Mu-Opioid Receptor – pAKT Signaling in the Ventral Tegmental Area is Critical for the Behavioral and Cellular Consequences of Social Stress

by

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ABSTRACT

Intermittent social defeat stress produces vulnerability to drugs of abuse, a phenomena known as cross-sensitization, which is proceeded by a corresponding upregulation of ventral tegmental area (VTA) mu-opioid receptors (MORs). Since VTA MORs are implicated in the expression of psychostimulant sensitization, they may also mediate social stress-induced vulnerability to drugs of abuse. Social stress and drugs of abuse increase mesolimbic brain-derived neurotrophic factor (BDNF) signaling with its receptor, tropomyosin-related kinase B (TrkB). These studies examined whether VTA MOR signaling is important for the behavioral and cellular consequences of social stress. First, the function of VTA MORs in the behavioral consequences of intermittent social defeat stress was investigated. Lentivirus-mediated knockdown of VTA MORs prevented social stress-induced cross-sensitization, as well as stress-induced social avoidance and weight gain deficits. Next it was examined whether VTA MOR expression is critical for stress-induced alterations in the mesocorticolimbic circuit. At the time cross-sensitization was known to occur, lentivirus-mediated knockdown of VTA MORs prevented stress-induced increases in VTA BDNF and its receptor, TrkB in the nucleus accumbens (NAc), and attenuated NAc expression of delta FosB. There was no effect of either stress or virus on BDNF expression in the prefrontal cortex. Since social stress-induced upregulation of VTA MORs is necessary for consequences of social stress, next activity dependent changes in AKT, a downstream target of MOR stimulation associated with sensitization to psychostimulant drugs, were investigated. Using fluorescent immunohistochemical double labeling for the active form of AKT (pAKT) and markers of either GABA or dopamine neurons in the VTA, it was determined that social stress significantly increased the expression of pAKT in GABA, but not dopamine neurons, and that this effect was dependent on VTA MOR expression. Moreover, intra-VTA inhibition of pAKT during stress prevented stress-induced weight gain deficits, while acute inhibition of VTA pAKT blocked the expression of cross-sensitization in subjects that had previously exhibited sensitized locomotor activity. Together these results suggest that social stress upregulates MORs on VTA GABA neurons, resulting in AKT phosphorylation, and that increased VTA MOR-pAKT

signaling may represent a novel therapeutic target for the intervention of substance abuse disorders.

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CHAPTER 1: INTRODUCTION AND BACKGROUND

1. INTRODUCTION

Substance abuse represents a broad reaching societal and economic problem, with 24.6 million Americans admitting to being 'current drug users' in 2013 (SAMHSA, 2014). In the 2012 fiscal year, the United States nationally spent over $24 billion dealing with the prevention, treatment, and criminal consequences of substance abuse (USDOJ, 2011), and when comorbid disorders are taken into account, addiction has some of the highest costs of any medical disorder (Kreek et al., 2005). While treatment has been the focus of much research, a majority of recovering addicts are likely to relapse, thus there is a need for research that focuses on reducing the occurrence of substance abuse (Sinha, 2011, NIDA, 2012). Vulnerability to substance abuse and addiction are influenced by a variety of genetic and environmental factors (Kreek et al., 2005). One environmental variable that can influence the transition from recreational drug use to abuse is stress, and it has been correlated with both increased substance abuse and relapse (Sinha, 2001, 2008, 2011, NIDA, 2012).

The neurobiological mechanisms of stress and addiction have been widely studied in humans and animals. In rodents, mild or intermittent experiences of stress induce changes in the mesocorticolimbic circuit that are similar to those seen after exposure to drugs of abuse (Nikulina et al., 2014). Much research has focused on increases in dopamine (DA) transmission from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and the changes observed in those regions. While the literature generally agrees that increases in VTA DA transmission are necessary for many of the behaviors observed after stress or sensitization to drugs of abuse, less attention has been paid to the involvement of VTA gamma amino-butyric acid (GABA) transmission. Drugs that activate mu-opioid receptors (MORs) have been shown to induce cross-sensitization to psychomotor stimulants, and in the VTA these receptors have been traditionally viewed as being localized to GABA neurons (see review of Nikulina et al., 2014). When inhibitory mu-opioid receptors (MORs) are activated on VTA GABA neurons, they reduce GABA transmission, thereby increasing VTA DA activity (Johnson and North, 1992, Margolis et al., 2014). In the case of social stress-induced cross-sensitization to amphetamine, there is a

corresponding increase in VTA mu-opioid receptor (MOR) expression (Nikulina et al., 2005, Nikulina et al., 2008). Taken together, it is possible that stress-induced increases in VTA MOR signaling in VTA GABA neurons may indirectly mediate the changes that occur in VTA DA neurons, and the behaviors associated with increased DA transmission to the NAc.

The goal of the experiments described herein is to determine whether VTA MORs play a functional role in the effects of social stress. The first experiment asks whether VTA MORs serve a functional role in social stress-induced behaviors, specifically stress-induced cross-sensitization to amphetamine. The second asks if VTA MORs are necessary for social stress-induced changes throughout the mesocorticolimbic circuit. Since MOR activation has been linked to phosphorylation of intracellular signaling kinase AKT (Polakiewicz et al., 1998, Iglesias et al., 2003, Russo et al., 2007, Mazei-Robison et al., 2011), the third experiment asks whether VTA MORs mediate stress-induced cross-sensitization through activation of intracellular AKT signaling in GABA neurons. The combined results of these experiments will not only improve our understanding of stress-induced cross-sensitization to psychomotor stimulants, but may also aid in the identification of potential new therapeutic targets.

1.1 Substance Abuse: A Role for Stress

In 2012, illegal drug use by United States citizens over age 12 reached its highest point in ten years, and worldwide, an estimated 183,000 drug-related deaths occurred (UNODC, 2014). Estimates from 2012 revealed that 3.5% – 7.0% of the world population had used an illegal drug in the last year (UNODC, 2014), while in the United States, an estimated 22.7 million people age 12 or older have a diagnosable substance abuse disorder (SAMHSA, 2014). Given that the majority of recovering addicts are likely to relapse (Sinha, 2011, NIDA, 2012), there is a need for research aimed at reducing the occurrence of substance abuse. Similar to the craving induced by drug cues, images depicting a stressor induce increases in cocaine craving and anxiety in recovering addicts (Sinha et al., 1999, Sinha et al., 2000), suggesting that stressful experiences are as potent as drug cues in potentiating relapse. While much research has targeted relapse and addiction treatments, the reality is that treatment can be costly and is not always readily available.

In fact, treatment for substance abuse was unavailable to 20.2 million of the 22.7 million people who were classified as having a substance abuse disorder in 2013 (SAMHSA, 2014), a problem which further emphasizes the importance of studying variables which confer vulnerability to drugs of abuse. While vulnerability to addiction likely has some genetic component, twin studies have shown that only 30-60% of the variance can be explained by heritability (Kreek et al., 2005), suggesting that environmental experiences also play a key role in determining susceptibility to drugs of abuse.

Stress is one variable that influences the transition from recreational drug use to abuse. In humans, stress has been associated with vulnerability to substance abuse and increased risk of relapse (Brewer et al., 1998, Sinha, 2001, 2008, 2011, NIDA, 2012). In rodents, the intermittent social defeat stress paradigm (Fig. 1.1) serves as a naturalistic model of social stress-induced changes in the behavior and pathology of humans, in particular, this paradigm has been shown to augment the effect of psychomotor stimulants, a phenomena termed 'cross-sensitization' (see reviews of Miczek et al., 2008, Nikulina et al., 2014). While many laboratory stress paradigms exist, a basic requirement of such models of *vulnerability* to addiction, is that the behavioral or biological feature being used to classify animals as vulnerable, must be manipulated and characterized prior to drug exposure (Piazza and Le Moal, 1996). In addition to satisfying this requirement, this model of social defeat stress has high face validity when compared to stress responses in humans (Covington and Miczek, 2005, Sinha, 2008).

Similar to the persistent sensitization induced by intermittent drug exposure (Paulson et al., 1991), this social defeat model induces persistent cross-sensitization to amphetamine lasting at least 2 months (Nikulina et al., 2004). The effects of stress and psychostimulant drugs are related, with both stimuli involving changes in stress-related hormones and trophic factors, specifically glucocorticoid hormones (cortisol in humans, corticosterone in rodents) and/or corticotropin releasing factor (Piazza and Le Moal, 1996, Sinha, 2001, Marinelli and Piazza, 2002). The literature strongly suggests that stress and drugs of abuse both act on the

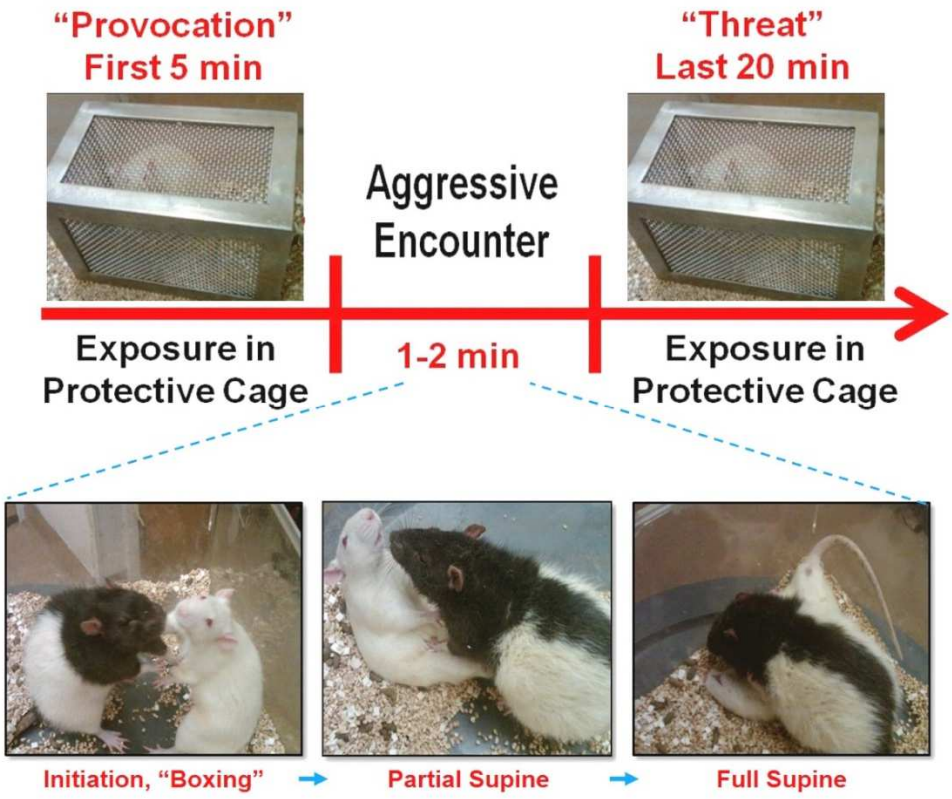

Figure 1.1. Schematic of Intermittent Social Defeat Stress. While models of intermittent social defeat stress can be daily or episodic, they all consist of a brief losing social interaction in the home cage of an aggressive resident. In the above schematic, the defeated intruder rat is a white Sprague Dawley, while the aggressive resident is a black and white Long Evans rat. To provoke attack by residents, for the first 5 min of stress procedures, the intruder is placed inside a protective cage that permits sensory contact (Top). During the physical aggressive encounter, the protective cage is removed, allowing the resident to attack (Bottom) until the intruder indicates that it has been defeated by display of the submissive supine position (Bottom far right). After having been defeated, the intruder is placed back inside the protective cage and exposed to threat of attack for an additional 20 min. In contrast to continuous social defeat models, at the conclusion of intermittent stress procedures, subjects are returned to home cages that are not in proximity to aggressive residents.

mesocorticolimbic brain circuit (Cleck and Blendy, 2008, Nikulina et al., 2014), thus the regions comprising this circuit serve as potential points of convergence for the effects of stress and drugs of abuse. In support of this, augmented dopamine transmission along the mesolimbic pathway (VTA-NAc) has been found to be associated with many of the effects of stress and drugs of abuse (Piazza and Le Moal, 1996, Sinha, 2001, Marinelli and Piazza, 2002, Cleck and Blendy, 2008, Nikulina et al., 2014).

1.2 Basic Anatomy of the Mesocorticolimbic Dopamine Circuit

The mesocorticolimbic circuit (Fig. 1.2), consisting of interconnected midbrain, limbic, and cortical areas, has been strongly implicated in the effects of both stress and drugs of abuse (Sinha, 2008, Morales and Pickel, 2012, Nikulina et al., 2014). Although neuroplasticity in the VTA, NAc, and prefrontal cortex (PFC), as well as other structures, is important for reward, stress, and reinforcing behaviors, it is well accepted that increased mesolimbic (VTA-NAc) DA transmission is crucial for many of the effects stress and drugs of abuse (see reviews of Fields et al., 2007, Nikulina et al., 2014). In fact, VTA DA transmission and release in the NAc is necessary for the induction of sensitization to both psychomotor stimulants and opiates (Joyce and Iversen, 1979, Vezina et al., 1987, Vezina and Stewart, 1989, Pierce and Kumaresan, 2006). The neurons found in the VTA are highly heterogeneous, with neuronal types differentially distributed across the rostocaudal and mediolateral axes of the VTA (Nair-Roberts et al., 2008, Morales and Pickel, 2012). The entire VTA region largely consists of dopamine neurons (50-65%), followed by GABA neurons (30-35%; Swanson, 1982, Oades and Halliday, 1987, Yamaguchi et al., 2007, Nair-Roberts et al., 2008), and glutamate neurons (2-3% Nair-Roberts et al., 2008). Study of this region has been complicated by the recent finding that a significant portion of glutamate neurons also express tyrosine hydroxylase (TH), the rate limiting enzyme in the synthesis of dopamine

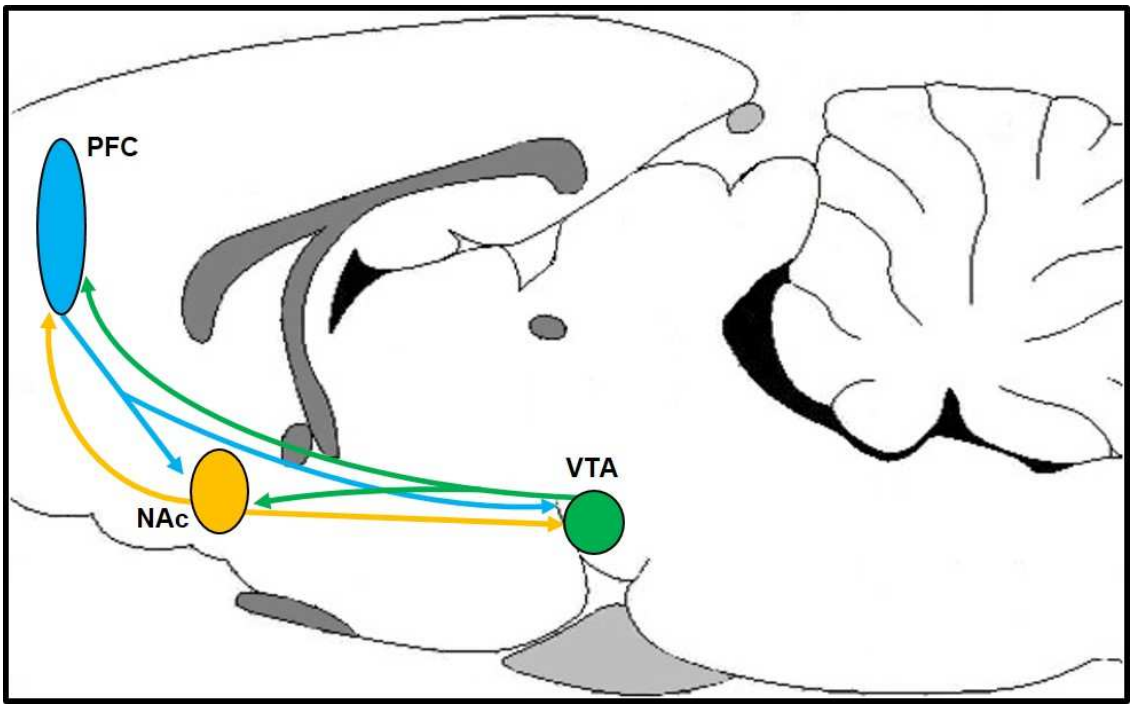

Figure 1.2. Schematic of Mesocorticolimbic Projections. The VTA (green), NAc (yellow), and PFC (blue) represent major nodes in the mesocorticolimbic circuit that are highly interconnected by afferent and efferent projections (Fields et al., 2007, Sesack and Grace, 2010, Morales and Pickel, 2012, Walsh and Han, 2014, Fields and Margolis, 2015).

(Fields et al., 2007, Morales and Pickel, 2012, Morales and Root, 2014). Based on this, VTA neurons can release GABA, DA, glutamate, or co-release glutamate and DA.

In addition to sending/receiving VTA excitatory/inhibitory projections to/from other areas of the mesocorticolimbic circuit (Fig. 1.2), the VTA also contains a population of GABA interneurons (Fields et al., 2007, Ting and van der Kooy, 2012). Excitatory glutamate projections from the PFC innervate the VTA and NAc, while the NAc sends inhibitory GABA projections to the PFC and VTA (Morales and Pickel, 2012, Fields and Margolis, 2015). Despite receiving GABA projections from the NAc, the rostromedial tegmental nucleus (tail of the VTA) is one of the primary sources of GABA release in the VTA and its neurons preferentially synapse with VTA DA neurons (see reviews of Ting and van der Kooy, 2012, Nikulina et al., 2014). Projections from VTA DA, GABA, and glutamate neurons synapse in both the NAc and PFC (see reviews of Fields et al., 2007, Morales and Pickel, 2012). Although VTA DA neurons project both to the NAc (mesolimbic pathway) and to the PFC (mesocortical pathway), far more DA transmission is associated with the mesolimbic than the mesocortical DA pathway (see review of Fields et al., 2007). While increased DA transmission along the mesolimbic pathway is associated with many of the effects of stress and drugs of abuse, this increase in activity of VTA DA neurons can be mediated by local inputs from GABA neurons (see reviews of Ting and van der Kooy, 2012, Nikulina et al., 2014), as well as extrinsic projections from the PFC and NAc (see reviews of Morales and Pickel, 2012, Fields and Margolis, 2015).

1.3 A Role for Mu-Opioid Receptors in the Regulation of Ventral Tegmental Area Dopamine Transmission

Decreased GABA release from local interneurons is one way in which the activity of VTA DA neurons can be increased. In the VTA, MORs are traditionally considered to be inhibitory on GABA neurons that project to VTA DA neurons (Johnson and North, 1992, Sesack and Pickel, 1995, Garzon and Pickel, 2002). When activated, these MORs inhibit GABA transmission, subsequently increasing release of DA in the NAc (Fig. 1.3) and facilitating response to drugs of

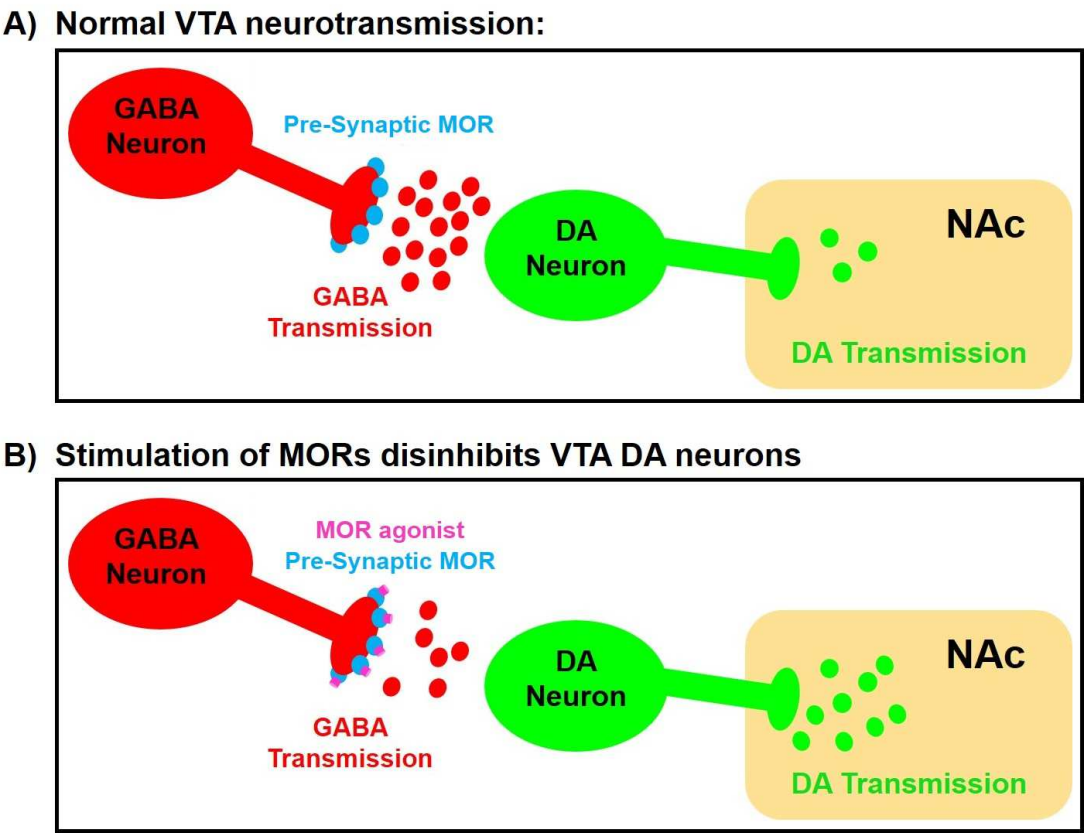

Figure 1.3. Stimulation of VTA MORs Disinhibits VTA DA Neurotransmission. (A) In the VTA, the majority of MOR have an inhibitory effect on VTA GABA neurons, in particular, a sub-population of GABA interneurons. In basal conditions, where VTA MORs are largely inactive, tonic GABA neurotransmission serves to inhibit local DA neurotransmission. (B) When VTA MOR receptors are stimulated on GABA neurons, either by an endogenous or exogenous agonist, they generally produce an inhibitory effect. This MOR-induced inhibition of VTA GABA neurons serves to disinhibit local DA neurotransmission (Johnson and North, 1992). While this schematic depicts pre-synaptic MORs, it is important to note that MORs can also be found post-synaptically, (see Chapter 1, Section 3.2 for details). Red circle: GABA neurotransmitter; green circle: DA neurotransmitter; blue circle: MOR; fuchsia cylinder: MOR agonist; number of red or green circles indicate degree of neurotransmission.

abuse (Fig. 1.3; Johnson and North, 1992, Bergevin et al., 2002; see also review of Dacher and Nugent, 2011). Some MORs have also been found on VTA DA neurons (Margolis et al., 2014), putting MOR signaling in a position to directly mediate the neurotransmission of both VTA DA and GABA neurons. Recent data have shown that some VTA MORs can actually be excitatory, and suggest that when activated separate receptors can simultaneously exert an excitatory and inhibitory effect on the same cell (Margolis et al., 2014). Ultimately, this means that VTA MORs are capable of influencing the changes in VTA DA transmission in two ways: through direct activity on DA neurons, or indirectly through modulation of local GABA transmission.

2. Effects of Social Stress

2.1 Behavioral Consequences of Social Stress

The rodent social defeat stress paradigm serves as a naturalistic model of stress-induced changes in the behavior and pathology of humans. While many stress paradigms serve to increase anxiety and depression-like behaviors, the social defeat paradigm is unique due to the face validity in the social nature of its stressor, as well as the lack of habituation associated with repeated exposure (Covington and Miczek, 2005). Social defeat stress has been shown to produce increases in anxiety in rodents, as evidenced by: increased acoustic startle response (Pulliam et al., 2010), ultrasonic vocalizations (van der Poel and Miczek, 1991, Vivian and Miczek, 1999), and time spent freezing (Venzala et al., 2012). Many of the changes seen after social defeat stress; including hyperalgesia to hot and cold temperatures (Marcinkiewcz et al., 2009, Hayashida et al., 2010) and increased analgesia (Siegfried et al., 1990, Williams et al., 1990, Teskey and Kavaliers, 1991, Rodgers, 1995), suggest that MOR play a critical role in social stress-induced long-term neuroplasticity.

The specific outcomes observed after social defeat stress are known to be influenced by the temporal pattern used during the administration of defeat (Miczek et al., 2008, Miczek et al., 2011a). In general repeated episodes of social defeat have been shown to increase social avoidance of a novel conspecific (Razzoli et al., 2009, Venzala et al., 2012) and to induce deficits in weight gain (Meerlo et al., 1996, Fanous et al., 2010, Pulliam et al., 2010, Fanous et al., 2011,

Venzala et al., 2012). In contrast, while continuous social defeat reduces preference for sweet rewards, only intermittent social defeat stress induces the behavioral sensitization that can lead to increased drug use (Miczek et al., 2011a). More specifically, intermittent social defeat stress consistently produces cross-sensitization to psychomotor stimulants (Covington and Miczek, 2001, Nikulina et al., 2005, Nikulina et al., 2012). Rodent studies have shown that repeated, non-continuous, social defeat stress consistently produces social avoidance (Razzoli et al., 2009, Fanous et al., 2011) and augments the effect of psychomotor stimulants, a phenomena known as 'cross-sensitization' (Covington and Miczek, 2001, Nikulina et al., 2004, Nikulina et al., 2012). In addition to psychomotor stimulants, repeated social stress also induces cross-sensitization to opiates, but does not increase binge-like responding for morphine (Cruz et al., 2011). Such behavioral sensitization to drugs of abuse is thought to reflect changes in the mesocorticolimbic circuit (Vanderschuren and Kalivas, 2000).

