LTP was significantly enhanced in the cocaine sensitized rats (1.53 ± 0.06, n=19(9)) when compared to the vH LTP in the naïve controls (1.28 ± 0.05; n= 13(6)) (Figure 3.3.C, left bars). However, cocaine conditioning did not induce any change in the dH LTP (1.37 ± 0.03, n=17(6)) when compared with the naïve control (1.42 ± 0.03; n=22(7)) (Figure 3.3.C, right bars). When the saline conditioning induced stress-like effect differentially affected LTP in both the sectors of hippocampus (refer to Figure 2.3), cocaine conditioning affected the LTP expression in hippocampus only in its ventral sector.

4) Blocking stress with Kappa opioid receptor antagonist did not prevent the cocaine conditioning-induced enhancement of LTP in vH

As described in chapter 2, saline conditioning induced stress enhanced vH LTP, which is the similar phenomenon observed in the cocaine conditioned rats as well. These cocaine conditioned rats were also subjected to the behavioral conditioning protocol similar to saline rats, where the combinatorial effect of stressors such as handling, novelty exposure, i.p. injections were all present. So it is indistinguishable whether the changes observed in the hippocampal LTP were specifically caused by cocaine or merely a conditioning effect. We were able to reverse the stress like effects on vH by administering the dynorphin-kappa opioid receptor antagonist, nBNI in the saline conditioned rats (Figure 2.7). Hence we administered nBNI in the cocaine conditioned rats one day prior to the behavioral protocol, so that influence of stress, if any, could be blocked.

Administration of nBNI (15 mg/kg, i.p.; n=9) failed to prevent the locomotor sensitization in rats towards cocaine challenge (Figure 3.4.A). Significant effect of day (activity vs. challenge) was revealed on a two-way repeated measures ANOVA ($F = 7.03$, $p < 0.001$), confirming that rats still exhibited locomotor sensitization to cocaine challenge despite of nBNI administration. Significant differences in the locomotor activity were present up to first 30 minutes between activity and challenge days (SNK post hoc analysis, $p < 0.001$).Thus kappa antagonist pretreatment did not prevent the subsequent induction and expression of cocaine-induced behavioral sensitization.

Furthermore, blocking the stress factor by nBNI in the nBNI/cocaine group did not change the LTP (1.55 ± 0.05, n=11(5)) in vH when compared to cocaine conditioned rats. (Figure 3.4.D; left bars). The effect of nBNI pretreatment did not result in any obvious change in

the dH (1.35 ± 0.04, n=13(5) as well when compared to that of the cocaine conditioned rats (Figure 3.4.D; right bars). Together, these results indicate that the metaplasticity induced by the repeated cocaine exposure in the vH is engaging a pathway independent of the dynorphin/KOR system and involves specific effects of cocaine in addition to the stress associated with behavioral conditioning.

5) Cocaine perfusion on the hippocampal slices enhanced LTP in vH, but not in dH

Cocaine's specific effect on the vH LTP was further confirmed by performing perfusion studies on the hippocampal slice preparations. Slices from naïve rats were perfused with cocaine (6 μM) for at least 20 min prior to LTP induction. It has been shown that compared to the dH, vH receives comparatively higher density of dopaminergic innervations from the midbrain dopamine cells. (Gasbarri et al 1996). This led us to hypothesize that cocaine's indirect dopamine agonist effect (i.e. to enhance LTP in the region, Swant et al., 2006) will be more pronounced in the vH than its effect in the dH. Indeed, the magnitude of LTP in cocaine-treated vH slices was significantly higher than the untreated controls (1.44 ± 0.08, n=8(5) vs. 1.28 ± 0.05; n= 13(6), $p<0.05$, Figure 3.5.A). In cocaine-treated dH slices, the magnitude of LTP was similar to that of the untreated controls (1.44 ± 0.04, n=17(7) vs. 1.42 ± 0.03; n= 22(7), Figure 3.5.B). These results were concurrent with those observed after the in vivo exposure of cocaine and suggest that the cocaine -induced metaplasticity is largely restricted to the ventral sector of the hippocampus. Hence, we will focus only on the vH in our further experiments to study cocaine induced metaplasticity in hippocampus.

6) Dopamine receptor $D_{1,5}$ and $D_{2\text{-like}}$ antagonists prevented cocaine induced locomotor sensitization, but only $D_{2\text{-like}}$ receptor antagonist prevented cocaine- induced metaplasticity in vH

Cocaine acts as an indirect dopamine agonist at the synapses, activating various types of dopamine receptors present at the synapse. We wanted to investigate the role of the two main types of dopamine receptors in cocaine's action on the hippocampal synaptic plasticity as well as the cocaine induced locomotor sensitization in rats.

Locomotor sensitization towards cocaine challenge was prevented when dopamine $D_{1,5}$ receptor antagonist SCH 23390 (0.5 mg/kg i.p., n=4) was administered along with cocaine during the conditioning protocol (Figure 3.6.A). However, SCH 23390 did not block the cocaine-induced enhancement of vH LTP (1.58 ± 0.05, n=8(4)) which remained significantly enhanced relative to the naïve group ($p<0.05$) (Figure 3.6.B). This shows that cocaine induced locomotor sensitization is a separate effect of cocaine which occurs independently from the cocaine's metaplastic effects on vH.

Similarly, locomotor sensitization in rats was prevented by dopamine $D_{2\text{-like}}$ receptor antagonist eticlopride (0.1 mg/kg, n=4) when administered along with cocaine injections during the conditioning protocol (Figure 3.7.A). Moreover, eticlopride significantly blocked the enhancement of LTP induced by repeated cocaine exposure in the vH (1.32 ± 0.03, n=10(4)) (# $p<0.05$, Figure 3.7.B). These results demonstrate that dopamine $D_{2\text{-like}}$ receptors are involved in the locomotor effects of cocaine as well as in the cocaine- induced metaplasticity in the vH.

Discussion

The important findings from the current sets of experiments are described below: First, repeated daily injections of cocaine induced sensitization in rats exhibited as enhanced locomotor

activity towards subsequent cocaine exposure. Second, conditioning with cocaine resulted in a persisting change in the magnitude of ventral hippocampal LTP. However, this change in the expression of LTP occurred without any obvious change in the basal synaptic transmission suggesting that cocaine was acting as a metaplastic trigger in the hippocampal neuronal circuitry. Preventing the influence of behavioral conditioning associated stress (Chapter 2) by nBNI did not reverse the effects of cocaine in vH, clearly demonstrating that cocaine exposure itself was specifically causing the metaplasticity. Moreover, the phenomenon of locomotor sensitization occurred independently from the metaplastic effects observed in the hippocampus, and was mediated by different molecular mechanisms. The potential interactions and importance of these findings are discussed below.

There has been a growing consensus for the segregation between the two sectors of hippocampus, both functionally and in terms of their anatomical connectivity (Fanselow & Dong 2010; Segal et al 2010). Anatomic integrity of vH with other limbic structures such as amygdala (Petrovich et al 2001) and reward pathway structures suggests the roles of vH in the formation of emotional memory and relapse to drug seeking behaviors respectively. However, dH is more associated with higher order cortical structures and mainly involved in cognition and spatial navigation. Electrophysiological investigations have revealed that the LTP magnitude in the dH is significantly higher than that of the vH (Papatheodoropoulos & Kostopoulos 2000a), the observation which we have successfully reproduced in the current study although with a different stimulation protocol. Additionally, other reports have also demonstrated the differences in the neuronal properties between vH & dH such as their intrinsic excitability (Dougherty et al 2012), density of voltage-(Dougherty et al 2012) and ligand- (Pandis et al 2006) gated ion channels.

Anatomical studies have shown that the vH receives prominent dopaminergic inputs from the midbrain ventral tegmental area cells, than that received by its dorsal counterpart (Gasbarri et al 1996; Verney et al 1985). In the current study, we found that acute cocaine perfusion on naïve hippocampal slices specifically enhanced LTP in the vH, but not in dH. This is in agreement with the afore-mentioned anatomical proof, and also demonstrating that endogenously released dopamine can have significant acute effects on the vH synaptic plasticity. Nevertheless, both ventral (Gasbarri et al 1996) and dorsal (Luo et al 2011) sectors of hippocampus were shown to be involved in the drugs of abuse associated neuroadaptations occurring in this the hippocampus. However, with the advent of newer technologies such as optogenetics and imaging, the key role of vH in modulating the reward pathway thereby contributing to addictive characteristics, is gaining prominence (Britt et al 2012). Despite of all these reports on the hippocampal synaptic properties being affected by cocaine, very few studies have attempted to characterize the effects of in vivo cocaine exposure on the hippocampal synaptic plasticity (del Olmo et al 2006b; Thompson et al 2004). The primary objective of the current study was to investigate the effects of repeated, intermittent and non-contingent cocaine exposures on the hippocampal synaptic plasticity along its septotemporal axis. In light of above observations, it would be expected that `the ventral zone of hippocampus would be more sensitive to cocaine when exposed in vivo.

Repeated i.p. injections of cocaine induced locomotor sensitization in rats towards a further challenge dose of cocaine (Figure 3.1.B), as evidenced by increased locomotor activity after the cocaine injections. When the hippocampal slices from the cocaine sensitized rats were monitored for basal synaptic transmission, we did not observe any change in dorsal or ventral slices (Figure 3.2), when compared to that of respective naïve slices. However, upon inducing LTP by high frequency stimulation, significantly greater LTP was expressed in the vH, but not in

dH, when compared to their respective naïve controls (Figure 3.3). Hence a form of induced metaplasticity is implicated in the hippocampal circuits which were exposed to repeated cocaine conditioning. Additionally, with respect to the sensitivity of vH to cocaine, the results from the in vivo cocaine exposure studies are in agreement with the effects of acute application of cocaine.

It is important to note that the cocaine conditioned rats were subjected to the behavioral protocol which was shown earlier to contain potential stressors such as novelty, handling and i.p. injections (Chapter 2). The saline conditioned rats which were taken through this protocol were shown haven subjected to stress-like effects on their hippocampal synaptic plasticity. Both saline- (Figure 2.3) and cocaine- conditioning (Figure 3.3) have specifically enhanced vH LTP. Hence it was unclear whether the metaplasticity observed in vH in the cocaine conditioned rats was due to stress, cocaine or a combination of both. Luckily, pretreatment with the dynorphin-kappa opioid receptor antagonist, nBNI, was successful in blocking stress-induced enhancement of vH LTP in the saline conditioned rats. Hence, we pretreated the cocaine conditioned rats with nBNI in an effort to block the potential stress effect and determine the cocaine-specific effects if any. Interestingly, nBNI pretreatment did not prevent the locomotor sensitization of rats to cocaine or the metaplasticity observed in the vH. This suggests that cocaine conditioning itself is the cause of metaplasticity observed in the current report, and that the nBNI- sensitive stress effect on vH might be altering the hippocampal synaptic properties via an independent pathway/mechanism.

Cocaine at the synapse acts as indirect dopamine agonist, and the involvement of dopamine receptor subtypes has been observed in the cocaine induced plasticity changes in the reward pathway and associated behaviors (Vanderschuren & Kalivas 2000). We have previously reported the involvement of $D_{2\text{-like}}$ receptors in the enhancement of LTP in the naïve slices upon

perfusing with cocaine (Thompson et al., 2005). To study the involvement of dopamine receptor subtypes in the cocaine-conditioning induced metaplasticity in vH, we co-administered $D_{1,5}$ or $D_{2\text{-like}}$ dopamine receptor antagonists in the cocaine conditioned rats. The involvement of both receptor subtypes in locomotor sensitization in rats was proven when both antagonists successfully blocked sensitization in rats (Figure 3.6.A & 3.7.A). However, co-administration of $D_{1,5}$ receptor antagonist SCH 23,390 did not prevent the cocaine-induced metaplasticity in vH. Conversely, $D_{2\text{-like}}$ dopamine receptor antagonist, eticlopride successfully reversed the cocaine-induced enhancement of vH LTP. Hence it could be argued that cocaine induced phenomena such as the locomotor sensitization and the metaplasticity in vH, are two independent effects and occur via different molecular mechanisms or distinct pathways.

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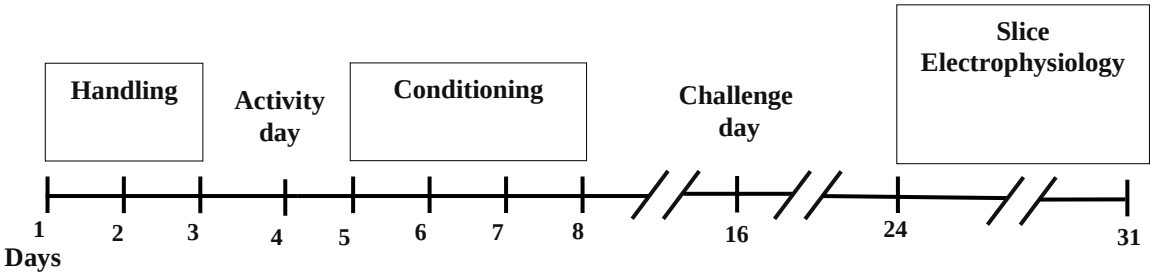

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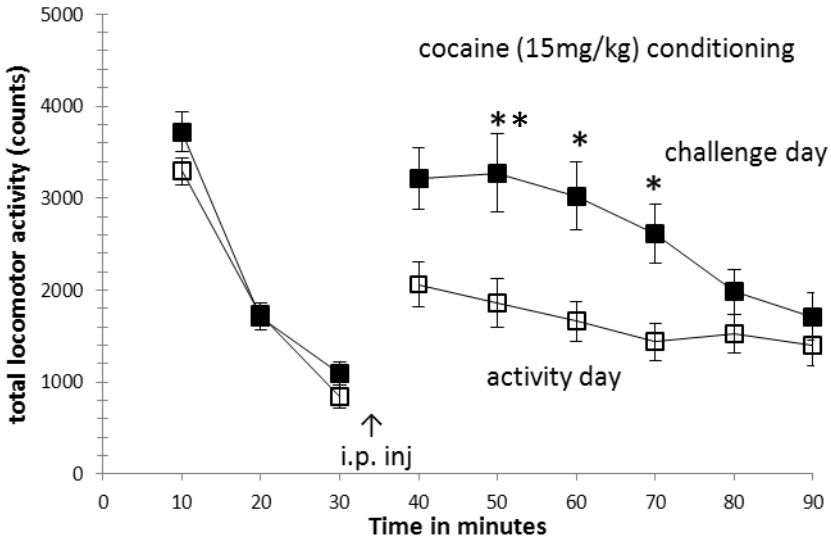


Figure 3.1. Rats exhibited locomotor sensitization to cocaine challenge **(A)** Experimental time line for the locomotor sensitization behavioral protocol and subsequent electrophysiological recordings from ex vivo hippocampal slices. **(B)** Effect of cocaine conditioning on the locomotor activity in rats during activity (□) and challenge (■) days. The x- axis shows time (in minutes) for which rats were tested for locomotor activity during pre-injection (first 30 minutes) and post-injection (last 60 minutes). The y- axis shows the locomotor activity (total photo-beam breaks) in the open field chamber. * significant difference in the locomotor activity between activity and challenge days at the given time points (two-way repeated measures ANOVA/SNK, $p<0.05$).

