MODULATION OF VENTRAL PERIAQUEDUCTAL GRAY DOPAMINE NEURONS

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum of Neurobiology

Chapel Hill 2013

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ABSTRACT

CHIA LI: Modulation of Ventral Periaqueductal Gray Dopamine Neurons (Under the direction of Dr. Thomas L. Kash)

Dopamine (DA) neurons within the ventral periaqueductal gray (vPAG) regulate reward, as well as negative emotional behaviors that often lead to addiction relapse. Due to the cell-type heterogeneity of the vPAG, little is known about the functional properties of these neurons, or how drugs of abuse, such as opioids and alcohol, modulate them. In these studies, transgenic mouse lines were used to evaluate the properties, projection, and functional modulation of vPAG DA neurons. Alcohol modulation of synaptic transmission was examined; acute alcohol had minimal effects on GABA transmission, but resulted in a robust enhancement of glutamatergic transmission onto vPAG dopamine neurons, as well as an increase in firing rate of these neurons. Interestingly, chronic intermittent alcohol exposure produced no significant alterations on either inhibitory or excitatory synaptic transmission, suggesting that alcohol has both region- and cell-typedependent effects on function. Negative emotional behaviors during withdrawal, a critical component of drug addiction, can often lead to relapse, making the study of such behaviors relevant and essential. The kappa opioid receptor (KOR) system has been implicated in disruption of affective behaviors including depression, anxiety, and drug abuse, some of which are mediated via dopamine signaling. Previous studies have shown that stress-induced dysphoria can augment KOR expression in dopamine-rich brain regions, providing a link between kappa opioid modulation and dopamine signaling. Due to the link between dopamine, drug abuse, and the behavioral relevance of the ventral periaqueductal gray (vPAG), this work aimed to elucidate the mechanisms underlying opioid modulation of GABAergic inputs onto vPAG DA neurons. In this study, activation of KOR significantly reduced GABAergic inhibition of vPAG DA neurons. In addition, mechanistic investigation suggested that this effect was mediated via presynaptic mechanisms of the G-protein coupled receptor $\beta\gamma$ subunit. Finally, this work demonstrated that the vPAG DA neurons are glutamatergic, and project to the bed nucleus of stria terminalis (BNST), a part of the extended amygdala that regulates stress-related behavior. Using viral tools, the potential of vPAG dopamine neurons in modulating behaviors, as well as activity of projection targets were demonstrated. Therefore, this work provides insight into the regulation of negative affective behavior for potential improvement in treatments for emotional disorders and drug abuse.

To my father, 李林峰, and my mother, 劉淑鎂, for their love and support.

感謝你們無私無條件的寵愛、教誨與支持。

此論文不足彰顯從小至今你們對我的付出,但我對你們的感謝及關愛永無止盡。

ACKNOWLEDGEMENTS

First and foremost, I wish to express my gratitude towards Dr. Thomas Kash for his infinite advice, patience, and support. The training I have received under his guidance will undoubtedly benefit me throughout my scientific career. My committee members, Dr. McCarthy, Dr. Hodge, Dr. Johns, and Dr. Criswell, have been very helpful offering their time, knowledge, insightful suggestions and guidance through the process of this dissertation. This work would not have been possible without the hard work and support from everyone in the Kash lab past and present, especially Nora McCall, Alberto Lopez, and Dr. Emily Lowery-Gionta, for advices towards the writing of this dissertation. Additional recognition is required for my undergraduate mentor Dr. Rick Bevins, who lead me into the world of research and guided me through the early stages of being a scientist; and throughout my graduate career, Dr. Darin Knapp has been a good friend, and a wonderful mentor. I would like to thank all my friends and family in Taiwan and the United states for their moral support throughout this process. I especially want to thank Brian Mehl for his unwavering support, patience, and love, and for sharing his life with me. The primary funding for this project came from a grant from the National Institute on Alcohol Abuse and Alcoholism (AA017668). The last academic year of my graduate program was additionally funded by the UNC Graduate School Dissertation Completion Fellowship.

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LIST OF ABBREVIATIONS

BNST	bed nucleus of the stria terminalis
cAMP	cyclic adenosine monophosphate
DA	dopamine
ERK	extracellular signal-regulated kinase
GABA	gamma-aminobutyric acid
GRK3	G-protein coupled receptor kinase3
JNK	c-Jun N-terminal Kinase
mEPSC	mini excitatory post-synaptic current
mIPSC	mini inhibitory post-synaptic current
PI3K	phosphoinositide 3-kinase
РКА	protein kinase A
РКСζ	protein kinase C zeta
SEM	standard error of the mean
sIPSC	spontaneous inhibitory post-synaptic current
TH	tyrosine hydroxylase
vPAG	ventral portion of the periaqueductal gray
VTA	ventral tegmental area

CHAPTER 1. GENERAL INTRODUCTION

DOPAMINE SIGNALING

For well over three decades, dopamine (DA) signaling has been known to play a critical role in reward and motivation, as well as the establishment of stimulus-reward association (1-3). Dopamine release in the striatal structures can be increased by reward, such as food, drug intake and sexual behavior, while dopamine antagonists can prevent these behaviors (4-7). Reward association, such as the ability to be trained to lever-press for naturally rewarding things as food, water or sexual contact, requires dopamine signaling, as reward association cannot be learned when dopamine function is impaired (8). Additionally, previously acquired stimulus-reward associative learning cannot be continued or maintained when dopamine systems are blocked (2, 9, 10). The DA pathways that modulate behavioral responses to environmental stimuli are central to the signaling of reward.

These pathways include dopamine neurons in midbrain nuclei such as the ventral tegmental area (VTA) and the substantia nigra pars compacta (SN) that project to striatal structures, (nucleus accumbens, NAc, and the dorsal striatum), limbic structures (amygdala and hippocampus), and cortical regions. Drug and food reward have been shown to lead to an increase in dopamine levels in the nucleus accumbens (11-13), suggesting that dopamine signaling to this nucleus from the VTA plays an important role

in the rewarding properties of these stimuli. Further, selective chemical lesions of NAcprojecting dopamine neurons lead to the attenuation of the rewarding effects of cocaine and amphetamine (14), while this effect was not found in animals with ablation of norepinephrine-expressing neurons. Dopamine pathways regulate motivational behaviors, demonstrated by motivational deficits in feeding and water drinking caused by dopamine lesions in the lateral hypothalamus (15). Striatal pathways regulate movement and reward: the dopamine D1-mediated direct pathway promotes movement and reward while the dopamine D2-mediated indirect pathway activation increases immobility and appears to serve as punishment (16, 17).

Dopamine is generally thought to play a critical role in the formation and maintenance of addictive behaviors in drug use (18-21), but despite the large body of evidence supporting this notion, the necessity of dopamine signaling in all drug reward remains controversial. Several drugs of abuse such as phencyclidine (22), morphine (23), and nicotine (24) have demonstrated both dopamine-dependent and dopamineindependent rewarding effects. Taken together, the importance of dopamine in rewardrelated behavior is undeniable; however, dopamine might not be required or limited to reward behaviors as studies find dopamine involvement in other complex emotional behaviors and conditions.

Many psychiatric and neurological disorders have been associated with a severe dysregulation of the brain reward system. There are strong correlations between movement, reinforcement, and reward in many neurological and psychiatric diseases, such as Parkinson's disease and Schizophrenia. Depression is a common symptom of Parkinson's disease (25) and is described in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR2000) by deficits in reward function, coupled with heightened punishment from negative consequences. Depressive individuals often display psychomotor retardation, consisting of slowing of speech, eye and body movement, as well as abnormal body posture and facial expressions (26, 27), overlapping with the symptoms of Parkinson's disease caused by dopamine deficiencies. Interestingly, these motor abnormalities caused by dopamine deficiencies in Parkinson's disease can be alleviated by dopamine agonists (28). In addition, medications for Parkinson's disease that improve motor deficits, such as dopamine agonists and monoamine oxide inhibitors, have also been found to relieve Parkinson-associated depression (29-31).

Depression is one of many negative emotional disorders modulated by dopamine; studies have shown that dopamine neurons can also modulate negative affective states such as anxiety. In these studies, select inhibition of VTA dopamine neurons induced depressive-like phenotype (32), and the ablation of kappa opioid receptors in dopamine neurons decreased anxiety-like effects in the open field and light/dark box behavioral assays (33). Additionally, dopamine D3 knockout animals display a decrease in depression-like behavior after immobilization stress (34). This evidence demonstrates the involvement of dopamine signaling in negative affective disorders, suggesting a regulatory role of dopamine signaling in not only reward, but also the development and maintenance of negative emotions.

The majority of dopamine research leans heavily on the mesolimbic and striatal pathways mentioned earlier. However, recent studies suggested that other populations of dopamine neurons in the midbrain are positioned to regulate both addiction and negative affective disorders. The following section discusses the periaqueductal gray and its role in dopamine signaling.

PERIAQUEDUCTAL GRAY

The periaqueductal gray is a highly heterogeneous brain region; in addition to the dopamine neurons, there are large populations of GABAergic and glutamatergic neurons (35). Due to the diversity of its cell types, the PAG also has a variety of functional outputs that modulate a wide array of behaviors, including reward and autonomic functions, as well as negative affective behaviors such as panic and aggression. The PAG's complex composition presents difficulties when trying to isolate neural networks and examine the functions of specific cell types. With the advent of transgenic animal models, as well as viral tools, the roles of periaqueductal gray neurons are slowly being unraveled.

The involvement of the PAG in both reward and negative affect position this structure in a critical role of modulating drug-seeking behaviors and the associated negative emotional behaviors that often accompany withdrawal. In support of this possibility, recent studies have revealed projections from the PAG to regions involved in reward circuits, including the VTA, and the SN (36). Such studies have also illustrated synaptic connections onto specific populations of VTA neurons, including dopaminergic neurons (37), further demonstrating a role of this projection in the rewarding effects of drugs. In addition to reward-related behaviors, recent studies suggest the existence of a PAG dopaminergic projection to brain structures known to regulate negative emotions. An example of such a structure is the bed nucleus of the stria terminalis (BNST), where dopamine and corticotropin-releasing factor (CRF) interact to regulate fear and anxiety

(38). Other studies have shown that modulation in the PAG can influence emotional behavior: antagonism of the GABA receptors in the PAG increases anxiety-like behavior (39). Further, the sensitization of the PAG has been shown to suppress positive affect in rats (40), suggesting that increased activity in the PAG is heavily engaged during production of negative emotional behaviors and the maintenance of negative affective states. The PAG is also an important site in the ascending nociceptive control (ANC) pathway projecting to the rostral ventral medulla and there by regulates pain-related behaviors (41). There is evidence that dopamine neurons in the PAG may potentially play a role in the ANC as opiate anti-nociception is attenuated upon chemical lesion of PAG dopamine neurons (42). The PAG DA-mediated negative emotional behaviors (44), and depression (45), have all been shown to exacerbate pain perception, and *vice versa*, suggesting that the regulation of these negative affects and their projections could be modulated in a similar fashion by certain groups of PAG DA neurons.

Another PAG dopamine-mediated behavior heavily linked to a negative emotional state is sleep. Selective chemical lesioning of dopamine neurons in the PAG leads to an increase in sleep time (46), illustrating the critical role of PAG dopamine projections to the prefrontal cortex in sleeping behavior. At the same time, deprivation of sleep is tightly associated with emotions such as stress (47), anxiety (48), depression (49), as well as pain (50), negative affective states already known to be influenced by PAG dopamine neurons. Given the knowledge of the cell-type diversity in PAG and its projections to a variety of brain regions linked to negative affects that are closely associated with one another, the PAG has become a region of interest for the treatment of affective disorders. Other behaviors mediated by PAG includes defensive behaviors (51), cardiovascular functions via its projections to the hypothalamus (52, 53), and lordosis behavior in sexual courtship (54).

As mentioned earlier, dopamine signaling mediates the rewarding properties of drugs of abuse, including alcohol. An abundance of evidence highlight the importance of the role the PAG plays in the negative affect specifically associated with alcohol abuse. Withdrawal following chronic ethanol exposure can induce hyperalgesia (55), suggesting a link between the PAG ascending nociceptive pathway and alcohol abuse. Further, dorsal PAG stimulation-induced freezing and escape was sensitized during ethanol withdrawal (56), and neurons in the PAG are critically involved in the neural circuitry for audiogenic ethanol withdrawal seizures (57). Taken together, this evidence illustrates that PAG-mediated behaviors can be altered by prolonged alcohol exposure. Additionally, alcohol can affect neural activity in the PAG, causing increased action potential frequency and hyper-excitability in PAG neurons during ethanol withdrawal (58). These studies provide essential information that links PAG function to alcohol abuse and related negative affects. It is important to note that the PAG has also been implicated in the withdrawal from diazepam (59) and opiates (60), both of which are popular substances of abuse. Given the recent findings that dopamine neurons in the PAG project to areas sensitive to drugs of abuse, it is compelling to investigate the modulation and functions of these A10dc dopamine neurons.

Several inputs of the PAG have been identified to play important roles in modulating PAG neural activity. Consistent with the knowledge that the PAG mediates negative emotional behaviors through projections to the extended amygdala (the BNST and central amygdala), a number of studies have demonstrated projections from the extended amygdala onto the PAG in regulating negative emotional behaviors as well (61-63). These extended amygdala projections to the PAG have been demonstrated to be GABA-mediated inhibitory inputs and modulate anxiety-like behaviors (38, 64, 65). In addition, studies have demonstrated a population of tonically active GABAergic neurons in the lateral portion of the PAG (66). Although local GABAergic interneurons have been shown to inhibit dopamine projection neurons in other dopamine-rich brain regions such as the VTA (67), the role of this local population of GABAergic neurons on vPAG dopamine neurons is unclear. The PAG also receives glutamatergic inputs from the lateral habenula (68, 69) that are associated with pain (70, 71), defensive behaviors (72), and sleep (73), and other projections to the PAG have also been found from the prefrontal cortex (74) and the brain stem (75, 76). These studies identified some projections that could contribute to the modulation of PAG-mediated behaviors; however, despite all the anatomical and synaptic studies conducted in the PAG, specific dopamine neuronmodulation has yet to be investigated due to the heterogeneity of the PAG. The investigation of how drugs of abuse modulate the PAG requires a thorough understanding of the actions of drugs, as well as their effects on neural networks and substrate alteration. The following section focuses on the effects of acute and chronic alcohol.

ALCOHOL

Alcohol addiction

Addiction is defined by the continuous use of a substance despite its negative consequences. Alcohol dependence is diagnosed based on three or more of the following criteria outlined by the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV, 1994): tolerance, withdrawal, drinking a larger amount or longer period than intended, inability to control use, a large amount of time spent in alcohol-seeking and related activities, missing important social/occupational/recreational activities due to alcohol use, as well as the continuation of use despite knowledge of having persistent physical or psychological problems caused by alcohol. Initially, alcohol consumption seems to be driven largely by its positive reinforcing effects (i.e. the euphoric effects of ethanol) (77). Over prolonged exposure, there is a proposed shift to a negative reinforcement, as negative affect is increased due to withdrawal. It is believed that the negative affective state drives an individual's consumption of ethanol to alleviate the symptoms (78), creating a vicious cycle strengthened by repeated exposure. The negative affective disorders induced by ethanol exposure include depression (79, 80), anxiety (81), panic disorder (82), stress (83-85), and pain (86). The manifestation of these psychiatric disorders has been shown to render an individual more prone to relapse, and could cause relapse even long after the withdrawal period (87, 88). Additionally, repeated ethanol withdrawal episodes enhance negative affective symptoms (89). Due to the causative relationship between negative mood states and ethanol use, individuals easily fall into a cyclic addictive behavior pattern. The high comorbidity of alcohol addiction and negative mood disorders often presents difficulties in the treatment of alcohol abuse; therefore, understanding the mechanisms underlying the formation of these negative affective symptoms and their modulation is critical for the treatment of alcoholism.