2.2 Stress-Induced Alterations Across the Mesocorticolimbic Circuit

Social defeat stress is known to have a significant impact on mesocorticolimbic expression of delta FosB and BDNF. Delta FosB is a transcription factor and the truncated form of FosB, a member of the Fos family of proteins. Much attention has been given to delta FosB because it gradually accumulates in response to repeated stimuli, such as stressor or drug, and can persist for long periods of time due to its high stability (see reviews of Nestler et al., 2001, Nestler, 2008, 2014). Generally BDNF is associated with brain regions rich in DA neurons, and in the VTA BDNF is known to co-localize with TH, a reliable marker for DA neurons (Gall et al., 1992, Seroogy et al., 1994). Interestingly, social stress not only increases BDNF and delta FosB in the NAc and PFC, but it also increases the co-expression of the two molecules (Nikulina et al., 2012). Both BDNF and delta FosB are thought to play an important role in responses to stress and drugs of abuse. As a key mediator of DA signaling in the VTA-NAc pathway, VTA BDNF is thought to mediate many stress- and drug-induced changes that occur in the NAc (see review of Nikulina et al., 2014). Although the expression of NAc delta FosB has not been shown to mediate mesolimbic DA transmission, this transcription factor has been well implicated as a downstream

indicator of augmented VTA DA activity (see reviews of Nestler et al., 2001, Nestler, 2008). Social defeat stress also induces a rapid and prolonged upregulation of VTA MORs (Nikulina et al., 2005, Nikulina et al., 2008). After repeat social defeat stress, stimulation of VTA MOR's by a MOR agonist has been shown to produce sensitized locomotor activity (Nikulina et al., 2005, Nikulina et al., 2008) during the same time period that social stress-induced cross-sensitization to psychomotor stimulants has been observed (Covington and Miczek, 2001, Nikulina et al., 2004, Nikulina et al., 2012). While it is unknown whether the expression of VTA MORs plays a role in stress-induced cross-sensitization to psychomotor stimulants, given that VTA MORs have the ability to mediate VTA GABA and DA transmission (Johnson and North, 1992, Margolis et al., 2014), it is very possible that they too play a role in mediating stress-induced sensitization to drugs. The effect of social defeat stress on the expression of these three proteins, and their respective relationships to the behavioral outcomes of such social stress, will next be discussed in turn.

2.2.1 Social Stress-Induced Expression of Delta FosB

Delta FosB is nuclear transcription factor and a truncated splice variant of FosB, as such it is a member of the Fos family of genes, which include: Fos, FosB, FosL1, and FosL2 (Dobrazanski et al., 1991, Morgan and Curran, 1995). Like other members of the Fos family, delta FosB heterodimerizes with proteins belonging to the Jun family, predominantly JunD, to form activator protein-1 (AP-1) complexes which augment gene expression by binding to AP-1 sites on promoter sequences. The C-terminal of the full form of FosB contains two degron domains that direct normal protein ubiquitination and degradated by proteasomal-dependent and -independent complexes (Dobrazanski et al., 1991, Carle et al., 2007). The delta FosB splice variant is a product of a premature stop codon, which produces the delta FosB variant that is missing the 101 amino acid C-terminus found in the full form of FosB (Dobrazanski et al., 1991). The C-terminus of FosB codes for the two degron domains, as such delta FosB lacks the two degron domains found in the missing C-terminus, which significantly prolongs the half-life of delta FosB (Carle et al., 2007). Further stability is conferred to delta FosB protein by phosphorylation of Ser27, by

either casein kinase 2 or calcium/calmodulin-dependent protein kinase II (CaMKII; Ulery et al., 2006, Robison et al., 2013). In contrast to the marked stability associated with delta FosB protein, its mRNA is fairly unstable and degrades quickly (Hope et al., 1994, Chen et al., 1995, Kelz and Nestler, 2000). Due to the prolonged stability of delta FosB, this transcription factor has been widely studied in the context of drug addiction and stress, and it is considered a prolonged marker of neuronal activation and plasticity in the brain (Nestler, 2008, 2014).

Repeated social defeat stress induces a prolonged increase of delta FosB throughout the mesocorticolimbic circuit (Nikulina et al., 2008, Nikulina et al., 2012). The time course of social stress-induced delta FosB expression in the mesocorticolimbic circuit has been well characterized and increased delta FosB expression persists for up to two weeks after the termination of stress procedures (Nikulina et al., 2008). Social stress-induced increases in delta FosB expression in the NAc generally last for around 10 days (Nikulina et al., 2012), a time period that corresponds to the expression of stress-induced amphetamine sensitization. However attempts to localize stress-induced changes in delta FosB expression in specific NAc structures have produced mixed findings. In particular, one study only detected a significant increase in delta FosB expression in the NAc shell (Nikulina et al., 2008), while another found significant increases in both the NAc shell and core at 10 days (Nikulina et al., 2012). Differences in delta FosB expression in the NAc core and shell after stress may be attributed to natural variability in the intensity of the social stress experience. Similar variability in delta FosB expression has been noted in the ventral striatum after cocaine self-administration, and it correlates with the intensity and duration of the drug stimulus (Larson et al., 2010). Of particular interest, the effects of delta FosB induction in the NAc appear to be specific to this splice variant, as overexpression of either the full form of FosB or the alternative splice variant delta2 delta FosB (Δ2FΔFosB), which is missing the 78 amino acid N-terminus, did not alter stress- or cocaine-induced behaviors in mice (Ohnishi et al., 2015).

Compared to the NAc, social stress seems to induce comparable changes in delta FosB expression across sub-regions of the PFC. Increased delta FosB expression has been detected for up to 7-10 days after the last episode of stress in the infralimbic, prelimbic, and anterior cingulate regions of the PFC (Nikulina et al., 2008, Nikulina et al., 2012). In contrast to both the

NAc and PFC, social stress does not seem to induce changes in delta FosB expression in the VTA with a recent study failing to note a significant increase in VTA delta FosB expression 10 days after the last episode of defeat (Nikulina et al., 2012). Given that 10 days after the termination of stress corresponds to the onset of psychostimulant sensitization (Nikulina et al., 2012) and that social stress increases mesolimbic DA activity (Tidey and Miczek, 1996), it is interesting that stress-induced increases in VTA delta FosB expression were not noted at this time (Nikulina et al., 2012). While delta FosB is largely unaffected in the VTA after social stress, the stress-induced expression of NAc delta FosB is mediated by the release of BDNF in the NAc (Wang et al., 2013, Wang et al., 2014), the source of which is presumably the VTA (Berton et al., 2006, Wang et al., 2013).

2.2.2 Social Stress-Induced Expression of BDNF

Social defeat stress is known to have huge impact on the expression of VTA BDNF, and the expression of this protein has been associated with the effects of both stress and drugs of abuse (see review of Nikulina et al., 2014). In fact, BDNF signaling with its receptor, tropomyosin-related kinase B (TrkB) in the NAc has been shown to mediate the behavioral and neurochemical changes associated with social defeat stress (Wang et al., 2014). In the NAc, social stress presumably increases the expression of TrkB receptor on medium spiny neurons, however it is also possible that the receptor is increased on DA projections that originate in the VTA, as their cell bodies express and release BDNF into the NAc. While TrkB receptor is also found in the VTA, and its expression on VTA GABA neurons is implicated in the effects of opioids (Vargas-Perez et al., 2009b, Vargas-Perez et al., 2014), it is not known whether social stress alters TrkB receptors in the VTA.

Increased VTA BDNF expression has been implicated as a long-term mediator of social stress-induced cross-sensitization (Nikulina et al., 2012), and in the VTA this increase persists for at least 2 weeks after the last social stress exposure (Fanous et al., 2010, Nikulina et al., 2012). Social stress also induces a rapid and transient increase of BDNF expression in the PFC, lasting about a week (Fanous et al., 2010, Nikulina et al., 2012). Due to the rapid and transient nature of

BDNF expression in the PFC, it has been difficult to determine the functional implications of its expression in this region. However it is likely that the PFC is one of the first mesocorticolimbic areas affected by social stress and that stress-induced alterations PFC neurotransmission mediate subsequent changes in its projection targets. By contrast, social stress-induced expression of VTA BDNF is better understood and VTA BDNF is known to play a critical role in social stress-induced cross-sensitization to amphetamine (Wang et al., 2013, Wang et al., 2014) and social stress-induced social avoidance (Berton et al., 2006, Fanous et al., 2011).

However, drawing similar conclusions across different models of social defeat stress in rats and mice warrants caution, as species differences have been noted. In particular, continuous social defeat stress reduces VTA BDNF in rats, while increasing it in mice (for details, see review of Nikulina et al., 2014), suggesting that BDNF may play a species-specific role in stress responses. Despite this, the expression of social stress-induced VTA BDNF in rats has been consistently correlated with cross-sensitization to amphetamine (Wang et al., 2013, Wang et al., 2014). Specifically, overexpression of VTA BDNF has been shown to exacerbate (Wang et al., 2013), while NAc knockdown of the BDNF receptor, TrkB, prevented social stress-induced cross-sensitization to amphetamine (Wang et al., 2014). Similarly, viral deletion of VTA BDNF prevented social stress-induced social avoidance (Berton et al., 2006, Fanous et al., 2011). Taken together, these findings suggest that increased VTA BDNF expression is one of the most functionally important changes induced after social stress.

2.2.3 Social Stress-Induced Upregulation of VTA MORs

A rapid and prolonged upregulation of VTA MOR can be found during the same time period that social defeat stress-induced increases in delta FosB, VTA BDNF and cross-sensitization to amphetamine occur (Nikulina et al., 2005, Nikulina et al., 2008, Nikulina et al., 2012). In the VTA, MORs are predominantly expressed by GABA neurons (Sesack and Pickel, 1995, Garzon and Pickel, 2002), which are hyperpolarized in response to MOR stimulation. Acute social defeat stress rapidly upregulates MOR mRNA expression in the VTA (Nikulina et al., 1999,

Nikulina et al., 2008), while repeated social stress exposure increases VTA MOR mRNA expression for up to 14 days after the last episode (Nikulina et al., 2008).

There is a high correlation between MOR mRNA expression and levels of protein binding (Mansour et al., 1994), suggesting that stress-induced increases in VTA MOR expression correspond to in endogenous mu-opioid peptide activity in the VTA. While it is unknown whether endogenous mu-opioid peptides are more abundant in the VTA after stress, rats exposed to repeated social defeat stress and then challenged with an intra-VTA infusion of a MOR-specific agonist exhibited sensitized locomotor activity (Nikulina et al., 2005, Nikulina et al., 2008). This VTA MOR agonist-induced sensitized locomotor activity was present during the same time period that social stress-induced cross-sensitization to psychomotor stimulants has been observed (Covington and Miczek, 2001, Nikulina et al., 2004, Nikulina et al., 2012), suggesting that the stress-induced upregulation of VTA MORs potentiates an increase in VTA DA activity (Fig. 1.3).

Expression of MORs is important for social stress-induced behavioral alterations, as genetic MOR knockout mice do not exhibit social avoidance following continuous social defeat (Komatsu et al., 2011). Taken together, these findings support the involvement of VTA MORs in the regulation of VTA DA transmission and suggest that MORs may play a critical role in the long-term neuroplasticity induced by social stress.

2.2.4 Interaction of Stress-Induced Expression of BDNF, MORs and Delta FosB in the Mesolimbic Pathway

While VTA MOR mRNA expression rapidly increases following social stress exposure (Nikulina et al., 2005, Nikulina et al., 2008), VTA BDNF expression is affected more slowly (Fanous et al., 2010). In contrast, the rapid increase of VTA MOR expression corresponds to social stress-induced mesocorticolimbic FosB expression (Nikulina et al., 2008). Similar to the prolonged expression of stress-induced delta FosB, VTA MOR expression is increased for roughly two weeks after stress termination (Nikulina et al., 2008). The stress-induced expression of these two proteins is related, as post-stress intra-VTA agonism of MORs exacerbated stress-induced increases in NAc delta FosB expression (Nikulina et al., 2008). In addition,

overexpression of VTA BDNF and knockdown of NAc TrkB receptor respectively enhance or attenuate social stress-induced expression of delta FosB in the NAc (Wang et al., 2013, Wang et al., 2014). Based on the respective relationships that exist between BDNF, delta FosB, and MORs in the mesolimbic pathway, it is possible that intermittent social defeat stress-induced increases in VTA BDNF and NAc delta FosB expression are related to upregulation of VTA MORs.

2.3 Neurological Correlates of Substance Abuse: Similarities to the Effects of Stress

Similar to social stress, many drugs of abuse induce changes in the mesocorticolimbic. In particular, increased mesolimbic DA transmission is thought to underlie many of sensitizing and reinforcing properties of drugs of abuse (Kalivas and Stewart, 1991, Pierce and Kumaresan, 2006, Thomas et al., 2008), and is implicated in the effects of social stress (Tidey and Miczek, 1996). The involvement of delta FosB, BDNF/TrkB receptor, and MORs have been studied in the context of both opiate and psychostimulant drugs. The drug-induced changes in the mesocorticolimbic expression of these proteins will be discussed next, with special emphasis on changes in the mesolimbic DA pathway.

2.3.1 Drugs of Abuse and Delta FosB Expression

An accumulation of delta FosB in the NAc is one of the most notable responses to repeated administration of drugs effecting DA transmission and in many cases disruption of delta FosB signaling inhibits the development of addiction-associated behaviors, including sensitized locomotor behaviors (see reviews of Nestler et al., 2001, Nestler, 2008). The expression of delta FosB is important for the effects of psychomotor stimulants, specifically, sensitization to cocaine increases delta FosB expression in subregions of the NAc shell in rats (Brenhouse and Stellar, 2006). By contrast, viral-mediated induction of delta FosB in the orbitofrontal cortex potentiates the locomotor stimulant properties of cocaine (Winstanley et al., 2009). Further support for the involvement of delta FosB in the effects of psychostimulant drugs comes from genetic knockout mice, notably, FosB knockout mice do not sensitize to cocaine (Hiroi et al., 1997). The expression

of delta FosB in the NAc has been consistently associated with the effects of psychostimulant drugs, specifically its expression is associated with acute cocaine treatment (Larson et al., 2010), and also withdrawal from chronic psychostimulant treatment (Murphy et al., 2003, Larson et al., 2010). CaMKII is also implicated in the effects of psychostimulant drugs and is of particular interest, as it is both upstream and downstream of delta FosB expression in the NAc. Specifically, CaMKII is necessary for the cocaine-induced accumulation of delta FosB in the NAc, while delta FosB is both necessary and sufficient for the induction of CaMKIIα gene expression in NAc D1 receptor-containing medium spiny neurons (Robison et al., 2013). In addition to conferring stability to delta FosB by phosphorylation of Ser27, CaMKII also phosphorylates delta FosB on Thr149, which regulates its transcriptional activity to control locomotor responses to cocaine (Cates et al., 2014). Specifically, phosphomimetic mutation of Thr149 increased AP-1 transcriptional activity, while viral-mediated manipulation of NAc delta FosB at Thr149 controlled locomotor responses to cocaine (Cates et al., 2014).

Opiate sensitization has also been associated with increased delta FosB expression in multiple brain regions associated with reward, learning, and stress. In particular, morphine sensitization has been found to increase the expression of delta FosB in the NAc core, as well as the infralimbic and prelimbic regions of the PFC (Kaplan et al., 2011). Additionally, overexpression of delta FosB in the NAc enhances sensitivity to the rewarding properties of morphine and exacerbates physical dependence (Zachariou et al., 2006). It is interesting that opposing behavioral phenotypes of morphine dependence and withdrawal have both been associated with increased delta FosB expression in nuclei of the brain stress system, including the NAc shell (Nunez et al., 2010). Of particular relevance to stress-induced changes in delta FosB and MOR expression, increased expression of delta FosB in the NAc shell of opioid-dependent rats was prevented by adrenalectomy (Garcia-Perez et al., 2012).

Other transcription factors have also been implicated in the effects of stress and drugs of abuse, such as cAMP response binding element (CREB), however in the NAc, CREB and delta FosB are thought to act in opposition to one another. Specifically CREB expression is thought to regulate a negative feedback mechanism through the induction of dynorphin, while delta FosB

expression in D1 receptor containing medium spiny neurons suppresses dynorphin and has pro-reward behavioral effects (see review of Nestler, 2013). Consistent with this view, in rats intermittent social defeat stress is known to increase delta FosB (Nikulina et al., 2008, Nikulina et al., 2012) and to reduce levels of phosphorylated CREB in the NAc (Yap et al., 2014). Despite this, it is important to note that phosphorylation of CREB at the *FosB* promoter is necessary for cocaine-induced NAc Fosb/delta FosB expression (Vialou et al., 2012), an effect which appears to mediated by histone H3 lysine 9 (H3K9me2), a repressive histone modification (Heller et al., 2014). Delta FosB is thought to act as either a transcriptional activator or repressor, depending on the nature of the target gene (Nestler, 2008, 2014). Although the expression of delta FosB does not necessarily mediate withdrawal or reinstatement, its expression is closely related to behavioral sensitization (Nestler et al., 2001, Kalivas and O'Brien, 2008, Nestler, 2008). Through regulating the expression of particular genes in the mesocorticolimbic circuit, delta FosB may represent one mechanism underlying changes associated with substance abuse (Nestler et al., 2001, Nestler, 2008).

2.3.2 Drugs of Abuse and BDNF – TrkB Receptor Signaling

The effects of different classes of drugs of abuse have been found to involve BDNF expression in the VTA. In particular, increased expression of VTA BDNF is frequently observed as a consequence of psychostimulant administration (Horger et al., 1999, Grimm et al., 2003, Bolanos and Nestler, 2004, Corominas et al., 2007, Thomas et al., 2008). Specifically, increases in VTA BDNF expression have been implicated in cocaine self-administration and withdrawal (Grimm et al., 2003), and BDNF infusion into the VTA enhances cocaine induced locomotion (Pierce et al., 1999). A single VTA infusion of BDNF at the end of cocaine self-administration enhances responding to cocaine-cues for up to 30 days after termination of cocaine use (Lu et al., 2004).

While less frequently reported, research has shown that chronic treatment with the exogenous opiates, such as morphine, also induces the expression VTA BDNF (Vargas-Perez et al., 2009b) . Additionally, increases in VTA BDNF expression have also been noted 7 days after

forced abstinence from morphine, but not at 2 h or 24 h (Mashayekhi et al., 2012). Of particular interest, in the context of opiates, BDNF seems to play different roles depending on drug history. Specifically, in drug-naïve rats, opiate effects are DA-independent, however VTA infusion of BDNF resulted in a shifted rats to a DA-dependent state associated with chronic morphine or heroin (Vargas-Perez et al., 2009b). It should also be noted that other studies have found that chronic morphine treatment either had no effect on VTA BDNF expression (Numan et al., 1998), or decreased its expression (Chu et al., 2007). However in light of the findings from the van der Kooy lab (Laviolette et al., 2004; Vargas-Perez et al., 2009, see also review by Ting and van der Kooy, 2012), it is possible that conflicting findings on opiate-induced VTA BDNF expression may be attributed to differences in drug history, as well as potential differences in the underlying endogenous VTA opioid system.

Expression of the BDNF TrkB receptor in the NAc is also important for the effects of opiate and psychostimulant drugs. In particular, over-expression of BDNF or TrkB receptor in the NAc has been shown to exacerbate chronic cocaine-induced psychomotor sensitization, conditioned place preference, and reinstatement, while these changes were inhibited by expression of a dominant negative form of TrkB receptor in the NAc (Bahi et al., 2008). The results of another study further suggest that while BDNF expression in either the VTA or NAc is important for the maintenance of cocaine reward, this effect is mediated by BDNF activation of TrkB receptors in the NAc (Graham et al., 2009). In addition to being crucial for the effects of chronic cocaine, activity of the BDNF's TrkB receptor is also necessary for the behavioral sensitization and conditioned place preference induced by a single injection of cocaine (Crooks et al., 2010). Using genome-wide interference of TrkB expression, this study found that cocaine increased TrkB receptor phosphorylation in the NAc (Crooks et al., 2010), which is consistent with the role of mesolimbic BDNF – TrkB receptor signaling in the effects of chronic cocaine (Bahi et al., 2008, Graham et al., 2009). In addition to its involvement in drug-induced changes, VTA BDNF has also been implicated as a long-term mediator of social stress-induced cross-sensitization to psychomotor stimulants (Nikulina et al., 2012). In fact, overexpression of BDNF exacerbates social stress-induced cross-sensitization to amphetamine (Wang et al., 2013), and

BDNF signaling with its receptor, TrkB, in the NAc is necessary for the induction of stress-induced sensitization (Wang et al., 2014).

In the medial PFC, BDNF expression was shown to increase 24 h after the last session of cocaine self-administration, however this increase was not present 7 days later (Fumagalli et al., 2013). Thus, while psychostimulants induce increases in PFC BDNF expression, these increases seem to be temporally and functionally different from those found in the mesolimbic pathway. For example, while increased mesolimbic BDNF enhances many effects of psychostimulants (see reviews of Ghitza et al., 2010, Nikulina et al., 2014), infusion of BDNF into the PFC actually reduces cocaine seeking after 1 or 6 days of withdrawal (Berglind et al., 2007). Similarly, for cocaine, context-induced reinstatement is attenuated by inactivation of the dorsal, rather than the ventral, portion of the medial PFC, while the opposite is true for heroin (Badiani et al., 2011).

2.3.3 Drugs of Abuse and MOR Expression and Activity

A role for MORs in the effects of both exogenous opiates, such as heroin and morphine, as well as psychomotor stimulants, such as cocaine and amphetamine, has been long supported been supported by the literature. In particular, systemic injections of morphine cross-sensitize animals to amphetamine (Vezina et al., 1989), and intra-VTA MOR agonism produce sensitization to cocaine and amphetamine (DuMars et al., 1988). Additionally, the expression of amphetamine sensitization is associated with persistent VTA MOR upregulation, and can be blocked by a treatment with MOR antagonist (Magendzo and Bustos, 2003, Trigo et al., 2010). Similarly MOR receptor interference in the VTA – substantia nigra regions blocks the rewarding and locomotor effects of the exogenous opiate, heroin (Zhang et al., 2009). Traditionally, VTA MORs are thought to be found on GABA neurons (Sesack and Pickel, 1995, Garzon and Pickel, 2002), which are hyperpolarized in response to MOR stimulation, thus disinhibiting local DA transmission (Fig. 1.3) and facilitating response to drugs of abuse (Johnson and North, 1992; see also review by Dacher and Nugent, 2011, Bergevin et al., 2002). Consistent with this view, MOR knockout mice exhibit reduced cocaine self-administration and increased VTA GABA transmission (Mathon et al.,

2005). In rats trained to seek cocaine and put through withdrawal, injection of heroin primes reinstatement of cocaine-seeking (Lu and Dempsey, 2004).

While VTA MOR activity seems to allow for an interaction between opiate and psychostimulant drugs, it is important to note that these drugs are not entirely interchangeable. For example, in rats escalated self-administration of cocaine is not predictive of heroin escalation, and escalated self-administration of heroin is not predictive of cocaine (Lenoir et al., 2012). Of particular importance, while repeated social defeat stress induces locomotor sensitization to both opiates and psychomotor stimulants (Miczek et al., 2008), binge-like drug consumption has only been associated with cocaine, and not heroin (Cruz et al., 2011).

2.3.4 Interactions Between Drug and Stress-Induced Alterations in Mesolimbic BDNF, MOR and Delta FosB Expression

Many of the mesocorticolimbic changes associated with drugs of abuse are also induced by social defeat stress (Fig. 1.4, Nikulina et al., 2014). The changes common to social stress and drugs of abuse may allow for a functional interaction between stress and drugs of abuse in the mesocorticolimbic circuit. As discussed in the previous two sections (1.2.2 and 1.2.3), the effects of stress, opiates and psychostimulants have been associated with increased mesolimbic BDNF/TrkB expression, VTA MOR expression, and NAc delta FosB expression. Stress- and drug-induced changes in the mesolimbic expression of these proteins are likely related at both the molecular and behavioral level.

Behaviorally, analgesia is induced after exposure to either opiate drugs (Zachariou et al., 2006, Solecki et al., 2008) or social stress (Siegfried et al., 1990, Williams et al., 1990, Teskey and Kavaliers, 1991, Rodgers, 1995), and in the case of morphine, analgesic tolerance is reduced in FosB knockout mice (Solecki et al., 2008), an effect which appears to be specific to the NAc (Zachariou et al., 2006). Additionally, after repeated social stress, delta FosB and BDNF are known to co-localize in the NAc and PFC 10 days after cessation of stress procedures

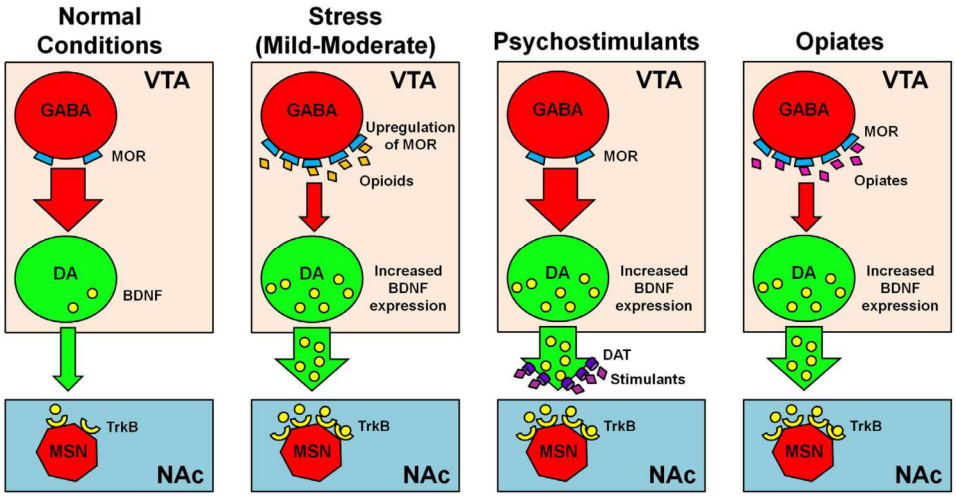

Figure 1.4. Effects of Drugs and Stress on VTA BDNF Expression and Mesolimbic Tone (from Nikulina et al., 2014). Under normal conditions, VTA GABA neurons express low levels of mu-opioid receptors (MORs) and tonically inhibit VTA dopamine (DA) neurons, producing low levels of DA neurotransmission to NAc medium spiny neurons (MSNs). After intermittent social defeat stress, MORs are upregulated on VTA GABAergic neurons, resulting in disinhibition of VTA DA neurons and enhanced BDNF expression (Johnston et al., 2015). In general, psychostimulant drugs increase DA in the synaptic cleft by blocking dopamine transporter (DAT) or releasing DA from pre-synaptic terminals which leads to induction of VTA BDNF and its release in the NAc (Graham et al., 2009, Wang et al., 2013, Wang et al., 2014), while opiates act on VTA GABA neurons, disinhibiting VTA DA signaling and increasing BDNF expression (Vargas-Perez et al., 2009a, Ting et al., 2013). In summary, stress, psychostimulants, and opiates all increase DA and BDNF signaling, albeit through different mechanisms. Size of red/green arrows represent strength of GABA or DA neurotransmission; large red circle: GABA neuron; large green circle: DA neuron; small blue trapezoids: MORs; orange diamonds: endogenous opioids; pink diamonds: exogenous opiates; purple diamonds: psychostimulant drugs; purple cylinders: DAT; small yellow crescents: TrkB receptors; small yellow circles: BDNF molecules; red heptagon: MSN.