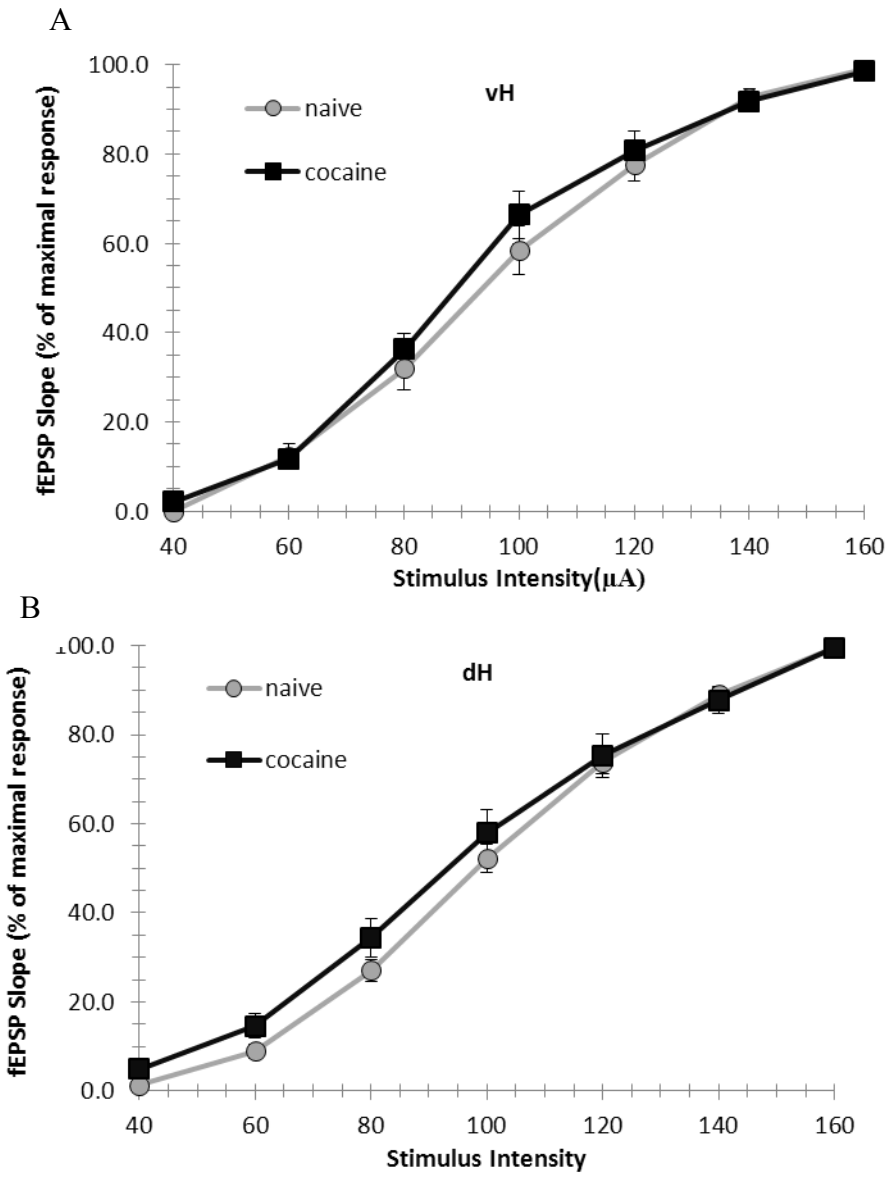


Figure 3.2. Cocaine conditioning did not affect the basal synaptic transmission in hippocampus. **(A)** Stimulus response curves obtained from the vH of naive (⊸●⊸) and cocaine (■) groups of rats. **(B)** Stimulus response curves obtained from the dH of naive (⊸●⊸) and cocaine (■) groups of rats. The x- axis represent stimulus intensities (µA) at which hippocampal slices are stimulated at the baseline frequency. The y-axis show the normalized slopes (normalized to the slope of maximal fEPSP response) of fEPSPs obtained at each stimulus intensities

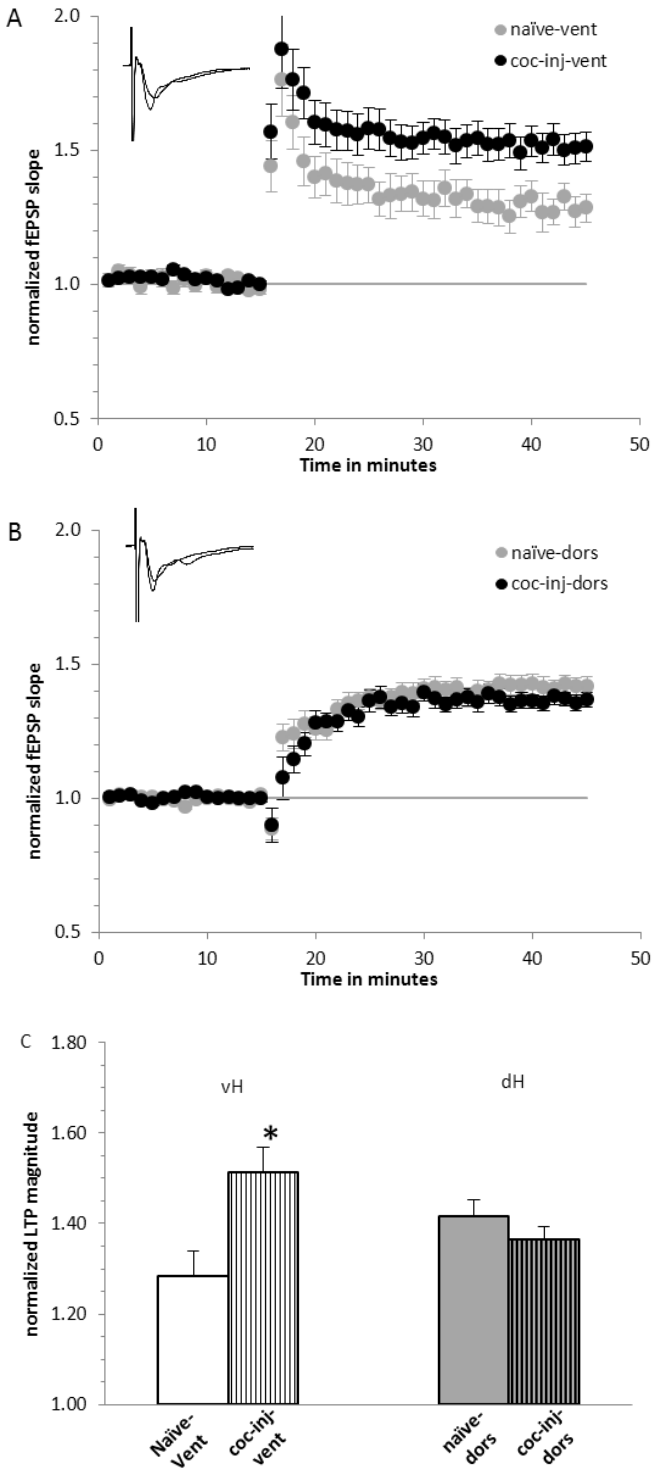
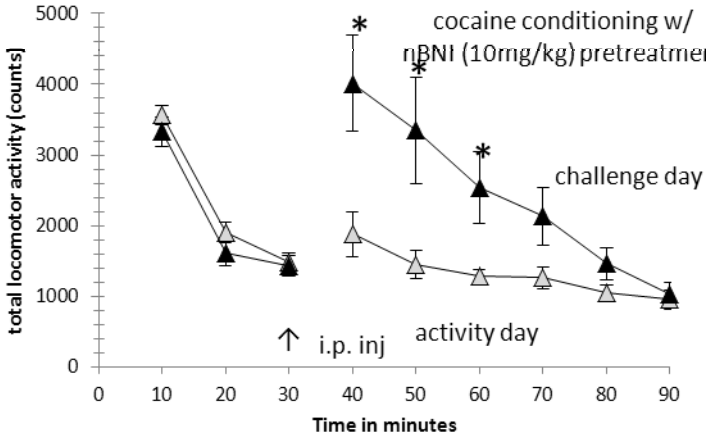


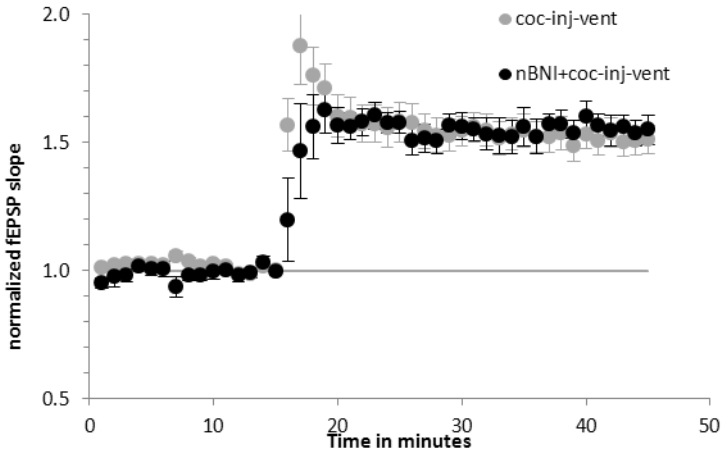
Figure 3.3. Cocaine induced metaplastic effects in vH, but not dH. **(A)** Summary plot of normalized fEPSP slope measurements of cocaine conditioned rats in the vH (●) in comparison

with respective vH in naïve group (○). **(B)** Summary plot of normalized fEPSP slope measurements of cocaine conditioned rats in the dH (●) in comparison with respective dH in the naïve group (○). The abscissas x- axis show the time (in minutes) for which the fEPSP slopes was recorded before and after tetanus. Inset shows the superimposed 50 ms representative fEPSP sweeps from the last 5 minutes of the pre tetanus baseline and 25-30 minutes post-tetanus in the cocaine conditioned rats. **(C)** Summary quantification of LTP magnitudes in the vH (left bars) and dH (right bars) comparing naive and cocaine conditioned groups of rats. * $p < 0.05$; Error bars ± SEM.

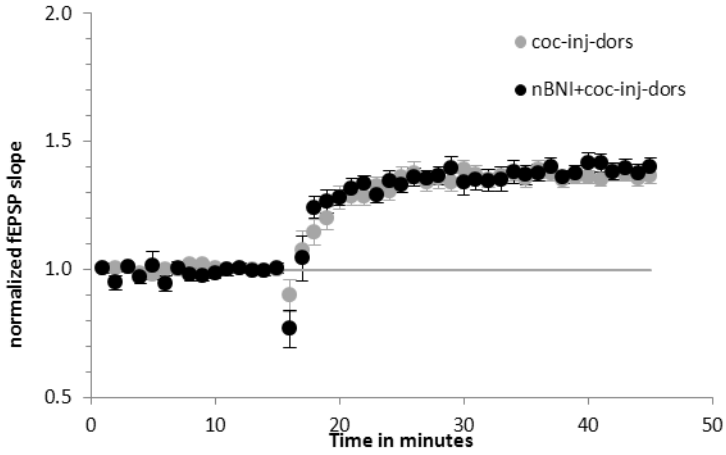
A


cocaine conditioning w/
nBNI (10mg/kg) pretreatmer
challenge day
activity day
i.p. inj
total locomotor activity (counts)
Time in minutes

B


coc-inj-vent
nBNI+coc-inj-vent
normalized fEPSP slope
Time in minutes

C


coc-inj-dors
nBNI+coc-inj-dors
normalized fEPSP slope
Time in minutes

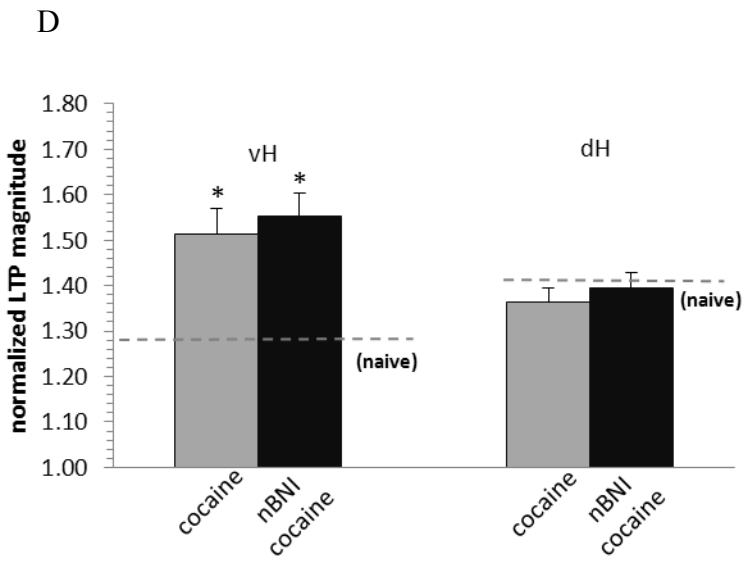


Figure 3.4. Blocking conditioning- induced stress by KOR antagonist did not affect the locomotor sensitization towards cocaine or cocaine- induced metaplasticity in hippocampus**. (A)** Effect of nBNI (kappa opioid receptor antagonist) administration 1 day prior to the cocaine conditioning on the locomotor activity of rats during activity (△) and challenge (▲) days. The x-axis shows time (in minutes) for which rats were tested for locomotor activity during pre-injection (first 30 minutes) and post-injection (last 60 minutes). The y- axis shows the locomotor activity (total photo-beam breaks) in the open field chamber. * indicates significant difference in the locomotor activity between activity and challenge days at the given time points (Two-way repeated measures ANOVA/SNK; $p<0.05$). **(B)** Summary plot of normalized fEPSP slope measurements in the vH of nBNI/cocaine group (●) in comparison with cocaine (●) group of rats**. (C)** Summary plot of normalized fEPSP slope measurements in the dH of nBNI/cocaine group (●) in comparison with cocaine (●) group of rats. The x- axis show the time (in minutes) for which the fEPSP slopes was recorded before and after tetanus. Inset shows the superimposed 50 ms representative fEPSP sweeps from the last 5 minutes of the pre tetanus baseline and 25-30

minutes post-tetanus**. (D)** Summary quantification of LTP magnitudes in the vH (left bars) and dH (right bars) comparing naïve, cocaine and nBNI/cocaine groups of rats. ***** significant difference between the naïve and the treatment groups; $p < 0.05$; Error bars ± SEM.