Acute Alcohol

Acute alcohol interacts with a variety of presynaptic and postsynaptic proteins to modulate synaptic activity, often altering the balance between inhibitory and excitatory

transmission. A large body of evidence shows that acute alcohol enhances the function of Cys-loop ligand-gated ion channels (LGIC) (90, 91). Among the various types of LGIC are the inhibitory glycine receptors and GABA_A receptors. While the majority of studies show alcohol-induced enhancement of LGIC functions, a few of exceptions show an inhibition of GABA_ARs (92, 93) and other LGICs (94, 95) upon acute application of alcohol. Putatively, alcohol enhances ion channels by either increasing agonist affinity (96) or encouraging the state of open channels (97). It has been shown that alcohol increases GABA-mediated inhibitory current amplitude and duration in the brain stem (98, 99), the basolateral amygdala (100, 101), the central amygdala (102), the hippocampus (103), and the cerebellum (104). Both glycine and GABAA receptors mediate inhibitory chloride currents, and both receptors are pentameric receptors that can be composed of a wide range of subunits and subunit compositions. Each subunit type can further be differentiated into numeric-variations, which can alter the pharmacological and electrophysiological properties of receptors. Acute alcohol can modulate channels both synaptically (98, 105, 106) and extra-synaptically (107-109); synaptic GABA_A receptors are composed of two α , two β , and one γ subunits, while extra-synaptic GABA_A receptors are high affinity receptors (110) and contain a δ subunit instead of γ . Alcohol has been reported to modulate GABA receptors containing specific subunit compositions, such as $\alpha/\beta/\gamma$ -subunit-containing receptors, as well as those containing $\alpha 4$ or $\alpha 6$ along with β and δ subunits (111-113). Potentiation of glycine-mediated tonic current by alcohol has been observed in the cerebellum (110), the hippocampus (114, 115), and the thalamus (116) in a subunit composition-dependent manner (117, 118).

Opposite from the impact on inhibitory transmission, acute alcohol has consistently been shown to exert inhibitory actions on excitatory cation-permeable ionotropic glutamate receptors: the AMPA receptors, the NMDA receptors, and the kainite receptors (119). Alcohol-inhibition on NMDARs has been observed in many brain regions, from the hippocampus to the amygdala, as well as the cerebellum (120-123). Much like the inhibitory receptors, alcohol also affects NMDA receptors differentially in a subunit-dependent manner. NMDA receptors are tetrameric and are composed of an obligatory NR1 subunit, in combination with at least one NR2 or NR3 subunit. The combination of different splice variations of the NR1 and accompanying NR2 can account for alcohol sensitivity (124), and although receptors containing NR3 subunit are relatively insensitive, the combination with NR2B could overcome the presence of NR3-insensitivity and enhance alcohol-induced inhibition (124). Glutamatergic AMPA receptors are inhibited by alcohol as well; however the impact of inhibition of AMPA receptors is not as robust as NMDA receptors (125-127). In addition, subunit composition of AMPA receptors has minimal influences on alcohol sensitivity (128), which could be due to alcohol-induced receptor desensitization (129). The above evidence suggests that acute alcohol has a consistent effect on ionotropic glutamatergic receptors and decreases glutamatergic currents.

Long-lasting changes in synaptic transmission could contribute to the formation of addiction (130), and are usually produced by repeated activation of specific pathways. The ability of acute alcohol to augment inhibitory tone to attenuate excitatory transmission explains the observation that long-term potentiation (LTP) in transmission is suppressed by alcohol in the hippocampus (131) and the amygdala (132). These data raise the potential of acute alcohol to induce synaptic plasticity in areas known to regulate learning and memory, as well as negative emotional behaviors, all of which are highly correlated with alcohol addiction (133).

Chronic Alcohol

Prolonged alcohol exposure often results in two behavioral alterations: tolerance and dependence. These changes make the treatment of alcoholism more difficult, as tolerance encourages overuse due to the physiological habituation to lower doses, and dependence is diagnosed during withdrawal when individuals experience negative affect such as pain, anxiety, sleep loss, and seizure in the absence of alcohol. Both of these behavioral modifications encourage continuing alcohol usage to achieve the desired intoxication level, as well as to avoid the negative affect of withdrawal. Changes in behavior arise due to shifts in synaptic transmission in neural pathways in a variety of brain regions. In this section, the effects of chronic alcohol are addressed in the context of the balance between inhibitory and excitatory transmission. It is important to note however, that there are many different chronic alcohol exposure and withdrawal paradigms. Depending on the brain region, experimental design, length of exposure, number of repeated withdrawals, as well as the time point during withdrawal, a large discrepancy can be observed in the results.

There has been some evidence that chronic alcohol can modulate presynaptic GABA release. Some evidences show that chronic alcohol can modulate presynaptic GABA release in brain regions such as the hippocampus where a reduction in long-term potentiation was observed via an increase in the evoked release of tritiated GABA without affecting uptake (134). Studies in the central amygdala (CeA) suggest that there

is an increase in evoked GABA release, as well as an enhancement of mIPSC in CeA neurons after chronic alcohol. Further, dependent animals show dramatically increased GABA release in the CeA upon in vivo microinjection of alcohol directly into the CeA relative to naïve animals, suggesting an increase GABAergic tone after chronic alcohol exposure (135, 136). In contrast, some studies show that voluntary drinking is associated with a significant increase in paired-pulse ratio, suggesting a decrease in GABA release probability in the dentate gyrus neurons of monkeys (137). Together, these studies demonstrate that chronic alcohol can alter presynaptic GABA release.

In contrast, a large body of studies has found a significant impact of chronic alcohol on postsynaptic GABAergic transmission. GABA receptor subunit expression has been found to change after chronic alcohol exposure and affect receptor composition, functions, and binding. For instance, in the cerebral cortex, chronic alcohol has been shown to differentially alter mRNA and protein expression. Specifically, levels of $\alpha 1$, $\alpha 2$, and α 3 were decreased after chronic alcohol exposure, while α 4, β 1, β 2, β 3, γ 1 and γ 2 subunit levels were increased (138, 139). Results in the cerebellum mRNA expression are consistent with decreases in the α 1 subunit and increases in the α 6 following chronic ethanol exposure (139, 140). Chronic ethanol administration also decreased polypeptide levels of the δ subunit in the rat cerebellum and hippocampus, but not in the cerebral cortex (141). Notably, the majority of results from hippocampus and cerebral cortex studies are exposure- and time point-dependent (139, 142). Likewise, in the VTA, $\alpha 1$ subunit immunoreactivity was decreased in a 12-week exposure paradigm, but not a 1-4 week paradigm (143, 144). Both α 1 and α 4 immunoreactivity decreased in the amygdala after two weeks of alcohol exposure, while in the nucleus accumbens, $\alpha 4$ decreased and

 α 1 levels remained unchanged (144). In addition to the alteration in receptor subunit expression, other proteins could play important modulatory roles in receptor initiation and trafficking, altering receptor functions after chronic alcohol. These modulatory proteins include clathrin and adaptor complex, which are crucial for GABA_A receptor internalization (145-147). Protein kinases such as PKA, PKC, and fyn could regulate GABA_A receptor trafficking, and chronic alcohol has been shown to decrease associate of PKC γ with the α 1 GABA_A subunit, and increase associate with α 4 in the cerebral cortex (148). This, however, was not observed in the hippocampus (145). The association between PKC γ and the α 4 subunit could lead to GABA_A receptor phosphorylation and disrupt recognition by the adaptor complex, thus preventing its internalization (147).

It is clear that receptor composition and expression can be altered by chronic alcohol exposure. However, it does so in a relatively region-specific manner, and is sensitive to the duration of ethanol exposure and time point during withdrawal. As GABA receptor properties is highly correlated with receptor functions, studies in the monkey hippocampus demonstrated a significant increase in paired-pulse facilitation of GABA-mediated IPSC following 18 months of daily alcohol intake (149). This finding is consistent with a decrease in release probability mentioned earlier, as well as the decrease in mIPSC frequency found in rats after chronic intermittent alcohol exposure (150). In the central amygdala, after 2-3 weeks of chronic alcohol vapor treatments, it was shown that evoked IPSCS in alcohol-dependent rats were significantly larger than those in naïve rats, suggesting a postsynaptic-mediated mechanism (135). As mentioned earlier, this research group also found a higher mIPSC frequency in alcohol-dependent animals, suggesting an increase in GABA release in the rat amygdala. Together, these data

suggest that both pre- and post-synaptic GABA transmission can be altered by chronic alcohol exposure.

In contrast to acute alcohol's effects on excitatory inputs, chronic alcohol exposure leads to a general increase in glutamatergic transmission (151, 152). However, similar to the acute effects of alcohol, NMDA receptors are modulated by chronic alcohol in a more robust fashion than other types of glutamatergic receptors (153, 154). Both in vivo and ex vivo chronic alcohol exposures induce increases in NMDAR functions, and subsequent increases in neuronal calcium (155) and nitric oxide (153) signals. The NMDAR subunit NR2B has been identified as important in the chronic alcohol-induced enhancement of NMDAR-mediated current (156-158). While chronic alcohol augments excitatory transmission, acute alcohol still produces glutamatergic-inhibition after chronic exposure, but to a smaller extent (159, 160). Some studies have shown increases of specific NMDA subunits after chronic alcohol exposure using immunohistochemistry, particularly the alcohol-sensitive NR2B, and different splice variation of the NR2A (161, Others have shown increases in mRNA expression levels of different splice 162). variants of the NR1 subunit (156, 163, 164). However, other studies do not report these NMDA receptor changes after chronic alcohol exposure. In addition to NMDA receptors, AMPA receptors have also been reported to increase, as evidenced by increases in mRNA (165) and protein expression (166), as well as increase in binding (167) following chronic alcohol exposure. Further, an enhancement of AMPAR functions has also been reported in the cerebellum (168) and the amygdala (152). Chronic alcohol-induced enhancement of kainate receptor function was found in the hippocampus (169) and the amygdala (170), but not in primary cortical neurons (166). Lastly, chronic alcohol has

been shown to increase mGluR-mediated signaling, as well as protein expression, particularly mGluR1 and mGluR5 (162, 171, 172).

The above section detailed chronic alcohol exposure's ability to modulate both inhibitory and excitatory transmission, as well as illustrated how the balance between inhibitory and excitatory inputs could be altered by chronic alcohol in brain region- and duration- dependent manners. It is clear that more studies are needed to further isolate the regulation of specific pathways and projections in order to better link them to the behavioral changes accompanied by chronic alcohol use in humans. The following section discusses the kappa opioid receptor and how it can modulate drinking behaviors.

KAPPA OPIOID RECEPTORS

As a member of the opioid receptor family, kappa opioid receptor (KOR) actions have been shown to play important roles in analgesia, mood modulation, and addiction. The KORs are widely spread throughout the brain, spinal cord, and peripheral systems, and are activated via opioid peptides derived from prodynorphin, such as dynorphin A (1-17) and dynorphin B (1-13) (173, 174). KOR activation involves coupling to the inhibitory G-protein Gi/o to inhibit adenylyl cyclase through the dissociation of the α subunit. The $\beta\gamma$ subunits modulate ion channels to increase potassium conductance and decrease calcium conductance (175). The kappa opioid receptors can also be phosphorylated and activate downstream signaling cascade (See Chapter IV for signaling cascade). Kappa opioid receptor ligands are inhibitory neuropeptides that hyperpolarize neurons (176, 177), as are other members of the opioid family. It has been shown that kappa opioid receptors can presynaptically inhibit both GABAergic inputs (178, 179), and glutamatergic inputs (180, 181), as well as the signaling of dopamine, norepinephrine, and serotonin (182-185). KORs have been shown to locate presynaptically on dopaminergic input to the amygdala, NAc, and PFC (186-188); on GABAergic inputs to the NAc and the extended amygdala (central nucleus of the amygdala and BNST) (189); on glutamatergic inputs to the VTA and NAc (189) and on serotonergic inputs to the NAc (182), as well as on noadrenergic inputs to the PFC (190). The KORs have also been found on the cell bodies of dorsal raphe serotonin (5-HT) neurons (182), locus coeruleus norepinephrine neurons (191), as well as VTA mesocortical dopamine neurons (192). Mechanistically, it has been shown that kappa opioid receptor activation has the ability to modulate dopaminergic neurons in the VTA. More specifically, a KOR agonist can inhibit the VTA DA neurons projecting to the prefrontal cortex, but not projections to the amygdala (192). Interestingly, while isolating GABA-mediated inhibitory post-synaptic currents (IPSCs) in the VTA dopamine neurons, the same research group found that opioid agonists also inhibited GABAergic transmission. The KOR system is widely distributed in the central nervous system and has been implicated in numerous pathophysiological disorders associated with mood and motivation (193-196), pointing to its potential as a putative therapeutic target for neuropsychiatric disorders.

Behavioral phenotypes of KOR activation are of particular interest due to its potential for serving as a target in the treatment of addiction and affective disorders. Effects of KOR activation include drug-seeking behavior (182) and analgesia (197), as well as dysphoric affect such as depression (198), stress and anxiety-like behaviors (199, 200), and most importantly, dependence-induced depressive-like behaviors (201). Many of the KOR-relevant emotional behaviors are modulated in the mid brain region, namely in the VTA, vPAG, and dorsal raphe. In particular, dopaminergic neurons and their regulation seem to play a critical role in these negative affective disorders. Studies have shown that KOR activation in the dorsal raphe, which also contains A10dc dopamine neurons and is just ventral of the vPAG, produces conditioned place aversion (202), and mediates the aversive effects of stress, and reinstates drug-seeking (182). In addition, KOR knockouts and conditional knockouts in dopamine-containing neurons produced anxiolytic-like effects and enhanced cocaine-induced plasticity (33). Depressive-like effects of the KOR agonist salvinorin A have also been shown associated with decreased phasic dopamine release in the NAc (203). Further, anti-nociception induced by oxytocin can be blocked by KOR antagonism in the PAG (204). The above evidence suggests that KOR actions can modulate negative emotions and drug-seeking behaviors, some of which are mediated via dopamine signaling. As mentioned earlier, dopamine signaling plays a critical role in reward and some negative emotional behaviors. In addition, the PAG is a brain region highly implicated in both drug addiction and negative affect. Given the evidence that kappa opioid receptors are distributed widely in the PAG (205), and that KOR actions can modulate dopamine signaling, the PAG becomes a critical brain region to study to better understand the mechanisms underlying drug addictionassociated mood disorders. More research on KOR modulation of neural activity in the PAG will allow us to better adapt strategies to develop treatment for negative mood disorders and alcoholism.

KAPPA OPIOID RECEPTORS AND ALCOHOLISM

Kappa opioid receptor activation not only produces negative affect similar to ethanol withdrawal, numerous studies provide direct links between KOR actions and ethanol intake. Interestingly, alcohol exposure can lead to an up-regulation in KOR signaling at the receptor level, as well as the ligand level (206). Microdialysis studies show that acute alcohol can stimulate the release of opiates, including dynorphin, in regions implicated in reward and affective disorders such as the VTA, the amygdala, and the nucleus accumbens (207-211). In addition, radioimmunoassay studies demonstrated that a 6-week chronic alcohol exposure significantly increased dynorphin B levels in the hypothalamus and substantia nigra, while it was decreased in the prefrontal cortex (212). *Vice versa*, KOR functions have also been shown to modulate alcohol-related behaviors. KOR activation mediates aversive effects produced by alcohol challenge (213), and systemic antagonism of KOR also attenuates dependence-induced excessive ethanol selfadministration in rats (214). It also seems that KOR systems can modulate dopamine mediated reward-seeking behavior, as acute KOR activation decreases the rewarding properties of alcohol (215) while decreasing operant responding to ethanol (215). Interestingly, one study using a dopamine neuron-specific prodynorphin knockout mouse found an increase in voluntary ethanol intake, higher blood ethanol levels, and higher ethanol-conditioned place preference (216). This result seems to oppose other studies mentioned earlier where KOR antagonism decreased dependence-induced drinking, however, this research group identified some critical receptor- and cellular modulation due to the genetic knockout. Specifically, they found that dopamine tyrosine hydroxylase expression was decreased and dopamine transporter levels were increased. In addition, proenkephalin (the endogenous precursor of the mu opioid ligand) expression decreased. This study showed the importance of dynorphin signaling in the maintenance of alcohol intake, as well as dopamine signaling. Further, other studies have shown that dynorphin plays a critical role in the modulation of the brain stress-response system after chronic alcohol exposure. Long-term alcohol exposure elevated foot-shock-induced c-Fos expression in the basolateral amygdala in wild type mice, but had the opposite effect in dynorphin-knockout mice (217). Just as opioid-inhibition of presynaptic GABAergic inputs onto VTA dopamine neurons can lead to the activation of dopamine neurons (67, 176), similar effects have been demonstrated by acute ethanol. In both the VTA (218) and the substantia nigra (SN) (219), it has been shown that acute ethanol stimulates the firing rate of dopamine neurons and inhibits GABAergic inhibitory currents. Recent data has suggested that ethanol differentially impacts populations of dopamine neurons by brain region. In a chronic vapor ethanol exposure paradigm, studies have shown that chronic alcohol exposure alters dopamine transporter (DAT) expression in the NAc, but not the striatum or the BNST (220), suggesting differential modulatory systems for dopamine neurons of different projections. Given the important role that KOR signaling plays in the development and maintenance of alcoholism, understanding the mechanisms underlying KOR modulation of dopamine signaling could shed some light onto the development of treatments for alcohol abuse, as well as battle against the vicious cycle encouraged by withdrawal-induced negative affects.