(Nikulina et al., 2012) and stress-induced increases in NAc delta FosB are dependent on local BDNF/TrkB receptor activity (Wang et al., 2014). Increased VTA BDNF expression is associated with both opiate and psychostimulant drugs of abuse, and also social stress (see review of Nikulina et al., 2014), and social stress-induced cross-sensitization to amphetamine is dependent on VTA BDNF – NAc TrkB receptor signaling (Wang et al., 2013, Wang et al., 2014). In addition, social stress-induced social avoidance is prevented by viral knockdown of VTA BDNF (Fanous et al., 2011), as well as in genetic MOR knockout mice (Komatsu et al., 2011). Moreover, augmented mesolimbic DA transmission is associated with sensitization to psychomotor stimulants and social stress (Fig. 1.4; Piazza and Le Moal, 1996, Sinha, 2001, Marinelli and Piazza, 2002, Cleck and Blendy, 2008, Nikulina et al., 2014), and both social stress and psychomotor stimulants increase the expression of MORs in the VTA (Magendzo and Bustos, 2003, Trigo et al., 2010). Furthermore, MORs are known to play a functional role in the behavioral consequences of social stress (Komatsu et al., 2011), the expression of NAc delta FosB (Zachariou et al., 2006), and opiate- (Mashayekhi et al., 2012) and psychomotor stimulant- (Magendzo and Bustos, 2003, Trigo et al., 2010) induced VTA BDNF expression. Taken together, it is possible that upregulation of VTA MORs may represent one mechanism underlying social stress-induced cross-sensitization and changes in mesocorticolimbic BDNF and delta FosB expression.

3. Ventral Tegmental Area Mu-Opioid Receptors

The term “opioid” is used to broadly describe any compound which binds to any of the known opioid receptor sub-types, while “opiates” are a subclass of natural opioids derived from the opium poppy plant, *Papaver somniferum* (Zollner and Stein, 2007, Trescot et al., 2008, Chartoff and Connery, 2014). Opioid receptors are found throughout both the central and peripheral nervous systems, with MORs being most densely located in the brainstem and medial thalamus (Trescot et al., 2008). As described in the previous sections, in the VTA one type of opioid receptor, the mu-opioid receptor (MOR) is in a position to mediate stress-induced cross-sensitization to drugs of abuse, as well as stress-induced changes across the mesocorticolimbic

circuit. Given their potential involvement in the consequences of social defeat stress, it is important to understand how MORs work and where they are found in the VTA. First, the general structure and properties of opioid receptors, with an emphasis on MORs, will be discussed. Next the traditional theory of MOR localization and activity in the VTA, as well as recent findings on novel MOR functions will be described. Finally, the implications of VTA MOR activity for sensitization and cross-sensitization to drugs of abuse will be discussed.

3.1 Structure and Function of Mu-Opioid Receptors

Opioid receptors belong to the superfamily of seven transmembrane-spanning (7TM) G-protein-coupled receptors (GPCRs; Connor and Christie, 1999, Law and Loh, 1999, Waldhoer et al., 2004, Zollner and Stein, 2007, Trescot et al., 2008, Pradhan et al., 2012). More specifically, opioid receptors belong to the Rhodopsin (class A) family of Gi/Go protein-coupled receptors (Connor and Christie, 1999, Law and Loh, 1999, Waldhoer et al., 2004, Zollner and Stein, 2007, Lopez and Salome, 2009). Opioid receptors consist of an extracellular N-terminal domain and 7TM helical domains (Connor and Christie, 1999, Law and Loh, 1999, Waldhoer et al., 2004, Zollner and Stein, 2007, Trescot et al., 2008, Lopez and Salome, 2009). The 7TM domains consist of three intracellular loops, three extracellular loops, and an intracellular C-terminal tail that is predicted to form a fourth intracellular loop with its putative palmitoylation sites (Law and Loh, 1999, Waldhoer et al., 2004, Zollner and Stein, 2007, Lopez and Salome, 2009).

There are three major sub-types of opioid receptor: mu-opioid receptor (MOR; Fig. 1.5.), delta opioid receptor (DOR) and kappa opioid receptor (KOR); and each type of opioid receptor is under the control of a different gene (Connor and Christie, 1999, Law and Loh, 1999, Zollner and Stein, 2007, Trescot et al., 2008, Pradhan et al., 2012). Although located on different chromosomes, these opioid receptors have similar genomic structures. The three types of opioid receptor are approximately 60% homologous, with the most similarity seen in the 7TM regions, they also exhibit diversity in their extracellular loops, and N and C terminals (Law and Loh, 1999, Waldhoer et al., 2004, Zollner and Stein, 2007, Pradhan et al., 2012). Additionally, while all opioid

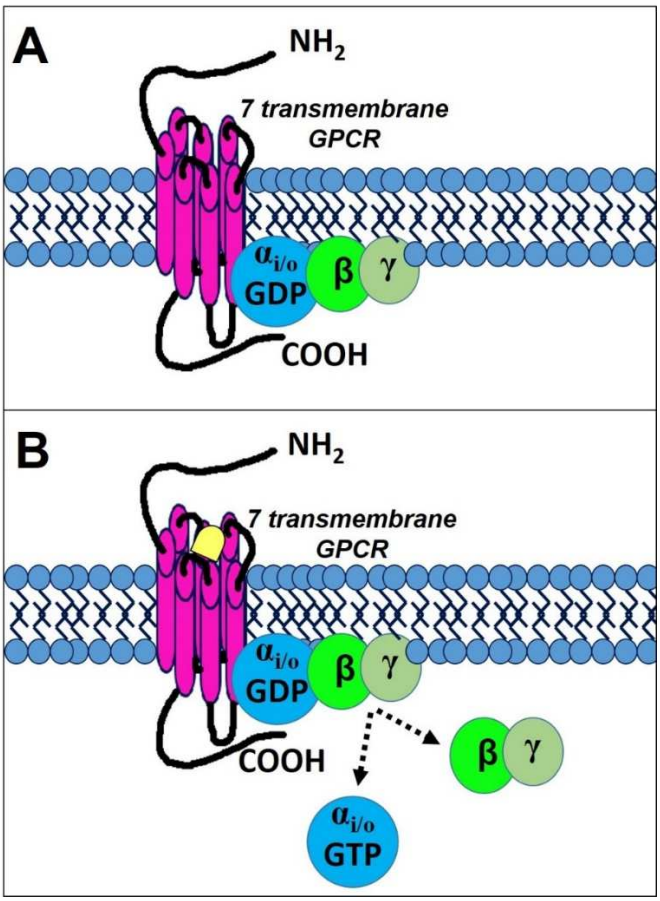

Figure 1.5. Inactive Versus Active MOR Structure and G-Protein Coupling. MORs are 7 transmembrane spanning GPCRs that consist of an extracellular NH_2-terminus (also, N-terminal or tail) and an intra-cellular COOH-terminus (also, carboxyl tail or C-terminal), and couple to $G\alpha_{i/o}$ and $G\beta\gamma$ G-protein subunits. (A) In their inactive state, MORs are coupled to their $G\alpha_{i/o}$ and $G\beta\gamma$ G-protein subunits. (B) Upon ligand binding (yellow), there is an exchange of GDP for GTP on the $G\alpha_{i/o}$ subunit, and the G-protein sub-units disassociate from the receptor to activate numerous intracellular signaling cascades (Connor and Christie, 1999, Waldhoer et al., 2004, Lopez and Salome, 2009). Blue circles with wavy lines: cell membrane; interconnected fuchsia cylinders: MOR receptor; yellow shape: MOR agonist; large blue and green circles: G-protein subunits; dotted arrows indicate disassociation of G-protein subunits from the ligand-bound receptor.

receptors consist of three exons, encoding the N-terminus and the 7TM structure, MORs differ from other opioid receptors in that they also contain a fourth exon that codes for 12 amino acids located at the tip of the C-terminus (Pasternak, 2014). While the greatest differences between MOR and DOR amino acid sequences are found in the C-terminal, exchange of this portion of the receptor does not give the MOR properties associated with the DOR (Law and Loh, 1999). These differences in opioid receptors are thought to underlie functional differences in the effects of opioid ligands. In particular, MOR- and DOR-agonists are rewarding and analgesic, while agonists selective for the KOR tend to be dysphoric (Waldhoer et al., 2004, Zollner and Stein, 2007).

While many features of the opioid-binding pocket are common to all opioid receptors, these receptors significantly differ in selectivity and affinity for both endogenous and exogenous compounds. The opioid-binding pocket is thought to be located within transmembrane helices 3, 4, 5, 6, and 7, with the cavity being partially covered by the extracellular loops (Waldhoer et al., 2004, Lopez and Salome, 2009). Differences in receptor conformation, due to the position of extracellular loops, are thought to play a part in mediating opioid receptor – ligand specificity (Law and Loh, 1999, Waldhoer et al., 2004). Ligand selectivity for the MOR has been attributed to the first and third extracellular loops, by contrast the second extracellular loop confers selectivity for KOR, while the third extracellular loops confer selectivity for DOR (Waldhoer et al., 2004). The endogenous opioid peptides are predominantly derived from several precursors: pro-opiomelanocortin, proenkephalin, and prodynorphin (Waldhoer et al., 2004, Zollner and Stein, 2007). After cleavage of these precursors by peptidases and post-translational modification, the active types of endogenous opioid ligands are generated for each receptor: endorphins (pro-opiomelanocortin), enkephalins (pro-enkephalin), and dynorphins (pro-dynorphin; Waldhoer et al., 2004, Zollner and Stein, 2007, Trescot et al., 2008). However in the case of the highly selective endogenous MOR peptides endomorphins 1 and 2, which are tetrapeptides structurally unrelated to other endogenous opioid peptides (Zadina et al., 1997), no precursor has been identified (see also reviews of Waldhoer et al., 2004, Zollner and Stein, 2007). The first four amino acid positions are identical for all endogenous opioid peptides, with the fifth position being either methionine (in

the case of Met-enkephalin and β-endorphin) or leucine (Leu-enkephalin and dynorphins; Pasternak, 2014). Specifically, the MOR has a high affinity for endomorphin (1 and 2) and β-endorphin, while the DOR has a high affinity for enkephalin (Leu and Met) and β-endorphin, and the KOR binds to dynorphin (A and B; Waldhoer et al., 2004, Pasternak, 2014). Despite differences in high affinity for particular endogenous opioid peptides, all three opioid receptors do show some affinity for non-preferred opioid peptides.

Investigation of the mechanisms underlying ligand-induced 7TM motions has suggested that binding to a GPCR results in exposure of the receptor's intracellular loops, making them more accessible and facilitating interactions with G-proteins (Law and Loh, 1999, Waldhoer et al., 2004, Zollner and Stein, 2007). In particular, intracellular loop three is considered a key determinant of coupling specificity to different G-protein alpha subunits, while intracellular loop two is thought to be more involved in the efficiency of G-protein activation (Gether, 2000). In the case of MORs, the conformational modifications following agonist binding involve the displacement of transmembrane helices 3, 6, and 7, which results in exposure of the intracellular loop domains and permits G-protein activation. The key determinant in MOR coupling specificity to G-proteins is intracellular loop three, while efficiency of G-protein activation is thought to be due to the second intracellular loop and C-terminal tail (Lopez and Salome, 2009). Similar to many other GPCRs, MORs display basal signaling activity in culture (Wang et al., 1994, Burford et al., 2000), and display increased constitutive activity following chronic morphine exposure (Wang et al., 1994).

Evidence indicates that the intracellular signaling pathways activated by MORs are ligand-specific (see review of Pradhan et al., 2012), and that some of this specificity may be attributed to differences in receptor conformation and G-protein coupling. Like other GPCRs, opioid receptor activation induces intracellular signaling through G-proteins consisting of an alpha subunit (G_{α}) and a beta/gamma subunit complex ($G_{\beta\gamma}$; Fig. 1.5). In particular, opioid receptors are predominantly coupled to pertussis toxin (PTX) sensitive, heterotrimeric Gi/Go alpha proteins, although coupling to pertussis toxin-insensitive Gs/Gz/Gq proteins has also been reported (Connor and Christie, 1999, Waldhoer et al., 2004, Lopez and Salome, 2009). While opioid

receptors do not vastly differ in terms of the G_{α} proteins that they couple with, they do exhibit respective differences in the degree of coupling to various G_{α} subunits (Connor and Christie, 1999), resulting in different G-protein complexes and possibly different effectors (Law and Loh, 1999). In the case of MORs, once activated by an agonist, they are thought to preferentially couple to $G_{\alpha o2} > G_{\alpha o1}$, and $G_{\alpha i3} > G_{\alpha i2}$ or $G_{\alpha i1}$ (Connor and Christie, 1999, Law and Loh, 1999). However more recently, others have noted different coupling preferences, with MORs preferentially coupling to $G_{\alpha o1}$, $G_{\alpha o2}$, $G_{\alpha i2}$, $G_{\alpha i3}$, and to a lesser extent, $G_{\alpha i1}$ (Saidak et al., 2006; see also review of Lopez and Salome, 2009). In their resting state, MORs exist as heterotrimers of α, β, γ G-protein subunits, and coupled to a G_{α} bound to GDP, βγ complex ($G_{\alpha}^{(GDP)}\beta\gamma$ complex; Lopez and Salome, 2009). Activation of MORs results in the catalytic exchange of GDP to GTP on G_{α} ($G_{\alpha}^{(GDP)}$ to $G_{\alpha}^{(GTP)}$), and disassociation of the G-protein complex into separate $G_{\alpha}^{(GTP)}$ and $G_{\beta\gamma}$ subunits (Fig 1.5). After activation, MORs have been reported to act through G-protein subunits to effect intracellular signaling and cell state. In particular, MORs inhibit adenylyl cyclases through $G_{\alpha i}$, while $G_{\beta\gamma}$ subunits stimulate some isoforms of adenylyl cyclases (Chakrabarti et al., 2005). Additionally, MORs activate G-protein-activated inwardly rectifying K^+ channels (GIRKs) and voltage-gated Ca^{2+} channels, indirectly activating phospholipase A_2 through the $G_{\alpha o}$ subunit (Connor and Christie, 1999, Clark et al., 2003, Waldhoer et al., 2004, Zollner and Stein, 2007, see also review of Lopez and Salome, 2009). The cell signaling that results through MOR activation is complicated by the receptor's ability to simultaneously or concurrently couple with different G-proteins, and by the lifetime of $G_{\alpha}^{(GTP)}$ complex, as return to receptor's inactive state is dependent upon restoration of the $G_{\alpha}^{(GDP)}{}_{\beta\gamma}$ complex (Lopez and Salome, 2009).

3.2 Classical View of Ventral Tegmental Area Mu-Opioid Receptor Effects: GABAergic Disinhibition of Dopamine

In general, MORs are perisynaptic and can be found near synapses, either postsynaptically on dendrites and cell bodies, or presynaptically on axon terminals (Williams et al., 2001, Bergevin et al., 2002, Steffensen et al., 2006, see also reviews of Chartoff and

Connery, 2014; Zollner and Stein, 2007). While pre-synaptic MORs can be found near nerve terminals (Bergevin et al., 2002), plasmalemmal MORs are not typically synaptic in the VTA (Garzon and Pickel, 2002, Steffensen et al., 2006), suggesting that when not situated near a synapse, VTA MORs are activated by release of endogenous opioids into the extracellular space. The literature largely suggests that opioid receptor activation typically depresses neuronal firing to inhibit neurotransmission. When located presynaptically, opioid receptor activation inhibits Ca^{2+} influx and subsequent release of neurotransmitter and peptides from primary afferent terminals (Bergevin et al., 2002, Zollner and Stein, 2007). Postsynaptic opioid receptors depress neuronal firing through GIRK-mediated hyperpolarization (Johnson and North, 1992, Zollner and Stein, 2007, Margolis et al., 2014). On dendrites or cell bodies, MORs regulate neuronal excitability and transduce receptor activation to downstream signal transduction pathways (Williams et al., 2001, Chartoff and Connery, 2014). The MORs found on axon terminals serve to inhibit neurotransmitter release by activation of K^+ conductance and/or inhibition of Ca^{2+} conductance (Williams et al., 2001, Chartoff and Connery, 2014).

In the VTA, MORs are thought to be concentrated on GABA neurons (Sesack and Pickel, 1995, Garzon and Pickel, 2002), where they inhibit GABA neurotransmission, consequently disinhibiting local DA neurons (Johnson and North, 1992). While predominantly localized to VTA GABA neurons, MORs can be act either pre-synaptically or post-synaptically. The original disinhibition hypothesis posited that MORs are post-synaptic and exert their effects through GIRK-mediated inhibition of VTA GABA neurons (Johnson and North, 1992). However rapidly inactivating K^+ current (A-type) was found to be more characteristic of the action potentials in DA neurons than GABA neurons (Koyama and Appel, 2006), suggesting that MOR-mediated decreases in Ca^{2+} influx (Zollner and Stein, 2007) may also contribute to MOR-induced inhibition of GABA.

The net result of MOR-mediated inhibition of GABA neurons is reduced GABA transmission at target sites, leading to disinhibition of excitatory targets, such as local DA neurons. Support for the role of VTA GABA neurons in the mediation of VTA DA transmission has been repeatedly validated. In particular, selective ablation of VTA GABA neurons increased

spontaneous locomotor activity (Shank et al., 2007), a sign of enhanced VTA DA transmission. Additionally, inhibition of VTA GABA neurons has been associated with increased basal activity of VTA DA neurons (Bocklisch et al., 2013). Conversely, optogenetic stimulation of VTA GABA neurons was shown to directly suppress the activity and excitability of neighboring DA neurons, and also reduced DA release in the NAc (van Zessen et al., 2012). These data show that VTA GABA neurons are capable of mediating the excitability of local DA neurons. Given that VTA MORs are concentrated on GABA neurons and have an inhibitory effect on neurotransmission, it is likely that MOR-mediated inhibition of VTA GABA transmission has a profound effect on VTA DA activity. This was demonstrated in a microdialysis study wherein VTA MOR agonism decreased GABA concentrations and increased DA overflow, while MOR knockout mice did not respond to MOR agonism, they exhibited increased levels of basal VTA GABA transmission (Chefer et al., 2009). This suggests that in the VTA endogenous MOR activity on GABA neurons may play a crucial role in the mediating tonic VTA GABA neurotransmission.

3.3 Novel Mu-Opioid Receptors: Excitatory and Localized to Dopamine Neurons?

While the canonical model of MOR action in the VTA proposed by Johnson and North (1992) is still largely upheld (Fig. 1.2), reports of conflicting findings are becoming more common. These recent findings disagree with the canonical model on two points, specifically: that VTA MOR expression is limited to GABA neurons, and that MOR activation produces an inhibitory effect (see review of Fields and Margolis, 2015). Together these contradictory reports highlight flaws in the original criteria used to classify neuronal types in the VTA. In particular, Johnson and North (1992) stated that neurons that were not inhibited by MOR stimulation were DA neurons. While these criteria have been widely used to delineate between DA and GABA neurons in the VTA, new evidence suggests that these criteria unreliable and produce false results.

The canonical theory of MORs in the VTA assumes that DA and GABA are the only two neuronal types in the VTA, however VTA glutamate neurons have recently been identified (see reviews of Morales and Pickel, 2012, Morales and Root, 2014). Thus while the criteria of Johnson and North (1992) state that cells that are not inhibited by MOR stimulation are DA neurons, they

could also be glutamate neurons. Of particular concern for the canonical theory that MORs *indirectly* disinhibit DA neurons, a subset of VTA DA neurons show *direct* MOR-mediated post-synaptic inhibition (Margolis et al., 2014). Moreover, in slice preparations, nearly half of all confirmed VTA DA neurons were inhibited by stimulation of MORs (Margolis et al., 2014). Thus, while the criteria of Johnson and North (1992) suggest that VTA cells inhibited by MOR stimulation are GABA neurons, they could also be DA neurons. It is unknown whether MORs also localize to the recently discovered VTA glutamate (Morales and Pickel, 2012, Morales and Root, 2014), or if their functions differ on neurons capable of co-releasing neurotransmitters.

The canonical model of Johnson and North (1992) also assumes that MOR activation always results in inhibition of neurotransmission. By contrast, a recent study beautifully illustrated that MORs can be either inhibitory or excitatory (Margolis et al., 2014). In fact 19% of VTA neurons are depolarized by bath application of the MOR agonist DAMGO, while 52% of neurons were inhibited by DAMGO (Margolis et al., 2014). The excitatory or inhibitory of function of VTA MORs is highly heterogenous, with both DA and non-DA neurons showing similar proportions of both types of receptor. Moreover, both excitatory and inhibitory MORs can be found on the same cell (Margolis et al., 2014). Thus the criteria of Johnson and North (1992), in terms of MOR-induced inhibition, is not a reliable marker for either GABA or DA neurons.

Taken together, these studies suggest that the traditional theory of MOR activity in the VTA is incomplete and more diverse than previously thought. Although the fact that MORs can have excitatory effects, and their presence on a small subset of VTA DA neurons, contradict the disinhibition hypothesis, they do not disprove it. It is important to note that tonic GABA release occurs in slice preparations, and that bath application of picrotoxin, a $GABA_A$ receptor antagonist, is sufficient to depolarize neurons (Margolis et al., 2014). Thus the machinery for disinhibition

exists within the VTA, and VTA MORs may act in a combination of ways, inhibitory or excitatory, to either directly or indirectly mediate VTA DA outputs (Fields and Margolis, 2015).

3.4 Mu-Opioid Receptor – Mediation of VTA DA Transmission: Implications for Substance Abuse and Cross-Sensitization

Both psychostimulants and social defeat stress upregulate the expression of VTA MORs, and social stress-induces cross-sensitization to psychomotor stimulants (Nikulina et al., 2014). Since VTA MORs can either directly or indirectly mediate DA transmission (see review of Fields and Margolis, 2015), it is possible that social stress-induced expression of VTA MORs may mediate the induction of stress-induced cross-sensitization to psychomotor stimulants. While this possibility is intriguing, it is unknown whether the MORs upregulated by social defeat stress are excitatory or inhibitory, and what type of neuron they are localized to.

If social stress increased the expression of inhibitory MORs on DA neurons, or excitatory MORs on GABA interneurons, the net effect would be reduced DA output. Decreased DA transmission has been associated with decreased response to psychostimulant drugs (Vezina and Stewart, 1989). In contrast, after social stress-induced upregulation, intra-VTA stimulation of MORs produces a sensitized locomotor response (Nikulina et al., 2005, Nikulina et al., 2008), suggesting that the upregulation of MORs potentiated VTA DA activity. Enhanced VTA DA activity is consistent with a potential upregulation of either inhibitory MORs on GABA interneurons, or excitatory MORs on DA neurons. It is important to note that while MORs are found VTA DA neurons, this appears to represent a small proportion of MORs (Garzon and Pickel, 2002), with an even smaller proportion of all VTA MORs being excitatory (< 20%; Margolis et al., 2014), suggesting that the net effect of VTA MOR activation is inhibitory on GABA neurons.

Given that social stress-induced increases in VTA MOR expression are associated with enhanced VTA DA activity (Nikulina et al., 2005, Nikulina et al., 2008), and that excitatory MORs are uncommon (Margolis et al., 2014), it is probable that social stress predominantly upregulates inhibitory MORs on VTA GABA neurons. If the net effect of social stress-induced upregulation of VTA MORs is inhibitory on GABA neurons, then social stress should also increase VTA DA

transmission. In fact, increases in DA release in the NAc have been found after repeated social stress (Tidey and Miczek, 1996, Miczek et al., 2011a) and are associated with stress-induced cross-sensitization to psychomotor stimulants. Given their role in modulating VTA DA output, the VTA MORs upregulated by social stress may play a critical role in the behavioral and cellular consequences of stress. Moreover, it is possible that preventing social stress-induced increases in VTA MORs will prevent stress-induced cross-sensitization to psychomotor stimulants. As such, VTA MORs may represent a novel therapeutic target for treating stress-induced vulnerabilities to drugs of abuse.

4. Ventral Tegmental Area Mu-Opioid Receptor – pAKT Signaling

Ventral tegmental MOR activity is important for the effects of both stress and drugs of abuse, thus it is possible some of these effects are mediated by MOR-activated intracellular signaling cascades. As previously discussed, the cellular inhibition typically associated with MOR-activation is thought to be largely mediated by GIRKs, likely through their beta/gamma G-protein subunit complex (Johnson and North, 1992, Margolis et al., 2014). However, in addition to their inhibitory effects, MOR activation likely mediates other cellular functions. MORs are GPCRs, which means that they are capable of activating numerous intracellular signaling cascades via their G-proteins. Of the intracellular signaling pathways activated by MORs, several have been well characterized, including: protein kinase A (PKA), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) – extracellular signal-regulated kinase (ERK) cascades (Williams et al., 2001).

While the functional implications remain to be fully elucidated, MORs also consistently activate the phosphoinositide 3-kinase (also, phosphatidylinositol 3-kinase; PI3K) signaling cascade (Polakiewicz et al., 1998, Iglesias et al., 2003). After activation of the intracellular PI3K signaling pathway, AKT is one of the first molecules to be phosphorylated (pAKT), and its phosphorylation is associated with alterations in GABA transmission (Wang et al., 2003). Moreover, changes in AKT phosphorylation have been implicated in stress- (Krishnan et al., 2007, Krishnan et al., 2008), psychostimulant- (Izzo et al., 2002, Iniguez et al., 2008), and opiate-

induced (Russo et al., 2007, Mazei-Robison et al., 2011) behavioral alterations. Since social defeat stress-induces an upregulation of VTA MORs at a time corresponding to the onset of cross-sensitization, it is possible that social defeat stress increases AKT phosphorylation downstream of MOR upregulation in the VTA.

4.1 Overview of AKT Phosphorylation

4.1.1 General Structure

The evolutionarily conserved gene for the AKT kinase was first identified in the mice infected with the AKT8 retrovirus, and is the cellular homolog of the v-akt oncogene transduced by the AKT8 retrovirus (Staal et al., 1977, Staal, 1987). Independent characterization and cloning of the AKT kinase has revealed that it exists in three isoforms that are encoded by different genes: AKT1, AKT2, and AKT3 (Scheid and Woodgett, 2001, Chong et al., 2005, Franke, 2008). Early on, AKT was also frequently referred to as protein kinase B (PKB), due to its similarity to protein kinases A and C (PKA and PKC respectively; Manning and Cantley, 2007, Franke, 2008, Pearce et al., 2010). All three isoforms of AKT are comprised of similar structures. In particular, they consist of an N-terminal regulatory domain, a hinge region, and a C-terminal region (Franke, 2008). The C-terminal is largely responsible for determining the induction and maintenance of AKT's kinase activity (Chong et al., 2005, Franke, 2008). The N-terminal of AKT includes a pleckstrin homology (PH) domain, which is connected to the kinase domain by the hinge region (Chong et al., 2005, Franke, 2008). AKT shares extensive homology with protein kinases A, G, and C, thus it is a member of the AGC superfamily of protein kinases and requires phosphorylation to achieve its active state (Scheid and Woodgett, 2001, Chong et al., 2005, Manning and Cantley, 2007, Pearce et al., 2010). Under normal conditions, AKT1 is expressed at low levels in the brain, however the expression of AKT greatly increases in neuronal cells following cellular stress or injury (Chong et al., 2005). Since AKT1 expression is dependent on changes in the cellular environment and because its actions have been well documented in the brain, particular emphasis will be devoted to this variant and discussion of site-specific

phosphorylation changes will reference AKT1. The kinase domain of AKT is specific for serine (Ser) and threonine (Thr), as such it has two phosphorylation sites: Ser473 and Thr308 for AKT1.