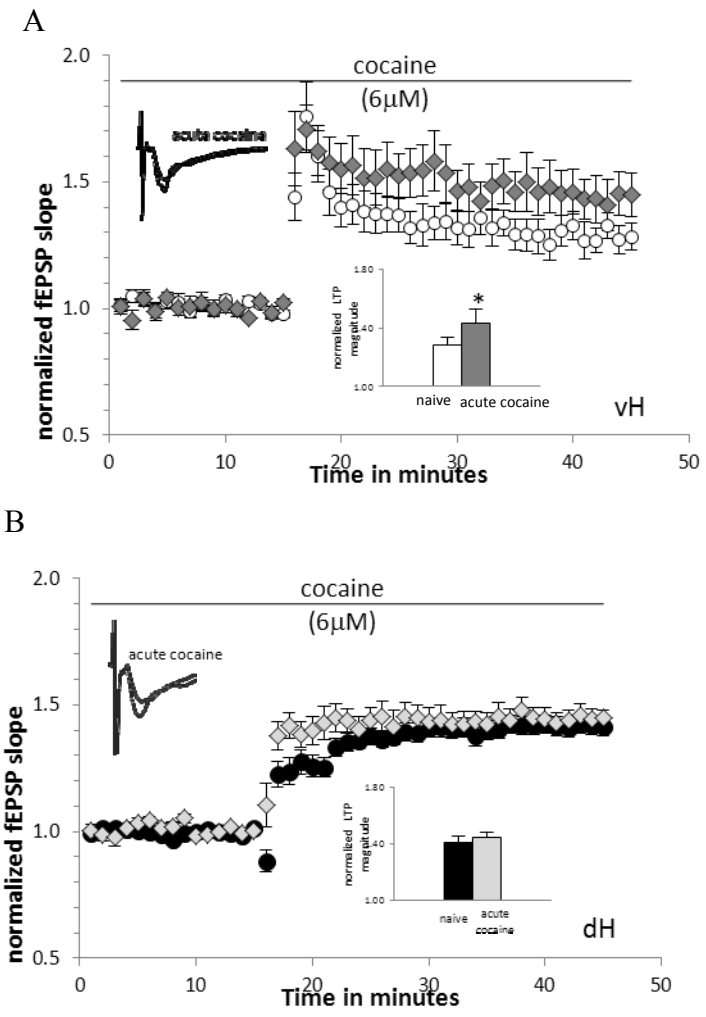


Figure 3.5. Acute cocaine perfusion enhanced LTP in the vH, but not in dH. **(A)** Summary plot of normalized fEPSP slope measurements in the cocaine perfused vH- (◆) slices compared with their respective no-drug controls (○). **(B)** Summary plot of normalized fEPSP slope measurements in the cocaine perfused dH- (◇) slices compared with their respective no-drug controls (●).The x- axis show the time (in minutes) for which the fEPSP slopes was recorded before and after tetanus. Inset shows the superimposed 50 ms representative fEPSP sweeps from the last 5 minutes of the pre tetanus baseline and 25-30 minutes post-tetanus. Inset bar graph shows LTP magnitudes of vH- (A) and dH- (B) slices with or without acutely perfused cocaine. * $p < 0.05$; Error bars ± SEM.

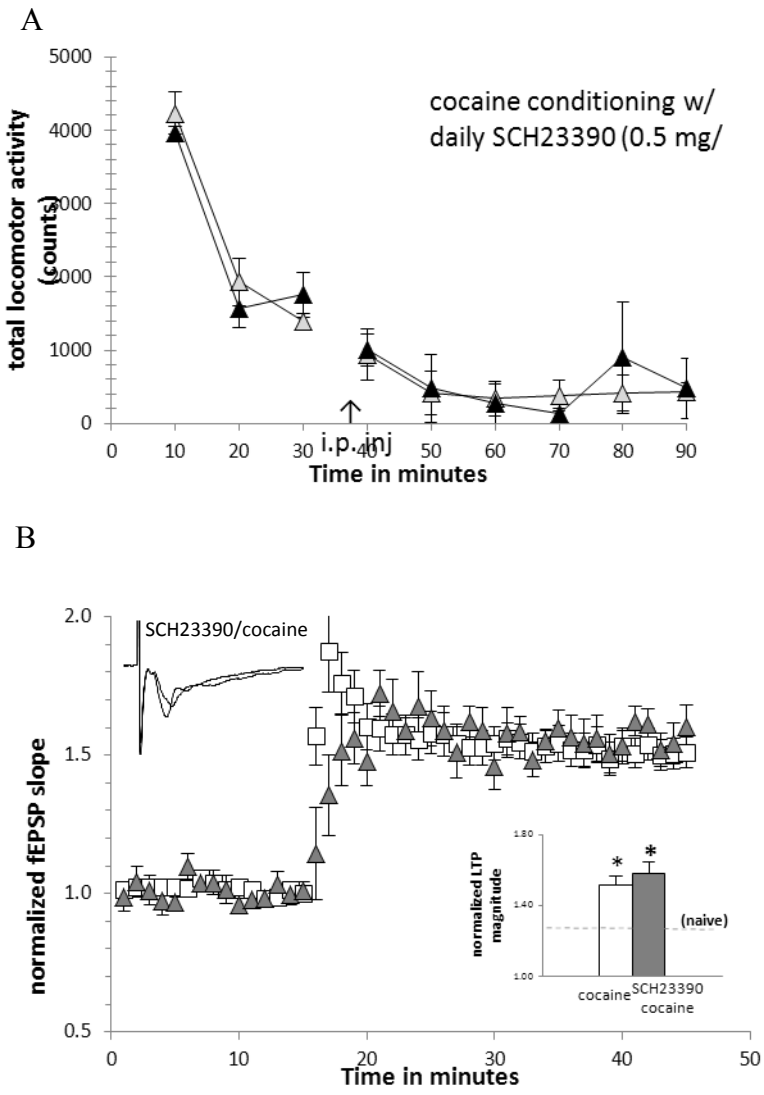


Figure 3.6. Effects of co-administration of dopamine receptor $D_{1/5}$ antagonist SCH 23,390 on the cocaine-induced locomotor activity in rats and the metaplasticity in vH. **(A)** Effect of co-administration of $D_{1/5}$ antagonist SCH 23,390 on the cocaine conditioned-induced locomotor activity in rats during activity (△) and challenge (▲) days. The x- axis shows time (in minutes) for which rats were tested for locomotor activity during pre-injection (first 30 minutes) and post-injection (last 60 minutes). The y- axis shows the locomotor activity (total photo-beam breaks) in the open field chamber. **(B)** Summary plots comparing the normalized fEPSP slope

measurements in vH-slices of cocaine conditioned rats (□) with SCH 23,390/cocaine (▲) group of rats. The x- axis show the time (in minutes) for which the fEPSP slopes was recorded before and after tetanus. Inset shows the superimposed 50 ms representative fEPSP sweeps from the last 5 minutes of the pre tetanus baseline and 25-30 minutes post-tetanus. Inset bar graph compares LTP magnitudes in vH of naïve & cocaine- conditioned rats with SCH 23,390/cocaine group. * significant difference between the treatment groups and the naïve; $p < 0.05$; Error bars ± SEM

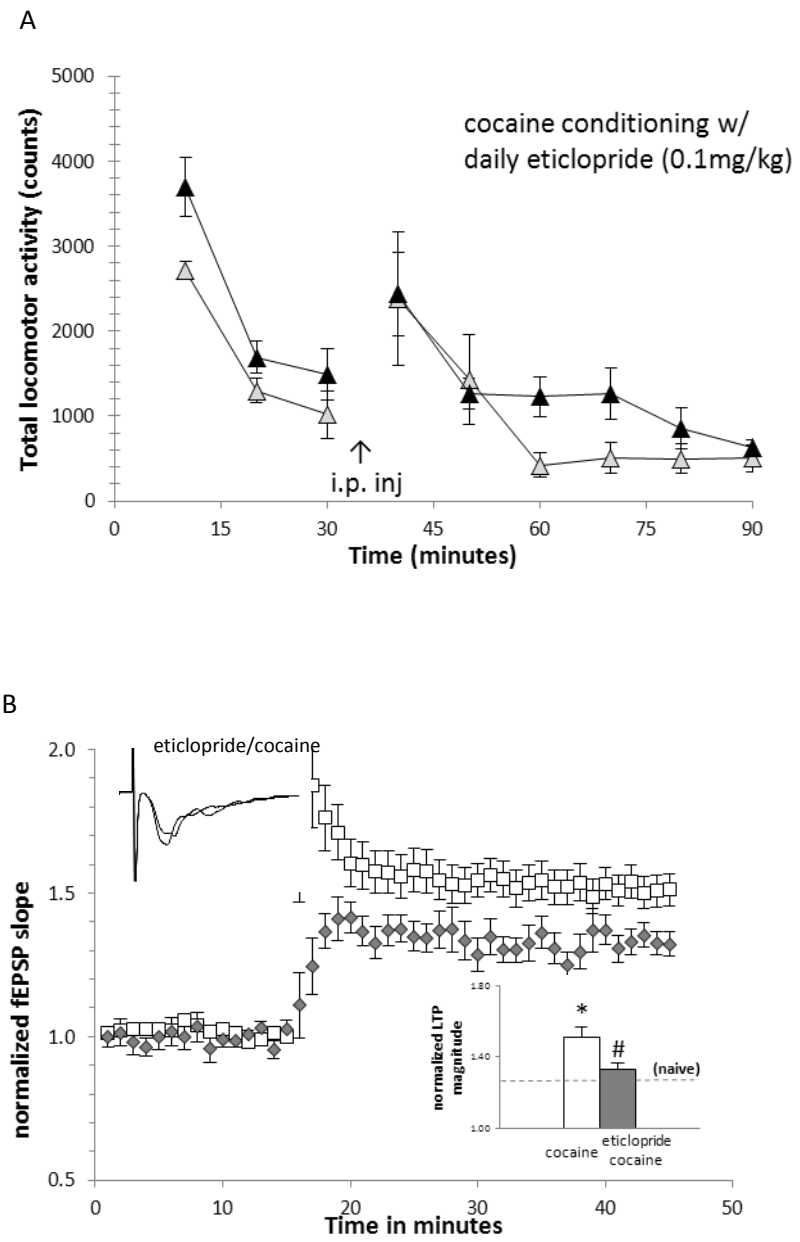


Figure 3.7. Effects of co-administration of $D_{2\text{-like}}$ dopamine receptor antagonist eticlopride on the cocaine-induced locomotor activity in rats and the metaplasticity in vH. **(A)** Effect of co-administration of $D_{2\text{-like}}$ antagonist eticlopride on the cocaine conditioned-induced locomotor activity in rats during activity (△) and challenge (▲) days. The x- axis shows time (in minutes) for which rats were tested for locomotor activity during pre-injection (first 30 minutes) and post-

injection (last 60 minutes). The y- axis shows the locomotor activity (total photo-beam breaks) in the open field chamber. **(B)** Summary plots comparing the normalized fEPSP slope measurements in vH-slices of cocaine conditioned rats (□) with eticlopride/cocaine (◇) group of rats. The x- axis show the time (in minutes) for which the fEPSP slopes was recorded before and after tetanus. Inset shows the superimposed 50 ms representative fEPSP sweeps from the last 5 minutes of the pre tetanus baseline and 25-30 minutes post-tetanus. Inset bar graph compares LTP magnitudes in vH of naïve & cocaine- conditioned rats with eticlopride/cocaine group. * significant difference between the treatment groups and the naïve; # significant difference between the treatment groups; $p < 0.05$; Error bars ± SEM

CHAPTER 4

EXTENDED ACCESS COCAINE SELF-ADMINISTRATION REGIMEN INDUCES PERSISTENT CHANGES IN THE VENTRAL HIPPOCAMPAL SYNAPTIC PLASTICITY

Introduction

Addiction to the psychostimulant cocaine has been a subject of intense investigation for over many years. Studies to find the molecular and electrophysiological changes induced by various drugs of abuse have mainly centered around the brain regions such as ventral tegmental area, nucleus accumbens, medial prefrontal cortex etc. which are part of the so called 'dopaminergic reward pathway'. Despite being able to elucidate the mechanism of action at the synaptic level, we still lag behind in our understanding of how these drugs, including cocaine, act at the circuit level in causing a subject to fall into the addictive phenotype. Many studies have employed in vivo rat models where the subjects were exposed to cocaine either via passive experimenter administration or short access self-administration protocols, or even ex vivo slice preparations. One major limitation of these studies is the lack of an adequate model of addiction. A typical rodent model of psychostimulant addiction exhibits the following characteristics – a) Compulsive binging, b) Seeking or taking drug even under adverse consequences, and c) extreme tendency to relapse (Ahmed & Kenny 2011). It has been shown that rats which were subjected to repeated extended access cocaine self-administration sessions show these characteristics (Wolf & Tseng 2012), and hence it is becoming accepted as an adequate model in addiction studies.

Drug- context influenced reward learning occurs during episodes of drug abuse. This type of associative learning causes consolidation of drug associated memories, which later when retrieved is believed to cause relapse / reinstatement of drug use in addicted subjects (Lee et al 2006). The hippocampus plays a major role in associative learning and in the processing, consolidation and storage of memory traces (Preston & Eichenbaum 2013). Functional heterogeneity of the different zones of hippocampus along its septotemporal axis has gained attention in the recent years. Dorsal zone of hippocampus (dH) is more functionally aligned with the higher order cortical structures and is thought to be involved mainly in the cognitive, declarative and spatial memory processing (Fanselow & Dong 2010). Meanwhile, the ventral zone of hippocampus (vH) is thought to associate more with the limbic and reward regions of the brain and hence is involved in emotional and salience associated memory processing (Fanselow & Dong 2010; Floresco et al 2001). Hence it is a reasonable premise to posit that hippocampus might also be involved in the processing and consolidation of drug associated memory traces, and in the retrieval of those memory engrams resulting in the reinstatement phenomenon.

Consistent with the above hypothesis, there are some evidences from behavioral and inactivation studies which suggest that hippocampus is involved in the reinstatement of drug use in rodents (Lasseter et al 2010; Rogers & See 2007; Sun & Rebec 2003). However, attempts to study the potential changes occurring in the hippocampal circuits in response to exposure to drugs of abuse have come up with variable results (Keralapurath et al 2014a; Thompson et al 2004; Ungless et al 2001). This variability could be attributed to the differences in the rodent model of drug exposure (ex vivo slice preparation vs. experimenter administered doses vs. short access self-administration sessions), the zone of hippocampus (dorsal vs. ventral) studied, and the length of time after the drug exposure that the study was conducted. Most of the studies

which examined hippocampal involvement in addiction had one or more of the following lacunas - 1) Lack of an adequate rodent model of addiction – in vivo exposure via passive (experimenter) administration or short-access self-administration sessions are inadequate to generate a true rodent model of addiction (Ahmed & Kenny 2011), 2) Most of the studies have focused on the changes happening in the dH (Del Olmo et al 2006a; Ungless et al 2001), while the evidence suggests vH to play a more influential role in addiction (Britt et al 2012; Floresco et al 2001; Vorel et al 2001), 3) Time/ stage of drug exposure at which the experiments were conducted – acute changes in the neuronal circuits in response to drug exposure might not always reflect the neuronal correlates responsible for inducing addictive characteristics (Wolf & Tseng 2012).