DISSERTATION

The focus of this dissertation will be to characterize the properties and functions of vPAG DA neurons and to evaluate alcohol and kappa opioid modulation of dopamine signaling, as well as the underlying mechanisms thereof. Clinically, related work has established the importance of understanding the negative affect produced from drug withdrawal, as they often lead to relapse, rendering treatment for addiction inefficient and the results short-lasting. Understanding how substances of abuse modulate emotional behavior circuits could shed light onto the development of more effective treatments. The dopamine signaling system mediates the rewarding properties of drugs of abuse. Evidence shows that alcohol exposure induces alterations in the signaling of dopamine-rich brain regions, such as the VTA. While the kappa opioid system is known to mediate negative affect, a rather novel population of dopamine neurons in the vPAG has been shown to project to regions regulating negative emotions such as stress, anxiety, and depression. Thus, it is likely that prolonged exposure to alcohol could increase vPAG dopamine signaling, consequently exciting stress and anxiety nuclei, and inducing stress- and anxiety-like behaviors.

The following work will investigate the impact of both acute and chronic intermittent alcohol on vPAG dopamine synaptic transmissions. Once the link between alcohol exposure and neural alteration is established, this study will investigate the modulation of vPAG dopamine neurons by KOR, putatively known to induce negative emotional behaviors when activated. Based on previous studies of opioid modulations, it is likely that KOR activation will inhibit GABAergic inputs presynaptic to vPAG dopamine neurons, hypothetically increasing their activity, as well as increasing dopamine release in their projection areas (i.e. anxiety and stress centers), thereby promoting negative affects. Following neural modulation, signaling mechanisms through which KOR activation alter neural transmission will be examined. KOR activation triggers an array of signaling cascades mediated through the Gi/o-protein coupled receptors; this work will discern the loci of mechanism by inhibition of individual signaling pathways. It was hypothesized that the MAP kinase ERK1/2 mediates the KOR activation-induced attenuation of inhibitory transmission, based on previous studies in the BNST. In addition, this work aims to examine the ability of vPAG DA neurons to modulate its projection area- the BNST. It has been shown in the VTA that inhibition of presynaptic GABA inputs on to dopamine neurons increases dopamine release in projection area such as the NAc. Thus, transmission in the BNST will be examined while stimulating vPAG DA inputs. The overall hypothesis of this dissertation is that both alcohol and KOR-activation modulate vPAG dopamine synaptic transmission and neural activity, inducing alterations in regulation of its projection area – the stress center BNST.

CHAPTER 2. EFFECTS OF ALCOHOL ON VPAG DOPAMINE NEURON*

INTRODUCTION

Alcohol use disorders are an enormous public health problem, and understanding the neurochemical systems involved in regulating the actions of alcohol can provide insight for the development of more effective treatments. Numerous reports have suggested that the subjective response to acute alcohol is related to the risk of development of alcohol use disorders (221). As such, understanding the actions of acute alcohol is an important area of investigation. Animal studies have shown that acute alcohol can induce locomotor stimulation (222-224) and is also anxiolytic (225, 226). Alcohol also modulates pain perception and the anti-nociceptive effects of opiates (227). Chronic alcohol exposure produces much different behavioral effects during withdrawal. Locomotor activity has been reported to decrease (228), and anxiety-like behaviors are observed (229) during withdrawal period. Other withdrawal-induced behaviors include aggression (230, 231), anhedonia (232), insomnia (233), and increased sensitivity to pain (55, 234). Given the diversity of these behavioral outcomes, there are likely multiple brain regions and their projection regions that are modulated by alcohol exposure.

A large body of evidence suggests that dopamine (DA) signaling plays a critical role in mediating the rewarding aspects of acute alcohol. Much of this research has

^{*} This chapter has been accepted to the journal Alcohol and will be published June of 2013. It has been placed into this dissertation after additional editing by the author.
focused on the mesolimbic DA system, which is composed of DA neurons in the ventral tegmental area (VTA) that project to the nucleus accumbens (235, 236). However, recent studies have shown that dopamine signaling in other brain regions critical to alcohol abuse, such as the extended amygdala (237), can play an important role in alcohol reward. Interestingly, the extended amygdala receives a strong dopaminergic projection from the A10dc DA neurons, located in the ventral periaqueductal gray (vPAG), and dorsal raphe nucleus (DRN) (38, 238). The vPAG is particularly relevant as a target for the acute actions of alcohol as it has been implicated in the regulation of arousal (231, 239), anxiety (240-242), sleep (46), pain (42, 243-245), and opiate reward (246). Despite the potential behavioral relevance of vPAG DA neurons to the actions of alcohol, there have been no studies examining either the properties of these neurons, or the ability of alcohol to modulate their function. In this study, we utilized a TH-eGFP transgenic mouse to selectively record from DA neurons in the vPAG and VTA for cell property comparison, as well as evaluating the impact of both acute and chronic alcohol on vPAG DA synaptic transmission.

With the knowledge that vPAG DA neurons project to the extended amygdala and pharmacological blockade of dopamine receptors in these regions can modulate alcohol drinking behavior, we investigated the effects of both acute and chronic alcohol on synaptic function in vPAG DA neurons. Briefly, we found that alcohol did not modulate mini inhibitory post-synaptic current (mIPSC) in vPAG DA neurons, but increased mini excitatory post-synaptic current (mEPSC) frequency. Consistent with these synaptic effects, we found that acute alcohol increased firing of vPAG DA neurons. Interestingly, chronic alcohol exposure did not alter synaptic function in these neurons.

MATERIALS AND METHODS

Animals and Husbandry

Adult male TH-eGFP Swiss Webster mice aged between 5 to 9 weeks were bred and used in accordance with an animal use protocol approved by the University of North Carolina – Chapel Hill (IACUC). Mice were group-housed in our colony room under a 12:12-hour light cycle, with lights on at 7:00 AM daily. Mice were given ad libitum access to rodent chow and water. Mating pairs of mice were created by GENSAT and obtained from the Mutant Mouse Regional Resource Center in North Carolina. In the TH-eGFP mouse line, the genome was modified to contain multiple copies of a modified BAC in which an eGFP reporter gene was inserted immediately upstream of the coding sequence of the gene for tyrosine hydroxylase (TH). Data presented here were obtained from the transgenic mice maintained in-house.

Electrophysiology Brain Slice Preparation and Slice Whole-Cell Electrophysiology

Mice were decapitated under isoflurane anesthesia and their brains were rapidly removed and placed in ice-cold sucrose artificial cerebrospinal fluid (ACSF): (in mM) 194 sucrose, 20 NaCl, 4.4 KCl, 2 CaCl2, 1 MgCl2, 1.2 NaH2PO4, 10.0 glucose, and 26.0 NaHCO3 saturated with 95% O2/5% CO2. Three hundred micron slices were prepared using a Leica VT1200 vibratome (Wetzlar, Germany). Brain slices containing the PAG were stored at approximately 30°C in a heated, oxygenated holding chamber containing ACSF (in mmol/L) 124 NaCl, 4.4 KCl, 2 CaCl2, 1.2 MgSO4, 1 NaH2PO4, 10.0 glucose, and 26.0 sodium bicarbonate before being transferred to a submerged recording chamber maintained at approximately 30°C (Warner Instruments, Hamden, Connecticut) Recording electrodes (3–5 MΩ) were pulled with a Flaming-Brown Micropipette Puller

(Sutter Instruments, Novato, CA) using thin-walled borosilicate glass capillaries. During inhibitory transmission experiments, recording electrodes were filled with (in mmol/L) 70 KCl, 65 K+-gluconate, 5 NaCl, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 QX-314, .6 EGTA, 4 ATP, .4 GTP, pH 7.4, 290 to 295 mOsmol. During excitatory transmission experiments, recording electrodes were filled with (in mmol/L) 117 D-gluconic acid, 118 CsOH, 20 HEPES, 0.4 EGTA, 5 TEA, 2 MgCl-2, 4 Na2APT, 0.4 Na2GPT, 2 QX-314. To measure Ih current, cells were held at -140mV for a 1second duration immediately after breaking into the membrane and analyzed for the final 100ms of this voltage step. In voltage-clamp experiments, cells were held at -70 mV and inhibitory post-synaptic currents (IPSCs) were pharmacologically isolated with 3 mmol/L kynurenic acid, to block α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptor-dependent post-synaptic current. Excitatory post-synaptic currents (EPSCs) were pharmacologically isolated by adding 25 μ mol/L picrotoxin to block γ -Aminobutyric acid (GABA) receptor-dependent current. To isolate miniature inhibitory post-synaptic currents (mIPSCs), tetrodotoxin (0.5 µmol/L) was added to the perfusing ACSF solutions described above. During chronic intermittent alcohol exposure electrophysiology experiments, mini inhibitory (cells held at +10mV) and excitatory (cells held at -55mV) transmission was examined within the same cells using the following internal solution (in mmol/L): 135 Cs-Methanesulfanate, 10 KCl, 10 HEPES, 1 MgCl2, 0.2 EGTA, 4 MgATP, 0.3 Na2GPT, 20 phosphocreatine. In firing rate experiments, we used cell-attached experiments where the cell membrane was not broken in with the seal maintained under 50M Ω , while recording electrodes were filled with (in mmol/L) 135 K+-gluconate, 5 NaCl, 2 MgCl2, 10 HEPES, 0.6 EGTA, 4 Na2APT, 0.4 Na2GPT. Signals were acquired via a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, California), digitized at 20 kHz, filtered at 3 kHz, and analyzed using Clampfit 10.2 software (Molecular Devices). Input resistance and access resistance were continuously monitored during experiments. Experiments in which changes in access resistance were greater than 20% were not included in the data analysis.

Chronic Intermittent Alcohol Vapor Paradigm

Adult mice were exposed to alcohol vapor or air in La Jolla Alcohol Research chambers for 16 hours per day (16 hours on, 8 hours off; from 4pm to 8am the following day) for 2 cycles of 4 days on, and 3 days off. Alcohol vapor was created by bubbling 95% alcohol in a vaporizer at a rate of 4-6 L/min air; the vapor was administered at a flow rate of approximately 15 L/min air to each individually sealed cage. Alcohol levels were adjusted to reach animal blood alcohol concentration (BAC) above 200 mg/dL, and monitored using a breathalyzer at the beginning and end of every exposure. To achieve intoxication BAC levels, alcohol-exposed mice were injected with 10ml/kg of 68.1mg/kg of the alcohol dehydrogenase inhibitor pyrazole combined with 1.5g/kg 20% (v/v) alcohol in saline; air-exposed mice received 10 ml/kg of 68.1mg/kg pyrazole in saline. Electrophysiology was performed 48 hours after exiting the chamber from the last cycle. *Immunohistochemistry*

Mice were anesthetized with Avertin and perfused trans-cardially with chilled 0.01M phosphate buffered saline (PBS), immediately followed by 4% paraformaldehyde phosphate buffered saline. Brains were extracted and post-fixed in 4% paraformaldehyde for 24 hours and immersed in a 30% sucrose solution for 48 hours, both at 4°C. Coronal sections 45um in thickness were collected using a Leica VT1000S vibratome (Leica

Microsystems, Nussloch, Germany) and stored in a 50% glycerol solution at -20°C until immunohistochemistry was performed. Slices were rinsed for 5 minutes in chilled PBS, followed by a 10-minute incubation in 0.1% Triton X-100 in PBS solution, two 5-minute PBS washes, and a 30-minute incubation in 0.5% Triton X-100 in PBS solution. After a 1-hour incubation in a blocking solution made of 0.1% Triton X-100/10% Normal Donkey Serum in PBS, the tissue was then incubated for 48 hours at 4°C with their respective primary antibodies diluted in 0.1% Triton X-100/10% Normal Donkey Serum in PBS blocking solution (anti-Tyrosine Hydroxylase, [1:500], Pel-Freez P60101-0, Lot 28632; anti-Green Fluorescent Protein, [1:500], Aves Laboratories, GFP-1020). Slices were rinsed three times for 10 minutes in chilled PBS before incubating for 24 hours at 4°C in their respective secondary antibodies diluted in PBS (Alexa Fluor 647 Donkey anti-Sheep, [1:800], TH; Alexa Fluor 488 Donkey anti-Chicken, [1:200], GFP; Jackson Immuno Research). Tissue was rinsed four times for 10 minutes in chilled PBS, and mounted using Vecta-Shield Mounting Medium (Vector Laboratories, Burlingame, CA, H-1000) prior to image collection.

Statistics

Effects of drugs during electrophysiological recordings were evaluated by comparing the magnitude of the dependent measure (mIPSC or mEPSC frequency and amplitude) between the baseline and wash-on (when drug had reached maximal effect at 10 minutes) periods using paired Student's t-tests. The effects of antagonists/blockers on the ability of drugs to modulate synaptic transmission were compared using t-tests during the washout period. All values given for drug effects throughout the article are presented as mean \pm SEM.

RESULTS

Basal Electrophysiology Properties: vPAG vs. VTA

We first wanted to confirm that the TH-eGFP mouse line would report correctly for DA neurons in the vPAG. To examine this, we performed dual label immunofluorescence and looked for overlap of TH and eGFP in the vPAG of the reporter (Fig2.1). We found that $69.6\pm4.5\%$ of eGFP positive neurons were co-localized withTH (n =2 animals). Having determined the fidelity of this TH-eGFP reporter line for DA neurons in the vPAG, we then examined the membrane capacitance and resistance in eGFP-positive neurons in the vPAG.

We used whole-cell voltage clamp to examine the Ih current, membrane capacitance, and membrane resistance in eGFP-positive neurons in both the vPAG and the well-characterized VTA. We found that the A10 DA neuron population in the VTA had a significantly (Student's t-test, p < 0.0001) lower membrane resistance (194.8 ± 52.9M Ω , n=10) compared to the A10dc DA neurons in the vPAG (866.7±72.5 M Ω , n=36)(Fig2.2B). Further, the DA neurons in the VTA also displayed a significantly (p < 0.0001) larger membrane capacitance (56.4±5.2 pF) than DA neurons in the vPAG (16.6±1.0pF) (Fig2.2A). Having established these differences in basic membrane properties, we next sought to determine whether the DA neurons in the PAG exhibit a hyperpolarization-activated current (Ih current). The presence of Ih current has been used as a marker for DA neurons in the VTA (247), however, this has been a topic of intense scrutiny (248). Consistent with previous findings, we found that putative DA eGFP-positive neurons in the VTA displayed a robust Ih current (-172.5± 46.3 pA, n=10), similar to those observed in previous studies (249). In contrast, the dopamine

neurons in the vPAG had a significantly smaller Ih current (-8.1 \pm 2.4 pA, n=36) (Fig2.2C and D), with most neurons in the vPAG lacking any measurable hyperpolarization current.

Acute alcohol modulation of GABAergic transmission in the vPAG

We next evaluated the effects of 50mM alcohol on GABAergic synaptic transmission. We selected a concentration based on previous studies that showed effective modulation of inhibitory currents in the VTA (250, 251). We found no effects (n=6) on miniature IPSC (mIPSC) frequency (100.4±27.6% of baseline. Fig2.3B), amplitude ($85.4\pm17.0\%$ of baseline, Fig2.3C), or decay ($98.4\pm4.7\%$ of baseline, Fig2.3D). Further, acute alcohol had no effects on evoked IPSC transmission (data not shown). Our data suggest that synaptic GABAergic transmission in vPAG DA neurons is not modulated by alcohol. However, previous studies have shown that alcohol can potently modulate extra-synaptic GABAA receptors (116). Thus, we examined the impact of 50 mM alcohol on holding current and the standard deviation of the noise, two measures that are related to tonic GABAA receptor-mediated currents (116). We found that 50 mM alcohol does not alter the holding current or cause a shift in the noise (Fig2.3E), suggesting that neither synaptic nor extra-synaptic GABAergic transmission are modulated by acute alcohol in the vPAG. As recent studies have found that the actions of alcohol on dopamine neurons can depend on intact network function (252), we next assessed the ability of alcohol to modulate spontaneous GABAergic transmission. In these experiments, GABAergic transmission was isolated by pharmacologically blocking glutamatergic transmission with 3mM kynurenic acid, but tetrodotoxin was excluded from the bath solution to allow for action potential dependent network activity.