4.1.2. Upstream Activators of AKT - PI3K Signaling

While the exact pathway leading to AKT activation differs slightly depending on the type of receptor stimulating AKT, given their homologous structures, it is not surprising that all three AKT isoforms utilize the same activation mechanisms. The PH domain of AKT is specific for 3'-OH (D3) phosphorylated phosphoinositide products of PI3K, as well as 3-phosphoinositide-dependent kinase-1 (PDK1; Chong et al., 2005, Franke, 2008, Okkenhaug, 2013). At the plasma membrane, PI3K phosphorylates phosphoinositides on the D3 position of the inositol ring, generating the second messengers phosphatidylinositol-3, 4-biphosphate ($PI(3,4)P_2$; also PIP_2) and phosphatidylinositol-3, 4,5-triphosphate (PIP_3; Chong et al., 2005; Franke, 2008; Okkenhaug, 2013). Although several classes of PI3K exist, Class 1A-B are most implicated in the receptor activation of AKT due to their generation of PIP_3 and $PI(3,4)P_2$ (Franke, 2008, Okkenhaug, 2013). In general, Class 1A PI3Ks are thought to mediate receptor tyrosine kinase (TRK) activation of AKT, while Class 1B PI3K is regulated by the βγ subunits of GPCRs (Franke, 2008, Iwanami et al., 2009). While p110 is the catalytic subunit of all PI3Ks, different isoforms of this subunit exist, with p110-α, -β, and -δ belonging to Class 1A and p110-γ belonging to Class 1B (Okkenhaug, 2013).

Class 1A isoforms of PI3K also consist of a Src homology (SH2)-containing regulatory subunit, and while there are different types, they can be collectively referred to as "p85" (Liu et al., 2009, Okkenhaug, 2013). The p85 SH2 domains of Class 1A PI3Ks recruit the p110 subunit to the membrane-associated proteins that have been phosphorylated by tyrosine kinases (Liu et al., 2009, Okkenhaug, 2013). In contrast the regulatory subunits of Class 1B PI3Ks, p101 and p84, bind to the βγ subunit of GPCRs and recruit p110γ to the activated receptor at the membrane surface (Liu et al., 2009, Okkenhaug, 2013). Like its Class 1B analog, p110β can also bind to the $G_{\beta\gamma}$ subunits to be activated by GPCRs, especially in cells that do not express high levels of p110γ (Okkenhaug, 2013). Particular types of PI3K are named according to their catalytic

subunit, and of these variants, only PI3K-β and PI3K-γ appear capable of phosphorylating AKT, and they may do so through either activation of RTKs or GPCRs (Okkenhaug, 2013). Since p110γ is mainly limited to immune cells, while p110β is broadly expressed in most cell types and can be efficiently activated by GPCRs in the absence of p110γ (Liu et al., 2009, Okkenhaug, 2013), it is likely that in the brain, AKT is phosphorylated downstream of PI3K-β.

4.1.3. Phosphorylation of AKT

While PI3K signaling is necessary, it is not sufficient for the full activation of AKT, and AKT does not reach full catalytic potential until it has undergone phosphorylation, locking the enzyme into its active conformation. Rather, increases in the phosphoinositide products of PI3K recruit AKT from the cytoplasm to the membrane, where it undergoes a conformational change, exposing its active loop and enabling phosphorylation (Fig 1.6; Stephens et al., 1998, Scheid and Woodgett, 2001, Chong et al., 2005, Franke, 2008, Iwanami et al., 2009). The phosphorylation sites of AKT are differentially located, with the residue for Thr308 being found in the kinase domain, while the residue for Ser473 is found in the hydrophobic motif of the C-terminal tail (Scheid and Woodgett, 2001, Franke, 2008).

The phosphoinositide products of PI3K interact with AKT, relaxing its conformation and allowing for phosphorylation of Thr308 by PDK1 (Scheid and Woodgett, 2001, Chong et al., 2005, Franke, 2008, Iwanami et al., 2009). Under normal physiological conditions, PDK1 is constitutively active at the cell membrane, however it cannot interact with AKT unless AKT has been recruited to the membrane by the phosphoinositide products of PI3K (Franke, 2008). Like AKT, PDK1 also contains a PH domain, however rather than being located within the N-terminal, PDK1's PH domain is located within its C-terminal (Franke, 2008). While the phosphoinositide products of PI3K can directly phosphorylate AKT at Thr308 through PIP_3 and $PI(3,4)P_2$, phosphorylation is more likely to occur through PDK1 (Fig. 1.6), as its PH domain, compared to that of AKT, has a much higher affinity for PI3K's phosphoinositide products (Franke, 2008).

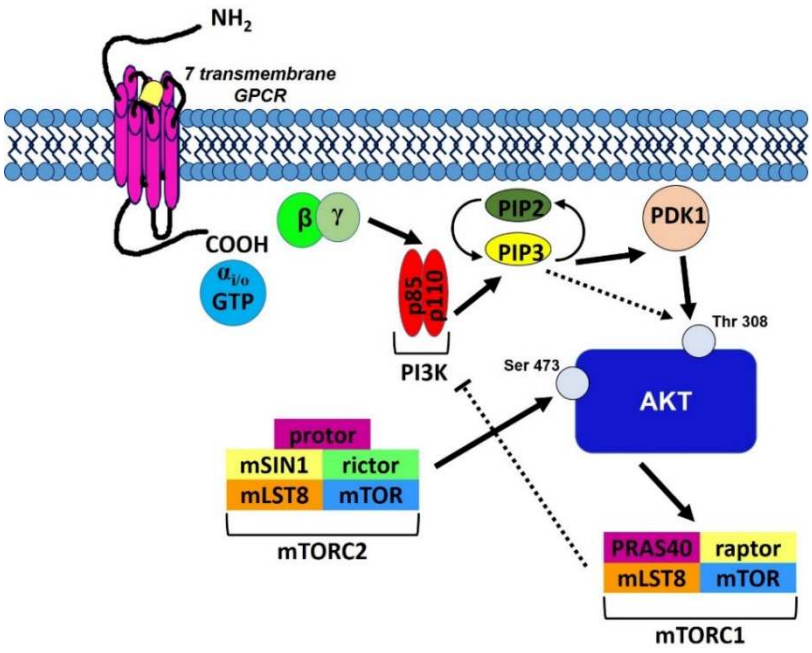

Figure 1.6. Overview of MOR-Mediated Activation of pAKT Signaling. In order for AKT to be activated, it must be phosphorylated at two distinct sites: Thr 308 and Ser 473. AKT is phosphorylated at Ser 473 by mTORC2, which exists as a complex of five proteins, and whose upstream regulation is poorly understood (Sarbassov et al., 2005, Franke, 2008, Iwanami et al., 2009, Liu et al., 2009, Okkenhaug, 2013, Lipton and Sahin, 2014). Phosphorylation of Ser 473 is necessary for the full catalytic activation of pAKT (Bellacosa et al., 1998, Scheid and Woodgett, 2001, Iwanami et al., 2009). AKT is phosphorylated at Thr 308 downstream of GPCR stimulation, as in the case of agonist-bound MORs, through induction of PI3K by βγ G-protein subunits. G-protein recruitment of PI3K to the cell membrane generates the phosphoinositide product, PIP3, which binds to PDK1 to phosphorylate AKT at Thr 308 (Chong et al., 2005, Franke, 2008, Iwanami et al., 2009, Liu et al., 2009, Okkenhaug, 2013). While Thr 308 of AKT is also responsive to PIP3, PIP3 has a much higher affinity to PDK1, which is considered the primary activator of pAKT at Thr 308 (Scheid and Woodgett, 2001, Chong et al., 2005, Franke, 2008, Iwanami et al., 2009). Upon phosphorylation, pAKT activates mTORC1, which exists as a complex of four proteins, which can indirectly inhibit PI3K through its downstream effectors to negatively regulate PI3K – pAKT activity (Vander Haar et al., 2007, Franke, 2008, Iwanami et al., 2009, Liu et al., 2009, Lipton and Sahin, 2014). Solid arrows indicate direct activation, while dotted lines indicate either indirect or inhibitory regulation of downstream targets; brackets indicate protein complexes.

Although PDK1 phosphorylates AKT at Thr308, it does not phosphorylate Ser473 (Chong et al., 2005). Importantly, phosphorylation of Ser473 always parallels the full activation of AKT, and it has been suggested that phosphorylation of Ser473 is necessary for the full catalytic activation of AKT (Bellacosa et al., 1998, Scheid and Woodgett, 2001, Iwanami et al., 2009). Since Ser473 is situated in the C-terminal, which determines the induction and maintenance of AKT activity (Franke, 2008), it is important to understand the mechanism by which Ser473 is phosphorylated.

4.1.4. Regulation of pAKT by Mammalian Target of Rapamycin Complexes

Until recently, the kinase that phosphorylated Ser473 was largely unknown and simply referred to as “PDK2” (Scheid and Woodgett, 2001, Chong et al., 2005, Franke, 2008). The serine threonine kinase mammalian target of rapamycin (mTOR) can act as both an activator and target of AKT (Fig. 1.6), the function of mTOR is dependent upon the specific complex it forms (Franke, 2008, Liu et al., 2009, Okkenhaug, 2013). There are two mTOR complexes: mTOR complex 1 (mTORC1), which is a target of AKT signaling, and mTOR complex 2 (mTORC2), which is upstream of pAKT (Franke, 2008, Liu et al., 2009, Okkenhaug, 2013). The mTORC1 complex is rapamycin-sensitive and consists of mTOR, proline-rich AKT substrate of 40 kDA (PRAS40), raptor, and mammalian lethal with sec12 protein 8 (mLST8, also known as GβL); while the mTORC2 complex is largely rapamycin-insensitive and consists of mTOR, rictor, protor, mammalian stress-activated mitogen activated protein kinase-interacting protein 1 (mSIN1), and mLST8 (Iwanami et al., 2009, Liu et al., 2009, Lipton and Sahin, 2014). While many had long suspected that PDK2 is really mTORC2, this theory was only recently proven (Sarbassov et al., 2005, Iwanami et al., 2009, Liu et al., 2009, Okkenhaug, 2013). Thus not only is mTORC2 upstream of pAKT, it directly phosphorylates AKT at Ser473 and enhances phosphorylation of AKT by PDK1 (Sarbassov et al., 2005; see also review of Franks, 2008).

In contrast to mTORC2, mTORC1 is a downstream target of AKT phosphorylation (Fig. 1.6). Activity of mTORC1 is controlled by a small GTPase, Ras homolog enriched in the brain (Rheb), which is under tonic inhibition by the tuberous sclerosis complex (also tuberin; TSC; Franke, 2008, Iwanami et al., 2009, Lipton and Sahin, 2014). Phosphorylation of AKT inhibits the

TSC1/TSC2 complex, disinhibiting Rheb and inducing mTORC1 activity (Franke, 2008, Iwanami et al., 2009, Lipton and Sahin, 2014). In addition to regulating mTORC1 activity through TSC2, pAKT can directly activate mTORC1 by inhibiting PRAS40 (Vander Haar et al., 2007, Liu et al., 2009, Lipton and Sahin, 2014). While mTORC1 has many of its own downstream signaling targets, of particular interest is its ability to negatively regulate PI3K – pAKT signaling (Fig. 1.6; Franke, 2008, Iwanami et al., 2009, Liu et al., 2009, Okkenhaug, 2013). Based on this, blocking AKT phosphorylation through inhibition of either PI3K or PDK2 could potentially depress this negative mTORC1 feedback loop, resulting in increased PI3K (and subsequent PDK1) activity and ultimately failure to prevent AKT phosphorylation. This suggests that the most successful way to prevent phosphorylation of AKT is to inhibit both mTORC2, preventing phosphorylation of Ser473, and mTORC1, preventing increased phosphorylation of Thr308. Cross-talk between mTORC2 – pAKT and pAKT – mTORC1 signaling has complicated study of AKT and mTORC intracellular signaling cascades, and while the signaling of mTORC1 is better understood, the upstream activator of mTORC2 is still unknown. One of the most interesting downstream effects of AKT phosphorylation, is the kinase's ability to insert $GABA_A$ receptors into the cell membrane (Wang et al., 2003), and to promote protein synthesis and long-term depression (LTD) through mTORC1 activation (see review of Lipton and Sahin, 2014). These findings are of particular interest, as they suggest that phosphorylated AKT is in a position to mediate neuronal excitability, possibly representing a mechanism by which pAKT interacts with drugs of abuse.

4.2 Support for MOR – pAKT/mTOR Signaling

As previously described in section 3, MORs belong to the Gi/Go family of GPCRs and activate intracellular signaling cascades through interactions with their G-protein subunits. Although PI3K – pAKT signaling is not a classical MOR-mediated intracellular signaling cascade, in recent years this pathway has been shown to be a consistent target of MOR activation (Polakiewicz et al., 1998, Iglesias et al., 2003, Russo et al., 2007, Mazei-Robison et al., 2011). In lymphocytes, morphine was found to increase phosphorylated PI3K and phosphorylated AKT, as well as the expression of MORs, in fact increases in MOR expression were dependent on PI3K –

pAKT signaling (Liu et al., 2010). That MOR stimulation increases AKT phosphorylation has also been demonstrated in other tissues, including cardiac cells (Xu et al., 2011) and cortical cells (Iglesias et al., 2003). As is the case with other GPCRs, MOR-induced pAKT is largely thought to occur after its βγ subunits disassociate from the G-protein complex and phosphorylate PI3K (Fig. 1.6). Thus it appears that all MORs and GPCRs may be capable of activating AKT signaling through their βγ G-protein subunits. Upon activation of PI3K, AKT is recruited to the membrane where it undergoes phosphorylation, after phosphorylation, pAKT has the ability to translocate into the cytoplasm or nucleus (Andjelkovic et al., 1997, Meier et al., 1997; see also review of Du and Tsichlis, 2005).

In the brain, the ability of opioid receptors to increase AKT phosphorylation has been most clearly demonstrated for the DOR. In the VTA DORs have been shown to upregulate $GABA_A$ receptor insertion into the membrane in a manner dependent on PI3K – pAKT signaling (Margolis et al., 2011). While this was shown with DORs and MORs have not been directly shown to increase AKT phosphorylation in brain tissues, given the large degree of homology between MORs and DORs, it is highly likely that MORs can utilize the same mechanisms to phosphorylate AKT and insert $GABA_A$ receptors into the membrane (Margolis et al., 2011). Although this study by Margolis et al. (2011), as well as the above cell culture studies, clearly indicate that opioid receptor activation induces AKT phosphorylation, the in vivo effects of MOR activation have not been as clear, likely due to differences in drug history and heterogeneity of cell types in Western blot measures of AKT activity. For example, using Western blot analyses, studies have suggested that chronic morphine downregulates AKT signaling in the VTA (Russo et al., 2007, Mazei-Robison et al., 2011). However these findings must be interpreted with caution, as their use of Western blot homogenates is biased towards DA neurons, which are the prevailing VTA cell type (50-65%), since Western blots represent a regional average, they are unlikely to detect changes in a small population of neurons, such as the VTA GABA neurons (30-35%) that contain higher densities of MOR (see review of Nikulina et al., 2014).

The *in vivo* study of MOR signaling in response to opiate drugs of abuse has been further complicated by differences in drug history, specifically differing histories of morphine exposure

produce different effects on AKT phosphorylation in the NAc (Muller and Unterwald, 2004). Given the work of Derek van der Kooy's laboratory in showing that mesolimbic signaling differs in the drug-naïve (DA-independent) vs. drug-dependent (DA-dependent) states (see review of Ting and van der Kooy, 2012), it is not surprising that activity of MOR intracellular signaling cascades also differ with drug history.

4.3 Implications of pAKT for Stress-Induced Amphetamine Sensitization

Repeated social defeat stress upregulates MOR expression in the VTA and produces cross-sensitization to psychomotor stimulants (see review of Nikulina et al., 2014), suggesting that MORs may mediate some of the behavioral consequences of social stress. In support of this, MOR knockout mice do not show social defeat stress-induced social avoidance (Komatsu et al., 2011). Since MORs appear to mediate at least some behavioral consequences of social stress, then it is possible that they do so through phosphorylation of AKT. In favor of this, signaling through pAKT – mTORC1 has been implicated in drug-induced sensitization to psychomotor stimulants. In particular, cocaine was associated with increases in mTORC1 activity, a downstream target of pAKT, in the mesocorticolimbic circuit, and systemic inhibition of mTORC1 was sufficient to block both the induction and expression of cocaine-sensitized locomotor activity (Wu et al., 2011). More specific manipulations have revealed that intracerebroventricular inhibition of PI3K, an upstream activator of pAKT, is necessary for the expression, but not the induction of sensitization to cocaine (Izzo et al., 2002). Social defeat stress upregulates VTA MORs, which are generally situated on GABA neurons and decrease GABA transmission, disinhibiting DA and increasing mesolimbic DA release (Fig. 1.4; see review of Nikulina et al., 2014). Since increases in mesolimbic DA release are also implicated in the effects of psychomotor stimulants, it is possible that repeated social stress-induced cross-sensitization to psychomotor stimulants may be due to upregulation of MOR and subsequent phosphorylation of its downstream kinase, AKT.

While MORs are clearly implicated in the behavioral consequences of social defeat stress (Komatsu et al., 2011), and its downstream targets PI3K – pAKT signaling have been well

implicated in sensitization to psychomotor stimulants (Izzo et al., 2002, Iniguez et al., 2008, Wu et al., 2011), one study suggests that VTA AKT phosphorylation may reduce the behavioral consequences of social defeat stress. In particular, this study blocked AKT phosphorylation in the VTA, which enhanced the negative behavioral outcomes of continuous social defeat stress in mice (Krishnan et al., 2008). When mice were classified as “susceptible” or “resilient” according to their post-stress behavioral phenotypes, Western blots of the VTA revealed that levels of phosphorylated AKT were reduced in susceptible mice (Krishnan et al., 2008). A major limitation of this study is its use of homogenate samples in Western blot, which precludes identification of neuron-type specific changes in AKT phosphorylation after stress. As such, the results of this study are heavily biased towards the majority population of DA neurons in the VTA, and must be interpreted with caution. While differences exist between the continuous and intermittent social defeat models, as well as in rats and mice (see review of Nikulina et al., 2014), the findings of Krishnan et al. (2008) suggest that intermittent social defeat stress will not increase AKT phosphorylation in VTA DA neurons. Since VTA MORs are largely localized to GABA neurons (Sesack and Pickel, 1995, Garzon and Pickel, 2002) and typically serve to indirectly disinhibit VTA DA neurons (Johnson and North, 1992), then it is possible that AKT phosphorylation may be increased in VTA GABA neurons, downstream of the social stress-induced upregulation of VTA MORs. Given that VTA MORs and pAKT phosphorylation have both been implicated in sensitization to psychomotor stimulants, it is possible that social defeat stress-induced cross-sensitization to psychomotor stimulants is mediated by stress-induced upregulation of MOR – pAKT signaling in VTA GABA neurons.

5. Research Objectives and Organization of Dissertation

Both globally and nationally, drug abuse represents a serious economic and societal problem. While a great deal of money is spent trying to intervene and treat substance abuse, treatment is not always available or affordable, and even with treatment, many addicts are likely to relapse. Based on these features, in addition to addiction itself, research on substance abuse also focuses on the variables that confer vulnerability to drugs of abuse. Most notably, stress

induces cross-sensitization to both opiates and psychostimulant drugs, the effects of which largely implicate the mesocorticolimbic circuit.

Through studying the functional effects of stress-induced changes in the mesocorticolimbic circuit, it is possible to develop new targets for the therapeutic intervention of substance abuse. The literature reviewed in this chapter clearly implicates MORs in the mediation of VTA DA transmission and the effects of drugs of abuse. Thus this dissertation will investigate the implications of VTA MOR signaling for the behavioral and cellular consequences of social stress, with emphasis on those changes related to stress-induced cross-sensitization to psychostimulant drugs.

The experiments described in the following chapters utilized the rat model of intermittent social defeat stress to investigate whether stress-induced VTA MOR upregulation is necessary for: **1)** the behavioral effects of social stress, namely cross-sensitization to amphetamine (Chapter 2), and **2)** stress-induced alterations in the mesocorticolimbic circuit, specifically, enhanced mesolimbic BDNF signaling (Chapter 3). This dissertation also investigated **3)** social defeat stress increases MOR – pAKT signaling in VTA GABA and DA neurons, and whether inhibition of VTA AKT phosphorylation is sufficient to prevent stress-induced cross-sensitization to amphetamine and deficits in weight gain (Chapter 4). The final chapter of this dissertation (Chapter 5) is a general discussion of the implications of the research described in Chapters 2 – 4.

CHAPTER 2: KNOCKDOWN OF VENTRAL TEGMENTAL AREA MU-OPIOID RECEPTORS MEDIATES EFFECTS OF SOCIAL DEFEAT STRESS: IMPLICATIONS FOR AMPHETAMINE CROSS-SENSITIZATION, SOCIAL AVOIDANCE, AND WEIGHT REGULATION [1]

ABSTRACT

Social defeat stress is naturalistic model that induces social avoidance and long-lasting cross-sensitization to drugs of abuse, as well as a corresponding upregulation of ventral tegmental area (VTA) mu-opioid receptor (MOR) mRNA. In the VTA, MOR activation is generally thought to inhibit a subpopulation GABA neurons thus disinhibiting VTA dopamine neurons and providing a role for VTA MORs in the regulation of psychostimulant sensitization. The present study determined the effect of lentivirus-mediated MOR knockdown in the VTA on the consequences of intermittent social defeat stress, a salient and profound stressor in humans and rodents. Social stress exposure induced social avoidance and attenuated weight gain in animals with non-manipulated VTA MORs, but both these effects were prevented by VTA MOR knockdown. Rats with non-manipulated VTA MOR expression exhibited cross-sensitization to amphetamine challenge (1.0 mg/kg, i.p.), evidenced by a significant augmentation of locomotion. By contrast, knockdown of VTA MORs prevented stress-induced cross-sensitization without blunting the locomotor-activating effects of amphetamine. Elucidating VTA MOR regulation of stress effects on the mesolimbic system may provide new therapeutic targets for treating stress-induced vulnerability to substance abuse.

INTRODUCTION

In humans, stress is one variable that influences the transition from recreational drug use to abuse, and it has been correlated with increased risk of substance abuse and relapse (Sinha, 2001, 2008, Razzoli et al., 2009, Sinha, 2011). Rodent studies have shown that repeated social

[1] All of the data in this chapter have been published, however this chapter does not include the immunohistochemical data included in Johnston et al. (2015) – to see this publication in its entirety, please refer to Appendix A.

defeat stress exposure consistently produces social avoidance (Berton et al., 2006, Krishnan et al., 2007, Fanous et al., 2011, Komatsu et al., 2011) and augments the effect of psychomotor stimulants, a phenomena known as 'cross-sensitization' (Covington and Miczek, 2001, Nikulina et al., 2004, Miczek et al., 2011a, Nikulina et al., 2012). Genetic mu-opioid receptor (MOR) knockout mice do not exhibit social avoidance following continuous social defeat (Komatsu et al., 2011), suggesting that MORs play a critical role in stress-induced changes in long-term neuroplasticity. In fact, even acute social defeat stress has been shown to rapidly upregulate MOR mRNA expression in the ventral tegmental area (VTA; Nikulina et al., 1999), while repeated social stress exposure increases VTA MOR mRNA expression for up to 14 days after the last episode (Nikulina et al., 2008).

In the VTA MORs are expressed by gamma-aminobutyric acid (GABA) neurons (Sesack and Pickel, 1995, Garzon and Pickel, 2002), which are hyperpolarized in response to MOR stimulation, thus disinhibiting local dopamine (DA) transmission and facilitating response to drugs of abuse (Johnson and North, 1992, Bergevin et al., 2002, Vargas-Perez et al., 2009b, Dacher and Nugent, 2011). Rats exposed to repeated social defeat stress, then challenged with an intra-VTA infusion of a MOR-specific agonist exhibited sensitized locomotor activity (Nikulina et al., 2005, Nikulina et al., 2008). This VTA opiate-induced sensitized locomotor activity was present at the same time point that social stress-induced cross-sensitization to psychomotor stimulants was observed (Covington and Miczek, 2001, Nikulina et al., 2004, Nikulina et al., 2012). Taken together, these findings indicate that increased VTA MOR expression might play a role in social stress-induced psychostimulant sensitization. Consistent with this view, MOR knockout mice exhibit reduced cocaine self-administration and increased VTA GABA transmission (Mathon et al., 2005). Furthermore, the expression of amphetamine sensitization is associated with persistent VTA MOR upregulation, and can be blocked by a treatment with MOR antagonist (Magendzo and Bustos, 2003, Trigo et al., 2010).

Although research has implicated VTA MORs in drug sensitization and social behaviors (Van Ree et al., 2000, Miczek et al., 2011l, Nikulina et al., 2014, Pitchers et al., 2014), it is unknown whether upregulation of VTA MORs causes the behavioral and biological effects of

social defeat stress exposure. To address this question, the present study used lentivirus-mediated gene transfer and RNA interference to knockdown MORs in the VTA, and then assessed social stress-induced cross-sensitization to amphetamine. Given that social avoidance is altered in MOR knockout mice after continuous social stress (Komatsu et al., 2011), the effect of VTA MOR knockdown on social stress-induced social avoidance was also examined. Finally, the effect of VTA MOR knockdown on stress-induced deficits of weight gain was investigated.

METHODS

2.1 Subjects

Experimental animals were male Sprague-Dawley rats (N = 71; Charles River Laboratories, Hollister, CA) weighing 200-250 g on arrival. Three days before social stress exposure, subjects were individually housed in standard plastic cages (25x50x20 cm^3). Twelve additional age-matched Sprague Dawley rats were group-housed 3 per cage and served solely as novel stimulus subjects during the social approach and avoidance test. Male Long-Evans rats (weighing 550-700 g), termed 'residents', were pair-housed with a tubal-ligated female in large plastic cages (37x50x20 cm^3). All rats were maintained on a 12-12 reverse light-dark cycle (lights out at 0900 h) with free access to food (Purina Rodent Diet, Brentwood, MO) and water. Residents were previously screened for aggressive behavior and were used to induce social defeat stress in experimental "intruder" rats. All experimental procedures were approved by the Institutional Animal Care and Use Committees at the Arizona State University and the University of Arizona. All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), and every effort was made to minimize pain and suffering, as well as the number of animals used.