In light of these observations, in the current study, we have investigated the effects of cocaine self-administration (protocol involving short access- followed by extended/long access-sessions) on the neuronal synaptic plasticity in the ventral hippocampus in rats. Rather than the acute changes, we were particularly interested in studying the persistent changes happening in hippocampus, and hence did all our recordings during 3 -5 weeks into the abstinence period in these rats. We investigated the effects of cocaine on glutamatergic and GABA-ergic neurotransmission at the Schaffer collateral – CA1 synapses in the vH. We found that repeated long-access cocaine self-administration persistently alter the excitatory as well as the inhibitory neurotransmission in the hippocampus, resulting in a mal-adaptive synaptic plasticity in the region.

Materials & Methods:

Drugs: Cocaine hydrochloride was obtained from NIDA (RTI international, NC, USA), and was dissolved in phosphate buffered saline (4 mg/ml solution) for the self-administration experiments. DL-AP5 and CNQX were purchased from Tocris, and picrotoxin from Sigma.

Animal maintenance & surgery: All procedures were approved by University of Georgia Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Harlan, IN) weighing approximately 300g were single-housed in clear plastic cages for 1 week (habituation) with ad libitum food and water under a 12 hour/ 12 hour light/dark cycle. On the day of surgery, animals were anesthetized by IP injecting Ketamine (70 mg/kg) & Xylazine (10 mg/kg) combination before implanting a silastic catheter into the right jugular vein. The other end of the jugular catheter was connected to a cannula which is stereotactically mounted and held firm on the top of the skull using dental cement and 4 screws. Post-operative antibiotic (gentamicin @ 5 mg/kg) was administered via the catheter for 5 days. On all subsequent days catheters were flushed with saline before and with heparin (100 USP/ml) after the self-administration protocol to test and maintain the patency respectively. A separate group of age-matched rats, termed as 'naïve', were maintained in their home cages.

Cocaine self-administration: 1 week after jugular catheterization surgery, rats were allowed to self-administer cocaine (0.5 mg/kg/infusion) or saline (0.125 ml/kg body weight) in well-lit and ventilated operant chambers (Med Associates, VT) equipped with active and inactive levers. Active lever presses were programmed to switch the house light off and turn the active lever-light on for a period of 30 seconds which is termed as the 'time-out' period. For the first 10 days rats were on an FR-1 (fixed-ratio 1) schedule, where one active lever press outside the 'time-out' period triggers single cocaine infusions. For the next 12days (day 11 – 22), rats had to press the active lever 3 times outside the 'time-out' period in order to get 1 infusion (FR-3). For the initial 16 days each rats were allowed to self-administer cocaine for a total of 90 minutes/day (Short access), while the last 6 days of the protocol (day 16 – 22) the experiment spanned for 6

hours duration each day (Extended access). The Med-PC software records the number of lever presses (active and inactive) and infusions during the entire period of experiment.

Slice Electrophysiology: Within 3 to 5 weeks after the self-administration protocol, 500 μm ventral hippocampal slices were obtained from the rats which were euthanized by decapitation after 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane) anesthesia. Slices were transferred to a submerged recording chamber were incubated for 1 hour at room temperature and another hour at 30 ^{o}C. During the incubation and subsequent recording periods, slices were continuously perfused (@1ml/min) with ACSF (120mM NaCl, 3mM KCl, 1.5mM $MgCl_2$, 1mM NaH_2PO_4, 2.5mM $CaCl_2$, 26mM $NaHCO_3$, 10mM glucose) saturated with 95% O_2/5% CO_2. Schaffer collateral fibers were stimulated using a bipolar electrode, and either whole cell recordings from the pyramidal cells or field potential responses in the stratum radiatum of the CA1 region were performed from each slice.

Whole-cell voltage clamp recordings were conducted using a pipette (3-5 mega Ohm) pulled using 1.5mm thin-wall borosilicate glass tubing (Sutter) in a P-97 horizontal pipette puller (Sutter). The following intracellular solution was used: 145mM $CsMeSO_3$, 2mM $MgCl_2$, 2mM EGTA, 0.2mM $CaCl_2$, 2mM Mg-ATP, 5mM QX-314, 0.5mM Mg-GTP, 2mM HEPES and at a pH of 7.2.

Antagonists for NMDA- (DL-AP5 @ 50μM) and AMPA receptors (CNQX @ 10μM) were bath applied on the slices to pharmacologically isolate and record monosynaptic GABA-ergic inhibitory post synaptic currents (GABA- IPSCs) at 0mV. Postsynaptic AMPA and NMDA currents were measured in the presence or absence of GABA antagonist (picrotoxin, 100 μM). In the experiments where picrotoxin was perfused (at least for 15 minutes before starting the recordings) to block inhibitory currents, after evoking a stable excitatory postsynaptic current

(EPSC) @ +40mV, DL-AP5 was bath perfused until the AMPA- EPSC was isolated (15 - 30 minutes). NMDA-EPSC was determined by subtracting (using Microsoft excel) the AMPA-EPSC from the total EPSC.

Unlike the usual protocol followed in the majority of studies which isolate NMDA & AMPA currents by blocking $GABA_A$ receptors using agents such as picrotoxin, in a separate set of experiments we did not block the local inhibitory GABAergic interneuronal activity (Michaeli et al., 2012). In such experiments, after evoking a stable mixed (total) postsynaptic current (PSC) @ +40mV, DL-AP5 was bath perfused to completely block NMDA-PSC. Following a stable post DL-AP5 response, CNQX was bath perfused to block AMPA component of the response. NMDA-PSC was determined by subtracting the post DL-AP5 response from the total PSC. AMPA- PSC was determined by subtracting the net remaining response from the post- DL, AP5 response. Voltage-clamp was applied using Multiclamp 700B amplifiers, all the recordings were digitized at 10 KHz and low-pass filtered at 1 KHz with Digidata 1322A and analyzed using Clampfit 10.2 software (Molecular Devices).

Field excitatory postsynaptic potentials (fEPSPs) were recorded using tungsten electrode. The long term potentiation (LTP) was induced by a high frequency stimulation (HFS) protocol consisted of 3 trains of 100pulses at 100 Hz separated by a 20 second interval. Data were digitized at 10 kHz, low pass filtered at 1 kHz, and analyzed with pCLAMP9.2 software (Axon Instruments). Synaptic responses were normalized by dividing all the fEPSP slopes by the average of five fEPSP slopes recorded 5 minutes prior to tetanus. LTP was calculated by averaging the slopes of five fEPSP responses from 26 to 30 minutes after the induction of tetanus.

Results

1) Cocaine/Saline self-administration:

The average number of infusions during the first 60 minutes of each self-administration session/day is shown in Figure 4.1. For the first 15 days, rats were maintained in the operant chambers for 90 minute sessions (short access) each day. Out of these, 10 days of self-administration protocol were under FR1- (fixed-ratio 1) schedule, where a single lever press gave the rats a single dose of either cocaine (0.5 mg/kg/infusion) or saline (0.125 ml/infusion) via their indwelling jugular catheters. From day 11 to 15 rats were on FR3- schedule, where they had to work hard (press the lever 3 times) for a single infusion of cocaine or saline. Rats were then transitioned into long access sessions (6 hours/day) on FR3-schedule on days 16 – 22. Rats which consistently took cocaine above a set threshold (>15 infusions/session/day for at least 2 consecutive days) during the short access sessions drastically increased their cocaine intake upon transitioning to long access schedule. Transitioning to the extended access sessions imparts the rats with the habit of compulsive binging resulting in the progressive escalation of drug intake, and sets the stage for the habitual- drug seeking behavior in the addictive subjects (Everitt & Robbins 2013; Koob & Volkow 2010).

Across all the self-administration trials, rats which were exposed to cocaine clearly fell into two subpopulations – those which took very low cocaine (classified under 'Low-Taker' group, n=10) or those which took significant to large doses of cocaine (classified under 'Taker' group, n=39). As mentioned above, rats which did not take more than 15 infusions (i.e. less than 2.4mg cocaine/day approximately) per day for at least 2 consecutive days were classified under 'Low-Taker' group. Comparable outcomes have been consistently reported upon employing self-administration protocols similar to what we have used in this study (Grigson & Twining 2002;

Puhl et al 2009). A separate group of rats were allowed to self-administer saline (n=11, 0.125 ml/infusion) under the same self-administration regimen as that of the cocaine- rats. Protocols similar to the current one have been proven to generate rats which exhibited at least 3 typical characteristics of psychostimulant addiction such as, 1) Compulsive binging, 2) seeking drugs even under aversive conditions, and 3) extreme tendency for reinstatement upon presenting drug associated cues, context or even priming with the drug (Ahmed & Kenny 2011).

2) Cocaine self-administration enhanced baseline excitatory synaptic transmission in the ventral hippocampal CA1 pyramidal cells

All the electrophysiological analyses were conducted in the ventral hippocampal slices from rats at least after 21 days (3 weeks) and within 35 days (5 weeks) of the last long access self-administration session in rats. Rats were maintained in their home cages, single-housed and with ad-lib food and water, during this period of abstinence.

In the whole-cell voltage clamp mode, EPSCs were isolated in the CA1 pyramidal cells after blocking the $GABA_A$ receptors by perfusing the slices with picrotoxin (100 μM) for at least 15 minutes prior to and continuing till the end of recordings. At +40mV, the input-output relationship was assessed to determine intrinsic excitability of the CA1 cells under the control- and cocaine- conditions. For this, Schaffer collaterals were stimulated at baseline frequency (0.017 Hz) with increasing current intensities (at the increments of 25 μA) and the EPSCs recorded from the CA1 pyramidal cells.

Amplitude of EPSCs were plotted as the function of stimulus intensities above the rheobase current, and comparisons made across the three groups of rats on each stimulus intensity (Figure 4.2.). Here the rheobase current was defined as the stimulus intensity (μA) at which a minimum of 20pA response could be evoked in the CA1 pyramidal cells (Purgianto et al

2013). At 3 consecutive stimulus intensities (+50, +75 and +100 μA) above the rheobase current, evoked EPSCs in the taker group (n= 9(9); 260.09 ± 33.28pA, 421.54 ± 63.35pA & 516.07 ± 78.25pA respectively) were significantly (One-way ANOVA, @ +50 μA: F=4.69, P=0.018; @ +75 μA: F= 5.60, p=0.009; @ +100 μA: F=4.95, p=0.014) enhanced over the EPSCs in the Control (n=9(12); 158.26 ± 12.81pA, 257.85 ± 15.70pA & 311.66 ± 24.57pA respectively) and the Low-taker (n= 10(10); 175.14 ± 28.31pA, 284.52 ± 24.64pA & 377.85 ± 32.54pA respectively) rats. These results suggest that the CA1 pyramidal cells in the 'taker' group of rats were more excitable, indicating that cocaine self-administration resulted in the potentiation of the Schaffer collateral - CA1 pyramidal cell excitatory synapses.

3) Cocaine self-administration caused enhanced AMPA/NMDA ratio in the ventral hippocampus

The main contributor to the excitatory conductance in the CA1 region of hippocampus is the glutamatergic neurotransmission, which is regulated by AMPA- and NMDA- receptors. In order to assess the contribution of these receptors to the enhanced baseline excitatory transmission observed in the 'taker' group, we measured the AMPA- and NMDA- EPSCs in slices. After recording the input-out characteristics in every slice, we isolated the AMPA-EPSCs from the total EPSC by perfusing the slice with a NMDA receptor antagonist, DL-AP5. NMDA-EPSCs were calculated by subtracting AMPA currents from the total EPSC using MS Excel.

We calculated the AMPA/NMDA ratio, which is widely recognized as a synaptic plasticity index, to assess the relative contribution of AMPA receptors to the total EPSC in the CA1 pyramidal cells. The control (no drug) group consisted of both naive (n= 6(7)) and saline self-administration (n= 8(8)) rats, and revealed an AMPA/NMDA ratio (0.80 ± 0.04) which was comparable to the values reported in earlier studies (Hsia et al 1998; Ji et al 2010). Mean AMPA/NMDA ratio of the taker (n= 13(13); 0.99 ± 0.07) group of rats was significantly (One-

way ANOVA, F=6.46, p=0.004) greater than that of the control and low-taker (n= 9(10); 0.65 ± 0.06) rats (Figure 4.3.). This finding suggests that enhancement in the baseline excitatory neurotransmission in the CA1 pyramidal cells subsequent to cocaine self-administration might have occurred due to an increase in the relative contribution of AMPA receptor- currents at the synapses.

4) Calcium-permeable AMPA receptors were increased (numerical trend) in the CA1 pyramidal cell synapses after cocaine self-administration

Stage-wise changes in the AMPA receptor density and subunit composition in the reward pathway are critical determinants in imparting typical behavioral manifestations associated with addictive phenotypes (Wolf & Tseng 2012). For e.g., initial increase in the AMPA- EPSC in the nucleus accumbens after cocaine self-administration is due to increase in the GluA2-containing (calcium impermeable, CI-AMPA) AMPA receptors, while the maintenance of craving is mediated by GluA2-lacking (calcium permeable, CP-AMPA) AMPA receptors which replace the CI-AMPA receptors initially formed at the synapses (Conrad et al 2008). Presence of CP-AMPA receptors are detected in the experimental conditions by the unique property of inward rectification in their I-V relationships. Ion channels with inward rectification properties allows ions (current) to pass into the cell, while outward ion movements are either partially or significantly blocked because of the structural properties of these channels. Intracellular polyamines block outward movements of ions (cations in the case of AMPA receptors) in the CP-AMPA receptors, hence less AMPA mediated current response could be evoked at higher depolarization potentials. The effect of dialysis of endogenous polyamines occurring during the whole cell patch configuration could be minimized by adding exogenous polyamines such as spermine or spermidine into the pipette solution, and detect for the presence of inwardly

rectifying AMPA receptors during the entire recording time. This could be evidenced by non-linear I-V relationship when CP-AMPA receptors are present at the synapses, while CI-AMPA receptors show linear I-V relationship.

To this end, after isolating AMPA-EPSCs, an I-V relationship was obtained by recording the AMPA currents at holding potentials ranging from -60 mV to +40 mV at 20 mV increments (Figure 4.4. A & B). As evidenced from the representative sweeps and the I-V plot, AMPA-EPSCs at depolarization potentials (+20 and +40 mV) in the Taker (n= 13(13)) group were comparatively smaller than the Control (n= 14(15)) & Low-Taker (n = 9(10)) groups. Rectification index was calculated by dividing the peak amplitude of AMPA-EPSC at -60mV by the peak amplitude at +40mV, after correcting for the current at 0mV holding potentials (Boehm J, Malinow R, 2006, Neuron). Figure 4.4.C shows a clear numerical increase in the rectification index of the Taker (3.25 ± 0.43) group of rats when compared to the Control (2.38 ± 0.1) and the Low-Taker (2.32 ± 0.70) rats (One-way ANOVA, $F=3.11$, $p= 0.057$). It is important to note that our intracellular pipette solution did not contain exogenous polyamines, hence the 'double rectification' phenomenon shown by CP-AMPA receptors demonstrated in other studies was absent in our recording conditions. In retrospect, we could argue that a robust inward rectification and statistically significant differences could have materialized had we included polyamines such as spermine in our intracellular pipette solution. Nevertheless, this result suggests that enhancement of AMPA-EPSC in the CA1 pyramidal cells after cocaine self-administration could be due to the increased expression of CP-AMPA receptors the SC – CA1 glutamatergic synapses.