We found that 10-minute bath application of 50mM alcohol (n=10) significantly (p<0.05) decreased the amplitude (88.7 \pm 15.5% of baseline. Fig2.4D) of spontaneous inhibitory post-synaptic current (sIPSC), but had no effect on frequency (87.3 \pm 42.2%. Fig2.4C). *Acute alcohol modulation of miniature excitatory post-synaptic current in the vPAG*

Having established that acute alcohol had minimal effects on inhibitory inputs to vPAG DA neurons, we next examined the impact of acute alcohol on excitatory glutamatergic synaptic function. We found that a 10-minute bath application of 50mM alcohol (n=7) significantly (p=0.02) increased mEPSC frequency (144.9 \pm 17.7% of baseline. Fig2.5C), but had no effects on amplitude (93.3 \pm 4.6% of baseline, Fig2.5D). Interestingly, this effect did not appear to reverse over the course of the 10-minute washout. Taken together, these results demonstrate that alcohol has no effects on inhibitory inputs, but facilitates excitatory inputs onto vPAG dopamine neurons via a presynaptic mechanism.

Acute alcohol modulation of vPAG dopamine neuron cell firing rate

After finding minimal effects of acute alcohol on inhibitory synaptic inputs but an increase in excitatory synaptic inputs, we investigated the effects of alcohol on overall firing rate using the loose cell-attached recording configuration. In these experiments, we did not block any synaptic transmission, and thus assessed the impact of alcohol on the circuits that regulate vPAG DA neuron firing. We found that the average firing rate of the last 5 minutes of the 10-minute 50mM alcohol ($2.5Hz\pm0.6$, n=7. Fig2.6B,C) bath application is significantly (p=0.04) higher ($32.6\%\pm10.1$ increase) when compared to baseline ($2.0Hz\pm0.5$. Fig2.6A). This effect was not reversible with a 15-minute washout, similar to our data in mEPSCs. In addition, alcohol application had no significant effects

(n=7. Fig2.6D) on firing rate in the presence of the excitatory AMPA receptor antagonist NBQX (10uM). These results suggest that acute alcohol increases the firing of vPAG DA neurons via an enhancement of excitatory inputs.

Chronic intermittent alcohol vapor exposure modulation of synaptic transmission in the vPAG

Having found that acute alcohol could enhance glutamatergic drive onto vPAG DA neurons, we next investigated the effects of chronic intermittent vapor alcohol exposure on both the mini inhibitory and mini excitatory inputs onto vPAG dopamine neurons. It has been previously shown that 2 cycles of chronic intermittent alcohol vapor exposure has both behavioral and electrophysiological effects. Specifically, mice undergoing this exposure paradigm exhibit enhanced alcohol seeking behavior (253, 254), as well as altered plasticity in the BNST (255, 256), a target of vPAG DA neurons. We recorded both mIPSC and mEPSC transmission within the same cell and compared the frequency, amplitude, and excitatory/inhibitory (E/I) ratio between air-exposed animals (n=8) and alcohol exposed animals (n=7). Surprisingly, our results showed no differences in basal frequency (Fig2.7C), amplitude (Fig2.7D), and E/I ratio (Fig2.7E) between control and alcohol groups. In addition, we examined basal cell properties (data not shown) of both groups and found no difference in membrane capacitance, resistance, and holding potential. Taken together, these results suggest that two weeks of chronic alcohol vapor does not alter the basal synaptic properties of vPAG DA neurons at this time point.

DISCUSSION

How alcohol modulates neurochemically-defined neurons in distinct brain regions is crucial to understanding alcohol's effects on behavior. This study focused on determining the actions of alcohol on a population of DA neurons in the vPAG that have been implicated in a wide variety of behaviors. We identified dopamine neurons using the TH-eGFP transgenic mouse line and found approximately 70% of GFP-positive cells were co-labeled with TH. While there is a concern for over-reporting, it is also a possibility that some GFP-positive cells express TH at levels that are too low to be observed through the immunofluorescent techniques. Further, it is critical to note that a recent paper from the Ungless group showed similar results (257). Having confirmed the fidelity of this reporter, we then characterized vPAG dopamine neuron membrane properties. Interestingly, we observed little to no hyperpolarization-activated currents, similar to what was observed in a recent study (257). Historically, researchers have used the presence of I_h current to identify dopamine neurons in the VTA (179, 258-260). While some studies were unable to establish a consistent relationship between dopaminergic neurons and I_h currents (248), other studies have suggested that the magnitude of the I_h current varies with target projection areas, as well as sub-regions of DA neuron-populated sites (179, 260). Recent studies have found dopamine neurons located in the medial portion of the VTA that display small Ih currents (261), similar to our results in vPAG dopamine neurons. Given the physiological and anatomical similarities between these populations of neurons, it is possible that they play similar roles in alcohol and stress-related behaviors. Indeed, preliminary results from our group suggest that similar to the neurons in the medial VTA, the TH-neurons in the vPAG also

express vGlut2. Future studies using optogenetic approaches will more clearly examine this interesting possibility.

Having characterized the basic properties of vPAG dopamine neurons, we next evaluated the effects of alcohol on synaptic transmission. We found that concentrations of bath-applied alcohol that had previously been shown to increase GABA release in the VTA, had no effect on mIPSCs on vPAG dopamine neurons. Our result contrasts with previous studies from the VTA that have demonstrated alcohol can enhance both spontaneous and miniature GABA transmission (251, 252). The ability of alcohol to enhance GABA transmission in the VTA, however, appears to depend on the sub region of the VTA, with anterior-VTA DA neurons showing an alcohol-induced increase in GABA transmission and posterior-VTA DA neurons showing a reduction in spontaneous but not miniature GABA transmission (252). Thus, our results demonstrating a lack of alcohol effects on mIPSCs in the vPAG are similar to posterior VTA DA neurons. Following this, we investigated how alcohol might impact extra-synaptic receptors. Curiously, we found that 50 mM alcohol did not alter holding current of these neurons, suggesting a lack of effect on extra-synaptic currents. While we did not see evidence for a modulation here, this could be due to the exquisite sensitivity of extra-synaptic receptors for alcohol. However, in a series of pilot experiments, we examined the ability of THIP, a compound that can selectively activate extra-synaptic receptors, to modulate holding current in vPAG DA neurons and found no effect, suggesting that these neurons do not express these receptors. Because of the lack of effect on mini IPSCs and holding current, we investigated the effect of alcohol on spontaneous IPSCs, as it was shown in the posterior VTA that alcohol significantly reduced sIPSCs. We found similar results as Guan et al., that alcohol caused a significant but minimal decrease in spontaneous inhibitory transmission; however, the decrease in amplitude we observed appears less robust than the effect in the posterior VTA. This could be due to some differences in effects of alcohol on the surrounding network and inputs. Taken together, these results suggest that vPAG DA neurons could be similarly modulated by alcohol as the posterior VTA DA neurons, and may have a partially overlapping set of GABA inputs, either from local interneurons or distal projections such as the BNST and the central amygdala.

We next examined the effects of alcohol on excitatory glutamatergic inputs in vPAG dopamine neurons. We found that bath application of alcohol increased mEPSC frequency, with no effect on amplitude, suggesting an increase in glutamate release. Numerous studies have shown that acute alcohol decreases glutamatergic transmission in many brain regions implicated in alcohol abuse, such as the central nucleus of the The ability of acute alcohol to enhance glutamatergic amygdala (CeA) (120). transmission in the vPAG was strikingly similar to that found in the VTA, where dopamine signaling mediates the enhancement of glutamatergic input by alcohol (262). Our observation of alcohol having minimal inhibitory effects on GABAergic inputs but an enhancement in glutamatergic transmission led us to investigate alcohol's effects on overall firing rate. We found an increase in firing rate upon application of acute alcohol, with this alcohol effect diminished in the presence of NBQX, suggesting this increase in firing rate is AMPA receptor-dependent. Taken together with our previous data, we speculate that via these alterations in function, acute alcohol may lead to increased dopamine release in the terminal regions, such as the BNST. This is interesting, as DA signaling in the BNST can modulate alcohol drinking behavior (237, 263).

To further investigate the role alcohol plays in modulating vPAG dopamine neurons and their target regions, we examined the synaptic effects of chronic intermittent alcohol vapor exposure for 2 cycles. This paradigm has been shown to enhance the development of long-term potentiation in the BNST, via modulation of the excitatory NMDA receptors extra-synaptically (255), as well as modulation of CRF-dependent increase of sEPSCs in the BNST (256). Given the knowledge that chronic alcohol modulates synapses and functions in the BNST, one of the extended amygdala structures that interact with the vPAG and impact behaviors critical to alcohol addiction, our findings were surprising. We found no effects of chronic intermittent alcohol exposure on either the inhibitory or excitatory inputs in the vPAG dopamine neurons. Perhaps a more profound systemic insult than the 2 cycles of intermittent alcohol exposure presented is required to affect the synaptic functions and to produce a detectable network While considering the possibility that the 2-cycle vapor alcohol exposure effect. paradigm could impact synaptic alterations differentially in a strain-dependent manner, it is interesting to note that during alcohol exposure, the Swiss Webster mice used in these experiments displayed a robust behavioral alteration towards aggression. Alternatively, it is possible that there are alterations in transmitter release in downstream regions such as the BNST that would engage CRF signaling leading to the alterations in function (256). While our study focused on changes in synaptic transmission, it is also possible that chronic alcohol is altering expression of neuromodulatory receptors in the BNST. Chronic alcohol exposure has been shown to alter opioid peptides in the vPAG (264), as well as in other brain regions that interact with the vPAG (265). This is interesting, as opioids have been shown to influence alcohol related behaviors (214, 266, 267). Thus,

although a direct network effect was not observed, chronic intermittent alcohol exposure could have an impact on modulatory peptides in the vPAG and how they regulate synaptic transmission.

It is currently unclear how alcohol modulation of vPAG DA neurons alters behavior. Given the prominent role that the vPAG plays in sleep and pain processing, it is tempting to speculate that alcohol actions on this circuit are involved in these processes. Future studies utilizing optogenetics to probe pathway-defined plasticity, as well as applying designer receptors exclusively activated by designer drugs (DREADD) to characterize pathway and cell type-specific modulation will likely shed light on this exciting possibility.

CONCLUSIONS

Here, we characterized the cell membrane properties as well as effects of alcohol exposure on vPAG DA neurons. Acute alcohol enhanced glutamate inputs and had inhibitory effects on GABA inputs, resulting in a net increase in firing rate. Chronic intermittent alcohol vapor exposure had no effects on GABA or glutamate transmission onto the vPAG dopamine neurons. Taken together, these results add to the growing body of literature pointing towards discrete effects of alcohol on defined cell-types in the brain.

FIGURES AND TABLES



Figure 2.1: The tyrosine hydroxylase-eGFP transgenic mouse model identifies dopamine neurons via fluorescence in the vPAG. (A) Immunohistochemistry can be used to label and verify validity of the reporter via (1) tyrosine hydroxylase fluorescence, (2) eGFP fluorescence, and the (3) co-localization of eGFP and tyrosine hydroxylase in dualpositive cells. No significant I_h current was observed in vPAG DA neurons (B) (1) eGFP and tyrosine hydroxylase-positive cells can be visualized under the microscope to selectively record from. Staining showed that the recorded cells loaded with (2) neurobiotin via diffusion from the patching pipette, are (3) tyrosine hydroxylase-positive.



Figure 2.2: The dopamine neurons in the VTA and in the vPAG display different electrophysiological membrane properties. (A) vPAG dopamine neurons have significantly lower membrane capacitance (p < 0.0001) than VTA dopamine neurons.
(B) vPAG dopamine neurons have significantly higher membrane resistance (p < 0.0001) than VTA dopamine neurons.
(C), (D) vPAG dopamine neurons display little to no hyperpolarization current (I_h) comparing to VTA dopamine neurons.



Figure 2.3: Acute bath-applied alcohol had no effect on mini inhibitory post synaptic currents (mIPSCs) in vPAG dopamine neurons. (A) Representative mIPSC traces of baseline control (i) and after 10 minutes of alcohol wash-on (ii). (B) Acute alcohol had no effect on mIPSC frequency. (C) Acute alcohol had no effects on mIPSC amplitude. (D) Acute alcohol had no effect on mIPSC decay. (E) Acute alcohol had no effect on mIPSC noise.



Figure 2.4: Acute bath-applied alcohol had no effects on spontaneous inhibitory post synaptic current (sIPSC) frequency in vPAG dopamine neurons. Representative sIPSC traces of (A) baseline control and (B) after 10 minutes of alcohol wash-on. (C) Acute alcohol had no effects on sIPSC frequency. (D) Acute alcohol significantly decreased sIPSC amplitude.



Figure 2.5: Acute bath-applied alcohol increased mini excitatory post synaptic currents (mEPSCs) in vPAG dopamine neurons. (A) Representative mEPSC trace of baseline control. (B) Representative mEPSC trace after 10 minutes of alcohol wash-on. (C) Acute alcohol increased mEPSC frequency. (D) Acute alcohol had no effects on mEPSC amplitude.



Figure 2.6: Acute bath-applied alcohol (50mM) increased firing rate of vPAG dopamine neurons in the cell-attached recording configuration. (A) Representative firing rate trace of baseline control. (B) Representative firing rate trace in the same neuron after 10 minutes of alcohol wash-on. (C) Alcohol increased firing rate in vPAG DA neurons. (D) Alcohol had no significant effect on firing rate in the presence of NBQX (10µM).



Figure 2.7: Chronic intermittent alcohol vapor exposure did not affect inhibitory and excitatory synaptic properties. (A) Representative mEPSC trace. (B) Representative mIPSC trace. (C) Chronic intermittent alcohol had no effect on mini frequency. (D) Chronic intermittent alcohol had no effect on mini amplitude. (E) Chronic intermittent alcohol had no effect on mini excitatory/inhibitory transmission ratio.

CHAPTER 3. MODULATION OF KAPPA OPIOID RECEPTORS ON VPAG DOPAMINE NEURONS

INTRODUCTION

The mesolimbic dopamine circuit has been well-characterized in reward and drug addiction; it includes dopaminergic projections from the VTA to the nucleus accumbens, and the nigrostriatal pathway projecting from the substantia nigra to the stiratum. However, recent studies (38) have suggested the involvement of another mid-brain population of dopamine neurons that is highly implicated in the rewarding properties of drug addiction, as well as the negative emotional behaviors that often accompany withdrawal. The A10dc group DA neurons project from the ventral periaqueductal gray (vPAG) to the extended amygdala - the bed nucleus of stria terminalis (BNST) and the central amygdala (CeA), areas known to regulate stress- and anxiety-related behaviors. In addition, the vPAG has been implicated in arousal and several negative emotional behaviors such as anxiety (241, 242, 268), ethanol withdrawal (56, 57, 269), fear conditioning and extinction (270-274), sleep (46), and pain (244, 245, 275, 276). These behavioral studies raise the possibility that functions of vPAG DA neurons may play a role in the negative affective state associated with drug abuse that often leads to relapse (84).

The PAG is a heterogeneous region in cell types, as well as in its projection targets implicated in various behaviors. For instance, the projection from the PAG to the prefrontal cortex (46), hypothalamus (73), and the dorsal raphe (46) has been shown to modulate sleeping behavior. Projections to the pons regulate cardiovascular and respiratory functions (277); projections to the hypothalamus regulate cardiovascular functions (52), as well as defense and fear conditioning (278); it as well as projects to the rostral ventral medulla that regulates nociception (279). Despite the large body of studies that have investigated PAG projections and functions, few have demonstrated cell-type specific properties. In this study, we took advantage of transgenic mouse lines, to specifically target vPAG dopamine neurons to examine how their synaptic transmission is modulated.

The following section describes some behaviors tightly linked to PAG activity, and vPAG dopamine signaling has been shown to mediate these behaviors. Of particular interest, the vPAG dopamine seems to regulate an array of negative emotional behaviors. Recent studies suggest that the vPAG dopamine projects to the BNST, where dopamine and corticotropin-releasing factor (CRF) interacts to regulate fear and anxiety (38). Modulation in the vPAG has also been shown to influence emotional behavior where antagonism of the GABA receptors in the vPAG increases anxiety-like behavior (39). Further, the sensitization of vPAG has been shown to suppress positive affect in rats (40). While nociception and negative emotional behaviors seem to exacerbate one another (43-45), studies show that the ventral PAG is also an important site in the ascending nociceptive control (ANC) pathway, projecting to the rostral ventral medulla (41). There is evidence that dopamine neurons in the PAG may potentially play a role in the ANC as opiate anti-nociception is attenuated upon chemical lesion of vPAG dopamine neurons (42), also suggesting a role of dopamine signaling in the mediation of opioid actions in the vPAG. Another PAG dopamine-mediated behavior heavily linked to negative emotional state is sleep, as sleep deprivation has been shown link to stress (47), anxiety (48), and depression (49). Evidence illustrated the critical role of PAG dopamine projections to the prefrontal cortex in sleeping behavior, as selective chemical lesion of dopamine neurons in the PAG leads to increase in sleep time (46). These studies show that the vPAG DA signaling is heavily engaged in negative affect, which overlaps with those behaviors previously identified to encourage drug relapse.