2.2 Experimental Design

2.2.1 General Procedure

Upon arrival, experimental rats were habituated to laboratory conditions for 7 days before surgery to manipulate regional MOR level. Rats were randomly assigned to one of four

experimental conditions: Non-Manipulated MOR+Handled, Non-Manipulated MOR+Stressed, MOR Knockdown+Handled, MOR Knockdown+Stressed. Three experiments were conducted in parallel (Fig. 2.1A); one group of subjects (n = 25) received an amphetamine challenge 10 days after the last episode of intermittent social stress or handling to study the effects of VTA MOR knockdown on social stress-induced cross-sensitization. Seven days later, VTA tissue from this group of subjects was flash frozen for radioligand binding to verify the efficacy of MOR knockdown. Social approach and avoidance testing was performed two days after termination of social stress or handling procedures in both the above rats, as well as another cohort of rats (n = 46). A separate cohort of rats of rats (n = 25) were weighed prior to each episode of intermittent social stress and handling, and again 10 days later to investigate the influence of VTA MOR knockdown on social stress-induced deficits in weight gain.

2.2.2 Bilateral VTA Infusion of Lentiviral Constructs

Rats assigned to control viral groups received infusions of lentivirus that expresses green fluorescent protein (GFP) and a short hairpin RNA (shRNA) that does not target any known rat gene, while rats assigned to VTA MOR knockdown groups received a lentivirus that expresses GFP and a shRNA that targets MOR (shMOR) for RNA interference. Lentiviral constructs were prepared as previously described (Lasek et al., 2007). The shMOR lentivirus reduces VTA MOR expression by 88-97% (Lasek et al., 2007). Therefore, the viral titre was diluted by 50% with cold sterile saline to reduce the efficacy. After random assignment to GFP or shMOR knockdown conditions, rats were anesthetized using isoflurane and positioned in a stereotaxic frame (Leica Angle Two; Richmond, IL). The appropriate lentiviral construct (1.0 µl each) was infused bilaterally into the VTA (AP -5.15, ML ±2.15, DV -8.7, Tilt 10°; Paxinos and Watson, 2007) at a flow rate of 0.1 µl/min, and allowed to diffuse for 10 min before withdrawal of the syringe (Hamilton; Model 7105 KH; 24 gauge tip; Reno, NV) . The accuracy of each infusion was later

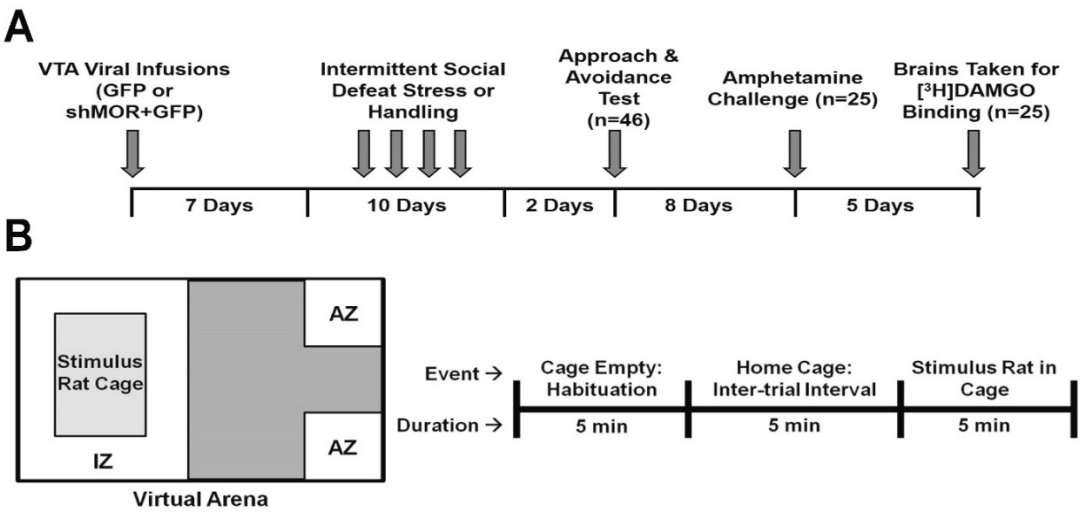

Figure 2.1. Timeline of Experimental Events and Schematic of Social Approach and Avoidance Test Procedure. (A) Two cohorts of rats were given 7 days to recover from surgery, and were then exposed to intermittent (4x in 10 days) social defeat or handling procedures. Two days after the last episode of defeat, all rats were given the social approach and avoidance test. Ten days after the last episode of defeat, one cohort of subjects was administered an amphetamine challenge. Five days after receiving the challenge, brains from these rats were removed and processed for *in vitro* [^{3}H]DAMGO autoradiography to verify the location and efficacy of MOR knockdown. (B) Subjects were assessed for social approach and avoidance using a procedure adapted from Berton et al. (2006). Left: Virtual arena dividing the chamber into 2 virtual zones: Interaction Zone (IZ), comprising of the 1019.35 cm^2 area immediately surrounding the containment cage, and Avoidance Zone (AZ), which comprised the two corners, combined 52.2 cm^2, opposite the containment cage. Right: Schematic of the timeline for the social approach and avoidance procedure.

verified using localization of GFP expression. Subjects were given 7 days to recover before the start of intermittent social stress or handling procedures (Fig. 2.1A).

2.2.3 Intermittent Social Defeat Stress and Handling Procedures

Social defeat stress was induced by a short confrontation between an aggressive resident and an experimental intruder rat, as previously described (Tidey and Miczek, 1996, Nikulina et al., 2004, Nikulina et al., 2012). After removing the female from the resident's home cage, an experimental rat was placed inside the resident's home cage for 5 min within the confines of a protective metal cage (15x25x15 cm^3). The protective cage was then removed, allowing the resident to attack the experimental intruder rat until it displayed supine posture for at least 4 sec (see Chapter 1, Fig. 1.1). Once submissive posture was exhibited, the experimental rat was placed back in the protective cage and exposed to threat from the resident for an additional 20 min before being returned to its own home cage. Intermittent social stress procedures were administered every third day for 10 days (Fig. 2.1A). At each corresponding time point, rats in the control groups were handled for approximately 2.5 min and then returned to their home cages.

2.3 Behavioral Assessments

2.3.1 Social Interaction

The social approach and avoidance test was conducted in a large plastic container (58x38x41 cm^3) equipped with a lightweight containment cage. Experimental rats were habituated to the empty test chamber for 5 min, then reintroduced when a novel stimulus rat was within the containment cage (Fig. 2.1B). The behavior of experimental rat was recorded for 5 min using TopScan (Clever Systems Inc.; Reston, VA). The software divided the chamber into virtual zones: Interaction, which comprised the area surrounding the containment cage, and Avoidance, which comprised the two corners opposite the containment cage (Fig. 2.1B; arena adapted from Berton

et al., 2006). The number of respective entries into the avoidance and interaction zones was recorded, as was the distance (cm) moved in each zone.

2.3.2 Amphetamine Challenge

A low dose d-amphetamine challenge was administered to test for social stress-induced cross-sensitization (Nikulina et al., 2004, Nikulina et al., 2012). For two days prior to the challenge, rats were injected with vehicle (0.9% sterile saline; 1.0 ml/kg, i.p.), and were acclimated in their home cage to the procedure room for 1 h. On the day of the challenge, rats were moved in their home cage to the procedure room, and locomotor activity was recorded at 10 min intervals using video tracking software (Videotrack, Viewpoint Life Sciences; Montreal, Canada). Locomotor activity was detected as the number of and distance travelled during movements (>10 cm) across 170 min consisting of 3 phases: Baseline, Saline, and Amphetamine. Baseline data were recorded for 30 min, after which a saline injection (1.0 ml/kg, i.p.) was given and locomotor activity was recorded for 60 min. Finally, rats received an injection of d-amphetamine sulfate (1.0 mg/kg, i.p.; Sigma-Aldrich; St. Louis, MO), and locomotor data were recorded for 80 min. Video tracking and data collection were paused during the administration of saline and amphetamine injections. Rather than stereotypical behaviors, this dose of amphetamine has been shown to primarily induce large ambulatory movements (Geyer et al., 1987). In order to quantify amphetamine sensitization, ambulatory movements (> 10 cm) were measured in terms of the number of movements initiated and the distance travelled (cm) during such movements.

2.4 Tissue Harvesting

2.4.1 Fresh Frozen VTA Tissue for Radioligand Binding

Rats were anesthetized with isoflurane, and their brains were rapidly removed and frozen in -35°C 2-methylbutane for 15 sec, then stored at -80°C prior to sectioning. On a cryostat, serial 20 µm sections through the VTA were collected (from AP -4.8 to -5.5; Paxinos and Watson, 2007) for radioligand binding and localization of GFP expression. Sections were thaw-mounted onto

glass microslides (Superfrost Plus; Fisher Scientific; Waltham, MA), dried in a vacuum chamber at 4°C, and stored at -80°C prior to processing. Separate slides were used to verify the accuracy and distribution of lentiviral infusions based on fluorescent detection of GFP expression.

2.5 [^{3}H]DAMGO Autoradiography

2.5.1 Radioligand Binding

Fresh frozen brain sections were used to verify shMOR knockdown in the VTA using tritiated [D-Ala^2,*N*-MePhe^4,Gly-ol^5] enkephalin ([^{3}H]DAMGO; NIDA Drug Supply Program; Bethesda, MD), as described by Zhou and Hammer (1995). Briefly, slides were placed in pre-incubation solution (15 mM Tris HCl, 150 mM NaCl, 1.0 mg/ml BSA) for 30 min at 4°C, then were incubated in 10 nM [^{3}H]DAMGO solution (50 mM Tris buffer, 3.0 mM Mn acetate, 1.0 mg/ml BSA) with or without the addition of naloxone (10 µM; NIDA Drug Supply Program) for 60 min at 22°C. Slides were washed with a 50 mM Tris buffer at 4°C, then dried and exposed on Kodak BIO Max MR X-ray film (Carestream; Sigma-Aldrich; St. Louis, MO) for 10 weeks at room temperature. Sections incubated in 1000-fold excess unlabeled naloxone were utilized to determine non-specific binding in subsequent autoradiography.

2.5.2 Autoradiography Analysis

Autoradiograpy film was developed and scanned at high resolution. In order to determine whether the shMOR viral construct infected regions outside of the VTA, the substantia nigra pars compacta (SNc) was chosen as a control region due to its close proximity to the VTA, and because social stress does not affect MOR expression in substantia nigra regions (Nikulina et al., 1999, Nikulina et al., 2005). The SNc, not to be confused with the medial terminal nucleus accessory optic tract (MT), contains a higher density of MOR labeling than either the substantia nigra pars reticulata (SNr) or VTA (Herkenham and Pert, 1982). Using this difference in expression, the SNc could be clearly demarcated on scans of autoradiographs by measuring the area directly above the SNr, lateral to the MT, and ventrolateral to the medial lemniscus. Optical densities for these regions were measured bilaterally in 2-3 sections using ImageJ (National

Institutes of Health, USA, http://imagej.nih.gov/ij), and then converted to μCi/g using calibrated [^{3}H] radiostandards (ART-123, ARC Inc.; St. Louis, MO) co-exposed with sections. For each subject, bilateral measurements were averaged across sections to yield total ligand binding in the VTA and SNc, respectively.

2.6 Statistical Analyses

The results of each measure are expressed as mean ± standard error (SEM) and a p value ≤ 0.05 was considered to be significant. All statistical analyses were run using SPSS software, version 18 (SPSS Inc., Chicago, IL), and Tukey's HSD was considered the preferred *post hoc* test across experiments. An exception was made in the case of the amphetamine challenge, where Fisher's LSD was used because violations of sphericity necessitated the use of a more conservative test of the main effects. Data from subjects were excluded only in the case of error during video tracking or loss of data due to damaged tissue sections: no statistical outliers were excluded. The locomotor and social approach and avoidance assays relied on automated video tracking systems, requiring that the animals be housed in black bedding to block light from reflecting off the cage bottom. However in some instances, rats exposed the cage floor while moving, causing illumination artifacts that necessitated the removal of individual bin data due to inaccurate tracking. More specifically in the locomotor and social approach and avoidance assays, which relied on automated video tracking systems, individual bin data were removed in those instances where reflection artifacts prevented accurate tracking. For analyses of mounted tissue sections, the sample size of each group was also reduced in cases where tissue was damaged in the course of processing.

2.7.1 Weight Gain Data

The initial weight obtained at the start of social stress procedures was used to normalize all subsequent data (n = 25) to weight gained from that time onward; no subjects were excluded from the analysis. A one-way analysis of variance (ANOVA) was performed to assess differences

in weight at each time point, and all significant main effects were analyzed using Tukey's test for *post hoc* comparisons among the means.

2.7.2 Social Interaction

Social approach and avoidance data were analyzed in terms of the number of entries to, and the distance travelled (cm) within the interaction and avoidance zones (Fig. 2.1B). Where illumination artifacts interfered with tracking, data were lost in a zone-specific manner. For example, avoidance zone entry data were analyzed from 40 subjects because illumination artifacts resulted in the exclusion of subjects from the following groups: GFP-Handled: 1; GFP-Stressed: 3; shMOR-Handled: 2. For distance travelled in the avoidance zone, an additional tracking error which occurred after a subject entered the zone further reduced the number of analyzed subjects to 35; subjects were excluded from the following groups: GFP-Handled: 2; GFP-Stressed: 1; shMOR-Handled: 5; shMOR-Stressed: 3. Illumination artifacts and tracking error reduced the number of subjects in the interaction zone to 37; subjects were excluded from the following groups: GFP-Stressed: 3; shMOR-Handled: 4; shMOR-Stressed: 2. A one-way ANOVA was run on data pertaining to each zone and any significant main effects were followed by an analysis of *post hoc* comparisons with Tukey's test.

2.7.3 Locomotor Activity

Locomotor data were analyzed using separate multivariate analysis of variance (MANOVA) for the mean number and distance (cm) travelled during ambulatory movements. In order to overcome violations of sphericity in the output of repeated measures ANOVA, MANOVA was used to analyze the number and distance of ambulatory movements exhibited throughout the amphetamine challenge. Significant multivariate effects were followed by univariate analyses to determine which time points produced significant group differences. Significant univariate effects were further analyzed for *post hoc* comparisons using Fisher's Least Significant Difference (LSD) test. Data were analyzed from 21 subjects for both dependent measures of ambulatory

movements. Some subjects' data were excluded from analysis due to the presence of illumination artifacts that interfered with tracking: GFP-Stressed: 2; shMOR-Handled: 1; shMOR-Stressed: 1.

2.7.4 MOR Binding

The results of radioligand binding with [^{3}H]DAMGO in the VTA and SNc were analyzed using separate one-way ANOVAs, and where necessary, significant main effects were followed by *post hoc* comparisons with Tukey's test. In the case of VTA [^{3}H]DAMGO results, a violation of homogeneity was corrected for with Welch's F test. Sample sizes were reduced after the target region was damaged during processing during [^{3}H]DAMGO binding for 1 shMOR treated subject. Consequently, receptor autoradiography was analyzed from 25 subjects in the VTA and 24 subjects in the SNc.

RESULTS

Verification of MOR Knockdown Using [^{3}H]DAMGO Autoradiography

Fluorescent detection of virally expressed GFP revealed that lentiviral infusions were specific to the VTA (Fig. 2.2A), and GFP was not detected in either SN region (data not shown). While lentiviral constructs were infused at AP -5.15, GFP expression indicated infusions to the target site varied by ±0.1 mm, and that the average spread of GFP was within AP -4.8 to -5.5 and Lateral 0.4 mm to 1.4 mm (Fig. 2.2B). Quantitative in vitro autoradiography with [^{3}H]DAMGO was used to determine the functionality of VTA MORs after lentivirus-mediated knockdown. Compared to the control GFP lentiviral construct, the subjects infused with the shMOR construct showed reduced [^{3}H]DAMGO binding (Fig. 2.2C). One-way ANOVA revealed that this effect was significant in the VTA (n = 25, $F_{1,20.13} = 102.46$, $p < 0.0001$), but not the SNc (n = 24, $F_{1,22} = 1.63$, $p > 0.22$; Fig. 2.2D). Thus, surgeries were accurate and bilateral shMOR knockdown selectively reduced VTA MOR binding density.

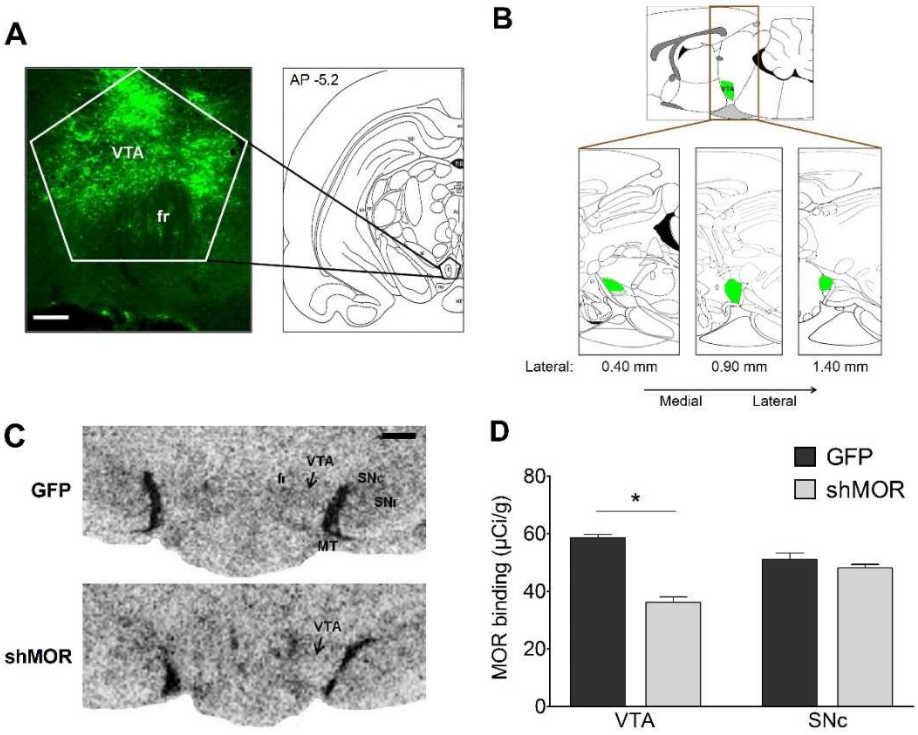

Figure 2.2. [^{3}H]DAMGO Autoradiography Revealed That the shMOR Construct, but Not the Scrambled GFP Construct, Significantly Reduced MOR Binding in the VTA, but Not the SNc. (A) Left: Representative image of reporter GFP expression in infected VTA cells (fr: fasciculus retroflexus; scale bar = 100 µm). Right: Plate 37, modified from Paxinos and Watson (2007). (B) Parasagittal illustrations showing the extent of GFP expression across the VTA drawn in green (lateral plates 0.40 - 1.4, modified from Paxinos and Watson, 2007). (C) Representative autoradiographs of [^{3}H]DAMGO binding in the VTA after infusion of either scrambled-GFP or shMOR lentiviral constructs (MT: medial terminal nucleus of the accessory optic system; scale bar = 500 µm). (D) The shMOR lentiviral construct (n = 14) significantly (* - $p < 0.0001$) reduced MOR binding in the VTA compared to the scrambled-GFP construct (n = 11), without affecting MOR binding of either the GFP (n = 11) or shMOR (n = 14) groups in the adjacent SNc ($p > 0.22$).

Effect of VTA MOR Knockdown on Intermittent Social Stress-Induced Deficit of Weight Gain

Weight gain data (n = 25) revealed a significant main effect during social stress exposure ($F_{3,21}$ = 10.15, p < 0.0003), and 10 days after the last stress episode ($F_{3,21}$ = 9.46, p < 0.0004; Fig. 2.3). *Post hoc* comparisons at this time point show that the GFP-Stressed group experienced less weight gain than either the GFP-Handled or shMOR-Handled groups (p < 0.006), while the shMOR-Stressed group only differed from the shMOR-Handled group (p < 0.02). Ten days after the final episode of social stress, the GFP-Stressed group showed significantly lower body weights compared not only to GFP-Handled and shMOR-Handled groups ($p \leq 0.006$), but also the shMOR-Stressed group (p < 0.05). These data suggest that social stress significantly reduces body weight, and that while VTA MOR knockdown attenuated this effect during social stress exposure, it rescued this effect 10 days after termination of stress.

Effect of VTA MOR Knockdown on Intermittent Social Stress-Induced Social Avoidance

The social approach and avoidance test revealed a main effect of experimental group on number of entries to the avoidance zone (n = 40, $F_{3,36}$ = 5.89, p = 0.002), with significantly more entries by GFP-Stressed rats compared to both GFP-Handled (p < 0.005) and shMOR-Stressed (p < 0.004) groups (Fig. 2.4A). Similarly, there was a significant main effect of experimental group on the distance traveled in the avoidance zone (n = 35, $F_{3,31}$ = 4.77, p = 0.008; Fig. 2.4B), with significantly more activity in the GFP-Stressed group than the GFP-Handled (p = 0.011), shMOR-Handled (p < 0.05), or shMOR-Stressed (p < 0.05) groups. There was no main effect of experimental group on the number of entries to the interaction zone (n = 37, $F_{3,26}$ = 1.14, p = 0.351; Fig. 2.4C). These data suggest that prior social stress exposure significantly increases social avoidance, and local VTA depletion of MOR prevents social stress-induced social avoidance without significantly altering social interaction.

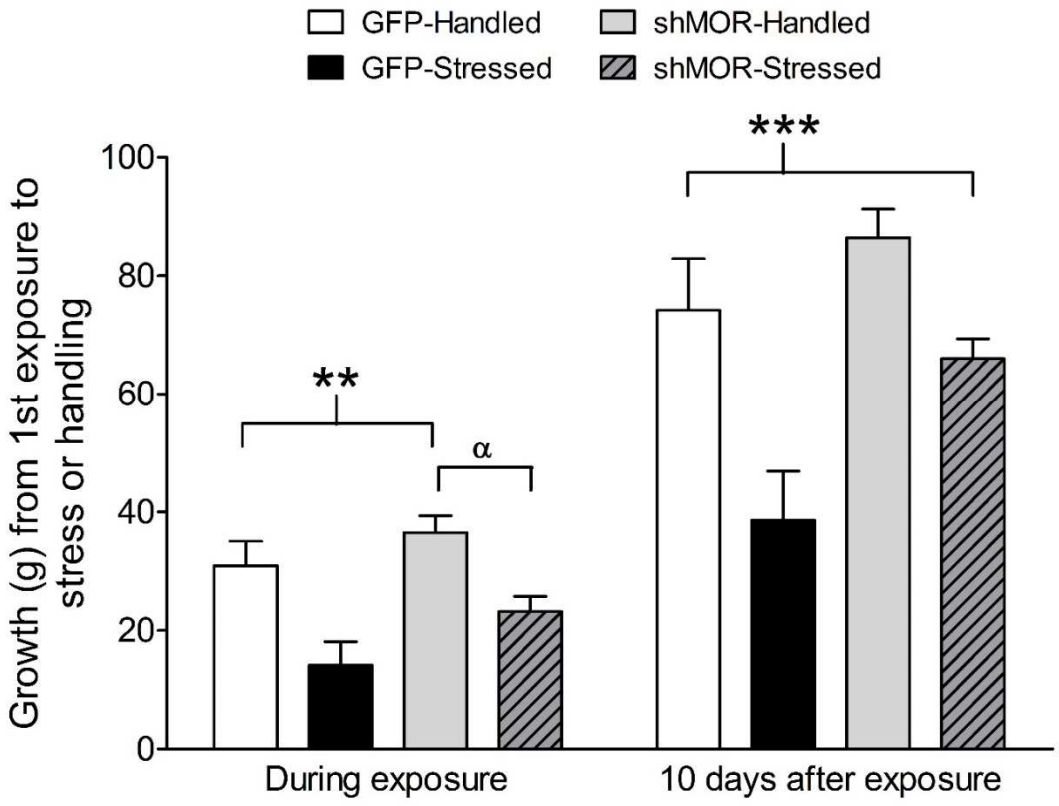

Figure 2.3. Knockdown of VTA MORs Prevents Social Stress-Induced Deficit of Weight Gain. While undergoing social stress or handling, GFP-Stressed rats (n = 5) exhibited significantly (** - $p < 0.05$) less weight gain than did GFP-Handled (n = 6) or shMOR-Handled (n = 7) rats. By contrast, shMOR-Stressed rats (n = 7) did not differ from GFP-Handled or -Stressed rats, showing significantly (α - $p < 0.05$) less weight gain than shMOR-Handled rats. Ten days after the last episode of exposure, GFP-Stressed rats had gained significantly (*** - $p < 0.05$) less weight than all other groups.

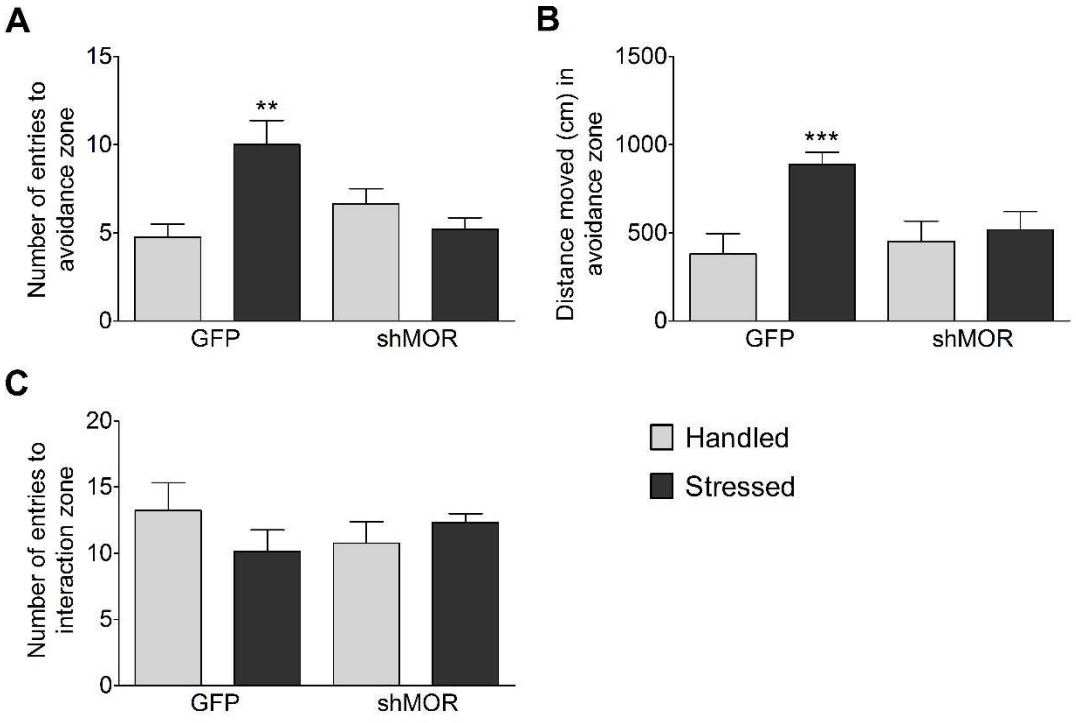

Figure 2.4. Knockdown of VTA MORs Prevents Social Stress-Induced Social Avoidance. (A) GFP-Stressed rats (n = 7) made significantly (** - $p < 0.005$) more entries to the avoidance zones than did GFP-Handled (n = 7) or shMOR-Stressed rats (n = 14). (B) GFP-Stressed rats (n = 9) were significantly (*** - $p < 0.05$) more active in the avoidance zones than GFP-Handled (n = 7), shMOR-Handled (n = 8), or shMOR-Stressed (n = 11) rats. (C) GFP-Stressed rats (n = 7) showed a slight tendency to spend less time in the interaction zone, but there was no significant ($p > 0.3$) main effect compared to GFP-Handled (n = 9), shMOR-Handled (n = 9), or shMOR-Stressed (n = 12) groups.