5) Cocaine self-administration reduced GABAergic inhibitory postsynaptic currents in vH

Cocaine induced suppression of postsynaptic GABAergic inhibition has been shown to occur in VTA (Bocklisch et al 2013; Liu et al 2005) and mPFC (Lu et al 2010). Studies from our lab have shown that exogenous application of dopamine D3 receptor agonists onto hippocampal slice preparations suppressed GABAergic IPSCs in the CA1 pyramidal cells (Swant et al 2008). Since cocaine at the synapse has a dopamine agonist-like effect, a similar effect on the inhibitory neurotransmission in the ventral hippocampus could potentially occur.

In separate trials, we isolated GABAergic IPSCs in the CA1 pyramidal cells by perfusing the slices with AMPA- and NMDA- receptor antagonists. Recordings were conducted at 0 mV holding potentials, and maximal evoked IPSCs were determined from each recorded cells. We found that compared to the Control (n = 12(12); 1310.72 ± 111.43 pA) group, cocaine self-administered rats (n= 14(16); 949.5 ± 121.18 pA) showed significant ($p < 0.05$; unpaired t-Test) reduction in the GABAergic IPSCs (Figure 4.5.). This result suggests that cocaine self-administration resulted in the suppression of inhibitory influence of GABAergic neurotransmission on the CA1 pyramidal cells in the vH. The detailed mechanism by which this could be possible, however, warrants further investigation.

6) Cocaine mediated enhancement of AMPA/NMDA ratio was exaggerated in the presence of local inhibitory activity

The above results show that extended access cocaine self-administration could persistently change the synaptic plasticity of ventral hippocampus by affecting both the excitatory and inhibitory circuits in the region. The hypothesis that the cocaine- induced changes in the inhibitory neurotransmission could influence its effect on glutamatergic activity in VTA has been proposed earlier (Michaeli et al 2012). They reported that AMPA/NMDA ratio

measured in the absence of picrotoxin (i.e. $GABA_A$ receptor not blocked) in the VTA slices were significantly greater than the ratio when measured in the presence of picrotoxin (i.e. $GABA_A$ receptor blockade). The discrepancies reported in the synaptic plasticity indices such as LTP and AMPA/NMDA ratio in VTA slices subsequent to cocaine exposures could have occurred due to presence (Argilli et al 2008; Ungless et al 2001) or absence (Liu et al 2005) of picrotoxin in the course of the recordings. This led us to investigate the effects of cocaine on the AMPA/NMDA ratio in the presence of intact local inhibitory influence, which can be approximated as a more physiologically relevant condition.

In a separate set of trials, when AMPA/NMDA ratio was calculated in the absence of picrotoxin (Figure 4.6.), hippocampal slices from the taker groups (n= 11(11); 1.81 ± 0.16) was significantly ($p< 0.05$, unpaired t-Test) greater than that of the no-drug control (n= 7(7); 0.84± 0.05). Importantly, it should be noted that the cocaine induced enhancement of AMPA/NMDA ratio was more pronounced in the absence of picrotoxin (1.81± 0.16) vs. the AMPA/NMDA ratio in the presence of cocaine (0.99 ± 0.07, Figure 4.3.).

7) Ventral hippocampal LTP was significantly suppressed in cocaine self-administered rats

Above results indicate that cocaine self-administration persistently changes the baseline tone of excitatory and inhibitory neurotransmission in the ventral hippocampus of rats. Any change in the baseline neurotransmission in hippocampus could alter the way hippocampal dependent processes are carried out. It is widely accepted that the hippocampal dependent learning and memory events are getting processed, consolidated and stored by cellular mechanisms involved in the phenomenon of LTP or LTD (Whitlock et al 2006). Hence we wanted to study whether cocaine affected the hippocampal ability to induce and express LTP subsequent to high frequency stimulation (HFS).

Slices used for the LTP experiments were taken from the subgroups of rats used for studying glutamatergic responses (Figures 4.2, 4.3, 4.4). Schaffer collaterals were stimulated using a bipolar electrode in current clamp mode and fEPSP responses were recorded from the S.radiatum layer of the CA1 region. High frequency stimulation consisted of 300 pulses at 100 Hz frequency was used to induce LTP in the slices. Figure 4.7 shows that LTP at the end of 30minutes post-HFS session in the naive (n= 10(26); 1.44 ± 0.02), saline self-administration (n=8(29); 1.44 ± 0.03) and the low-takers (n= 5(9); 1.39± 0.02) were significantly greater (One-way ANOVA, F= 9.997, p=0.001) than the LTP observed in the Taker (n= 5(11); 1.26 ± 0.03). Despite an increase in the excitatory baseline activity and a simultaneous suppression of GABAergic inhibition in the ventral hippocampus, LTP in the region was occluded. This indicates that cocaine self-administration might have induced a saturation of synaptic potentiation in the SC- CA1 synapses or resulted in impairment in mechanisms required for the proper expression of LTP.

Discussion

The current study demonstrates persistent changes in the synaptic plasticity occurring in the ventral hippocampus subsequent to cocaine self-administration in rats. The self-administration protocol that we employed consisted of 2 weeks of short-access sessions which were followed by 1 week of long-access sessions. It was shown previously that similar protocols were essential to generate rats with exclusive characteristics of addiction such as compulsive cocaine seeking, binge consumption and a higher tendency to relapse. We found that extended access cocaine enhances the baseline excitatory synaptic transmission in the s. radiatum layer of the CA1 region in the ventral hippocampus. The increased excitability in this region was associated with greater AMPA/NMDA ratio and a simultaneous reduction in the GABAergic

IPSCs in the postsynaptic CA1 pyramidal cells. However, despite a potentiated glutamatergic neurotransmission and a concurrently suppressed inhibitory activity, LTP was suppressed in the region. This occlusion of LTP suggests that chronic, voluntary intake of cocaine causes persistent maladaptive changes in the hippocampal neurotransmission which could impair further synaptic adaptations to other external stimuli.

Drugs of abuse induced changes in neurotransmission are extensively studied in the regions of the mesolimbic reward pathway such as ventral tegmental area, nucleus accumbens and medial prefrontal cortex. The molecular and electrophysiological observations made in these regions in milieu of exposure to drugs of abuse were successfully correlated with behavioral manifestations exhibited by the addictive subjects during various stages of addiction. One of the main research focuses in the addiction field is to track changes in the excitatory glutamatergic neurotransmission within the reward pathway, mediated mainly by AMPA- and NMDA-receptors. The AMPA/NMDA ratio is widely accepted as an index of synaptic plasticity and has been used to measure the strength of the synaptic conductance and receptor expression. Changes in the expression or conductance of AMPA receptors is the major regulator of this index, hence AMPA receptor mediated glutamatergic neurotransmission is a major focus in the studies of neuronal synaptic plasticity. Stage dependent changes in AMPA receptor density, subunit expression and conductance have been demonstrated in the reward pathway in response to various drugs of abuse.

An enhanced activity of dopamine neurons causing increased DA neurotransmission locally and at the afferent synapses of VTA is believed to cause the ‘feeling of high’ (euphoria) during drug episodes. The amplified influence of DA in the reward pathway is thought to be associated with the dopamine D2 autoreceptor subsenstivity (Marinelli et al 2003) or with the

strengthening of glutamatergic synapses on the DA cells in VTA (Borgland et al 2004; Mameli et al 2009). To this end, the potentiation of glutamatergic neurotransmission due to an increased AMPA/NMDA ratio was demonstrated in the DA cells in response to cocaine self-administration in rats (Chen et al 2008) . These changes occurring in VTA mark the stage for the induction of reward related synaptic adaptations in the brain (Wolf & Tseng 2012). Changes observed in nucleus accumbens, one of the afferent targets of VTA, were shown to associate with the induction and maintenance of incubation of craving during the periods of abstinence in rodents (Conrad et al 2008). Within a few minutes of cocaine administration a transient decrease in AMPA/NMDA ratio was noticed in the nucleus accumbens due to NR2B- subunit of NMDA receptor- induced generation of silent synapses in the medium spiny neurons (MSNs). As time progresses, this ratio was enhanced due to increase in the surface expression and glutamatergic conductance by AMPA receptors which marks the induction of incubation of craving. At this stage the rise in AMPA receptor expression was mainly contributed by the increased insertion of GluA2-containing (calcium impermeable) AMPA receptors into the silent synapses. However, almost one month into the abstinence, these calcium impermeable AMPA (CI-AMPA) receptors were replaced by GluA2-lacking subunits containing AMPA receptors which are calcium permeable (CP-AMPA), which in turn are responsible for the persistence of the phenomenon of craving (Conrad et al 2008). The long lasting enhanced expression of CP-AMPA significantly alters the properties of synaptic conductance by increasing calcium entry into postsynaptic neurons. Increased calcium conductance triggers activation of multiple signal pathways at transcriptional level triggering changes in the synaptic receptor protein density, their expression and activity at the postsynaptic neurons.

An important impediment in the treatment of substance abuse disorder is the extreme tendency to relapse even after prolonged abstinence. Impaired glutamate homeostasis in the nucleus accumbens plays a major role in contributing to the withdrawal symptoms during the abstinent periods, and triggers relapse in addictive subjects (Kalivas 2009). Nucleus accumbens MSNs forms glutamatergic synapses with many afferent pathways arising from mPFC, amygdala, hippocampus etc (Groenewegen et al 1987; Lu et al 2010; Sesack & Pickel 1990; Stuber et al 2011). One way of modulating the strength of a population of glutamatergic synapses and altering glutamate homeostasis in the nucleus accumbens occurs via mPFC – nucleus accumbens pathway (Moussawi et al 2009). However, a recent optogenetics assisted exploration of glutamatergic pathways on to nucleus accumbens revealed that the MSN synapses formed with the glutamatergic afferents arising from the ventral hippocampus was significantly potentiated after cocaine exposure (Britt et al 2012). Indeed, many behavioral and lesion studies had previously indicated the importance ventral hippocampus in the context of addictive behaviors. For example, electrical stimulation in the subicular region of vH in rats triggered reinstatement of cocaine seeking behavior (Taepavarapruk & Phillips 2003; Vorel et al 2001). Conversely, chemical inactivation of ventral hippocampus resulted in the attenuation of reinstatement of cocaine seeking in rats (Lasseter et al 2010; Rogers & See 2007; Sun & Rebec 2003). Furthermore, it was shown that the ventral- subicular/hippocampal stimulation modulated VTA DA neuronal activity eventually leading to increased DA efflux in the nucleus accumbens (Floresco et al 2001). However, no changes were observed in the dorsal sector of hippocampus consequent to cocaine exposure (Ungless et al 2001). The above observations suggest that vH is more sensitive to and involved in the drugs of abuse- induced modifications compared to the dH. Indeed the functional and anatomical connectivity differences between these regions are gaining

increased appreciation. When compared to its dorsal counterpart, vH also receives significantly greater dopaminergic innervation from midbrain DA cells (Gasbarri et al 1996; Verney et al 1985), a strong anatomical evidence for its putative role in modulating the reward pathway. In fact, we recently reported that repeated i.p. injections of cocaine specifically enhance LTP in the vH and not in dH(Keralapurath et al 2014a). All this evidence points to the fact that it is specifically the ventral hippocampus which plays a major role in modulating reward related behaviors, especially the reinstatement of drug seeking behavior, in the addictive subjects.

In the current study we demonstrated that repeated cocaine self-administration persistently enhances the AMPA/NMDA ratio at the CA3-CA1 synapses in the ventral hippocampus (Figure 4.3). This observation was consistent with the finding that the baseline excitatory synaptic transmission at these synapses was also enhanced after repeated cocaine exposures (Figure 4.2). Subunit composition of AMPA receptors are critical determinants of the functional properties of neurons. Even in the absence of intracellular polyamines in our pipette solution, an inward rectification trend was observed in the input-output curve of AMPA- EPSCs in rats self-administered cocaine (Figure 4.4). Inward rectification for the AMPA conductance indicates the presence of CP-AMPA receptors at the synapses. These observations are concurrent with the changes in AMPA receptor conductance and subunit composition observed in nucleus accumbens subsequent to cocaine exposures (Britt et al 2012; Conrad et al 2008). Hence we speculate that demonstrated potentiation at the nucleus accumbens – vH glutamatergic synapses (Britt et al 2012) could be due to an activity dependent strengthening of neurotransmission occurring one step upstream at the level of hippocampal pyramidal cell soma and dendrites. Meanwhile, it is strikingly interesting to note that when cocaine self-administration enhanced the basal synaptic transmission in the ventral hippocampus, repeated non-contingent doses of

cocaine failed to do so in the same species of age-matched rats (Keralapurath et al 2014a). This indicates that in addition to the drug induced changes in the neuronal circuits/synapses, the animal actively relies on the hippocampal dependent associative learning process to acquire and consolidate drug associated memories during the voluntary self-administration sessions.

In the current study, we found that cocaine self-administration suppresses evoked GABAergic IPSCs in the CA1 pyramidal cells (Figure 4.5), although the mechanistic details were not investigated further. Cocaine induced suppression of postsynaptic GABAergic inhibition was demonstrated in the mPFC pyramidal neurons (Lu et al 2010) as well as in the VTA DA cells (Bocklisch et al 2013; Liu et al 2005; Niehaus et al 2010; Steffensen et al 2008). Nevertheless, the mechanisms by which cocaine suppresses GABAergic inhibition in VTA and mPFC cells are apparently different. Cocaine induced disinhibition is occurring in VTA (Bocklisch et al 2013), while a decrease in the surface expression of $GABA_A$ receptors was demonstrated in the mPFC pyramidal cells (Liu et al 2005). Meanwhile, we had previously reported that dopamine D3 receptor activation in the hippocampal pyramidal cells causes suppression of evoked postsynaptic GABAergic IPSCs via dynamin dependent- endocytosis of $GABA_A$ receptors (Swant et al 2008). Hence it seems that cocaine, at the pyramidal cell synapses, suppresses postsynaptic GABAergic inhibition by reducing the surface expression of $GABA_A$ receptors, even though confirmation warrants further investigation. The property of cocaine to influence inhibitory neurotransmission could explain some of the discrepancies in results obtained from separate studies despite employing similar experimental set ups. For example, a few hours after i.p. injections of cocaine, (despite having almost similar experimental design), a steady depolarization protocol could induce (Liu et al 2005) or failed to induce (Argilli et al 2008) LTP in the VTA DA cells. One obvious difference between these studies is that Liu

et al, had conducted their LTP experiments in VTA slices in the presence of $GABA_A$ antagonist picrotoxin, while the latter group left the inhibitory transmission unblocked. In light of these observations, Michaeli et al., (2012) measured the AMPA- and NMDA- currents in the mid brain slices from rats chronically exposed to cocaine after leaving the GABAergic activity in the slices intact, which is in contrast to the conventional methods where glutamatergic currents are isolated by blocking GABAergic activity. They found that AMPA/NMDA ratio in the VTA DA cells measured in the absence of $GABA_A$ receptor antagonist picrotoxin was significantly greater than the ratio measured in the presence of antagonist (Michaeli et al 2012). A concurrent observation was made in this study as well, where we found that AMPA/NMDA ratio measured in the absence of picrotoxin (Figure 4.6) was significantly amplified when compared to a moderate increase in the ratio measured in the presence of picrotoxin (Figure 4.3). These observations suggest that cocaine's modulation of excitatory neurotransmission becomes further apparent under more physiological conditions, and that the changes in synaptic plasticity observed is an end result of its two-pronged effects on the excitatory and inhibitory neurotransmission in the region.