As mentioned in chapter I, dopamine signaling has been closely associated with the rewarding properties of drugs of abuse, and the kappa opioid receptor activity can modulate drinking behavior, as well as negative emotions. Kappa opioid receptor (KOR) activation mediates aversive effects produced by alcohol challenge (213), and systemic antagonism of KOR also attenuates dependence-induced excessive ethanol selfadministration in rats (214). It would also seem like KOR systems can modulate dopamine mediated reward-seeking behavior, as acute KOR activation decreases the rewarding properties of alcohol (215) while decreasing operant responding to ethanol (215). Together, these studies demonstrated that the kappa opioid receptor (KOR) system plays a critical role in negative emotional disorders, as well as drug abuse, possibility via the modulation of dopamine signaling.

Dysphoria induced by KOR activation includes depression (198), stress and anxiety-like behaviors (199, 200), and most importantly, drug abuse-induced depressivelike behaviors (201). Many of the KOR-relevant behaviors have been shown to be modulated in mid brain regions such as the VTA, vPAG, and dorsal raphe. In particular, dopamine signaling seems to play a critical role in these negative affective disorders. Studies have shown that KOR activation in the dorsal raphe, also containing A10dc dopamine neurons just ventral of the vPAG, produces conditioned place aversion (202), mediates the aversive effects of stress, and reinstates drug-seeking (182). Further, antinociception induced by oxytocin can be blocked by KOR antagonism in the PAG (204). Understanding of the mechanisms underlying KOR's effects on behavior in the PAG will allow us to better adapt strategies while seeking treatment for negative mood disorders and drug addiction. Mechanistically, it has been shown that kappa opioid receptor activation has the ability to modulate dopaminergic neurons in the VTA. More specifically, a KOR agonist inhibits the VTA DA neurons projecting to the prefrontal cortex (192). Interestingly, while isolating GABA-mediated inhibitory post-synaptic currents (IPSCs) in the VTA dopamine neurons, the same research group found that opioid agonists inhibited GABAergic transmission. GABAergic neurons have been shown to inhibit projection dopamine neurons in other dopamine-rich brain regions such as the VTA (67). Similar to the VTA, a population of tonically-active GABAergic neurons have been found in the PAG (66). Although KORs have been shown to distribute widely in the PAG (205), specific dopamine neuron-modulation has yet to be investigated due to the heterogeneity of the PAG. Given the strong implications of the vPAG in both negative affect and drug addiction, the existing behavioral and electrophysiological findings together lead us to the hypothesis that kappa opioid receptors could serve a role in modulating GABAergic inputs on the dopaminergic neurons in the vPAG.

Physiologically, the activation of receptors of the opioid family putatively inhibits presynaptic transmission. Opioid receptor-activation inhibits glutamate transmission presynaptic to neurons in the hypothalamus (181), as well as presynaptic to the brainstem catecholamine neurons (280). Opioid receptor-activation has also been shown to presynaptically inhibit GABAergic transmission in the VTA, resulting in disinhibition of the VTA dopamine neurons, increasing their activity (67, 281). The above evidence raises the possibility that kappa opioid receptor activation could modulate vPAG dopamine neurons, as well as the activities of their output regions. With the knowledge that vPAG DA neurons project to the BNST that regulates stress and negative affect, understanding of kappa modulation on these neurons could provide insight onto the regulation of emotional behaviors. In this study, we utilized a tyrosine hydroxylase (TH)-eGFP transgenic mouse to selectively record from DA neurons in the vPAG and pharmacologically evaluated the impact and mechanisms of KOR activation on GABA-mediated inhibitory inputs onto vPAG dopamine neurons.

As a member of the opioid receptor family, kappa opioid receptor actions have been shown to play important roles in analgesia, mood modulation, and addiction (198-201, 282). The KORs are widely spread throughout the brain, spinal cord, and peripheral systems, and are activated endogenously via the opioid peptide dynorphin (173, 175, 283, 284). KOR-activation involves the inhibition of cyclic AMP production (285), mediated through the coupling of the inhibitory G-protein Gi/o (286) (Fig3.1). Dissociation of the α subunit recruits beta arrestin and activates downstream mitogen-activated protein (MAP) kinases such as ERK1/2 (287) and p38 that affect transcription factor expression. The $\beta\gamma$ subunits has been shown to directly bind and inhibit calcium channel, as well as increase potassium channel conductance (175). These direct effects on ion channel conductance have been found in several brain regions, ranging from the hippocampus to the dorsal root ganglia (288), In addition, the phosphorylated KOR activates ERK1/2, as well as phosphoinositide 3 (PI₃) kinase and protein kinase A (PKA). Evidence suggests ERK signaling mediates KOR activation-induced attenuation in evoked IPSC response in the BNST (178). In addition, studies have shown that p38 signaling mediates the effects of KOR-induced conditioned place preference, and is required for negative affective behaviors that can be blocked by KOR antagonists (289). In the peripheral nervous system, KOR has been shown to inhibit inflammatory analgesia via the PI3 Kinase signaling pathway (290). These results suggest an array of possible downstream signaling cascades upon KOR activation. Therefore, in this study, we used pharmacological manipulations to investigate downstream KOR signaling cascade through which the presynaptic inhibition was mediated.

MATERIALS AND METHODS

Animals and Husbandry

Adult male TH-eGFP mice on a Swiss Webster background (aged between 5 to 9 weeks) were bred and used in accordance with an animal use protocol approved by the University of North Carolina – Chapel Hill (IACUC). Mice were group-housed in our colony room under a 12:12- hour light cycle, with lights on at 7:00 AM daily. Mice were given ad libitum access to rodent chow and water. Mating pairs of mice were created by GENSAT and obtained from the Mutant Mouse Regional Resource Center in North Carolina. In the TH-eGFP mouse line, the genome was modified to contain multiple copies of a modified BAC in which an eGFP reporter gene was inserted immediately upstream of the coding sequence of the gene for tyrosine hydroxylase (TH). Data presented here were obtained from the transgenic mice maintained in-house.

Electrophysiology Brain Slice Preparation

Mice were decapitated under isoflurane anesthesia and their brains were rapidly removed and placed in ice-cold sucrose artificial cerebrospinal fluid (ACSF): (in mM) 194 sucrose, 20 NaCl, 4.4 KCl, 2 CaCl2, 1 MgCl2, 1.2 NaH2PO4, 10.0 glucose, and 26.0 NaHCO3 saturated with 95% O2/5% CO2. Three hundred micron slices were prepared using a Leica VT1200 vibratome (Wetzlar, Germany).

Slice Whole-Cell Electrophysiology

Brain slices containing PAG were obtained and stored at approximately 30°C in a heated, oxygenated holding chamber containing artificial cerebrospinal fluid (ACSF) (in mmol/L) 124 NaCl, 4.4 KCl, 2 CaCl2, 1.2 MgSO4, 1 NaH2PO4, 10.0 glucose, and 26.0 sodium bicarbonate before being transferred to a submerged recording chamber

maintained at approximately 30°C (Warner Instruments, Hamden, Connecticut) Recording electrodes $(3-5 \text{ M}\Omega)$ were pulled with a Flaming-Brown Micropipette Puller (Sutter Instruments, Novato, CA), using thin-walled borosilicate glass capillaries. During inhibitory transmission experiments, recording electrodes were filled with (in mmol/L) 70 KCl, 65 K+-gluconate, 5 NaCl, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 QX-314, .6 EGTA, 4 ATP, .4 GTP, pH 7.4, 290 to 295 mOsmol. In experiments where post-synaptic GPCR signaling was blocked, GDPBs was used to replace GTP in All experiment were conducted under the voltage clamp the internal solution. configuration, cells were held at -70 mV and inhibitory post-synaptic currents (IPSCs) were pharmacologically isolated with 3 mmol/L kynurenic acid, to block α -amino-3hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptor-dependent post-synaptic current. To isolate miniature inhibitory postsynaptic currents (mIPSCs), tetrodotoxin (0.5 µmol/L) was added to the perfusing ACSF solutions described above. Signals were acquired via a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, California), digitized at 20 kHz, filtered at 3 kHz, and analyzed using Clampfit 10.2 software (Molecular Devices). Input resistance and access resistance were continuously monitored during experiments. Experiments in which changes in access resistance were greater than 20% were not included in the data analysis.

Statistics

Effects of drugs during electrophysiological recordings were evaluated by comparing the magnitude of the dependent measure (mIPSC frequency and amplitude) between the baseline and wash-on (when drug had reached maximal effect at 10 minutes)

periods using paired Students t-tests. The effects of antagonists/blockers on the ability of drugs to modulate synaptic transmission were compared using t-tests during the washout period. All values given for drug effects throughout the article are presented as mean \pm SEM.

Drugs

Dynorphin A (300 nM) and Norbinaltorphimine (Nor-BNI, 100 nM) were from Tocris (Ellisville, MO), and were both dissolved in distilled water. BAPTA-AM (50µM), Gallein (100µM), and Wortmannin (1µM) were from Tocris and dissolved in DMSO. Nor-BNI is an opioid receptor antagonist highly selective for the kappa opioid receptor (3): 4-[4-(4-Fluorophenyl)-2-[4-(methylsulfinyl)phenyl]-1H-imidazol-5-yl]pyridine (SB203580, 20 µM) and 4-aminopyridine (4-AP, 100µM) were from Ascent and dissolved in distilled alpha-[Amino[(4-aminophenyl)thio]methylene]-2water; (trifluoromethyl)benzeneacetonitrile (SL327, 10 µM) was from Ascent and dissolved in DMSO. Tetrodotoxin citrate (TTX, 500nM) and kynurenic acid (3mM) were from Abcam and dissolved in water. GDP_{βs} (4mM) and RP-Adenosine 3',5'-cyclic monophophorothioate triethylammonium salt hydrate (RP-camps, 10µM) were from Sigma-Aldrich and dissolved in water. EGTA (100µM) was obtained from Fisher Scientific and dissolved in 1M NaOH.

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RESULTS

Endogenous KOR agonist Dynorphin A attenuates GABAergic input onto vPAG DA neurons via presynaptic mechanisms

We first examined the effects of KOR activation on GABA synaptic transmission via bath application of the endogenous ligand dynorphin A (300nM). A 10-minute bath application of dynorphin A significantly attenuated the mini inhibitory post-synaptic current (mIPSC) in the vPAG dopamine neurons (Fig3.2A, n=5). Specifically, a decrease was seen in mIPSC frequency (69.5±7.4% of baseline, p=0.03, Fig3.2B,D), but not amplitude (99.3±4.8% of baseline, Fig3.2C,E), indicating the possibility of a pre-synaptic mechanism. To further confirm that the dynorphin effect observed was mediated through KOR activation, we incubated the slices in a selective KOR antagonist, nor-BNI (100nM) for 40 min before and during dynorphin wash-on (n=5). In the presence of nor-BNI, dynorphin A application failed to produce effects on mIPSC frequency (93.3±10.3% of baseline, Fig3.2F) or amplitude (89.4±6.1% of baseline, Fig3.2G). To assess the level of tonic KOR functions, we investigated the effects of nor-BNI alone (n=6) and found no effects in sIPSC frequency (100.3±5.2% of baseline, Fig3.2H) and amplitude (84.8±7.7%) of baseline, Fig3.2I), suggesting that there is no tonic level of KOR activation in the vPAG DA neurons. These mini data suggest a pre-synaptic effect of dynorphin in the vPAG DA neurons. We further tested this by blocking post-synaptic GPCR functions via the replacement of GTP with GDP_{βs} in the recording pipette, which disrupts the exchange of GTP and GDP, thus interfere with the downstream signaling cascade upon GPCR activation. With post-synaptic GPCR functions impaired, the application of dynorphin A still decreased mIPSC frequency (54.7±5.8% of baseline, n=6, p=0.04,

Fig3.3A,C), but not amplitude (97.8 \pm 11.3% of baseline, Fig3.3B, D), providing additional support that dynorphin attenuates inhibitory input onto vPAG DA neurons via a pre-synaptic mechanism. Unpublished results from the Kash lab have shown GDP β s effective in blocking the postsynaptic effects of a NPY Y1 agonist (Leu-pro NPY) in the BNST.

Dynorphin effects on GABA are not mediated through MAP kinase signaling

We next investigate the role of MAP kinases ERK1/2 and p38 in KOR modulation of GABAergic transmission. As mentioned earlier, ERK1/2 antagonist has been shown to mediate KOR activation-induced attenuation in evoked IPSC response in the BNST (178). We incubated the slices in either a selective MEK inhibitor (SL327, 10 μ M, n=6), or p38 inhibitor (SB203580, 20 μ M, n=5) for 40 min before and during dynorphin wash-on. In the presence of the MEK inhibitor SL327, dynorphin A significantly decreased mIPSC frequency (67.2±10.6% of baseline, p=0.04, Fig3.4A), but not amplitude (97.1±4.3% of baseline); In the presence of the p38 inhibitor SB203580, dynorphin A significantly decreased mIPSC frequency (46.6±9.9% of baseline, p<0.05, Fig3.4B) and amplitude (83.2±3.6% of baseline, p=0.02, Fig3.4B). Together, these results suggest that the dynorphin-induced attenuation of GABAergic input onto vPAG DA neurons was not mediated through MAP kinase signaling.

Dynorphin effects on GABA are not mediated through calcium and potassium ion channel conductance

As mentioned earlier, the dissociation of the $\beta\gamma$ subunit of the GPCR can directly influence the conductance of ion channels. Thus, we investigated the roles calcium and potassium channels play in the dynorphin modulation of GABA-mediated IPSC. To eliminate the role of calcium channels, we incubated the slices in calcium-free ACSF and the selective calcium chelators BAPTA-AM (50µM) and EGTA (100µM) for 1-2 hours before recording, and continued to record from the slice in calcium-free ACSF in the presence of just EGTA, or EGTA plus 4-AP (100µM) to block potassium channels. In calcium-free experiments, dynorphin A significantly decreased mIPSC frequency (71.9±8.6% of baseline, p=0.02, n=6, Fig3.5A), but not amplitude (102.3±9.6% of baseline, Fig3.5A). In calcium-free experiments where potassium channels were blocked with 4-AP, dynorphin A significantly decreased mIPSC frequency (77.8±8.0% of baseline, p < 0.05, n = 8, Fig3.5B), but not amplitude (107.5 \pm 5.7% of baseline, Fig3.5B). These data suggest that the change in conductance of these ion channels does not mediate the KOR activation effect on IPSCs. To further investigate the role of KOR $\beta\gamma$ subunits, we incubated the slices in gallein (100 μ M), an inhibitor of G protein $\beta\gamma$ subunitdependent signaling. The KOR activation-induced attenuation of GABA transmission was blocked in the presence of gallein (n=6), with no significant difference in mIPSC frequency (107.1±7.1% of baseline, Fig3.5C) or amplitude (102.8±7.4% of baseline, Together these data suggest that the effects of KOR on GABAergic Fig3.5C). transmission were mediated via $\beta\gamma$ subunit signaling, but not the ion channels.

Dynorphin effects on GABA are not mediated through PI3 kinase or PKA signaling

Having established the possibility of $\beta\gamma$ -mediation of KOR-induced decrease in GABA function, we further investigated other possibilities of KOR activation, the PI3 kinase and PKA signaling. We incubated the slices in the PI3 kinase inhibitor wortmannin (1µM), or the PKA inhibitor RP-camps (10µM), wortmannin did not block the previously observed decrease in GABA transmission frequency (72.4±7.1% of

baseline, n=6, Fig3.6A), and no effects on amplitude remains (94.2 \pm 5.7% of baseline, Fig3.6B). RP-camps did not block the KOR-induced decrease in GABA transmission; Dynorphin frequency (73.0 \pm 7.0% of baseline, n=5, p=0.051, Fig3.6B) was lower than baseline with no effects on amplitude (89.8 \pm 6.1% of baseline, Fig4B). These data suggest that PI3 kinase and PKA do not mediate KOR activation-induced decrease in GABA function.