Effect of VTA MOR Knockdown on Intermittent Social Stress-Induced Cross-Sensitization

There were significant main effects of experimental group on the number of ambulatory movements (n = 21, *Wilks' λ* = 3.78×10^{-7}, $F_{51.0,17} = 10.57$, $p = 0.019$, $\eta^2 = 0.993$, *observed power* = 0.87) and distance travelled during ambulatory movements(n = 21, *Wilks' λ* = 1.26×10^{-6}, $F_{51.0,17} = 7.03$, $p = 0.039$, $\eta^2 = 0.989$, *observed power* = 0.87) across all time points. The number of movements differed significantly only, at 30 ($F_{3,17} = 3.66$, $p = 0.034$), 40 ($F_{3,17} = 3.36$, $p = 0.043$), and 50 ($F_{3,17} = 4.46$, $p = 0.017$) min after amphetamine injection, but there were no differences across groups before or after saline injection ($p > 0.05$ at all other time points). *Post hoc* testing (Fig. 2.5A) showed that the GFP-Stressed group exhibited significantly greater number of movements compared to GFP-Handled ($p < 0.005$) and both shMOR-Handled and -Stressed groups ($p < 0.05$) at 30 min after amphetamine injection, compared to GFP-Handled ($p < 0.01$) 40 min post-amphetamine, and compared to GFP-Handled ($p < 0.002$) and both shMOR-Handled and -Stressed groups ($p < 0.03$) 50 min after amphetamine.

Similarly, distance travelled exhibited significant main effects only 20 ($F_{3,17} = 3.51$, $p = 0.038$), 30 ($F_{3,17} = 6.83$, $p = 0.003$), and 40 ($F_{3,17} = 4.86$, $p = 0.013$) min after amphetamine injection. *Post hoc* analyses (Fig. 2.5B) showed that the GFP-Stressed group moved a significantly greater distance compared to the GFP-Handled, shMOR-Handled, and shMOR-Stressed groups ($p < 0.02$) 20 min after amphetamine injection, compared to GFP-Handled and both shMOR groups ($p < 0.002$) 30 min post-injection, and compared to the GFP-Handled and both shMOR groups ($p < 0.03$) groups 40 min post-amphetamine. Thus, the GFP-Stressed group showed social stress-induced cross-sensitization following amphetamine challenge, but the shMOR-Stressed group did not.

DISCUSSION

These data show that lentivirus-mediated overexpression of shMOR successfully reduced MOR binding activity in the VTA, and that the affected region was limited to the VTA. Furthermore, the results of this study indicate that intermittent social stress induction of VTA

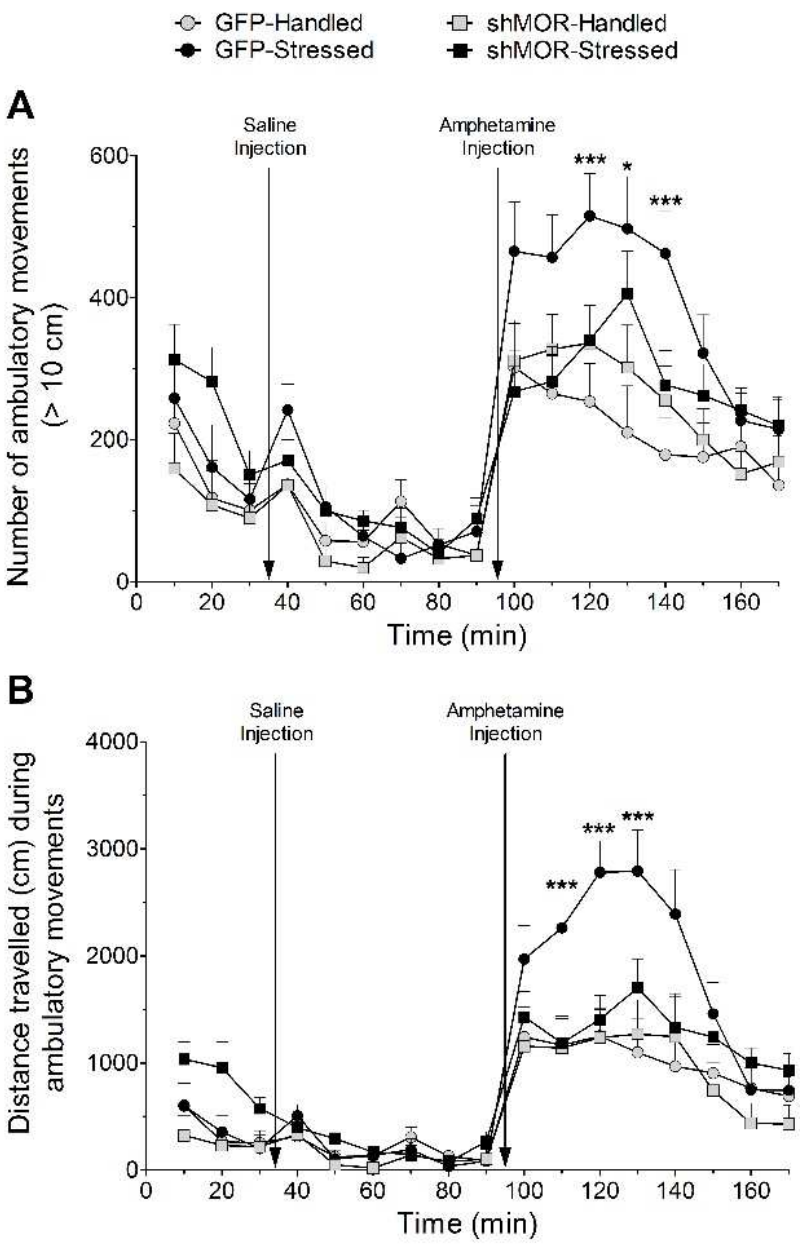

Figure 2.5. Knockdown of VTA MORs Prevents Social Stress-Induced Amphetamine Cross-Sensitization Without Affecting Baseline Activity. Multivariate analyses revealed that the only significant main effects occurred during the amphetamine phase of the assay. Data collection and video tracking were paused to administer saline and amphetamine, vertical arrows denote the time point when injection occurred. (A) GFP-Stressed rats (n = 4) exhibited significantly (*** - $p < 0.05$) more movements at 120 and 140 min compared to GFP-Handled (n = 5), shMOR-Handled (n = 6), and shMOR-Stressed (n = 6) rats, and differed significantly (* - $p < 0.02$) from GFP-Handled rats at 130 min. (B) GFP-Stressed rats travelled a significantly (*** - $p < 0.03$) greater distance at 110, 120, and 130 min compared to all other groups.

MORs is required for various stress-induced changes. For example, lentivirus-mediated knockdown of VTA MORs blocks intermittent social stress-induced social avoidance, cross-sensitization to amphetamine, and deficit of weight gain.

VTA MOR Upregulation is Necessary for Intermittent Social Stress-Induced Weight Gain Deficits

Exposure to social stress attenuated weight gain both during and 10 days after social stress exposure, which is consistent with previous findings (Meerlo et al., 1996, Pulliam et al., 2010, Fanous et al., 2011, Venzala et al., 2012). VTA MOR knockdown rescued the deficit of weight gain 10 days after the last episode of stress, but not during stress exposure. That knockdown of VTA MORs attenuated and promoted recovery from social stress-induced weight gain deficit is consistent with a report of increased body weight in MOR *knockout* mice (Han et al., 2006). Another study using the same lentiviral construct in the VTA (Lasek et al., 2007) also showed no significant effect on weight, indicating that VTA MOR knockdown is not sufficient to alter weight gain in the absence of social stress.

The role of MORs in the regulation of food intake and weight gain is complex, making it difficult to separate MOR effects on food palatability, food intake, and a more general increase of hedonic value. Pharmacological stimulation of MORs has frequently been associated with increased hedonic value of food and drug stimuli (Badiani et al., 1995, Nathan and Bullmore, 2009), while MOR antagonism has been associated with decreased consumption of highly palatable food (Segall and Margules, 1989), as well as decreased sensitivity to natural reward (Pitchers et al., 2014). Stimulation of VTA MORs has been found to facilitate food consumption in a dopamine D_1 receptor-dependent manner (Badiani et al., 1995, MacDonald et al., 2004), while antagonism reduced consumption of palatable foods (Segall and Margules, 1989). Based on this, one might expect that VTA MOR knockdown would further reduce weight gain by altering feeding behaviors. By contrast, the present data show that VTA MOR knockdown rescues the stress-induced deficit in weight gain without affecting normal weight gain.

If knockdown of VTA MORs rescued the stress-induced reduction of weight gain by attenuating the psychological effects of stress, one might expect to see signs of increased reward

or hedonic value in the amphetamine challenge or social approach and avoidance test. However, compared to GFP-Handled rats, subjects in the shMOR-Handled group did not show increased, or impaired response to amphetamine, or differ in social interaction. That subjects with VTA MOR knockdown, regardless of stress treatment, did not exhibit significant differences in weight gain compared to control GFP-Handled subjects, suggests that the rescue of weight gain is likely due to the prevention of downstream stress-induced changes in the mesolimbic circuit. For example, social stress also increases VTA brain-derived neurotrophic factor (BDNF) expression, however this begins at after upregulation of VTA MORs (Nikulina et al., 2008, Fanous et al., 2010, Nikulina et al., 2012). Since VTA BDNF expression is also necessary for the stress-induced weight gain deficit (Fanous et al., 2011), it is possible that knockdown of VTA MORs rescued the stress-induced weight gain deficit by augmenting VTA BDNF expression.

The current study did not measure food consumption, so it is not possible to ascertain whether altered food intake contributed to the weight gained after stress, with or without VTA MOR knockdown. However, if the stress-induced deficit of weight gain were related to VTA MOR-mediated changes in food intake, one would expect both Handled- and Stressed-shMOR knockdown groups to show significant differences in weight gain compared to GFP-Handled rats, which was not the case. There is some evidence to suggest that that MOR activity can alter weight gain without producing deficits in food consumption. In particular, daily morphine injection for 8 days had no effect on weight gain or food intake, while a parallel group of subjects that received escalating doses of morphine exhibited reduced weight gain without significant any significant effect on food consumption (Ren et al., 2013). In the same study, injections of escalating doses of morphine led to activation of cAMP responsive binding element protein (pCREB) in the VTA, implicating this region in MOR-mediated reduction of weight gain, but not food intake. Based on this, it is possible that escalating endogenous mu-opioid activity in the VTA underlies the weight gain deficit seen after social stress.

Upregulation of VTA MORs is Necessary for Intermittent Social Stress-Induced Social Avoidance

Rodents with non-manipulated VTA MORs and a history of social stress engaged in significantly more social avoidance (Berton et al., 2006, Fanous et al., 2010, Komatsu et al., 2011). However, MOR knockout mice do not show social avoidance after continuous social stress (Komatsu et al., 2011), just as knockdown of VTA MORs prevented intermittent social stress-induced social avoidance in the current study. MORs have been implicated in the rewarding components of social behavior, while MOR antagonists are associated with reduced social play (Vanderschuren et al., 1997) and experience-induced facilitation of sexual behavior (Pitchers et al., 2014), allowing for the possibility that VTA MOR knockdown might alter normal social interaction. However, the current results showed that VTA MOR knockdown in handled rats did not alter any measures of social interaction, suggesting that VTA MORs affect social behavior only upon the impact of stress exposure.

Previous research has also indicated that social history alone (isolation vs. social housing) or in conjunction with a social interaction test has a profound effect on MOR expression (Vanderschuren et al., 1995). Specifically, long-term social isolation increased MOR binding density in the VTA, while an acute social interaction reduced VTA MOR binding. Taken together, it is possible that positive and negative social situations alter VTA MOR expression, respectively decreasing or increasing VTA MOR activity.

Knockdown of VTA MORs Prevents Intermittent Social Stress-Induced Cross-Sensitization

Stressed rats with non-manipulated VTA MORs exhibited significantly greater locomotor activity after a low dose amphetamine challenge, confirming prior reports that intermittent social stress induces amphetamine cross-sensitization 10 days after the last stress episode (Covington and Miczek, 2001, Nikulina et al., 2012). By contrast, knockdown of VTA MORs prevented social stress-induced cross-sensitization without blocking amphetamine-induced locomotion. VTA MORs are presynaptically expressed by GABA neurons (Sesack and Pickel, 1995, Garzon and Pickel, 2002), and when activated, reduce GABAergic inhibition of VTA DA neurons (Johnson and North, 1992, Bergevin et al., 2002, Vargas-Perez et al., 2009b, Trigo et al., 2010, Dacher and

Nugent, 2011) and facilitate response to psychomotor stimulants. Thus, if stimulation of MORs in the VTA indirectly increases VTA DA activity by reducing GABA transmission, then it is likely that knockdown of VTA MORs increases GABA release. In fact, MOR knockout mice showed enhanced VTA GABA release onto local DA neurons, resulting in reduced cocaine self-administration (Mathon et al., 2005).

While this theory of inhibitory MOR activation is well accepted, it was recently demonstrated that MORs can also be found on VTA DA neurons, and can even have excitatory functions (Margolis et al., 2014). Given that intra-VTA agonism of MORs, after stress-induced upregulation, produces a sensitized locomotor response (Nikulina et al., 2005, Nikulina et al., 2008) that is associated with increased VTA DA activity, thus it is unlikely that social stress upregulates inhibitory MORs on DA, or excitatory MORs on GABA. However if stress increased the expression of excitatory MORs on VTA DA neurons, it would produce a direct increase in DA activity, similar to the indirect increase associated with inhibitory MORs on VTA GABA neurons. Thus it is possible that knockdown of VTA MORs mediated VTA DA output in two ways: either through direct excitation of DA neurons, or classical disinhibition of DA via inhibitory MORs on GABA neurons.

Although treatment with a MOR antagonist has been shown to abolish amphetamine responses (Magendzo and Bustos, 2003), this study showed that knockdown of VTA MORs did not block psychomotor activation following amphetamine challenge. This suggests that knockdown of VTA MORs does not produce unnatural alterations of mesolimbic tone. The results of this study reveal that VTA MOR upregulation is necessary for intermittent social stress-induced cross-sensitization to amphetamine. As such, in the VTA social stressors may function to increase endogenous MOR activity on GABA neurons, thus reducing the GABAergic inhibition of local DA neurons and facilitating behavioral sensitization to psychostimulant drugs.

Concluding Remarks

Since increased mesolimbic BDNF – TrkB receptor activity has been associated with the behavioral consequences of social stress, as well as stress- and drug-induced sensitization (see

review of Nikulina et al., 2014), it is possible that VTA MOR knockdown prevented the behavioral effects of social stress by augmented stress-induced changes in mesolimbic BDNF – TrkB receptor signaling. Given that in the present study, VTA MOR knockdown likely functioned to block stress-induced increase of dopaminergic tone, this manipulation also might prevent stress-induced changes in the NAc, such as increases in TrkB receptor expression. Future studies are needed to determine whether VTA MOR knockdown alters stress-induced increase of mesolimbic BDNF – TrkB receptor activity.

In conclusion, the results of this study indicate that social stress exposure increases VTA MOR activity, potentially disinhibiting VTA dopaminergic tone to facilitate response to drugs of abuse. The present data suggest that upregulation of VTA MORs following social stress exposure may underlie vulnerability to psychostimulant drugs in some individuals, thereby providing a potential target for therapeutic intervention during abuse of these drugs.

CHAPTER 3: KNOCKDOWN OF VENTRAL TEGMENTAL AREA MU-OPIOID RECEPTORS: IMPLICATIONS FOR SOCIAL STRESS-INDUCED CHANGES ACROSS THE MESOCORTICOLIMBIC CIRCUIT [2]

ABSTRACT

Social defeat stress induces prolonged cross-sensitization to drugs of abuse, as well as a corresponding upregulation of ventral tegmental area (VTA) mu-opioid receptor (MOR) mRNA. Previously lentivirus-mediated knockdown of VTA MORs was found to prevent the behavioral consequences of social stress. The present study used lentivirus-mediated interference to investigate whether intermittent social stress-induced upregulation of VTA MORs mediates stress-induced changes throughout the mesocorticolimbic circuit. At the time point corresponding to amphetamine challenge, immunohistochemical analyses were performed to examine the effect of VTA MOR knockdown on stress-induced brain-derived neurotrophic factor (BDNF) expression in the VTA and prefrontal cortex (PFC), as well as delta FosB and tropomyosin-related kinase B (TrkB) receptor expression in the nucleus accumbens (NAc), all of which have been implicated in stress-induced drug sensitization. Prior stress exposure increased the expression of BDNF in the VTA and its receptor TrkB in the NAc of rats with non-manipulated VTA MOR expression, while VTA MOR knockdown prevented stress-induced expression of VTA BDNF and NAc TrkB receptor. Although social stress alone significantly increased the expression of delta FosB in the NAc core, this effect was only somewhat attenuated by VTA MOR knockdown. By contrast, there was no effect of either stress or VTA MOR knockdown on BDNF expression in the PFC. Taken together, these results suggest that upregulation of VTA MOR is necessary for increased mesolimbic expression of BDNF and its TrkB receptor. These data extend what is known about

[2] The BDNF immunohistochemistry performed in this chapter was published in Johnston et al. (2015), to see this manuscript in its entirety, please refer to Appendix A. In-text references to Johnston et al. (2015) refer to the behavioral data presented in Chapter 2.

the directionality of stress-induced signaling in the VTA, and suggest that VTA MORs may serve as the starting point for the effects of stress in the mesolimbic pathway.

INTRODUCTION

Stress is one variable that influences the transition from recreational drug use to abuse in humans, and it has been correlated with both increased risk of substance abuse and relapse (Sinha, 2001, 2008, 2011). Rodent studies have shown that intermittent social defeat stress consistently confers vulnerability to drugs of abuse, a phenomena known as stress-induced cross-sensitization (Covington and Miczek, 2001, Nikulina et al., 2004, Nikulina et al., 2012). Moreover social stress-induced upregulation of ventral tegmental area (VTA) mu-opioid receptors (MORs) was recently shown to be necessary for the development of cross-sensitization to amphetamine (Johnston et al., 2015). In the VTA, MORs are generally expressed by gamma-aminobutyric acid (GABA) neurons (Sesack and Pickel, 1995, Garzon and Pickel, 2002), which are hyperpolarized in response to MOR stimulation, and disinhibit local dopamine (DA) transmission to facilitate response to drugs of abuse (Johnson and North, 1992, Bergevin et al., 2002, Dacher and Nugent, 2011). However it was recently shown that MORs can have either inhibitory or excitatory functions in both VTA GABA and DA neurons (Margolis et al., 2014). Despite this, rats exposed to repeated social defeat stress and then challenged with an intra-VTA infusion of a MOR-specific agonist, exhibit sensitized locomotor activity (Nikulina et al., 2005, Nikulina et al., 2008), which is typically thought to reflect increased VTA DA transmission. This suggests that net effect of social stress-induced MOR upregulation is to enhance VTA DA transmission, and given that only a small proportion of MORs are excitatory on VTA DA neurons (Sesack and Pickel, 1995, Garzon and Pickel, 2002, Margolis et al., 2014), implies that the VTA MORs upregulated after stress are predominantly inhibitory on GABA interneurons.

Delta FosB is a nuclear transcription factor that gradually accumulates with repeated exposure to stimuli, such as stress or drugs of abuse (Nestler et al., 2001, Nestler, 2008, 2014). Generally speaking, delta FosB is thought to act as either a transcriptional activator or repressor, depending on the nature of the target gene, and an accumulation of delta FosB in the nucleus

accumbens (NAc) is one of the most common responses to drugs that effect VTA DA transmission (Nestler et al., 2001, Nestler, 2008). In particular, NAc delta FosB expression is increased following acute cocaine treatment (Larson et al., 2010), as well as during withdrawal from chronic psychostimulant administration (Murphy et al., 2003, Larson et al., 2010). Similar to drugs of abuse, social stress also significantly increases the expression of delta FosB in the NAc (Nikulina et al., 2008, Nikulina et al., 2012), and the expression of this protein seems to be related to mesolimbic brain-derived neurotrophic factor (BDNF) – tropomyosin-related kinase B (TrkB) receptor signaling. In fact, viral-mediated overexpression of VTA BDNF exacerbates social stress-induced NAc delta FosB expression (Wang et al., 2013), while viral-mediated depletion of NAC TrkB, the BDNF receptor, prevents delta FosB induction (Wang et al., 2014).

Consistent with its effects on NAc delta FosB expression, social stress-induced cross-sensitization to psychomotor stimulants has also been shown to involve increased mesolimbic BDNF – TrkB receptor signaling (Wang et al., 2013, Wang et al., 2014). Specifically, viral-mediated overexpression of VTA BDNF exacerbated (Wang et al., 2013), while knockdown of TrkB receptor in the NAc prevented stress-induced amphetamine cross-sensitization (Wang et al., 2014). In addition to increasing VTA BDNF, social stress also rapidly induces BDNF expression in the prefrontal cortex (PFC; Fanous et al., 2010), however it is unknown whether PFC BDNF expression is effected by manipulations of the mesolimbic circuit. Increased VTA BDNF expression has been implicated as a long-term mediator of social stress-induced cross-sensitization (Nikulina et al., 2012), and in the VTA this increase persists for at least 2 weeks after the last social stress exposure (Berton et al., 2006, Fanous et al., 2010, Nikulina et al., 2012).

Although VTA MOR mRNA expression rapidly increases following social stress exposure, VTA BDNF expression is affected more slowly and its expression persists beyond that of VTA MORs (Nikulina et al., 2005, Nikulina et al., 2008, Fanous et al., 2010). Since both VTA BDNF and MOR expression have been implicated in social stress-induced cross-sensitization to amphetamine (Wang et al., 2013, Wang et al., 2014, Johnston et al., 2015), and since increases in VTA MOR expression occur prior to increases in VTA BDNF expression, it is possible that knockdown of VTA MORs may respectively prevent social stress-induced increases in VTA

BDNF and NAc TrkB receptor expression. Given that increased mesolimbic BDNF – TrkB signaling is associated with the induction of delta FosB expression in the NAc after stress, it is possible that VTA MORs may also mediate this effect, either through indirect augmentation of mesolimbic BDNF – TrkB signaling, or through direct mediation of GABA and DA projections to the NAc. In addition to sending direct GABA projections to the NAc, the VTA also innervates the PFC (Sesack and Grace, 2010, Morales and Pickel, 2012, Walsh and Han, 2014), thus it is possible that knockdown of VTA MORs may also have reciprocal effects in the PFC.

While it has been established that social stress-induced increases in VTA MORs are critical for the behavioral consequences of social defeat stress (Johnston et al., 2015), it unknown whether upregulation of VTA MORs mediates the effects of social stress on the mesocorticolimbic circuit. To address this question, the present study used lentivirus-mediated gene transfer and RNA interference to knockdown MORs in the VTA, and then at the time point that stress-induced cross-sensitization to amphetamine is known to be present, assessed for social stress-induced expression of BDNF in the VTA and PFC, as well as for its TrkB receptor and delta FosB in the NAc.

METHODS

3.1 Subjects

Subjects were male Sprague-Dawley rats (N = 21; Charles River Laboratories, Hollister, CA) weighing 200-250 g on arrival. Three days before social stress exposure, subjects were individually housed in standard plastic cages (25x50x20 cm^3). Male Long-Evans rats (weighing 550-700 g), termed 'residents', were pair-housed with a tubal-ligated female in large plastic cages (37x50x20 cm^3). All rats were maintained on a 12-12 reverse light-dark cycle (lights out at 0900 h) with free access to food (Purina Rodent Diet, Brentwood, MO) and water. Residents had previously been screened for aggressive behavior and were used to induce social defeat stress in experimental subjects. All experimental procedures were approved by the Institutional Animal Care and Use Committees at the Arizona State University and the University of Arizona. All studies were conducted in accordance with the Guide for the Care and Use of Laboratory

Animals (National Research Council, 2011), and every effort was made to minimize pain and suffering, as well as the number of animals used.

3.2 Experimental Design

3.2.1 General Procedure

Before to surgery to manipulate VTA MOR levels, subjects were habituated to laboratory conditions for 7 days and randomly assigned to one of four experimental conditions: Non-Manipulated MOR+Handled, Non-Manipulated MOR+Stressed, MOR Knockdown+Handled, MOR Knockdown+Stressed. All subjects were perfused 10 days after the last episode of intermittent social stress (Fig. 3.1) to study the effects of Stress and MOR knockdown on stress-induced changes in the mesocorticolimbic circuit during the time that cross-sensitization to amphetamine is known to be occur.

3.2.2 Bilateral VTA Infusion of Lentiviral Constructs

Rats assigned to control viral groups received infusions of lentivirus that expresses green fluorescent protein (GFP) and a short hairpin RNA (shRNA) that does not target any known rat gene, while rats assigned to VTA MOR knockdown groups received a lentivirus that expresses GFP and a shRNA that targets MOR (shMOR) for RNA interference. The spread and efficacy of these lentivirus constructs was previously established in the rat VTA (Johnston et al., 2015) and were prepared as described in (Lasek et al., 2007). After random assignment to GFP or shMOR knockdown conditions, rats were anesthetized using isoflurane and positioned in a stereotaxic frame (Leica Angle Two; Richmond, IL). The appropriate lentiviral construct (1.0 µl each) was infused bilaterally into the VTA (AP -5.15, ML ±2.15, DV -8.7, Tilt 10°; Paxinos and Watson, 2007) at a flow rate of 0.1 µl/min (Hamilton syringe; Model 7105 KH; 24 gauge tip; Reno, NV), and allowed to diffuse for 10 min. The accuracy of each infusion was later verified using localization of GFP expression (data not shown). Subjects were given 7 days to recover before the start of intermittent social stress or handling procedures (Fig. 3.1).