Despite an increase in the baseline excitatory synaptic transmission and potentiation at the synapses with a simultaneous suppression of the inhibitory circuits, LTP in the hippocampal slices were occluded in the cocaine self-administered rats (Figure 4.7.). The phenomenon of occlusion of synaptic plasticity mechanisms in hippocampal circuits was demonstrated previously. Synaptic adaptations occurring during spatial learning tasks which rely on hippocampal dependent associative learning are thought to share similar molecular mechanisms with the synaptic modifications occurring during electrically induced stimulation protocols. This rationale led to an interesting argument that if there is a limit to the neuronal synaptic plasticity,

then synaptic potentiation induced by external electrical stimulation should occlude a natural learning process, and vice versa. The supporting evidence for this hypothesis came when separate groups reported that LTP induced in hippocampus in live rats via electrical stimulation further impaired spatial learning tasks in these animals (Moser et al 1998; Worley et al 1993). Conversely, natural learning was also shown to induce saturating synaptic plasticity in neocortical circuits which further impeded the expression of LTP upon electrical stimulation (Rioult-Pedotti et al 2000). Furthermore, a single inhibitory avoidance learning in rats produced enhanced baseline excitatory transmission and greater field potential responses in such a way that further attempts to induce LTP by a HFS was prevented (Whitlock et al 2006). Occlusion was also demonstrated in the reward pathway regions in the context of addiction in rodents. Neither spike timing dependent- (Argilli et al 2008; Luu & Malenka 2008; Mameli et al 2011) nor electrically evoked-(Ungless et al 2001) stimulation protocols were able to induce LTP in VTA in animals previously exposed to cocaine. High frequency stimulation failed to induce LTP in the MSN synapses in the nucleus accumbens despite an enhancement of basal glutamatergic synaptic transmission (Moussawi et al 2009) during the withdrawal period after cocaine self-administration. Similarly, in the current study we observed that an HFS stimulation failed to induce a normal magnitude LTP at the CA3-CA1 synapses in the rats that experienced extended access cocaine self-administration sessions. This occlusion of LTP observed in these synapses could occur due to a saturation of synaptic potentiation or maladaptive synaptic modification induced by the repeated exposures to cocaine. Either way, the mechanism of synaptic plasticity is getting hindered in these circuits, indicating that chronic cocaine is hijacking the hippocampal neuronal circuits impeding further learning and memory formation. A concurrent finding was recently reported in rats where cocaine self-administration diminished the neuronal encoding

process in the nucleus accumbens resulting in poor learning ability of second- order pavlovian associations (Saddoris & Carelli 2014).

Rat models of psychostimulant addiction exhibiting typical addictive phenotypes- such as compulsive binging, seeking and taking of drugs despite the adverse consequences, and extreme tendency to relapse, could be generated by employing extended access self-administration protocols (Wolf & Tseng 2012). The synaptic plasticity changes observed in the VTA and NAc were more persistent in the rats which experienced extended access cocaine self-administration, when compared to those rats which received cocaine via experimenter administered injections or short access self-administration sessions (Chen et al 2008; McCutcheon et al 2011). Lack of effects on the hippocampal basal synaptic transmission in the previous reports (Keralapurath et al 2014a; Ungless et al 2001) in response to experimenter injected passive doses of cocaine also suggests the requirement to devise a judicious rodent model to study various aspects of addiction. In the light of results from the current study, it could be argued that extended access drug sessions subserve an opportunity for the hippocampal dependent memory system for a robust acquisition and consolidation of contextual memory associated with the drug episodes. Hence the changes observed in vH synaptic plasticity could be implicated in the persistence of drug associated memories and reflect a true cause-effect relationship implying the importance of hippocampus in the induction of reinstatement behaviors in addictive rats.

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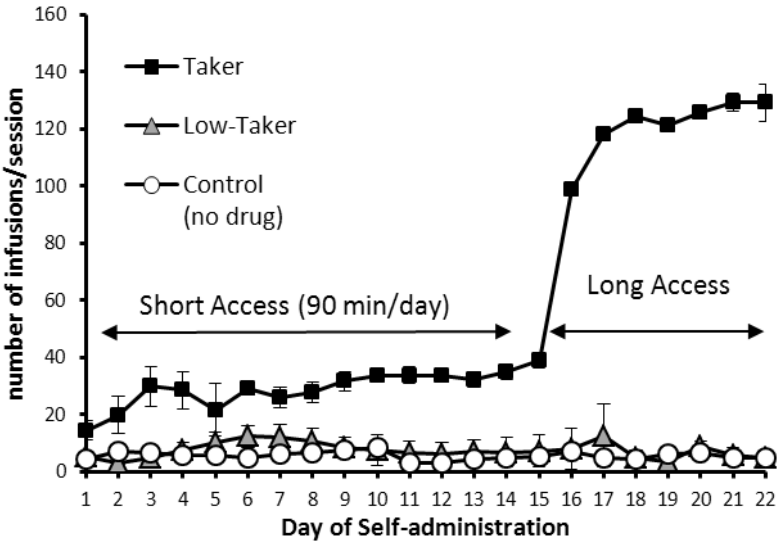


Figure 4.1. Self-administration behavior in rats. Average number of infusions per day on each cocaine (0.5mg/kg per infusion) self-administration session by the Taker- (■, n= 39) and Low-Taker- (▲, n=10) group of rats. A separate control group (no drug/saline (○, n=11)) group of rats were allowed to self-administer saline (0.125 ml/infusion). Days 1-15 were 90 minute- short-access sessions, while days 16 – 22 were 6 hour- long-access sessions. Transition to the long-access sessions resulted in an escalation of cocaine infusions only in the 'taker' groups of rats. Error bars ± SEM.

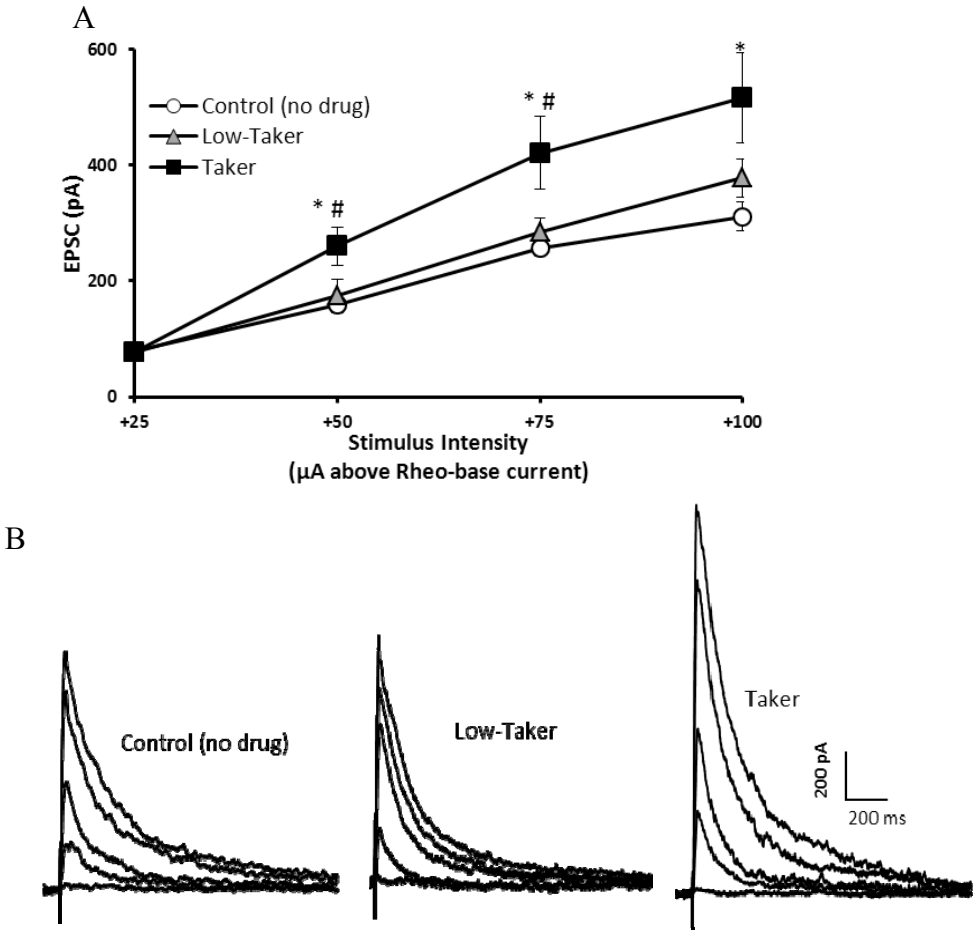


Figure 4.2. Cocaine enhanced baseline excitatory synaptic transmission in vH**.(A)** EPSCs evoked at baseline frequency at increasing stimulus intensities (25 µA increments) above the rheo-base current. EPSC amplitudes at +50 and +75 µA of Taker group (■, n= 9(9)) were significantly greater than the EPSCs of Controls (○, n= 9(12)) and Low-Taker (▲, n= 10(10)) at the same intensities, while EPSC at +100 µA of taker group was significantly greater than the saline group. * Significant difference between Taker vs. Control; # significant difference between Taker vs. Low-Taker ($p<0.05$, One way ANOVA followed by SNK post-hoc test, error bars ± SEM). **(B)** Representative EPSC sweeps at intensities ranging from 0 to +100 µA above the rheo-base current in the Control (left panel), Low-Taker (middle panel) and Taker (right panel) groups of rats.

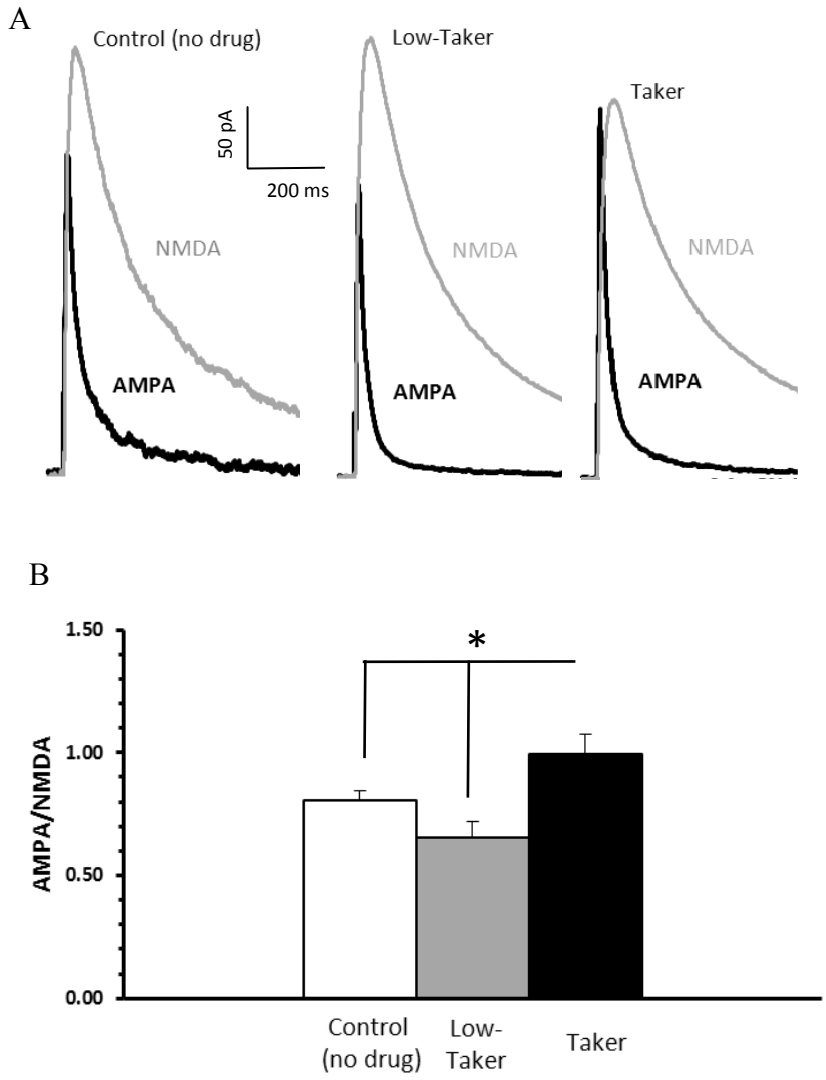


Figure 4.3. AMPA/NMDA ratio was enhanced in vH after cocaine self-administration**. (A)** Representative traces showing AMPA- (dark) and NMDA- (light) EPSCs recorded from the CA1 pyramidal cells of the ventral hippocampal slices from Control (left panel), Low-Taker (middle panel) and Taker- (right panel) groups of self-administration rats. **(B)** Summary quantification of the AMPA/NMDA ratio in the Control (n= 14(15)), Low-Taker (n= 9(10)) and Taker (n= 13(13)) rats. * Significant difference between the Taker group with Control or Low-Taker groups. One way ANOVA followed by SNK, $p < 0.05$; Error bars ± SEM.