DISCUSSION

Ventral PAG dopamine neurons have been implicated in a variety of emotional behaviors. How these mid-brain dopamine neurons are modulated by kappa opioid functions could be a crucial piece of information in understanding the regulation of addiction, as well as the associated negative affect that often leads to relapse. This study focused on determining the effects of kappa opioid receptor-activation on the inhibitory synaptic transmission onto vPAG dopamine neurons, in addition, the downstream signaling mechanisms through which the effects take place.

We frist investigated the effects of KOR activation on GABA-mediated mini IPSC and found that KOR activation by its endogenous ligand dynorphin A attenuated inhibitory transmission by frequency but not amplitude, suggesting a presynaptic mechanism-mediation. This KOR effect is consistent with previous studies in the BNST (178), and was blocked in the presence of a KOR antagonist nor-BNI in both regions, suggesting KOR-selective mediation. Although all vPAG DA neurons recorded in this study showed a decrease in GABA-mediated IPSCs, studies in the VTA demonstrated that KOR attenuates inhibitory transmission onto dopamine neurons in a projection target-dependent manner (179, 291). Recent evidence showed that GABA afferent region could also play a role in the differential modulation of IPSC by KOR activation (292). Interestingly, KOR antagonist nor-BNI alone had no effects on spontaneous inhibitory transmission, implying no tonic constitutive kappa modulation on vPAG DA neurons. In order to verify that the attenuation on GABAergic transmission was indeed mediated by presynaptic signaling of KOR, and not postsynaptic, we blocked GPCR signaling in the recorded postsynaptic cell. The blockade of postsynaptic GPCR was
achieved by the addition of GDP β s in the patching pipette, filling the recorded cell by diffusion. GDP β s is a non-hydrolyzable GDP analogue; it blocks postsynaptic GPCR signaling by preventing the exchange of GDP to GTP, thus preventing G protein activation. We found that the attenuation of mIPSCs caused by dynohpin A persisted in the presence of postsynaptic GDP β s, suggesting that the postsynaptic KOR does not play a role in the effects seen. Using GDP β s to block postsynaptic GPCRs has been shown effective in the Kash lab where postsynaptic NPY Y1 receptor agonist effects were blocked in its presence. The above evidence demonstrated KOR mediates inhibitory inputs onto vPAG DA neurons presynaptically. This is not surprising as KOR agonists have been shown to inhibit presynaptic GABA release in other brain regions, such as the BNST (178), and in the hypothalamus (293).

We next investigated the downstream signaling cascade that mediates the KOR activation-induced attenuation of IPSCs in vPAG DA neurons. Previous studies in the Kash lab demonstrated in the BNST that KOR-induced decrease in GABAergic current was mediated presynaptically via the classic MAP kinase ERK1/2 pathway. In this study, the blockade of ERK1/2 signaling by inhibitors U0126 and SL327 both blocked the KOR effects on GABAergic transmission that was previously seen. However, the current investigation showed that inhibition of ERK1/2 signaling via SL327 did not block dynorphin-induced decrease in GABAergic transmission, suggesting site-specific actions of KOR activation-triggered ERK1/2 signaling in the inhibition of presynaptic GABA. No previous study has shown a correlation between the MAP kinase P38 and modulation of synaptic transmission. However, is it a possible target as studies have shown it critical in KOR-mediated behaviors such as stress, reinstatement of drug-seeking, and dysphoria

(182, 289). We hypothesized KOR activation attenuates inhibitory transmission input, allowing dopamine neuron activity to be enhanced, increasing in signaling in projection areas such as the BNST and amygdala to increase anxiety- and stress-like behaviors, making p38 a likely candidate for the attribution of decreased inhibition. However, in the presence of the p38 inhibitor SB203580, diminished GABAergic current persisted. Curiously, both mIPSC frequency and amplitude decreased upon application of the KOR agonist dynorphin A, implying perhaps SB203580 modulated postsynaptic properties that makes vPAG dopamine neurons sensitive to dynorphin A.

We then examined ion channels that are directly regulated by the $\beta\gamma$ subunit of the KOR. Opioids depress transmission at many central synapses (294); many studies have implicated an kappa opioid-mediated inhibition of presynaptic calcium channels (189, 295) or the activation of presynaptic potassium channels (296, 297). However, other studies have ruled out the involvement of either channel (181, 291, 298). Our data demonstrated that in the vPAG dopamine neurons, neither of these ion channels contributed to the presynaptic inhibition of GABAergic release. However, inhibition of G protein By subunit-dependent signaling successfully prevented dynorphin-induced decrease in mIPSC frequency, suggesting that other actions of the $\beta\gamma$ subunit can contribute to this effect. Because gallein has been shown to not only inhibit $\beta\gamma$ subunit, but also activate PI₃ kinase activity (299), we sought to clarify the role of PI₃ kinase with wortmannin, a PI₃ kinase inhibitor. Our results suggested that KOR-induced reduction of presynaptic inhibition lies outside of the actions of PI_3 kinase. Together, we have confirmed that KOR-activation reduces inhibitory input via a presynaptic mechanism. In addition, with the removal of calcium, the effect persists, indicating that this inhibition

occurs downstream of calcium entry and is calcium-independent. These results raise the possibility that dynorphin A could be activating the KOR and directly affect the presynaptic release machinery in the GABAergic inputs onto the vPAG dopamine neurons. This data is similar to studies on KOR presynaptic inhibition of glutamatergic inputs in the hypothalamus (181). Although we did not identify the molecular target through which dynorphin inhibits presynaptic GABA, our results are consistent with studies proposing direct modulation of the exocytosis release machinery by the $\beta\gamma$ subunit of the Gi/o-coupled GPCR (300, 301). In addition, we continued to rule out the possibility of the actions of phosphorylated KOR, inhibiting PKA and the generation of cAMP. We found a close to significant (p=0.051) decrease in mIPSC frequency upon the application of dynorphin A, consistent with other results that suggest KOR does not inhibit through that actions of PKA.

In this current study, we found that KOR activation presynaptically attenuates inhibitory inputs potentially via the modulation of release machinery. However, KOR influences on excitatory inputs of vPAG dopamine neurons are unclear. Some studies have shown the KOR does not modulate EPSCs onto other dopamine-rich regions, such as the VTA (302) while others have shown the opposite (303). over all cell excitability in the VTA has been shown to be modulated in a projection-specific manner (192) where only mPFC-projecting VTA dopamine neurons are hyperpolarized by KOR activation, but not NAc-projecting dopamine neurons. It is currently unclear how KOR modulation of vPAG DA neurons alters behavior. Given the prominent role that the vPAG plays in pain and negative affect processing, as well as the correlation of KOR functions and emotional behaviors, it is tempting to speculate that KOR actions on this circuit are involved in these processes. Under the hypothesis that KOR presynaptically inhibits GABAergic inputs, disinhibiting the vPAG dopamine neurons to potentially modulate projection areas and related behaviors, further elucidation is needed regarding how the KOR modulates the glutamatergic inputs, as well as the overall effect on activity of the vPAG DA neurons. Future studies utilizing optogenetics to probe pathway-defined plasticity, as well as applying designer receptors exclusively activated by designer drugs (DREADD) to characterize pathway and cell type-specific modulation will likely shed light on this exciting possibility.

FIGURES AND TABLES



Figure 3.1: KOR-mediated signal transduction. Receptor activation by a KOR-selective ligand can result in activation of several kinase cascades. Arrows refer to activation steps, T line refer to blockers or inhibition of functions. Abbreviations are as follows: α , G-protein alpha subunit; $\beta\gamma$, G-protein beta-gamma subunit; cAMP, cyclic adenosine monophosphate; ERK1/2, extra-cellular signal-regulated kinase; GRK3, G-protein coupled receptor kinase3; JNK, c-Jun N-terminal Kinase; p38, p38 MAPK; p, phosphorylation;PI₃K, phosphoinositol 3-kinase; PKCζ, protein kinase C zeta.



Figure 3.2: KOR agonist, dynorphin A (300nM), decreases mIPSCs in vPAG DA neurons via KOR actions. (A) Representative mIPSC traces of baseline control (i) and after 10 minutes of 300nM dynorphin A wash-on (ii). (B) Dynorphin A significantly

decreased mIPSC frequency. (C) Dynorphin A had no effect on mIPSC amplitude. (D) Cumulative frequency of mIPSC was shifted towards longer interevent intervals by Dynorphin A. (E) Cumulative probability of mIPSC amplitude was not affected by dynrophin A. (F) Dynorphin A no longer decreased mIPSC frequency in the presence of nor-BNI (100nM). (G) Dynorphin A had no effect on mIPSC amplitude in the presence of nor-BNI. (H) nor-BNI alone had no effect on mIPSC frequency. (I) nor-BNI alone had no effect on mIPSC amplitude.



Figure 3.3: The postsynaptic GPCR inhibitor, GDPβs (4mM), defused into the recorded vPAG DA neuron did not block dynorphin A from attenuating mIPSC, suggesting a presynaptic mechanism. (A) Inhibition of postsynaptic GPCR did not block dynorphin A from attenuating mIPSC frequency. (B) Dynorphin A had no effects on mIPSC amplitude in the presence of GDPβs. (C) GDPβs did not block dynorphin A's ability to shift the mIPSC cumulative frequency towards longer interevent intervals. (D) Dynorphin A had no effect on mIPSC amplitude cumulative probability.



Figure 3.4: The ERK1/2 inhibitor SL327 (10 μ M) and the p38 inhibitor SB203580 (20 μ M) did not block dynorphin A from attenuating mIPSC. (A) Dynorphin A significantly decreased mIPSC frequency in the presence of SL327. (B) Dynorphin A had no effects on mIPSC amplitude in the presence of SL327. (C) Dynorphin A significantly decreased mIPSC frequency in the presence of SB203580. (D) Dynorphin A significantly decreased mIPSC amplitude in the presence of SB203580.



Figure 3.5: KOR inhibition does not require calcium and potassium ion channels. The GPCR $\beta\gamma$ subunit inhibitor blocked dynorphin A from attenuating mIPSC. (A) KOR/dynorphin A inhibition persisted even when all Ca²⁺ was removed by incubating slices in 0mM Ca²⁺/4mM Mg²⁺, 100µM EGTA with 50µM BAPTA-AM. (B) KOR/dynorphin A inhibition persisted even when all Ca²⁺ was removed and K⁺ channels are blocked with 4AP (100µM). (C) Gallein (100µM) prevented dynorphin A from inhibiting mIPSC frequency. mIPSC amplitude remained unaffected.



Figure 3.6: The PI₃ kinase inhibitor wortmannin $(1\mu M)$ and the PKA inhibitor RPcamps $(10\mu M)$ did not block dynorphin A from attenuating mIPSC. (A) Dynorphin A significantly decreased mIPSC frequency but had no effect on amplitude in the presence of wortmannin. (B) Dynorphin A decreased mIPSC frequency and had no effect on amplitude in the presence of RP-camps.



Figure 3.7: KOR presynaptically inhibit GABA release potentially via the disruption of functions in SNARE complex of the release machinery by the $\beta\gamma$ subunit of the KOR GPCR.

CHAPTER 4. VPAG DOPAMINE MODULATION ON PROJECTION AREA AND ITS BEHAVIORAL IMPLICATIONS

INTRODUCTION

Previous studies in the Kash lab have shown that both acute alcohol and KOR activation can modulate the synaptic transmission onto vPAG DA neurons; and these neurons have been implicated in the negative emotional behaviors contributing to drug relapse (84, 87, 88). Specifically, acute alcohol increased vPAG DA neuron firing rate via an increase in excitatory mEPSC, while it had no effect on inhibitory mIPSC (Chapter 2). In addition, we showed that the activation of KOR by its endogenous ligand dynorphin A significantly decreased mIPSC measured in the vPAG DA neurons, via the presynaptic actions of the $\beta\gamma$ subunit of the KOR GPCR, potentially through altering the functions of the release machinery (Chapter 3). While both alcohol exposure and KOR activation have been shown to produce negative emotional behaviors that contribute to the relapse of alcohol use, the potential of an alteration in vPAG DA neuron activity to modulate the synaptic transmission of projection regions has yet to be addressed. Both the VTA and vPAG dopamine neurons have been shown to project to the stress center, the BNST (38, 304, 305). Previously, we demonstrated distinct differences in physiological membrane properties of these two populations of dopamine neurons (Chapter 2). Specifically, the vPAG DA neurons displayed significantly lower

membrane capacitance and significantly higher resistance. In addition, the commonly observed hyperpolarization-activated I_h current in the VTA DA neurons was not observed in the vPAG DA neurons. Based on these differences in cell properties, these two distinct groups of DA neurons could potentially modulate activity in the projection region differentially.

Previous studies have shown that dopamine from the VTA to the BNST leads to a CRF-dependent increase in glutamate release in the BNST (256, 304), while dopamine signaling in the BNST has been suggested to be involved in the regulation of anxiety-like behaviors in a potential CRF-dependent fashion (38). This indirect effect on glutamatergic transmission in projection areas indicates that dopamine signaling has the potential of modulating the excitability of projection areas. In addition, the VTA dopamine projection terminals co-release dopamine and glutamate in the nucleus accumbens (261, 306). These findings raised a possibility that activity of the vPAG DA neurons could serve also as an excitatory input to target regions, such as the BNST. Interestingly, other studies have suggested that dopamine neurons could also express GABA as co-transmitters. Specifically, activation of dopaminergic neurons in the striatal slices rapidly inhibited action potentials in both the direct and indirect pathway via the release of GABA (307). However, the ability of vPAG dopamine neurons to co-release excitatory or inhibitory transmitters has not been assessed, nor is its ability to modulate target regions known. In this study, we used viral approaches to address how vPAG DA neurons can modulate target regions. Recent technique development allows researchers to simultaneously address both cell type-specificity and projection-specificity while examining neural networks and their behavioral implications. This study utilized two

cutting edge chemical genetic tools – optogenetics and designer receptors exclusively activated by designer drugs (DREADD), to address the modulatory role of the vPAG dopamine neurons in the BNST.

Optogenetics utilizes light-sensitive proteins found in algae and bacteria to activate or inhibit neural activity. The follow three types of proteins provide activation and inhibition in sub-second time scale and are most commonly incorporated: the neural-activating cation channel channelrhodopsin-2 (ChR2) (308, 309), the neural-inhibiting halorhodopsin (NpHR) (310, 311), the neural-silencing proton pump archaerhodopsin (Arch) (312). These proteins change the excitability of cells by regulating membrane potential according to their protein properties, and are activated by exposure to light of different wavelengths. In neurons, a large current is required to depolarize the cell to trigger an action potential, thus functional ChR2 must be expressed at a high level in order to reach a cumulative current large enough to reach action potential. Such a high density expression can be achieved via the infection of an adeno-associated virus (AAV). In vivo studies have demonstrated effective control of activation or inhibition of neural networks, as well as behavior, via optogenetics (313).

In addition to the light-sensitive ion channels, the Designer receptors exclusively activated by designer drugs (DREADDs) are modified G-protein coupled receptors (GPCRs) that have no basal constitutive activity, and respond to a synthetic ligand that had no natural targets within the organism. The DREADD can be coupled with the modified human muscarinic receptors (M1-M4) to activate the inhibitory Gai and the stimulatory Gaq and Gas (314-316). These receptors are activated via a synthetic ligand, the pharmacologically inert metabolite of clozapine, clozapine-N-oxide (CNO). CNO

demonstrated the lack of appreciable affinity to any receptor but the modified hM1D-hM4D (316). In addition, studies have shown that not only can CNO activate G α signaling; the activation of downstream signaling pathways that involve multiple effectors is also preserved in the modified human muscarinic DREADD receptors, such as β -arrestin and downstream MAP kinase signaling (315).

Both optogenetic light-activated cation channels and DREADDs are packaged as cre-inducible viral constructs and are expressed in specific cell populations via transgenic Cre mouse lines. In this study, we used the tyrosine hydroxylase-cre (TH-cre) and vesicular glutamate transporter 2 (vGlut2-cre) mouse lines to specifically express ChR2 or DREADD with a fluorophore for the identification and activation of dopamine neurons and glutamatergic neurons in the vPAG. We hypothesize that vPAG DA neuron activation can lead to excitation of BNST neurons and their signaling.