Figure 3.1. Timeline of Experimental Events. Rats were given 7 days to recover from surgery, and then were exposed to intermittent (4x in 10 days) social defeat stress or handling procedures. Ten days after the last episode of defeat, subjects were perfused to for immunohistochemical analyses of the effect of VTA MOR knockdown on stress-induced changes throughout the mesocorticolimbic circuit.

3.2.3 Intermittent Social Defeat Stress and Handling Procedures

Social defeat stress was induced by a short confrontation between an aggressive resident and an experimental intruder rat, every third day for 10 days (Fig. 3.1), as previously described (Tidey and Miczek, 1996, Nikulina et al., 2004, Nikulina et al., 2012, Johnston et al., 2015; see also Chapter 1, Fig. 1.1). Briefly, females were removed and an experimental rat was placed inside the resident's home cage for 5 min within the confines of a protective metal cage (15x25x15 cm^3). Then the protective cage was removed, allowing the resident to attack until the subject displayed supine posture for a minimum of 4 sec. After which, the experimental rat was placed back in the protective cage and exposed to threat from the resident for an additional 20 min. During this time, rats in the control groups were handled (~2.5 min), at the end of defeat or handling procedures, all subjects were returned to their home cages.

3.3 Perfusion for Immunohistochemistry

As previously described (Fanous et al., 2010), rats were anesthetized with sodium pentobarbital (100 mg/kg, i.p.; Euthasol, Virbac Co., St. Louis, MO) and perfused transcardially with 4% paraformaldehyde 10 days after the last episode of stress or handling (Fig. 3.1). Brains were then removed, post-fixed for 1.5 hours at 4°C, and placed in graded sucrose solutions. Frozen brain tissue was sectioned on a sliding microtome (20 µm) and serial VTA sections were mounted onto slides from 0.05 M phosphate buffer (pH = 7.4). Adjacent slides from each brain were processed for chromogen immunohistochemistry.

3.4 Immunohistochemistry and Quantification

3.4.1 Chromogen Immunohistochemistry

Immunohistochemistry was performed using polyclonal BDNF, FosB, and TrkB receptor antisera as described previously (Fanous et al., 2010, Wang et al., 2013, Wang et al., 2014). Briefly, blocking solution (10% normal goat serum/0.5M KPBS/0.4% Triton X-100) was applied to sections for 1 h at room temperature, then the respective primary antibody diluted in blocking solution was applied for 48 hr at 4°C (BDNF 1:1000 dilution, SP1779, Millipore/Chemicon,

Temecula, CA; FosB 1:7000 dilution, SC-48, Santa Cruz Biotechnology Inc., Santa Cruz, CA; TrkB receptor 1:7000 dilution, Biosensis, 23-36, Thebarton, S. Australia). Sections were then incubated for 1 h with a biotinylated rabbit secondary antibody, treated with avidin/biotin complex solution for 45 min (Vectastain Elite ABC Kit; Vector Laboratories; Burlingame, CA), and developed using a diaminobenzidine (DAB) peroxidase substrate kit with nickel intensification (Vector Laboratories). Tissue sections cover slipped and imaged using a Zeiss Axioskop microscope with a 20x objective, and digitalized using a color digital camera. It should be noted that the FosB antibody used here targets both FosB and delta FosB proteins, due to the similarity in their N-terminal. However given the transient nature of FosB vs. delta FosB's propensity to accumulate (Perrotti et al., 2004), and the timeline of prolonged absence from stress used in the current study (Fig. 3.1), the FosB labeling seen in this study predominantly reflects delta FosB expression and will be discussed as throughout.

3.4.2 Modified Stereological Cell Counts

Immunolabeled cells were quantified using Stereo Investigator software (MBF Biosciences; Williston, VT) and ImageJ (National Institutes of Health, USA, http://imagej.nih.gov/ij), and the analysis was conducted using the modified stereology counting procedure described in Nikulina et al. (2012). Briefly, a grid of squares (0.0075 mm^2) was overlaid on each of 2-3 sections from each subject. Immunolabelled cells were counted in half the grid squares, the precise squares being randomly determined. Immunolabelled cells had a blue-black reaction product and were counted such that cells crossing the bottom or right lines of each square were included, while cells crossing the top or left lines of the square were excluded from analysis. For each subject, estimates of total labeling density (mm^2) were calculated by averaging the bilateral counts of labeled cell profiles across sections, and then dividing the total number of cell profiles by the total area assessed.

3.5 Statistical Analyses

The results of each measure are expressed as mean ± standard error (SEM) and a p value ≤ 0.05 was considered to be significant. All statistical analyses were run using SPSS software, version 18 (SPSS Inc., Chicago, IL). No statistical outliers were excluded, sample sizes were only reduced when the site of interest was damaged during the course of processing. Since a previous study reported no significant differences between GFP-Handled and shMOR-Handled rats (Johnston et al., 2015), a t-test was run for each data set and confirmed that there were no significant differences between groups ($p > 0.05$). Based on this, GFP-Handled and shMOR-Handled groups were combined into one Control Handled group for subsequent statistical analyses of immunohistochemical data. Immunohistochemistical cell counts were analyzed using separate univariate ANOVA's, and where necessary, significant main effects were followed by *post hoc* comparisons with Tukey's test.

Due to tissue damage during mounting and processing for immunohistochemical processing, sample sizes were reduced by 2-3 subjects in the VTA and NAc. Specifically, in the case of TrkB staining in the NAc, tissue resulted in the analysis of data from 18 subjects in both the NAc core and shell. For FosB staining in the NAc shell, data were analyzed from 19 subjects, while additional damage in the NAc core limited analysis to 18 subjects. In the VTA, BDNF expression was analyzed in 19 subjects, while PFC cortex sections did not have significant damage and consequently data were analyzed from all subjects (N = 21). In the analysis of FosB in the NAc core, a violation of homogeneity required a correction, and so Welch's test was employed to adjust the degrees of freedom and F-value.

RESULTS

Effect of VTA MOR Knockdown on BDNF Expression After Intermittent Social Stress Exposure

There was a significant main effect of stress on VTA BDNF expression (n = 19, $F_{2,16}$ = 33.87, $p < 0.0001$). Specifically, the GFP-Stressed group had significantly greater VTA BDNF

expression compared to Control Handled and shMOR-Stressed groups ($p < 0.0001$, Fig. 3.2), while there were no differences between the shMOR-Stressed and the Control Handled groups ($p > 0.74$). In contrast, there was no significant main effect on BDNF expression in either the prelimibic (PL; n = 21, $F_{2,18} = 1.12$, $p = 0.348$) or infralimbic (IL; n = 21, $F_{2,18} = 0.235$, $p = 0.793$) areas of the PFC (Fig. 3.3). Taken together, VTA MORs are necessary for the social stress-induced increases in VTA, but not PFC BDNF expression 10 days after the last episode of stress or handling.

Effect of VTA MOR Knockdown on NAc TrkB Receptor and Delta FosB Expression After Intermittent Social Stress Exposure

There was a significant main effect of stress on TrkB receptor expression in both the NAc core (n = 18, $F_{2,15} = 9.95$, $p = 0.002$) and shell (n = 18, $F_{2,15} = 9.95$, $p < 0.0001$, Fig. 3.4). Specifically, in both the NAc core and shell, GFP-Stressed rats had significantly higher levels of TrkB receptor ($p < 0.003$) than either Control Handled, or shMOR-Stressed rats. By contrast, TrkB receptor expression in shMOR-Stressed rats did not significantly differ from Control Handled subjects ($p > 0.23$). Taken together, this is evidence that VTA MORs are necessary for the social stress-induced increase of TrkB receptor expression in the NAc.

There was also a significant main effect of stress on delta FosB expression in the NAc core (corrected with Welch's test; n = 18, $F_{2,8.98} = 22.64$, $p < 0.001$), but not the NAc shell (n = 19, $F_{2,16} = .77$, $p > 0.47$). In the NAc core, GFP-Stressed rats expressed significantly more delta FosB than Control Handled subjects ($p = 0.01$; Fig. 3.5). By contrast, although the difference between shMOR-Stressed rats and GFP-Stressed rats approached significance ($p = 0.071$), the difference between the shMOR-Stressed and Control Handled groups was highly non-significant ($p > 0.58$). These data show that VTA MORs attenuate, but do not significantly prevent the effect of social stress on delta FosB expression in the NAc core.

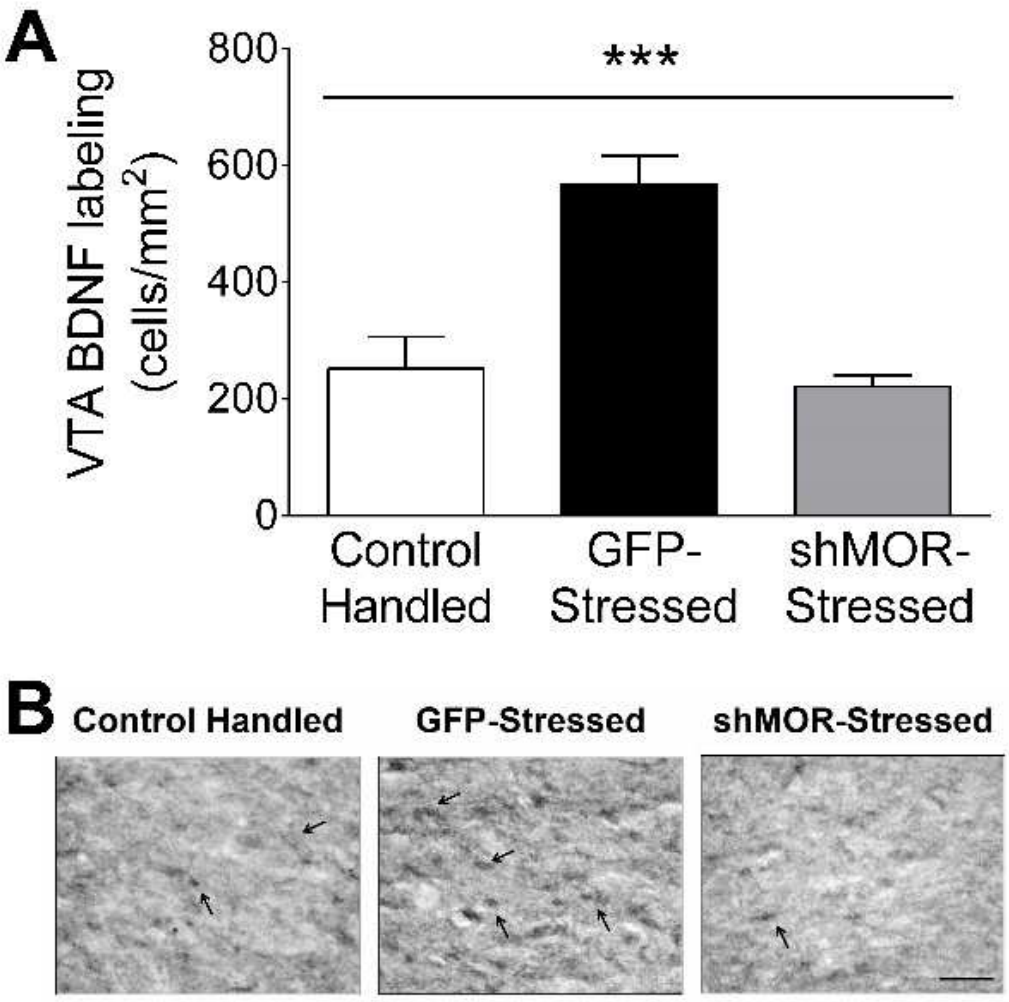

Figure 3.2. Knockdown of VTA MORs Blocks Social Stress-Induced Increase of VTA BDNF Expression. (A) The GFP-Stressed group (n = 5) exhibited significantly (*** - $p < 0.0001$) more VTA BDNF immunolabeling than either Control Handled (n = 8) or shMOR-Stressed (n = 6) groups. Numbers of labeled cells did not significantly differ between Control Handled and shMOR-Stressed rats ($p > 0.74$). (B) Representative images of BDNF labeling in the anterior VTA. More BDNF labeled cells (identified by arrows) are visible in the GFP-Stressed group than in any others. (Scale bar = 100 μm).

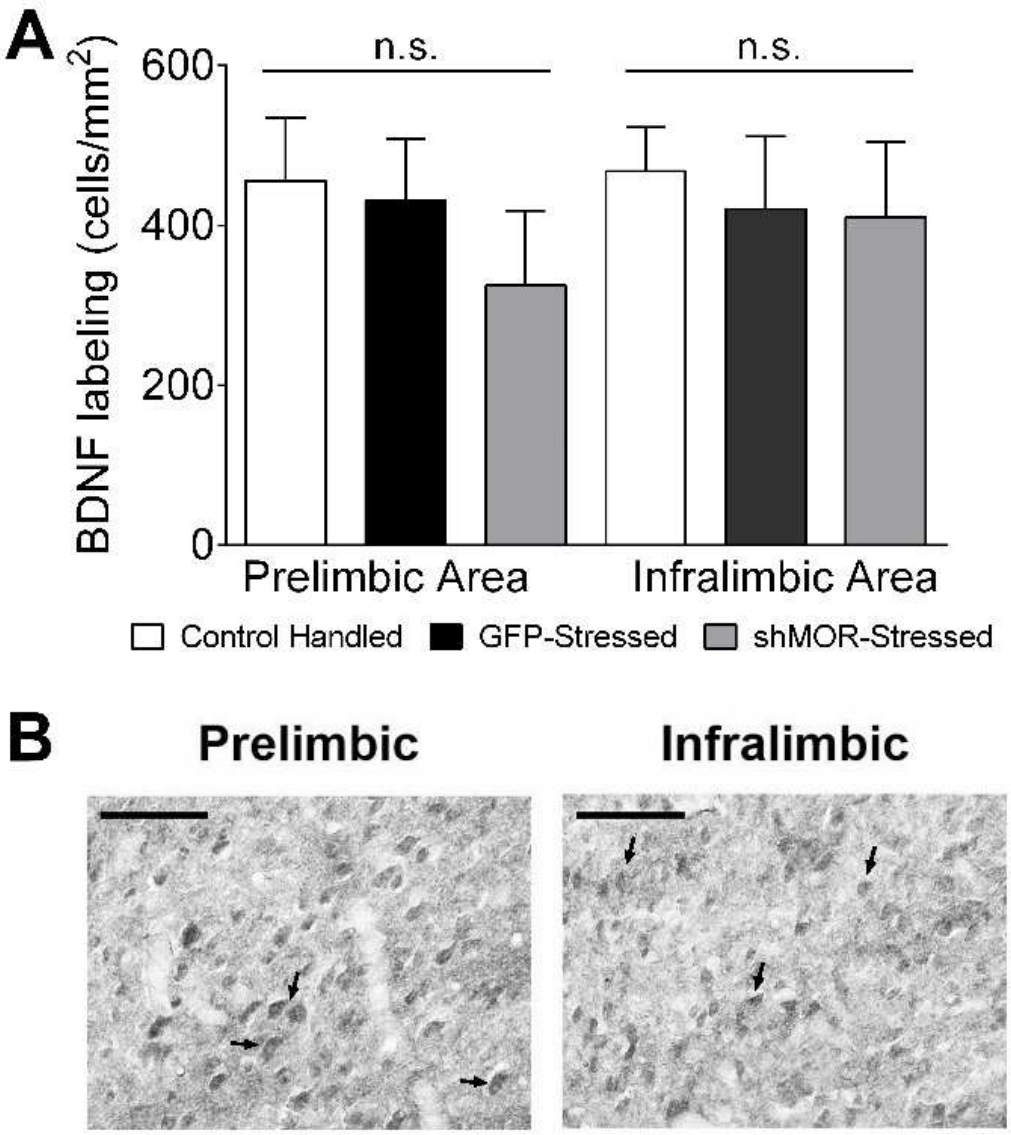

Figure 3.3. There Was No Effect on BDNF Expression in the PFC Ten Days After the Last Episode of Stress. (A) Neither social stress nor VTA MOR knockdown (N = 21) had an effect on BDNF expression in the prelimbic or infralimbic areas of PFC ($p > 0.34$). (B) Representative images of BDNF labeling in prelimbic and infralimbic regions, labeled cells are indicated by arrows (Scale bar = 100 μm).

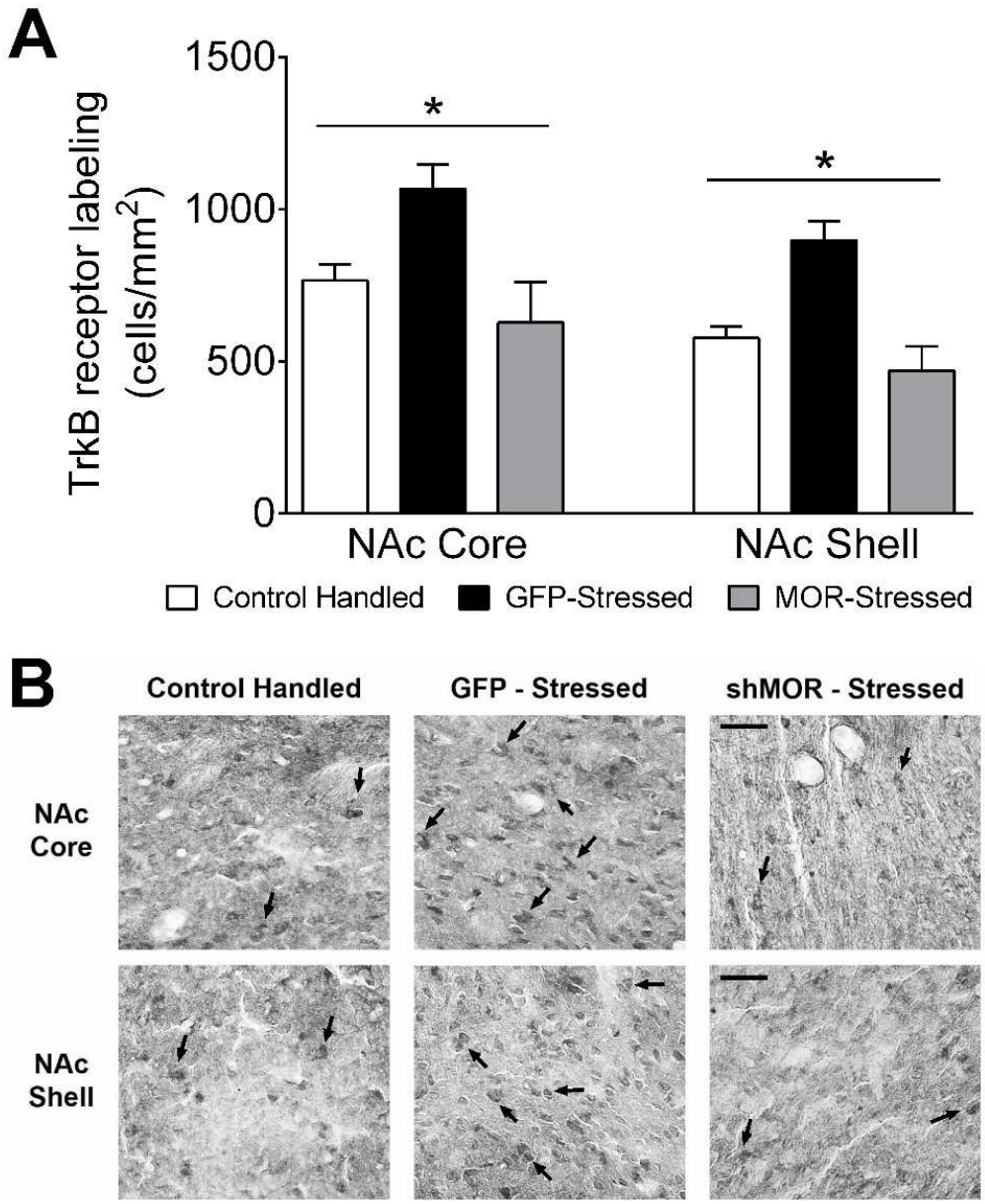

Figure 3.4. Knockdown of VTA MORs Blocks Social Stress-Induced Increase of TrkB Receptor in the NAc. (A) In both the NAc core and shell, the GFP-Stressed group (n = 5) exhibited significantly (* - $p < 0.003$) more immunolabeling for TrkB receptor than either Control Handled (n = 8) or shMOR-Stressed (n = 5) groups. Numbers of labeled cells did not significantly differ between Control Handled and shMOR-Stressed rats ($p > 0.23$). (B) Representative images of TrkB receptor labeling in the NAc core and shell. More TrkB receptor labeled cells (identified by arrows) are visible in the GFP-Stressed group than in any other (Scale bar = 50 μm).

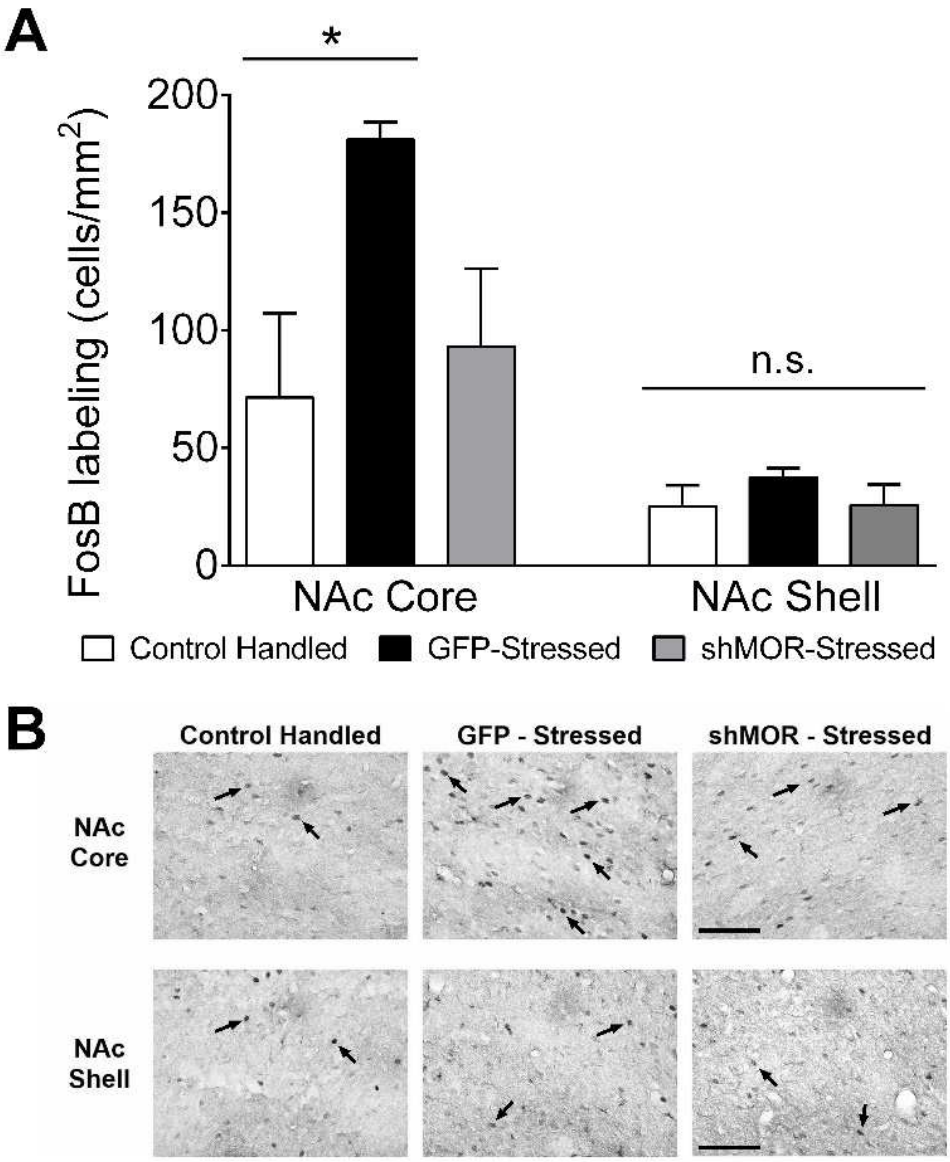

Figure 3.5. Knockdown of VTA MORs Attenuated the Stress-Induced Increase of Delta FosB in the NAc Core. (A) The GFP-Stressed group (n = 4) exhibited significantly more delta FosB labeling than Control Handled rats (n = 9) in the NAc core (* - $p < 0.04$). Although shMOR-Stressed rats (n = 6) did not differ from the Control Handled group ($p > 0.58$), they did tend to have lower levels of delta FosB compared to the GFP-Stressed group, however this effect was not significant ($p > 0.06$). In contrast, there was no effect of either stress of VTA MOR knockdown on delta FosB expression in the NAc shell ($p > 0.47$). (B) Representative images of delta FosB labeling in the NAc core and shell. In the NAc core, more delta FosB labeled cells (identified by arrows) are visible in the GFP-Stressed group than in any others (Scale bar = 100 μm).

DISCUSSION

These data show that induction of VTA MORs by intermittent social defeat stress is necessary for social stress-induced increases in mesolimbic BDNF and TrkB receptor, but not for social stress-induced increases in delta FosB in the NAc. In particular, lentivirus-mediated knockdown of VTA MORs blocked the stress-induced induction of VTA BDNF and NAc TrkB receptor expression, but only attenuated the stress-induced increase of NAc delta FosB. There was no effect of either stress or VTA MOR knockdown on BDNF expression in the PFC.