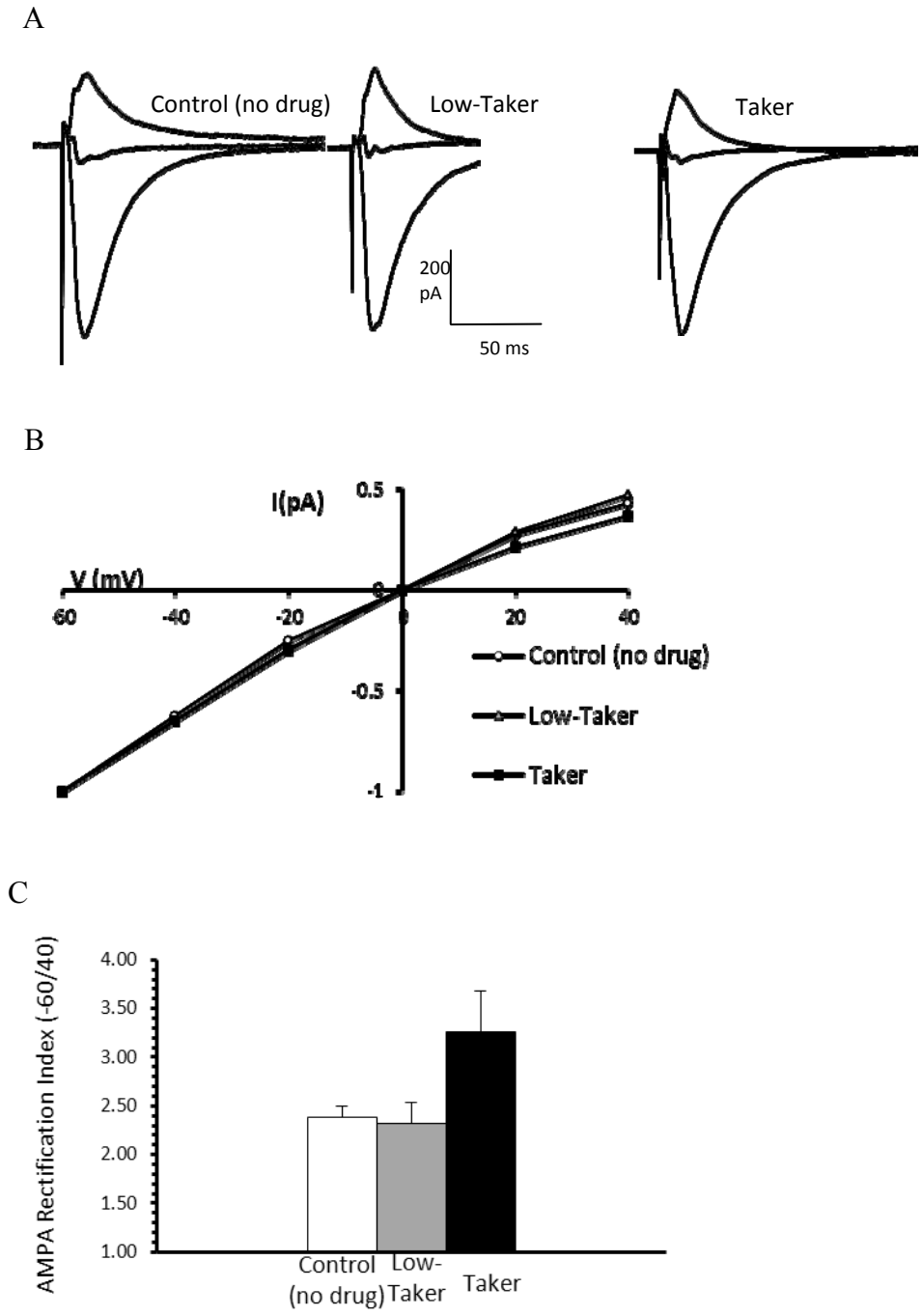


Figure 4.4. AMPA receptors showed inward rectification in the CA1 pyramidal cells after cocaine self-administration**.(A)** Representative traces of AMPA- EPSCs recorded at +40mV (top trace), 0mV (middle trace) and -60mV (bottom trace) from Control (left panel), Low-Taker (middle panel) and Taker- (right panel) groups of self-administration rats**.(B)** Normalized AMPA-EPSC amplitudes

plotted against the holding potentials at which responses were evoked. Taker (■, n= 13(13)) rats showed rectification trend at positive holding potentials while the current-voltage relationship was linear in the Low-Taker (▲, n= 9(10)) and Control (○, n= 14(15)) rats**.(C)** AMPA rectification index ($EPSC_{-60mV}/EPSC_{+40mV}$) showing a clear trend (not statistically significant) of increased inward rectification in the Taker groups when compared to the Control and Low-Taker rats.

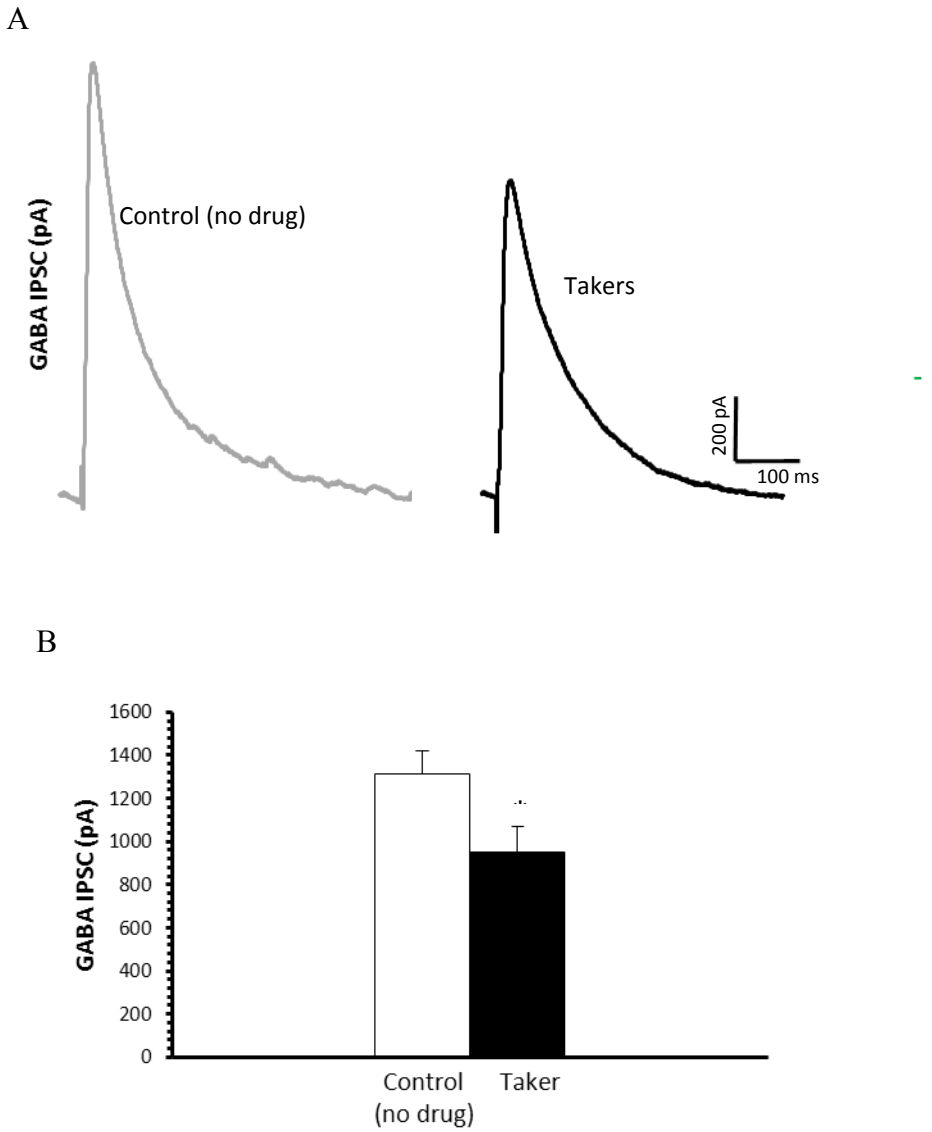
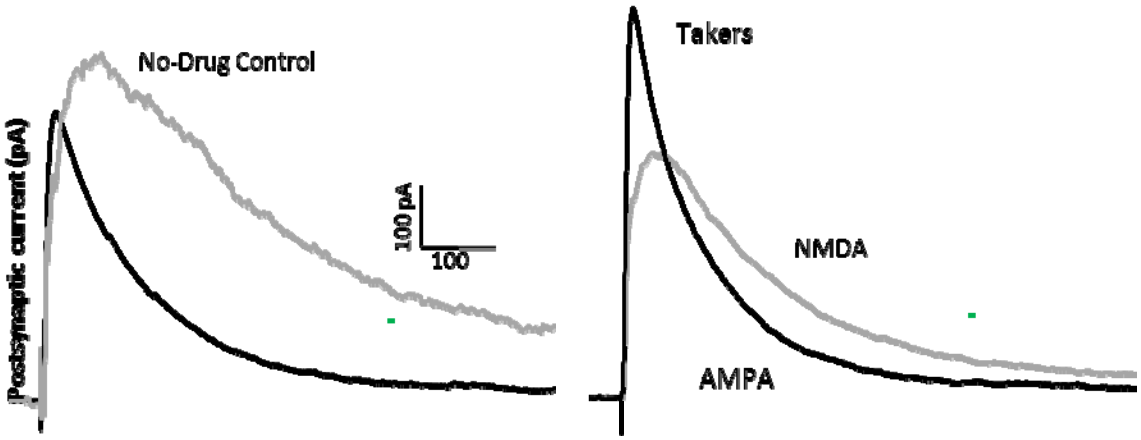


Figure 4.5. Cocaine self-administration suppressed GABAergic inhibition on the CA1 pyramidal cells. **(A)** Representative traces of GABAergic evoked IPSCs isolated at 0mV in the Taker (dark trace, right panel, n= 14(16)) group was significantly reduced when compared to the Control (grey trace, left panel, n= 12(12)). **(B)** Bar graphs showing mean amplitudes of evoked IPSCs in the Control and Taker groups of rats. * $p < 0.05$; unpaired t-Test, Error bars ± SEM.

A



B

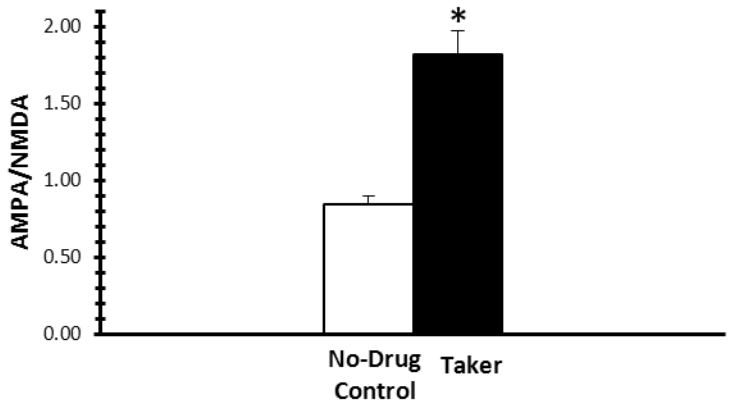
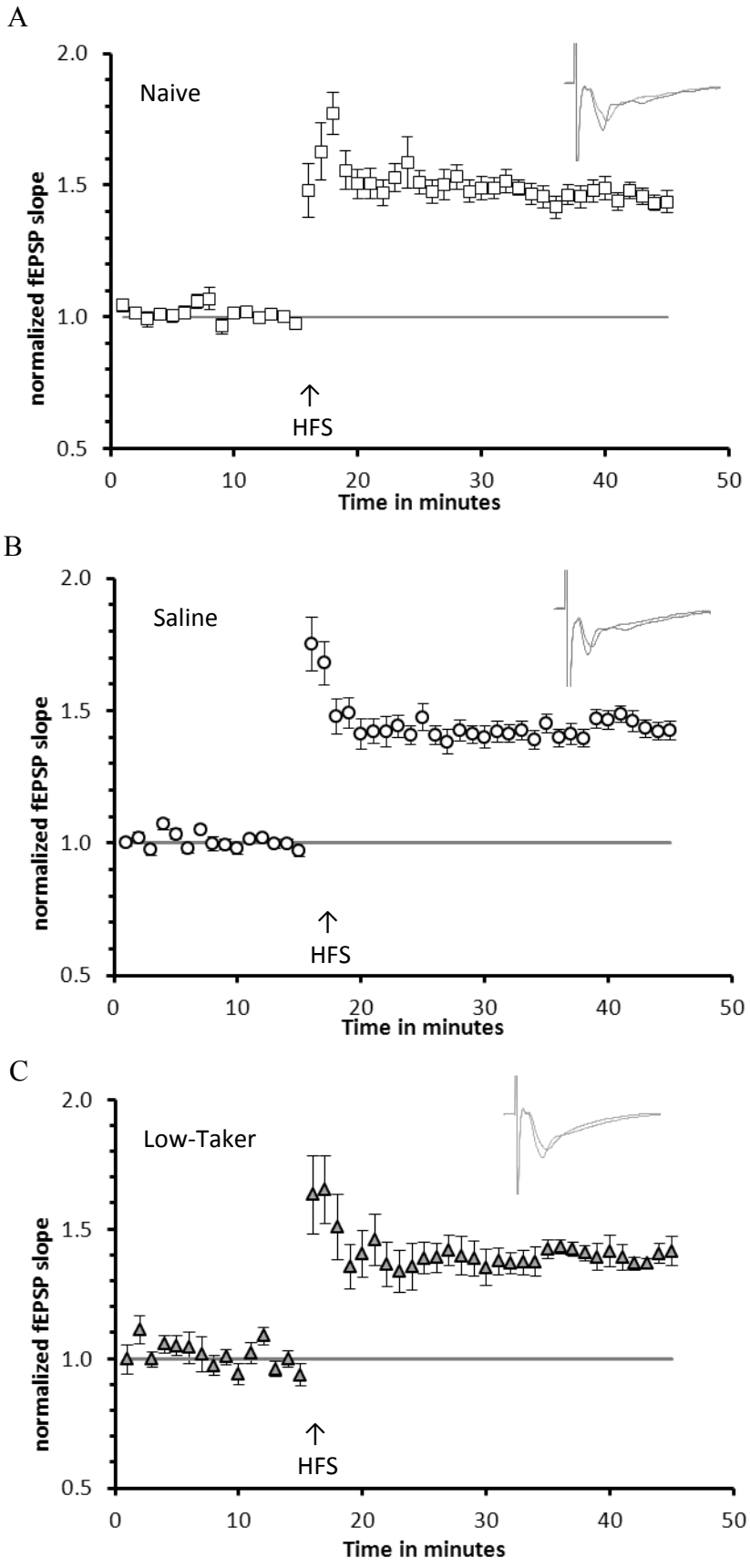


Figure 4.6. Cocaine mediated enhancement of AMPA/NMDA ratio was more pronounced in the presence of intact local inhibition in the vH. **(A)** Representative traces of AMPA- (dark trace) and NMDA- (light trace) EPSCs recorded at +40 mV in the presence of GABAergic inhibitory activity (i.e. $GABA_A$ antagonist picrotoxin was not used) in the Control- (left panel) and Taker- (right panel) groups**. (B)** Bar graphs showing AMPA/NMDA ratio in the Taker (n = 11(11)) rats was significantly enhanced that the Control group (n = 7(7)). Please note that this difference between the groups is less pronounced when $GABA_A$ activity was blocked with picrotoxin (refer to Figure 3.3). * $p < 0.05$; unpaired t-Test, Error bars ± SEM.