MATERIALS AND METHODS

Animals and Husbandry

Adult male TH-cre mice on a C57 (aged between 5 to 9 weeks) were bred and used in accordance with an animal use protocol approved by the University of North Carolina – Chapel Hill (IACUC). Mice were group-housed in our colony room under a 12:12- hour light cycle, with lights on at 7:00 AM daily. Mice were given ad libitum access to rodent chow and water. Mating pairs of mice were purchased from The Jackson Laboratory. In the TH-cre male mouse line, the tyrosine hydroxylase (TH) promoter directs expression of Cre recombinase to catecholaminergic cells; in the vesicular glutamate transporter 2 (vGlut2-cre) mouse line, the vGlut2 transporter promoter directs expression of Cre recombinase to a subset of glutamatergic neurons. Data presented here were obtained from the transgenic mice maintained in-house.

Immunohistochemistry

Mice were anesthetized with Avertin and perfused trans-cardially with chilled 0.01M phosphate buffered saline (PBS), immediately followed by 4% paraformaldehyde phosphate buffered saline. Brains were extracted and post-fixed in 4% paraformaldehyde for 24 hours and immersed in a 30% sucrose solution for 48 hours, both at 4°C. Coronal sections 45um in thickness were collected using a Leica VT1000S vibratome (Leica Microsystems, Nussloch, Germany) and stored in a 50% glycerol solution at -20°C until immunohistochemistry was performed. Slices were rinsed four times for 5 minutes in chilled PBS, followed by a 30-minute incubation in 50% methanol, a 5- minute incubation in 3% hydrogen peroxide, three 10-minute PBS washes, a 30-minute incubation in 0.5% Triton X-100 in PBS solution, and a 10-minute PBS wash. After a 1-

hour incubation in a blocking solution made of 0.1% Triton X-100/10% Normal Donkey Serum in PBS, the tissue was then incubated overnight (~18 hours) at room temperature with their respective primary antibodies diluted in 0.1% Triton X-100/10% Normal Donkey Serum in PBS blocking solution (anti-Tyrosine Hydroxylase, [1:1000], Immunostar 22941-907001; anti-Green Fluorescent Protein, [1:500], Aves Laboratories, GFP-1020). Slices were rinsed three times for 10 minutes in chilled PBS before incubating for 2 hours in their respective secondary antibodies diluted in PBS (Alexa Fluor 647 Donkey anti-Mouse, [1:200], TH; Alexa Fluor 488 Donkey anti-Chicken, [1:200], GFP; Jackson Immuno Research). Tissue was rinsed four times for 10 minutes in chilled PBS, and mounted using Vecta-Shield Mounting Medium (Vector Laboratories, Burlingame, CA, H-1000) prior to image collection.

Stereotaxic Surgery

Mice were anesthetized with isoflurane and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). A microinjection needle (32 gauge) connected to a 1µL Hamilton syringe mounted onto a digital micro-infusion pump (KD Scientific, Holliston, MA) was inserted at a 20° angle to aim for the vPAG (coordinates from Bregma: -4.5 AP, 0.0 ML, -2.8 DV). Dopaminergic neurons were transduced with 250nL of AAV5 virus coding for double floxed inverted ChR2(H134R)-mcherry under control of the EF1 α promoter, mixed with 250nL of AAV8 virus coding for double floxed inverted hM3D(Gq) DREADD-IRES-mCitrine driven by the human synapsin (hSYN) promoter at a rate of 0.1µL/min followed by 5 minutes to allow diffusion of viral particles away from the injection site. Mice were allowed to recover for 3-4 weeks to achieve maximal

infection and protein expression before DREADD behavioral assays, and 4-6 weeks prior to electrophysiological experiments.

Viral vectors

DNA plasmids coding double floxed-ChR2-mcherry were obtained from the laboratory of Karl Deisseroth. Plasmid DNA were grown and collected using a standard plasmid maxiprep kit (Qiagen, Hilden, Germany). Following plasmid purification and restriction digest, and sequencing to assure DNA fidelity, purified AAV was produced, using calcium phosphate precipitation methods by the UNC Vector Core facilities (AAV serotype 2; University of North Carolina at Chapel Hill). Viral titers used were > 1012 g.c. (genome copies) /mL. DREADD virus was obtained from Dr. Bryan Roth at UNC-Chapel Hill, who developed the construct.

Electrophysiology Brain Slice Preparation

Mice were decapitated under isoflurane anesthesia and their brains were rapidly removed and placed in ice-cold sucrose artificial cerebrospinal fluid (ACSF): (in mM) 194 sucrose, 20 NaCl, 4.4 KCl, 2 CaCl2, 1 MgCl2, 1.2 NaH2PO4, 10.0 glucose, and 26.0 NaHCO3 saturated with 95% O2/5% CO2. Three hundred micron slices were prepared using a Leica VT1200 vibratome (Wetzlar, Germany).

Slice Whole-Cell Electrophysiology

Brain slices containing the vPAG were obtained and stored at approximately 30°C in a heated, oxygenated holding chamber containing artificial cerebrospinal fluid (ACSF) (in mmol/L) 124 NaCl, 4.4 KCl, 2 CaCl2, 1.2 MgSO4, 1 NaH2PO4, 10.0 glucose, and 26.0 sodium bicarbonate before being transferred to a submerged recording chamber maintained at approximately 30°C (Warner Instruments, Hamden, Connecticut)

Recording electrodes (3–5 M Ω) were pulled with a Flaming-Brown Micropipette Puller (Sutter Instruments, Novato, CA) using thin-walled borosilicate glass capillaries. In vPAG light-evoked firing experiments, light pulses of 2ms were introduced at varying frequencies of 1, 5, 10, 20, and 40Hz. We used cell-attached experiments where the cell membrane was not broken in with the seal maintained under 50M Ω , while recording electrodes were filled with (in mmol/L) 135 K+-gluconate, 5 NaCl, 2 MgCl2, 10 HEPES, 0.6 EGTA, 4 Na2APT, 0.4 Na2GPT. During BNST light-evoked GABAergic and glutamatergic electrophysiology experiments, inhibitory (cells held at +10mV) and excitatory (cells held at -55mV) transmission was examined using the following internal solution (in mmol/L): 135 Cs-Methanesulfanate, 10 KCl, 10 HEPES, 1 MgCl2, 0.2 EGTA, 4 MgATP, 0.3 Na2GPT, 20 phosphocreatine. Signals were acquired via a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, California), digitized at 20 kHz, filtered at 3 kHz, and analyzed using Clampfit 10.2 software (Molecular Devices). Input resistance and access resistance were continuously monitored during experiments. Experiments in which changes in access resistance were greater than 20% were not included in the data analysis.

Behavioral Assays

Ten Mice injected with Gq DREADD were allowed 5 weeks for transfection (5 TH-cre, 5 wild type). The following behavioral assays were conducted with 1 resting day in between each assay: Von Frey hyperalgesia, open field test, elevated plus maze test, and light/dark box test. On each test day, each mouse was weighed and injected with CNO (1mg/kg) 30 minutes before the start of behavioral session.

Von Frey hyperalgesia

Ten Mice injected with Gq DREADD were allowed 5 weeks for transfection (5 TH-cre, 5 wild type). Starting day 1 of habituation, mice were weighed and injected with saline 30 minutes prior to being placed individually into plexiglass cages on a wire-mesh table positioned approximately 1 ft above a standard laboratory bench. Animals were given approximately 25 min to acclimate to the testing apparatus. Three hyperalgesia test habituation days were allow prior to 2 baseline days and 1 CNO-injected (1mg/kg) test day. During the hyperalgesia test, the monofilament was placed perpendicularly onto the midplantar region of the left and right hind paws through the holes in the wire-mesh table, and pressure was applied just until the point of deflection of the monofilament, after which the monofilament was immediately removed. A von Frey monofilament (Stoelting, Wood Dale, IL) with a calibrated bending force of 1.4g was presented to the hind paw 10 times, with a 1 s duration and a 1 s inter-stimulus interval for each presentation. The percent responses to the frequency test were calculated as the # paw withdrawals/10. Mechanical hyperalgesia was defined as an increase in the response rate, since this monofilament does produce some withdrawal behavior in normal mice. The results of the left and right paw did not differ, thus, we averaged the response of both paws of the five animals in each condition.

Open field test

Mice were placed into the corner of an open field box measuring 20"x20" for a 30-minute session. The center and surrounding space of the open field box were physically identical, but defined during analysis. Time spent in each defined space and total distance traveled were tracked with the software EthoVision XT by Noldus. The

center space was defined by the 50% area in the center, and the other 50% area accounted for the surrounding space.

Elevated plus maze test

Mice were placed onto the center of the elevated plus maze facing an open arm for a 5-minute session. The maze consisted of 2 open arms and 2 closed arms, each measuring 13.5"x2.5". Time spent in each compartment and entry number were analyzed with the software EthoVision XT by Noldus.

Light/dark box test

Mice were placed into the dark half of the Light/Dark box for a 15-minute session, with each compartment measuring 11"x5". The light half of the chamber was illuminated with LED lights to reach a brightness of 400 lux, while the dark side was maintained at nearly 0 lux. Time spent and entry number in each compartment were analyzed with the software EthoVision XT by Noldus.

Statistics

Effects of behavioral assays were evaluated by comparing the parameters mentioned above between the wild type and TH-cre animals using unpaired Student's t-tests. All values given for behavioral effects throughout the article are presented as mean \pm SEM.

RESULTS

vPAG dopamine neurons and their projection to the BNST

We first aimed to confirm the prior studies that found that vPAG DA neurons project to the BNST. To examine this, we injected the retrograde tracer fluorogold (FG) into the BNST and allowed ten days for the uptake and trafficking by neuronal terminals prior to perfusing the animals for tissue. TH was stained for in coronal sections from the vPAG and VTA. We found co-localization of TH and FG in both sites (Fig4.1A-B), indicating that dopamine neurons in these regions indeed project to the BNST. As mentioned earlier, dopamine neurons in the VTA have been shown to co-express glutamate. To characterize the dopamine neurons in the vPAG, we stained for TH and GFP in a vGlut2-ai3 mouse line and found that while only a small subset of vGlut2positive neurons co-expressed TH, nearly all dopamine neurons were also vGlut2positive (Fig4.1C-D). In addition, a TH-cre mousseline injected with an AAV5-DIOeYFP cre-inducible virus, images from the BNST revealed that vPAG DA neurons appear to project to a sub-region of the dorsal BNST that highly resembles the oval nucleus (Fig4.1E). Some striatal dopamine neurons have been shown to co-express GABA, thus we assess this possibility in the vPAG using a GAD67-eGFP mouse line to satin for TH. We found that GABAergic neurons are located laterally and distinct from the TH-positive dopamine neurons (Fig4.2).

Optogenetics – vGlut2-cre animals

Next we used an optogenetic approach to assess the potential of vPAG DA neurons to modulate activity in the BSNT. We first used vGlut2-cre mice, as we reasoned that this would be a more robust projection, and would allow us to assess the

feasibility of this approach. In a vGlut2-cre mousseline, we injected AAV-DIO-ChR2mCherry into the vPAG and allowed 8 weeks for transfection. Transfected cell bodies in the vPAG that expressed mcherry were visualized and recorded from. Blue light stimulation pulses (2ms each) varying in frequency were presented, we found that the vPAG dopamine neurons fired in a time-locked manner at corresponding frequencies of 1, 5, 10, 20, and 40Hz (Fig4.3), indicating a successful and functional ChR2 transfection. To assess the ability of vPAG DA neurons to modulate BNST transmission, we targeted BNST neurons in proximity to clusters of mCherry-expressing terminals and attempted to light-activate ChR2-expressing terminals. Blue light stimulation pulses were presented in the same fashion as were in the vPAG. We measured light-evoked glutamatergic currents in the BNST neurons (n=4) at corresponding frequencies of 1, 5, 10, 20, and 40Hz (Fig4.4), indicating vPAG DA neuron-activation can trigger glutamate release in the projected BNST. Interestingly, on occasions, we also found light-evoked GABAergic currents (n=2) with a longer delay between stimulation artifact and current onset $(11.5\pm4.9\text{ms})$ comparing to that of the light-evoked glutamate current $(2.115\pm0.16\text{ms})$.

Optogenetics – TH-cre animals

Using the same approach as the above section, TH-cre animals were injected with AAV-DIO-ChR2-mCherry into the vPAG and allowed 8 weeks for transfection. We targeted BNST neurons in proximity to clusters of mCherry-expressing terminals and attempted to light-activate ChR2-expressing terminals. Blue light stimulation pulses (2ms each) were presented in pairs and measured light-evoked GABAergic and glutamatergic currents. We found measureable light-evoked glutamatergic currents (Fig4.5B) in the BNST (n=3). Interestingly, we also found light-evoked GABAergic

currents (n=5) (Fig4.5A) in some BNST neurons. Similar to the findings in vGlut2-cre virus-injected animals, the GABA currents have a significant longer delay between stimulation artifact and current onset (8.0 ± 1.3 ms) comparing to that of the light-evoked glutamate current (3.1 ± 0.2 ms)

Von Frey hyperalgesia test

We next evaluated the behavior implications of the vPAG DA neurons in nociception. We used a Von Frey filament that produced around 60-70% pawwithdrawal response out of the ten filament stimulations given. The data of the right foot and left foot were pooled together as they were not different. We found that during the two baseline days, the responses of the two groups were not different (Baseline day1: TH-cre 6.2 ± 0.3 , WT 6.3 ± 0.4 ; baseline day2: TH-cre 6.2 ± 0.3 , WT 6.3 ± 0.4 , Fig4.6). On the CNO injection day, both groups received injections of 1mg/kg CNO. The TH-cre animals displayed a robust decrease in response (TH-cre 3.0 ± 0.3 , WT 7.1 ± 0.3), indicating a robust anti-nociceptive effect of activated vPAG DA neurons via Gq-DREADD.

Open field test

To evaluate anxiety-like behaviors, we next conducted the open field test upon DREADD activation of vPAG DA neurons. We found no significant difference in locomoter defined by total distance moved between wild type (9769.4 \pm 302.6cm, Fig4.7A) and TH-cre (8607.1 \pm 815.3cm) animals. In addition, we found no significant differences in time spent in center (WT 167.6 \pm 24.7s, TH-cre 201.1 \pm 42.8s, Fig4.7B) and entrance number to center (WT 72.8 \pm 4.5, TH-cre 73.8 \pm 12.9, Fig4.7C), suggesting no change in anxiety-like behavior.

Elevated plus maze test

We next conducted another anxiety assay using the elevated plus maze. We found no significant differences in time spent in either open arms (WT 54.8 \pm 7.3s, TH-cre 57.8 \pm 2.2s, Fig4.8A) or closed arms (WT 187.0 \pm 6.7s, TH-cre 193.1 \pm 2.8s, Fig4.8C). We also found no significant differences in entry number to either open arms (WT 9.4 \pm 1.5, TH-cre 10.6 \pm 0.7, Fig4.8B) or closed arms (WT 20.0 \pm 1.1, TH-cre 22.0 \pm 2.0, Fig4.8D), suggesting no change in anxiety-like behavior.

Light/dark box test

We additionally tested anxiety using the light/dark box assay. We found no significant differences in time spent in either light (WT 240.7 \pm 67.3s, TH-cre 374.2 \pm 32.6s, Fig4.8A) or dark compartment (WT 646.7 \pm 67.9s, TH-cre 513.0 \pm 33.1s, Fig4.8C). We also found no significant differences in entry number to either light (WT 23.8 \pm 5.6, TH-cre 26 \pm 2.8, Fig4.8B) or dark compartment (WT 25.6 \pm 5.0, TH-cre 27.2 \pm 3.1, Fig4.8D), suggesting no change in anxiety-like behavior.

DISCUSSION

The vPAG DA neurons project to extended amygdala nuclei that are identified to regulate negative emotions such as stress and anxiety. However, whether vPAG DA activities can modulate synaptic transmission in these projection regions is unknown. This study focused on the potential synaptic modulation of the BNST, a known projection area of the vPAG DA neurons, upon activation of the cell bodies in the vPAG. First, we confirmed dopaminergic projections to the BNST via the injection of a retrograde tracer into the BNST. We found that both VTA and the vPAG dopamine neurons project to the BNST, consistent with previous findings (38). Once this projection was established, we next probed the possibility of vPAG dopamine neurons co-expressing vGlut2 via immunohistochemistry in a vGlut2 reporter mouse line stained for TH. We found that nearly all of vPAG dopamine neurons also expressed vGlut2, similar to results from other studies showing co-expression of TH and vGlut2 in the VTA axons and synapses via immunocytochemical labeling (317). Further, at the VTA projection terminals, dopamine neurons co-release dopamine and glutamate in the nucleus accumbens (261, 306). These findings raised a possibility that activity of the vPAG DA neurons could serve also as an excitatory input to target regions, such as the BNST. Interestingly, other studies have suggested that dopamine neurons could also express GABA as co-transmitters. Specifically, activation of dopaminergic neurons in the striatal slices rapidly inhibited action potentials in both the direct and indirect pathway via the release of GABA (307). This release was shown to be directly from dopaminergic axons, independent of the vesicular GABA transporter vGAT. Instead, GABA was released via the vesicular monoamine transporter vMAT2. In our vGAT reporter line stained for TH, vPAG displayed a clear distribution of GABAergic and TH-positive dopaminergic neurons, distinctly separated from one another, proposing that the dopamine neurons co-express glutamate, but not GABA.

We further showed that these vGlut2/DA neurons project to the stress center – BNST, suggesting a potential modulatory role of vPAG DA neurons in the regulation of negative stress affect. In addition, our BNST vGlut2 staining highly overlapped with a sub-region known as the oval nucleus (Allen Brain Atlas), where dopamine signaling has been linked with cocaine self-administrating behavior (318). As mentioned in earlier chapters, activities in the vPAG are linked to the negative affect of drug addiction, as well as the rewarding properties of drugs of abuse, increasing the likelihood that the vPAG DA neurons modulate these behaviors through signaling in the oval nucleus of the BNST. Our results raised the possibility that the activation of vPAG DA neuron projection could positively modulate activities in the BNST by releasing glutamate.

We next utilized both TH-cre and vGlut2-cre mouse lines with channelrhodopsin viral vector injected into the vPAG to identify and record from dopamine neurons in the vPAG. We were able to visualize dopamine cell bodies that are transfected with the virus and expressing the fluorophore mCherry. Using cell-attached recording, we demonstrated the success of functional ChR2 transfection that can be activated by light. While measuring cell firing, vPAG DA neurons fired in a time-locked manner as the presentation of the blue light-stimulation pulses were varied in frequency. The confirmation of excitability of vPAG DA neurons triggered by blue light suggested that activity in ChR2-expressing processes and terminals should as well be capable of being stimulated for release by blue light presentations. Indeed, while targeting BNST neurons

in close proximity to clusters of terminals and axonal processes expressing mCherry, we measured time-locked EPSC stimulation by blue light pulses. This response was consistently abolished in the presence of the AMPA receptor blocker NBQX, demonstrating light-evoked glutamate release onto the recorded BNST neurons synapsing with excited vPAG DA projections.

On occasions, we observed light-evoked GABAergic current in BNST neurons. However, there seemed to be a significant delay between light stimulation artifact and the GABAergic current measured compared to the light-evoked glutamatergic currents measured. A possible explanation for this observation is that vPAG DA/vGlut2 neurons synapses onto local GABAergic interneurons, while light stimulation isn't spatially specific, the light-evoked inhibitory input could be elicited from local interneurons. Via ultra-violate uncaging of glutamate, a recent study in the BNST demonstrated a dense intra-regional connection that is mostly GABAergic (319). This information compels more investigation on the differential distribution of DA projections onto the BNST from the VTA and vPAG, as well as the cell types on which they synapse. It has been suggested that BNST-projecting VTA dopamine neurons release dopamine into the BNST, enhancing local CRF activity to modulate BNST projection neurons (256). Additional, other studies suggested a role of BNST-projecting vPAG DA neurons in the modulation of CRF-dependent moods and affective disorders using the acoustic startle behavioral paradigm (38). The above data urges us to examine dopamine release in the BNST by vPAG DA neurons, and the modulation of local neurons follow suit.

While assessing behavioral influences of the vPAG dopamine neurons via DREADD-injections into the vPAG in both TH-cre and wild type animals, we conducted

assays to assess anxiety-like behavior, such as open field test, elevated plus maze, light/dark box, in addition to a pain perception assay via the Von Frey hyperalgesia test. We found no significant difference between TH-cre and wild type groups in anxiety assays upon activation of DREADD in vPAG DA neurons via the injection of CNO. However, we found significant differences in the Von Frey hyperalgesia assay. The THcre mice displayed a robust anti-nociception effect comparing to the wild type animals, suggesting that the activation of vPAG DA neurons is analgesic. This is consistent with other studies where selective dopamine lesions or dopamine antagonism in the vPAG attenuates opioid-induced analgesia (42, 320), and microinjections of DA agonist in the vPAG also cause a robust anti-nociception that can be inhibited by antagonists. However, we found no significant anxiety-like effects upon activation of the vPAG DA neurons. One possibility to this observation is that the vPAG DA neurons could be synapsing onto neurons that dive anxiolysis. The kinetics of DREADD activation could also contribute to this observation where CNO activation of DREADD could be dispersed over time, and not sufficient or robust enough to drive anxiety-like behavior. Taken together, we found that vPAG DA activation could potentially modulate the projection area, BNST, by increasing excitatory transmission. In addition, vPAG DA plays an important role in the ascending nociceptive control (ANC) pathway, and can produce analgesia effects when excited.

It is currently still unclear how vPAG DA neurons influence negative emotional behaviors such as anxiety and depression through the modulation of projection areas. Given the prominent role of BNST in stress-related behaviors and our findings that vPAG DA neuron activation could release glutamate onto BNST neurons, it is tempting to speculate that vPAG DA actions on this circuit are involved in these processes. Future studies utilizing optogenetics and fast-scan cyclic voltammetry to probe the role of dopamine release from specific pathways and cell type-specific modulation will likely shed light on this exciting possibility.

FIGURES AND TABLES







Figure 4.2: GABAergic and dopaminergic cell body distribution in the vPAG. A GAD67-eGFP animal co-stained for TH demonstrated that dopaminergic neurons are located ventral and medial to the aqueduct, and GABAergic neurons are situated laterally to the aqueduct. Further, the GABAergic and dopaminergic neurons are distinct from one another and do not co-express.















Figure 4.3: Light-evoked action potential in the vPAG DA neurons. A vGlut2-cre mouse was injected with a viral vector of double inverted channelrhodopsin(ChR2)-mcherry into the vPAG. Transfected cell bodies in the vPAG that expressed mcherry were visualized and recorded from. Blue light stimulation pulses varying in frequency were presented while the vPAG dopamine neurons fired in a time-locked manner at corresponding frequencies of 1, 5, 10, 20, and 40Hz.


















Figure 4.4: Light-evoked glutamatergic current in the BNST neurons. A vGlut2-cre mouse was injected with a viral vector of double inverted channelrhodopsin(ChR2)-mcherry into the vPAG. Transfected vGlut2 terminals projected from the vPAG expressed mcherry and could be visualized in the BNST. Neurons in proximity to clusters of mcherry-expressing terminals were recorded from. Blue light stimulation pulses varying in frequency were presented while glutamatergic current were evoked in BNST neurons at corresponding frequencies of 1, 5, 10, 20, and 40Hz.



Figure 4.5: Light-evoked GABAergic and glutamatergic currents in the BNST neurons. A TH-cre mouse was injected with a viral vector of double inverted channelrhodopsin(ChR2)-mcherry into the vPAG. Transfected TH projections from the vPAG expressed mcherry and could be visualized in the BNST. Neurons in proximity to clusters of mcherry-expressing terminals were recorded from. Blue light stimulation pulses were presented and both (A) evoked-GABAergic and (B) evoked-glutamatergic currents were observed in BNST neurons.



Figure 4.6: Activation of vPAG TH-positive dopamine neurons produced a robust antinociceptive effect. TH-cre and wild type animals were injected with viral vectors of double inverted Gq-coupled DREADD into the vPAG. Upon activation of vPAG dopamine neurons via the injection of CNO prior to the Von Frey hyperalgesia test, THcre animals displayed a significant decrease in response to the hyperalgesia test than the wild type animals.



Figure 4.7: Activation of vPAG TH-positive dopamine neurons had no significant effects on open field assay. Upon activation of vPAG dopamine neurons via the injection of CNO prior to the open test, there was no significant differences in A) total distance traveled, B) time spent in center, and C) entrance number to center between TH-cre and wild type animals.







Figure 4.9: Activation of vPAG TH-positive dopamine neurons had no significant effects on light/dark box assay. Upon activation of vPAG dopamine neurons via the injection of CNO prior to the open test, there was no significant differences in A) time spent in light compartment, B) entry number to light compartment, C) time spent in dark compartment, and D) entry number to dark compartment between TH-cre and wild type animals.

CHAPTER 5. GENERAL DISCUSSION

Substance abuse, a global threat to health and productivity, has become an increasingly difficult problem to solve, as access to commercially available uncontrolled substances and legal prescription drugs are easy to acquire, and many of which are highly addictive, putting average families at risk. The vPAG, a somewhat neglected brain region in the research of drug abuse, is ideally positioned to regulate drug-seeking behaviors and the negative affects that are so often associated with drug withdrawal. Previous chapters provide evidence that link vPAG functions to many negative emotional behaviors, as well as demonstrate the role vPAG DA neurons play in the rewarding properties of drugs of abuse. Despite their demonstrated relevance to both emotional behaviors and drug abuse, little has been done to characterize vPAG dopamine neurons. To better speculate the role of vPAG DA neurons in drugs of abuse and negative affects, it is necessary to understand the what, how and where of this cell population: what are the differences between vPAG DA neurons and dopamine neurons in other regions? How are they modulated by neighboring networks and drugs of abuse? To where do they project and how do they influence target areas? Although the heterogeneity in the vPAG has made it difficult to isolate neural systems to examine their synaptic properties the modulation of functions, this dissertation utilizes genetic tools and cutting edge

techniques that allow us to address cell-type specific and projection-specific functions and modulation.

First, we examined the effects of alcohol in the vPAG DA neurons. As mentioned previously, both acute and chronic alcohol modulate synaptic transmission in VTA dopamine neurons; however, the vPAG DA neurons, an extended population of the VTA DA neuron group, displayed a surprising resilience to alcohol exposure. Concentrations of alcohol that commonly induce synaptic alterations in other brain regions had no effect on vPAG DA inhibitory transmission regardless of the method of administration and measurement. Acute alcohol administration, whether through *in vivo* i.p. injections or *ex vivo* bath application, had no influence on electrically evoked and spontaneous IPSCs. Given that GABA receptors display subunit-dependent sensitivity to alcohol, it is possible that GABAergic receptor-composition in the vPAG could be drastically different than that in other brain regions, buffering the effects of alcohol in the vPAG. Further examination of GABA subunit expression in the vPAG could provide more information on the unique resilience to alcohol in this brain region.

We also observed no difference in synaptic transmission after 2 cycles of alcohol exposure. While other studies have found this paradigm sufficient to induce synaptic changes in C57BL/6J mice, the Swiss Webster mice used in these experiments displayed no alteration in response to alcohol exposure. A longer exposure period could be required for the Swiss Webster background mice to produce synaptic changes; however, we did observe a robust increase in aggressive behavior, contrasting with the generally docile demeanor of Swiss Webster mice. Alcohol-induced aggression has been reported across species (230, 231), and interestingly, some of the proposed sites for defensive and

aggressive behaviors include the lateral habenula glutamatergic inputs onto the dorsal PAG, where raphe serotonergic neurons also innervate (72, 278), as well as the dorsal PAG projections to the hypothalamus/thalamus. Together, this evidence suggests a potential role of vPAG mediating alcohol-induced aggression for future investigation.

Having investigated the effects of alcohol on vPAG DA neurons, we next examined the modulation of these neurons by kappa opioid receptors, which mediate both drinking behaviors and negative emotional behaviors. We demonstrated that KORactivation induced a significant decrease in GABA-mediated inhibitory inputs (mIPSC), and that this attenuation in transmission is mediated through a presynaptic KOR mechanism. Through a series of experiments to eliminate possible signaling transduction pathways, we concluded that the by subunit of the GPCR mediated the observed presynaptic KOR-inhibition, possibly through a change in the release machinery. Although our data clearly demonstrated that KOR-activation attenuates inhibitory transmission onto vPAG DA neurons, the ways in which KOR activity can influence excitatory transmission and overall excitability remain unknown. However, with such a significant decrease in inhibitory input, it is likely that vPAG DA neurons could be disinhibited, subsequently increasing in firing rate and signaling in projection regions. Given that vPAG DA neurons project to regions mediating negative affect such as stress and anxiety and that KOR actions mediate dysphoria, it is possible that KORdisinhibition of vPAG DA neurons enhances target region signaling, increasing negative emotions. While mu-opioid receptor (MOR)-activation is generally known to produce euphoric effects (321), it would be interesting to probe this hypothesis and examine MOR effects on the vPAG DA neurons for possible differential modulation.

This dissertation made one of the first attempts to characterize the membrane properties of vPAG dopamine neurons. We demonstrated that although the vPAG DA neurons and the well-studied VTA DA neurons share a common projection region, the stress center BNST, they display distinct differences in membrane properties. This finding emphasizes the possibility that vPAG and VTA dopamine neurons could have distinct synaptic properties, corresponding with our finding that vPAG DA neuron IPSCS are resilient to alcohol exposure while other studies show VTA DA neurons susceptible. Using retrograde tracer injection into the BNST, we demonstrated that both the vPAG and VTA project to the BNST, however, their respective distribution is unknown. Given that dopamine neurons in these two regions are different in membrane properties and response to alcohol, it is highly possible that they regulate activities in the BNST in a diverging manner.

Our studies also show that vPAG DA neurons co-express vGlut2, and via optogenetic approach, light-stimulation of TH terminals originating from the vPAG can evoke glutamatergic currents in the BNST, supporting the hypothesis that vPAG DA neurons can modulate the projected region. The TH-cre mice injected with a creinducible eYFP illustrated a dense and localized glutamatergic projection to the oval nucleus of the BNST; however, projection-specificity is undefined as some VTA DA neurons also co-express glutamate. The projection-specific distribution in the BNST could be addressed via the injections of AAV-DIO- vectors expressing different fluorophores into the VTA and vPAG of TH-cre mice, resulting in the expression of different fluorophores, representing two distinct dopamine populations. Similar approach could be taken by injecting two AAV-DIO-ChR2 vectors that are activated by different spectral wavelengths. Via this approach, we could distinctly activate one projection verses another, while examining BNST synaptic transmission during light activation. This would provide us with the necessary information in understanding how dopamine neuron projections to the BNST from two distinct regions modulate target region activity.

Another method to acquire both cell type- and projection-specific activation is the Flp-FRT recombination, analogous to the Cre-lox recombination. In the TH-cre animals, the retrograde cre-inducible Flp vector (HSV-DIO-Flp) is injected into the projection site (BNST) to be taken up by the terminals of cre-expressing cell types, while the cre- and Flp-inducible FRT-ChR2-(fluorophore) is injected into the cell body region (vPAG). Because the injected Flp is cre-inducible, the recombination of Flp-FRT can only occur in cre-expressing cells, only TH-positive neurons will be expressing the light-activated ChR2 for manipulation. The few different viral vectors mentioned above are all suitable to address cell type- and projection-specificity both ex vivo and in vivo. Although our in vivo Gq-DREADD data demonstrated a robust vPAG DA neuron-activation-induced anti-nociception, this experiment was activating all of vPAG DA projections. Our data suggested that both alcohol and KOR activation have the potential of increasing vPAG DA neuron activities. In addition, both alcohol withdrawal and KOR activation have been shown to increase negative emotions such as stress and anxiety. Thus, it is important to investigate the meaning of vPAG DA neuron activation in terms of addiction-related behaviors and associated negative affect. To address the role vPAG DA activity plays in mediating stress and anxiety, one of optogenetic approaches mentioned above can be applied in vivo in combination with an array of behavioral assays.

This work characterized vPAG DA neurons and investigated their modulation by alcohol and KOR activity. Acute alcohol increased excitatory glutamatergic inputs and overall activity of the vPAG DA neurons, but had no effect on inhibitory GABAergic inputs. In addition, KOR activation attenuated GABAergic transmission onto vPAG DA neurons via presynaptic GPCR $\beta\gamma$ subunit signaling. In support of the original hypothesis, evidence showed that vPAG DA neurons co-express glutamate, and project to the stress center, BNST. Activation of this vPAG DA input to the BNST produced glutamatergic currents, suggesting synaptic transmission modulation by the vPAG. This work indicated a strong potential of vPAG DA neurons to directly modulate negative affective disorders that are often associated with alcohol and drug abuse, rendering treatment ineffective or short-lived. While further investigation is necessary, our results provided critical information on the mechanisms and modulation of vPAG DA neurons that could assist in the development of treatment for both drug abuse and emotional disorders.

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