A
Naive
normalized fEPSP slope
2.0
1.5
1.0
0.5
HFS
0
10
20
30
40
50
Time in minutes
B
Saline
normalized fEPSP slope
2.0
1.5
1.0
0.5
HFS
0
10
20
30
40
50
Time in minutes
C
Low-Taker
normalized fEPSP slope
2.0
1.5
1.0
0.5
HFS
0
10
20
30
40
50
Time in minutes

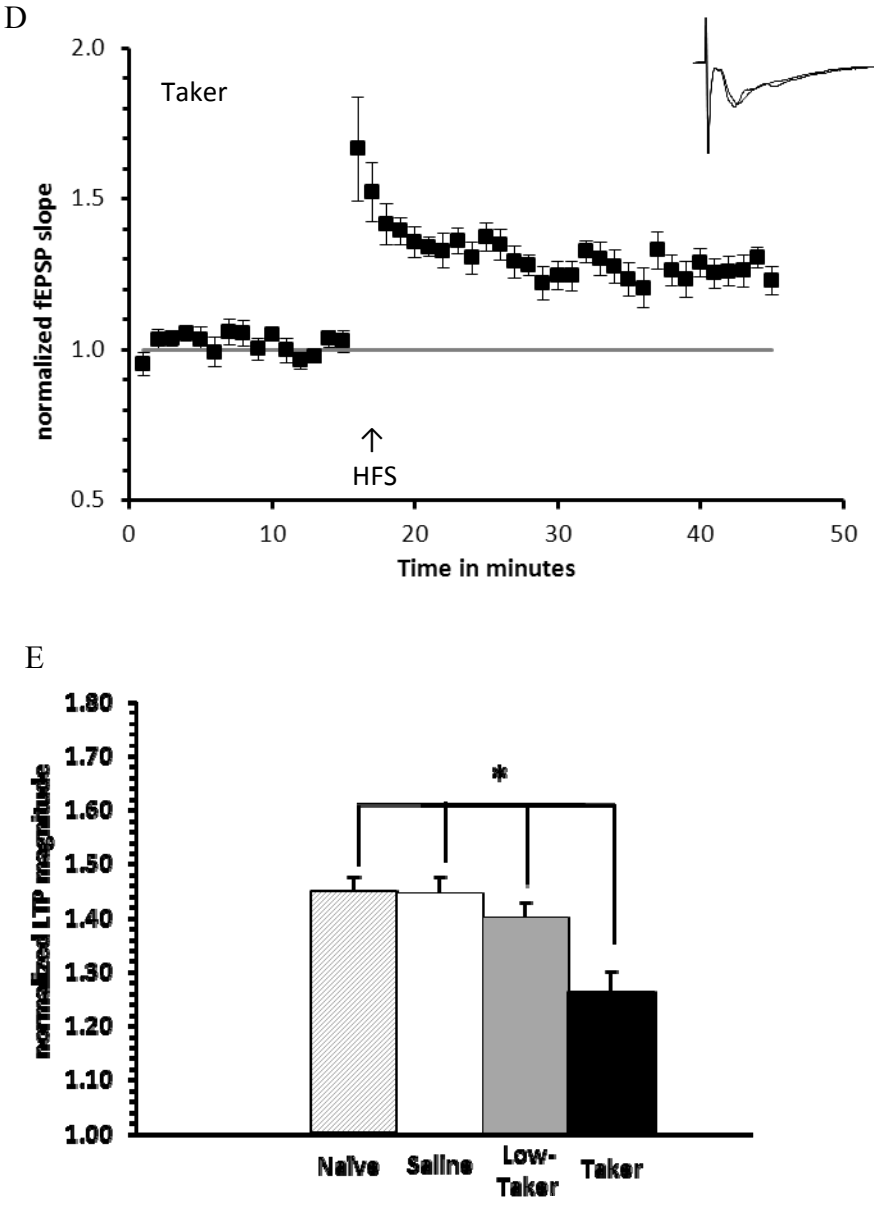


Figure 4.7. Cocaine self-administration occluded LTP in vH. Summary plot of normalized fEPSP slope measurements in the naïve (**A**, n = 10(26)), saline (**B**, n= 8(29)), Low-Taker (**C**, n= 5(9)) and Taker (**D**, n= 5(11)) groups of rats. X-axis shows time (minutes) for which fEPSP slope was measured prior - (15 minutes) and post- (30 minutes) tetanus. Inset shows the superimposed 50 ms representative fEPSP sweeps from the last 5minutes of the pre-tetanus

baseline and 25-30 minutes of the post-tetanus baseline. **(E)** Summary graphs showing the LTP magnitudes of the above groups which indicate LTP was significantly suppressed in the Taker rats. * $p < 0.05$; One-way ANOVA followed by SNK post-hoc test, Error bars ± SEM

CHAPTER 5

SUMMARY

The hippocampus is well understood as a learning and memory structure in the brain, being involved in the acquisition, processing and storage of various types of memory. Its roles in memory associated functions such as cognition, spatial navigation, novelty detection etc., have been recognized decades ago. However, now it is accepted that most of these functions ascribed to hippocampus are primarily dorsal sector-specific. In the last 20 years, with the advent of newer technologies and technical access to deeper tissues in the brain, the importance and functional distinction of the ventral sector of hippocampus from its dorsal counterpart have been receiving greater appreciation. Anatomical connections of the dorsal sector of hippocampus with higher order cortical structures involved in perception and memory imply its functional role in cognition, navigation, exploration etc.(Fanselow & Dong 2010). Meanwhile, anatomical integrity of the ventral hippocampus to the limbic and reward centers of the brain indicate its role in emotion and reward processing (Fanselow & Dong 2010). This current document has described studies conducted to further our understanding of how stress and exposure to the psychostimulant cocaine can affect synaptic plasticity in these two sectors of the hippocampus.

Addiction is a context dependent phenomenon, where previously drug-paired environments or paraphernalia trigger the 'wanting' of the drug leading to the relapse to drug seeking. One major hurdle in the successful treatment of addiction is the difficulty to prevent relapse (reinstatement in rodents) in human addicts. Behavioral and inactivation studies have demonstrated the role of hippocampus in the reinstatement of drug use in rodents (Rogers & See

2007; Sun & Rebec 2003). Electrical stimulation of ventral hippocampal formation has been shown to reinstate the drug seeking behavior in cocaine exposed rats (Vorel et al 2001). Anatomical and functional evidence shows the prominent connectivity of ventral hippocampus to the reward pathway (Britt et al 2012; Floresco et al 2001). Despite all this evidence, molecular and synaptic changes happening in the hippocampus in response to drugs of abuse exposure are understudied. Here we showed the electrophysiological evidence for the changes in the synaptic plasticity occurring in hippocampus in response to cocaine exposure. Moreover, we also demonstrate how the non-contingent and self-administration (contingent) modes of cocaine administration differentially affect the hippocampal synaptic transmission.

Conditioning associated stress and hippocampal plasticity

To investigate cocaine's effect on hippocampus, we conducted acute cocaine perfusion studies on *ex vivo* slice preparations, or used rat models to study the persisting effects of *in vivo* exposure to cocaine. The first set of *in vivo* studies employed a common behavioral protocol typically used to study the locomotor sensitization in rodents towards drugs (Chapter Three). In this non-contingent cocaine exposure protocol, rats were subjected to repeated daily i.p. injections of cocaine and subsequently tested for their locomotor activity. The behavioral manipulations involved in these procedures involves many potential stressors such as novelty exposure, repeated handling and injection procedures (Keralapurath et al 2014b). Though usually neglected, these factors were shown to induce acute stress effects in various regions of the brain, including hippocampus. So the results obtained from such experiments could be a combinatorial effect of stress and the drug under study.

Indeed, we showed that just by experiencing the locomotor sensitization protocol induces stress-like effects on the hippocampal synaptic plasticity in the saline-conditioned rats (Chapter

2). LTP magnitudes in the dorsal and ventral sectors were altered in such a way that dorsal hippocampal LTP was suppressed and ventral LTP was increased, simulating a stress-like effect (Maggio & Segal 2010). These effects stood persistent for at least 1-2 weeks after the last behavioral manipulations. Altered LTP was detected along with no observed changes in the basal synaptic transmission in the region. This suggests that minor stressors such as novelty, repeated handling and i.p. injections involved in the protocol are not strong enough triggers to cause direct changes in the synaptic plasticity, but could somehow prime the hippocampal circuit to express aberrant responses towards a future stimuli (in this case our LTP- inducing high frequency stimulation). Hence we could argue that stress-induced changes in the hippocampal LTP was metaplastic (plasticity of plasticity) phenomena. The results from our study could suggest that even mild degrees of stress which is not sufficient to elicit a physiologically prominent response, would confound the interpretation of experimental read-outs. There is evidence that physiological stress responses could interfere with the accurate estimation of toxicological endpoints such as LC_{50} (Pottinger & Calder 1995). Such insights are also necessary in experiments where the results cannot be directly quantified and where interpretations could be made only by comparing with the control groups in the study. Furthermore, if the animal groups designed as behavioral or injection controls are under the influence of stress sufficient to modulate the neuroendocrinological and synaptic mechanisms, the reliability of treating such groups as real controls is under question.

Cocaine & hippocampal plasticity

Cocaine conditioning itself also resulted in altered hippocampal LTP, which was independent of the stress-induced effects (Chapter 3). However, cocaine specifically enhanced the LTP only in the ventral hippocampus, with no change observed in the dorsal sector.

Cocaine's specific targeting of the ventral hippocampus was further confirmed by the acute perfusion studies where cocaine perfused onto the naïve slices specifically enhanced LTP in the ventral slices, but not in the dorsal slices. Similar to the conditioning effect, cocaine exposure did not cause changes in the basal synaptic transmission in any of the sectors, and hence it could be also called as a metaplastic phenomenon. Stress and cocaine induced metaplastic phenomena were found to be functioning through different signaling pathways. This was clear when the kappa opioid antagonist could successfully block the stress-induced metaplasticity, but not cocaine's effect on the ventral hippocampal LTP. Even further, we also found that cocaine induced metaplasticity in the ventral hippocampus was a phenomenon independent of the locomotor sensitization towards cocaine in rats. Locomotor sensitization in rats towards cocaine could be blocked by dopamine $D_{1/5}$ and $D_{2\text{-like}}$ receptor antagonists, while the metaplasticity in ventral hippocampus was blocked only by the $D_{2\text{-like}}$ receptor antagonists.

Though the locomotor sensitization model helps to study the effects of *in vivo* cocaine exposure on the hippocampal neurotransmission, it does not sub-serve the purpose of generating a true rodent model of addiction. In the non-contingent model of drug administration, the experimenter, and not the animal, determines the dose and frequency of drug intake. One main attribute of addiction, especially in the initial stages of drug use, is the motivation-driven drug seeking and taking. However, in the non-contingent drug protocol, there is no measurable motivational component involved. This is a major caveat for such rodent models of addiction. Lack of a true rodent model of addiction in many studies has impeded the efforts to find the hippocampal involvement in the development of addiction to the drugs of abuse (Ungless et al 2001). The most widely accepted model is the one which shows typical addictive behaviors such as compulsive seeking and taking drugs despite adverse consequences, frequent binging and an

extreme tendency to relapse (Ahmed 2012; Wolf & Tseng 2012). Such models could be generated by employing self-administration protocols where the animals are exposed to drugs of abuse for longer duration (approximately 6 hours each day). Additionally, the effect of behavioral conditioning induced stress is very minimal in these self-administration models.

Our cocaine self-administration protocol was comprised of short- access sessions (90 min/session/day) for 15 days, followed by extended (long) -access self-administration sessions for 1 week (6hr/session/day, Chapter Four). The initial short sessions simulate the acquisition phase of cocaine abuse, where the addictive humans begin to use the drugs. The long- access sessions results in the escalation of drug use in rats, which is similar to the compulsive binging phase in human addicts (Everitt & Robbins 2005). We investigated the effects of such cocaine exposure on the hippocampal glutamatergic and GABAergic synaptic plasticity 3-5 weeks following the last cocaine session. Interestingly, we observed a significant enhancement in the basal synaptic transmission in the CA1 region of ventral hippocampus of rats which self-administered cocaine over longer sessions. This was different from the unaltered baseline synaptic transmission which we observed in the non-contingently exposed group, indicating that the repeated self-administration sessions of cocaine are acting as strong enough triggers to alter the synaptic plasticity in the ventral hippocampus. Additionally, the learning and motivational components in the self-administration protocol might be actively relying upon hippocampal dependent associative learning processes to acquire memories of drug episodes.

The enhancement of baseline synaptic transmission was found to be mediated by augmented glutamatergic neurotransmission in the region as evidenced by significantly higher AMPA/NMDA ratio in the cocaine taker groups. Furthermore, we found that the baseline GABAergic transmission was significantly suppressed in the cocaine taker rats. However,

despite the activation of excitatory transmission and a simultaneous suppression of inhibitory activity, the LTP expression in the ventral hippocampus of cocaine takers was significantly suppressed. This could mean that the repeated binging of cocaine over a period of 3 weeks might have resulted in a saturating level of potentiation at the CA3-CA1 synapses, which occluded a further potentiation by our tetanus stimuli. Another possibility exists that cocaine exposure resulted in some maladaptive changes in the circuit which impeded further synaptic plasticity. Either way, hippocampal learning and memory dependent plasticity processes seem to be impaired after cocaine self-administration. This is in concurrence with a recent study showing that cocaine self-administration induced changes in nucleus accumbens that impaired the animal's ability to learn second-order Pavlovian associations (Saddoris & Carelli 2014).

Implications in addiction

According to the 'glutamate homeostasis' hypothesis, impaired glutamate neurotransmission in the nucleus accumbens is contributing to many of the behavioral attributes such as withdrawal symptoms, craving and relapse associated with addiction (Kalivas 2009). Nucleus accumbens medium spiny neurons synapse with many glutamatergic afferents arising from medial prefrontal cortex, amygdala, and hippocampus (Britt et al 2012; MacAskill et al 2012). Medial prefrontal cortex glutamatergic regulation of the reward pathway has been known for many years now (Lu et al 2010). However the appreciation for the involvement of the hippocampal glutamatergic pathway in the reward associated behaviors is on the rise (Britt et al 2012; Floresco et al 2001). Britt et al., found that nucleus accumbens synapses with ventral hippocampal glutamatergic afferents were potentiated after the cocaine exposure. Here we demonstrated that the pyramidal cell synapses in the ventral hippocampus are also potentiated, with a general enhancement of baseline synaptic transmission in the region. We argue that the

potentiation observed at the MSN- glutamatergic fiber synapses (Britt et al 2012) is consequent to the potentiation and enhanced activity at one synapse upstream - at the CA1 pyramidal cells that we demonstrated in the current report. Hence, we could argue that cocaine- induced persistent alteration of synaptic plasticity in the ventral hippocampus could be a key process in the development of addiction and crucial for its involvement in the relapse/reinstatement behaviors in addicts.

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