Knockdown of VTA MORs Attenuates Intermittent Social Stress-Induced Delta FosB Expression in the NAc

Social stress-induced increases in NAc delta FosB expression have been well documented (Nikulina et al., 2008, Nikulina et al., 2012), consistent with these reports, the current study found social stress significantly increased the expression of delta FosB in the NAc core. By contrast, the present finding that social stress did not significantly increase delta FosB expression in the NAc shell is inconsistent with the significant increase reported in Nikulina et al. (2012). In the context of drugs of abuse, such increases in NAc delta FosB are thought to reflect changes in VTA DA transmission, as they are associated with enhanced locomotor activity (see reviews of Nestler et al., 2001, Nestler, 2008). The social stress-induced increase of NAc delta FosB was attenuated, but not prevent by knockdown of VTA MORs. More specifically, shMOR-Stressed rats did not significantly differ from rats in either the Control Handled or GFP-Stressed groups. Thus VTA MOR only *attenuated* stress-induced delta FosB expression in the NAc core, which is interesting, since knockdown significantly prevented stress-induced changes in mesolimbic BDNF – TrkB receptor expression. Moreover, previous studies have shown that expression of VTA BDNF and NAc TrkB receptor mediate delta FosB expression in the NAc (Wang et al., 2013, Wang et al., 2014). Since VTA MOR knockdown blocked stress-induced increases in mesolimbic BDNF – TrkB receptor expression, which are associated with delta FosB expression in the NAc (Wang et al., 2013, Wang et al., 2014), it is surprising that knockdown of VTA MORs did not produce a statistically significant effect on NAc delta FosB expression. The VTA contains both

local GABA interneurons, as well as primary GABA neurons that innervate medium spiny neurons in the NAc (Sesack and Grace, 2010), and while MORs are largely localized to VTA GABA neurons (Sesack and Pickel, 1995, Garzon and Pickel, 2002), a subpopulation of VTA MORs have been localized to DA neurons (Margolis et al., 2014). Thus it is possible that the MORs upregulated by stress are localized to both GABA inter- and primary neurons, as well as potentially some DA neurons. Based on this, VTA MORs may have differential effects in the NAc, depending on whether they indirectly or directly mediate DA transmission, and whether they are on primary GABA neurons that directly project to and influence neuronal activity in the NAc. Thus VTA MOR knockdown may have had differential effects on NAc delta FosB expression, partially cancelling one another out, such that net effect was attenuation, rather than prevention.

Of particular interest, when mice were classified as “susceptible” or “resilient” according to their post-social stress behavioral phenotypes, resilient mice exhibited increased delta FosB labeling in only D1 receptor-containing medium spiny neurons, while susceptible mice showed increased delta FosB labeling in only D2 receptor-containing medium spiny neurons (Lobo et al., 2013). Such differential induction of delta FosB in subtypes of medium spiny neurons in susceptible and resilient rodents may help to explain conflicting reports of stress-induced delta FosB expression in the NAc. When VTA neurons were phasically stimulated, delta FosB was increased in both types of medium spiny neurons in the NAc core, but only increased the expression of delta FosB in D1 receptor-containing neurons of the NAc shell (Lobo et al., 2013). Moreover, stimulation of D2 receptor-containing medium spiny neurons promotes susceptibility to subthreshold social defeat stress in mice, and these neurons show enhanced excitatory inputs after stress (Francis et al., 2015). A subset of medium spiny neurons were shown to form functions synapses on non-dopamine neurons in the VTA, and the terminals of these medium spiny neurons are sensitive to mu-opioids and act via $GABA_A$ receptors (Xia et al., 2011) (Xia et al., 2011). Taken together, this finding and the finding that activity of D2 receptor-containing medium spiny neurons confers susceptibility to social stress (Francis et al., 2015), it is possible

the NAc is a potential source of the endogenous mu-opioids that activate, and potentially underlie, social stress-induced MOR expression in the VTA.

There Is No Effect of Either Stress or VTA MOR Knockdown on BDNF Expression in the PFC

Although a previous study noted a significant increase in PFC BDNF expression that persisted for up to 10 days (Nikulina et al., 2012), the present study failed to note a significant effect of stress on BDNF in these areas of PFC at the same time point. In addition to being transiently expressed in the PFC, BDNF is rapidly upregulated within 2 h of social stress, and levels are known to return to normal at most 23 days after the last episode of social stress (Fanous et al., 2010). In light of this, the present finding that social stress did not increase BDNF expression in the PFC suggests that 10 days may be near the upper limit of this transient stress-induced increase of PFC BDNF expression. This theory is consistent with reports that intra-BDNF infusion to the medial PFC attenuates cocaine seeking (Berglind et al., 2007) and cocaine-induced neuroadaptations in the NAc (Berglind et al., 2009). Additionally, another study showed that chronic unpredictable stress attenuates cocaine-induced increase of PFC BDNF expression, and also noted that in repeatedly stressed rats given saline, PFC BDNF levels did not differ from saline treated controls (Fumagalli et al., 2009). Taken together, these studies suggest that increased BDNF expression in the PFC might not be expected to accompany, and may even attenuate, stress-induced cross-sensitization to psychomotor stimulants. In which case, given that the present study collected tissue samples at the time that cross-sensitization is known to be present (Johnston et al., 2015; see also review of Nikulina et al., 2014), it is not surprising that there was no effect of stress on BDNF expression in the PFC.

The VTA receives dense innervation from PFC glutamate projections, a role for which has been well established in behavioral sensitization to drugs of abuse (Vanderschuren and Kalivas, 2000), additionally the PFC also receives VTA GABA projections that contain MORs (Morales and Pickel, 2012). Taken together, these features of the mesocortical pathway allowed for the possibility that VTA MOR knockdown may have altered BDFN expression in the PFC by either direct projections or indirect feedback mechanisms, regardless of stress treatment. In

contrast, data from this study show that VTA MOR knockdown had no effect on BDNF expression in either the infralimbic or prelimbic areas of the PFC. While the VTA receives glutamate projections from the PFC, it has efferent DA and GABA projections that innervate the PFC (Sesack and Grace, 2010, Morales and Pickel, 2012, Walsh and Han, 2014). The present finding that VTA MOR knockdown does not alter PFC BDNF expression suggests that social stress does not upregulate MOR expression on VTA neurons that project to the PFC, and that knockdown of VTA MORs does not activate a feedback loop to this region. However given the rapid stress-induced upregulation of BDNF in the PFC (Fanous et al., 2010) and that the present study did not note an effect of stress in the PFC at this time point, it is possible that knockdown of VTA MORs may have augmented stress-induced changes in PFC BDNF expression at an earlier time point than was analyzed in this current study. Namely, it is possible that knockdown of VTA MORs augmented DA and/or GABA transmission in the PFC and induced transient changes that were not detectable 10 days after the last episode of stress. Thus the possibility that the PFC is involved in VTA MOR-mediated effects of stress in the mesocorticolimbic circuit cannot be ruled out, and future studies will need to be conducted to determine if the effects VTA MOR knockdown vary at different time points after stress.

VTA MORs Are Necessary for Induction of VTA BDNF by Intermittent Social Stress

The two-fold increase of VTA BDNF expression which was observed in the VTA is consistent with previous reports (Berton et al., 2006, Fanous et al., 2010, Nikulina et al., 2012). More importantly, these results show that knockdown of VTA MORs prevented social stress-induced VTA BDNF expression. That knockdown of VTA MORs blocks social stress-induced increase of BDNF labeling suggests that VTA BDNF induction after social stress exposure is dependent on local MOR upregulation. In fact, increased MOR activity in hippocampus also induces local BDNF mRNA (Zhang et al., 2006). While others have suggested that VTA BDNF modulates the function of local MORs (Vargas-Perez et al., 2009b, Koo et al., 2012), the current study shows that VTA MORs can regulate the local expression of BDNF. These reciprocal findings may be attributed to differences between exogenous opiate and endogenous opioid

functions, as well by differences in the VTA input systems recruited by exposure to morphine and social stress.

Although VTA BDNF is predominantly thought to be found in DA neurons (Gall et al., 1992, Seroogy et al., 1994), it is possible that MORs may control the transmission of VTA GABA neurons to indirectly produce subsequent changes in local DA neurons. Specifically, if MOR activity on GABA neurons increases the excitability of local DA neurons (Mathon et al., 2005), then the subsequent reduction in VTA GABAergic tone allows for MORs to affect BDNF expression in VTA DA neurons. A recent study showed that a subset of VTA MORs are actually excitatory and found on VTA DA neurons (Margolis et al., 2014). Thus it is also possible that social stress may upregulate excitatory MORs on VTA DA neurons, to directly increase VTA DA transmission and BDNF expression. Additional research using electrophysiology will be needed to determine whether social stress differentially increases the expression of excitatory vs. inhibitory MORs in the VTA.

VTA MORs Are Necessary for Intermittent Social Stress-Induced Increases in Mesolimbic BDNF-TrkB Receptor

Previous studies have indicated that social stress increases the release of VTA BDNF in the NAc (Krishnan et al., 2007, Wang et al., 2013, Walsh et al., 2014), and have further implicated it's receptor TrkB in the behavioral consequences of social stress (Wang et al., 2014). Taken together, these studies suggest that social stress increases the expression of TrkB receptor in the NAc in response to increased release of VTA BDNF. In support of this, the present study found that intermittent social stress-significantly increased TrkB receptor expression in both the NAc core and shell of subjects that also exhibited stress-induced expression of VTA BDNF. Similar to the preventative effect of VTA MOR knockdown on VTA BDNF, the present study also found that VTA MOR knockdown blocked social stress-induced TrkB receptor expression in both regions of the NAc. Taken together, these data extend on previous findings, suggesting that not

only does social stress increase mesolimbic BDNF-TrkB receptor expression, but that this effect is mediated by MORs in the VTA.

Implications of VTA-MOR Augmentation of Social Stress-Induced Increases in Mesolimbic BDNF – TrkB Receptor Signaling

Recently it was reported that social stress-induced upregulation of VTA MORs is critical for the behavioral consequences of intermittent social defeat stress (Chapter 2; see also Johnston et al., 2015). In particular, knockdown of VTA MORs prevented stress-induced cross-sensitization to amphetamine (Johnston et al., 2015), a phenomena that has been linked to mesolimbic BDNF – TrkB receptor signaling (Wang et al., 2013, Wang et al., 2014). Additionally, upregulation of VTA MOR occurs early in the cross-sensitization process (Nikulina et al., 1999, Nikulina et al., 2005, Nikulina et al., 2008), prior to a persistent increase of VTA BDNF expression (Fanous et al., 2010). In light of these findings, it is not surprising that the present results demonstrated that VTA MORs are necessary for social stress-induced increases in VTA BDNF and its TrkB receptor in the NAc. Prevention of social stress-induced expression in BDNF by VTA MOR knockdown suggests that stress-induced increases in VTA BDNF are a consequence of MOR-dependent increases in VTA GABAergic tone. In support of this, and consistent with the preventative behavioral effects VTA MOR knockdown (Johnston et al., 2015), is the study by Mathon et al. (2005), in which genetic MOR knockout mice showed increased GABAergic input onto local VTA DA neurons and decreased cocaine reinforcement. Thus it is likely that social stress upregulates MOR expression on VTA GABA neurons to facilitate BDNF expression in local DA neurons, while VTA MOR knockdown may increase VTA GABAergic tone, preventing alterations in the mesolimbic expression of BDNF and its receptor TrkB.

Concluding Remarks

In summary, knockdown of VTA MORs prevented social stress-induced increases in VTA BDNF and TrkB receptor in the NAc. By contrast, knockdown of VTA MORs only attenuated the effect of stress on NAc delta FosB expression, while there was no effect of either stress or MOR

knockdown on BDNF expression in areas of PFC. In rats, continuous social stress suppresses cocaine reward and decreases VTA BDNF expression (Miczek et al., 2011a), however it is unknown whether continuous social stress alters VTA MOR expression. It is possible that continuous social stress reduces cocaine reward and VTA BDNF expression as a function of downregulated VTA MOR expression, which would suggest that VTA MORs may mediate a switch between the sensitizing effects seen with intermittent social stress and the suppressed cocaine reward observed after continuous social stress.

In conclusion, the present results indicate that social stress-induced upregulation of VTA MORs mediates subsequent increases in mesolimbic BDNF – TrkB receptor signaling, all of which have been functionally implicated in stress-induced cross-sensitization to amphetamine (Wang et al., 2013, Wang et al., 2014, Johnston et al., 2015). Knockdown of VTA MORs alone did not significantly alter the expression of BDNF, TrkB receptor, or delta FosB in the mesocorticolimbic circuit, yet significantly prevented stress-induced alterations in their expression, and is known to prevent stress-induced cross-sensitization. Taken together, these findings suggest that VTA MORs may represent a potentially specific target for the therapeutic intervention of stress-related substance abuse.

CHAPTER 4: LOCALIZATION OF PHOSPHORYLATED AKT IN VTA GABA NEURONS AFTER SOCIAL STRESS EXPOSURE: IMPLICATIONS FOR AMPHETAMINE CROSS-SENSITIZATION AND WEIGHT REGULATION [3]

ABSTRACT

Intermittent social defeat stress produces long-lasting cross-sensitization to psychomotor stimulants, an effect that is known to depend on the expression of mu-opioid receptors (MORs) in the ventral tegmental area (VTA). Stimulation of MORs is known to activate a variety of intracellular signaling cascades, including phosphorylation of AKT (pAKT) by phosphoinositide 3-kinase (PI3K). Additionally, AKT phosphorylation is critical for drug-induced sensitization to psychomotor stimulants. It is unknown whether social stress increases pAKT downstream of MOR upregulation, or whether AKT phosphorylation is related to the behavioral outcomes of social stress. To address these questions, two experiments were conducted. In the first, to determine whether social stress increases MOR – pAKT signaling, after lentivirus-mediated manipulation of VTA MORs and intermittent social defeat stress, fluorescent immunohistochemical double labeling of pAKT with markers for DA or GABA neurons was conducted in the VTA. The results show that social stress significantly increased pAKT co-localization with GABA, but not DA neurons in the VTA. Knockdown of VTA MORs prevented this effect, suggesting that social stress-induced increases in AKT phosphorylation are dependent upregulation of VTA MORs. In experiment 2, intra-VTA inhibition of pAKT during to stress was used to assess whether stress-induced AKT phosphorylation mediates the behavioral consequences of social defeat stress. When administered an amphetamine challenge (1.0 mg/kg, i.p.), rats assigned to Saline-Stressed conditions exhibited significant induction of cross-sensitization compared to Control Handled subjects, this induction was attenuated in rats who received intra-VTA infusions of inhibitor during stress. To determine whether AKT

[3] None of the data in presented in this chapter have been published, in-text references to Johnston et al. (2015) are referring to the behavioral data presented in Chapter 1 or the results of VTA BDNF immunohistochemistry presented in Chapter 2 of this dissertation. To see the published manuscript in its entirety, please refer to Appendix A.

phosphorylation is necessary for the expression of cross-sensitization, a reversal was conducted using Saline-Stressed rats that previously exhibited cross-sensitization. Prior to a second amphetamine challenge, these rats were given intra-VTA infusions of pAKT inhibitor. Acute inhibition of VTA pAKT was sufficient to block the expression of stress-induced cross-sensitization, without blunting the locomotor-activating effects of amphetamine. Taken together, these data suggest that AKT phosphorylation represents a novel target for the treatment of substance abuse disorders.

INTRODUCTION

Stress has been correlated with increased risk of substance abuse and relapse in humans (Sinha, 2001, 2008, 2011). In rats, intermittent social defeat stress procedures in rats consistently result in enhanced response to psychomotor stimulants, a phenomena known as "cross-sensitization" (Covington and Miczek, 2001, Nikulina et al., 2004, Nikulina et al., 2012). Additionally, social defeat stress rapidly upregulates mu-opioid receptors (MORs) in the ventral tegmental area (VTA), an effect which persists for up to 14 days (Nikulina et al., 2008). Of particular importance, a recent study showed that social stress-induced upregulation of VTA MORs is necessary for stress-induced weight gain deficits, cross-sensitization to amphetamine, and BDNF expression in the VTA (Johnston et al., 2015). Given that increases in VTA MORs are necessary for both the behavioral and cellular consequences of social stress, it is important to look at activity dependent changes in VTA MOR-mediated intracellular signaling cascades. MORs activate numerous intracellular pathways, including the pathway that phosphorylates AKT (pAKT; Iglesias et al., 2003, Mazei-Robison et al., 2011).

Of particular interest, MOR activation has been shown to directly increase phosphoinositide 3-kinase (PI3K) – pAKT signaling, an effect that can be disrupted with a PI3K/AKT inhibitor, but not an inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), another MOR-induced intracellular signaling cascade (Iglesias et al., 2003). This suggests that MOR-induced AKT phosphorylation is unrelated to MAPK/ERK signaling and suggests that it might have distinct functional effects in neurons. In fact, AKT

phosphorylation has been implicated in the mediation of GABA transmission through insertion of $GABA_A$ receptor in the cell membrane (Wang et al., 2003). A functional role for pAKT has also been demonstrated in drug-induced sensitization to cocaine, wherein systemic or intracerebroventricular inhibition of AKT phosphorylation prevented sensitized responses (Izzo et al., 2002, Wu et al., 2011)

While phosphorylation of AKT is crucial for drug-induced sensitization to cocaine (Izzo et al., 2002, Wu et al., 2011), it is unknown whether AKT phosphorylation is important for stress-induced cross-sensitization to drugs of abuse. Although one study found a regional decrease of VTA pAKT after continuous social defeat stress (Krishnan et al., 2008), MORs are only expressed in a subpopulation of VTA cells (Sesack and Pickel, 1995, Garzon and Pickel, 2002). Thus if stress-induced MOR activity increased pAKT phosphorylation in a subset of VTA cells, it is not likely to be detected by the Western blot analyses employed by Krishnan et al. (2008). Moreover, in rats continuous and intermittent social defeat stress are known to produce opposite effects on response to psychostimulant drugs and VTA BDNF expression (Miczek et al., 2011a), therefore while pAKT may be reduced after continuous social stress, it is possible that it increased by intermittent social stress.

Although research has implicated AKT phosphorylation in sensitization to psychostimulant drugs (Izzo et al., 2002, Wu et al., 2011), it is unclear whether the VTA is the site action and whether AKT phosphorylation is relevant to stress-induced cross-sensitization. To address these questions, two separate experiments were conducted. In the first experiment, lentivirus constructs were infused to the VTA to manipulate MOR expression, after recovery, subjects were put through intermittent social defeat stress procedures. In order to investigate the effects of social stress and VTA MOR knockdown on local pAKT expression, subjects were sacrificed to quantify pAKT expression in VTA DA and GABA neurons using fluorescent immunohistochemical double labeling. The second experiment was conducted to investigate whether VTA AKT phosphorylation mediates social stress-induced weight gain deficits and cross-sensitization. Subjects were bilaterally implanted with intra-VTA cannula and a pAKT inhibitor was delivered prior to each episode of stress, 10 days later an amphetamine challenge was

administered to assess for the induction of cross-sensitization. To determine whether AKT phosphorylation is necessary for the expression of stress-induced cross-sensitization, one week later a reversal was conducted wherein intra-VTA infusions of pAKT inhibitor were administered prior to a second amphetamine challenge. Finally, the functional role of VTA AKT phosphorylation in social stress-induced long-term weight gain deficits was investigated.

METHODS

4.1 Subjects

Experimental subjects were male Sprague-Dawley rats (N = 41; Charles River Laboratories, Hollister, CA) weighing 200-250 g on arrival. Three days before social stress exposure, subjects were individually housed in standard plastic cages (25x50x20 cm^3). Male Long-Evans rats (weighing 550-700 g), termed 'residents', were pair-housed with a tubal-ligated female in large plastic cages (37x50x20 cm^3). All rats were maintained on a 12-12 reverse light-dark cycle (lights out at 0900 h) with free access to food (Purina Rodent Diet, Brentwood, MO) and water. Residents were retired breeders, previously screened for aggressive behaviors, and were used to induce social defeat stress in experimental subjects. All experimental procedures were approved by the Institutional Animal Care and Use Committees at the University of Arizona. All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), and every effort was made to minimize pain, suffering and the number of subjects.

4.2 Experimental Design

Two experiments were conducted in parallel, the first investigated whether increased AKT phosphorylation is a downstream target of social stress-induced MOR upregulation in the VTA. Next the second experiment investigated the involvement of AKT phosphorylation in the induction and expression of social stress-induced cross-sensitization to amphetamine. In both

experiments, after arrival, experimental rats were habituated to laboratory conditions for 7 days before surgery to either knockdown VTA MORs or to implant cannulas above the VTA.

In the first experiment (Fig. 4.1), subjects were randomly assigned to one of four conditions: Non-Manipulated MOR+Handled, Non-Manipulated MOR+Stressed, MOR Knockdown+Handled, MOR Knockdown+Stressed. At the time that amphetamine cross-sensitization is known to be present, 10 days after the last episode of stress or handling, all subjects (N = 21) were perfused to study the effects of Stress and MOR knockdown on the co-localization of pAKT with DA and GABA neurons of the VTA.

In the second experiment (Fig. 4.1), rats (N = 28) were randomly assigned to one of four experimental conditions: Saline+Handled, Saline+Stressed, pAKT Inhibitor+Handled, pAKT Inhibitor+Stressed. In order to study the effect of VTA AKT phosphorylation on the *induction* of social stress effects, subjects (N = 28) received an amphetamine challenge 10 days after the last episode of stress or handling. Additionally, to study the effect of pAKT on the *expression* of amphetamine cross-sensitization, a reversal was conducted 8 days later, wherein Handled+Inhibitor and Saline+Stressed rats were given a second amphetamine challenge (N = 15). All subjects were perfused 23 days after the last episode of defeat or handling for verification of infusion sites with Nissl stain. To investigate the influence of VTA pAKT inhibition on social stress-induced deficits in weight gain, all subjects were weighed prior to each episode of defeat/handling, and again prior to perfusion.

4.3 Surgical Procedures and Infusions

4.3.1 Bilateral VTA Infusion of Lentiviral Constructs

The control lentiviral construct induced expression of green fluorescent protein (GFP) and a short hairpin RNA (shRNA) that does not target any known rat gene, while the lentiviral construct used to induce MOR knockdown expressed GFP and a shRNA that targets MOR (shMOR) for RNA interference. Lentiviral constructs were have been previously validated (Johnston et al., 2015) and were prepared as previously described (Lasek et al., 2007). After random assignment to either GFP or shMOR knockdown conditions, rats were anesthetized with

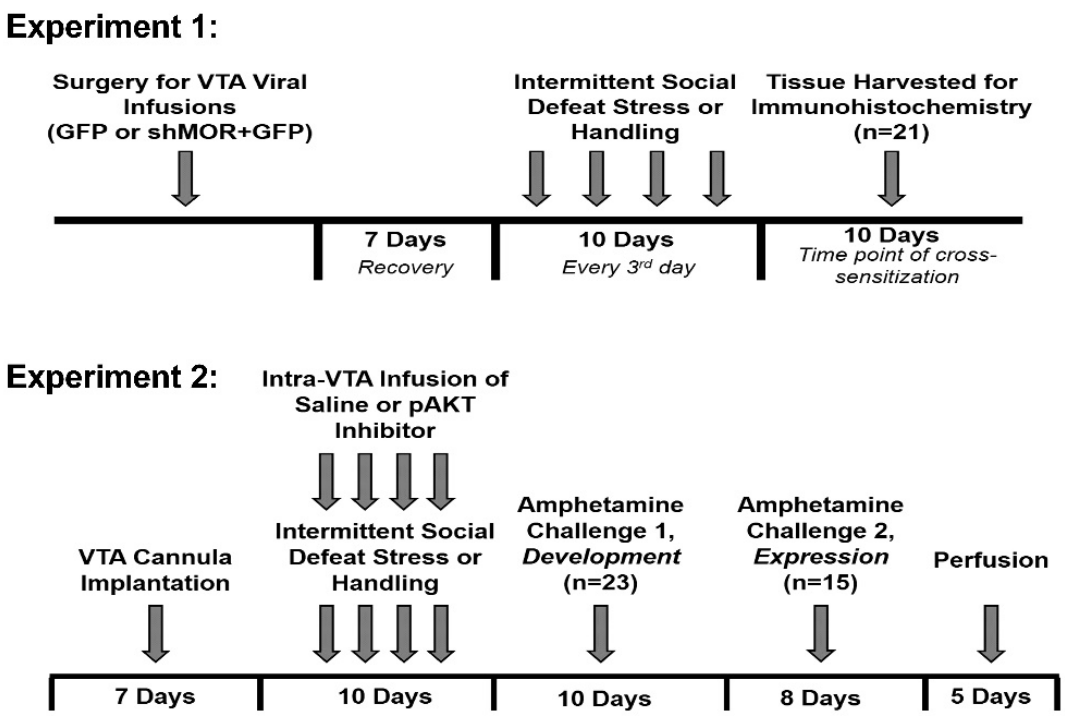

Figure 4.1. Schematic of Experimental Timelines. In experiment 1, rats were given 7 days to recover from surgery to manipulate VTA MOR levels, and were then exposed to intermittent (4x in 10 days) social defeat stress or handling procedures. Ten days after the last episode of stress, at a time when stress-induced cross-sensitization is observed (Nikulina et al., 2012, Johnston et al., 2015), subjects were perfused for fluorescent immunohistochemical double labeling of pAKT expression in VTA GABA and DA neurons. In experiment 2, subjects were bilaterally implanted with intra-VTA cannula and then a pAKT inhibitor was infused prior to each episode of stress or handling. Ten days after the conclusion of stress and handling procedures, subjects were given an amphetamine challenge to test for the induction of stress-induced cross-sensitization. To test whether acute inhibition of VTA pAKT blocks the expression of stress-induced cross-sensitization, eight days later subjects that had previously showed cross-sensitization, intra-VTA infusions of pAKT inhibitor were delivered prior to a second amphetamine challenge. Five days later, all subjects were perfused to verify locations of cannula.

isoflurane and positioned in a stereotaxic frame (Leica Angle Two; Richmond, IL). Bilateral VTA (AP -5.15, ML ±2.15, DV -8.7, Tilt 10°; Paxinos and Watson, 2007) infusions of the appropriate lentiviral construct (1.0 µl each) were administered at a flow rate of 0.1 µl/min, and allowed to diffuse for 10 min before withdrawal of the syringe (Hamilton; Model 7105 KH; 24 gauge tip; Reno, NV) . The accuracy of each infusion was later verified using localization of GFP expression (data not shown). Subjects were given 7 days to recover before the start of intermittent social stress or handling procedures (Fig. 1A).

4.3.2 Bilateral VTA Implantation of Cannulas and Infusions of pAKT Inhibitor

Rats were anesthetized using isoflurane and positioned in a stereotaxic frame (Leica Angle Two; Richmond, IL). Two small stainless steel screws (Tx2-4, Small Parts Inc., Miami Lakes, FL) were inserted near bregma to stabilize the skull, and then guide cannulas (24 gauge, 6 mm long, C316G, Plastics One, Roanoke, VA) were implanted above the VTA (AP -5.15, ML +/-2.15, Tilt 10 degrees; Paxinos and Watson, 2007). Everything was cemented into place (Duralay, MD-1300, Reliance Dental Mfg. Co., Worth, IL) and then guide cannula were fitted with a dummy cannula (6.7 mm long, C316DC, Plastics One) to prevent clogging and contamination. During each set of infusions, dummy cannula were removed and infusion cannula (31 gauge, 7.7 mm long, C316I, Plastics One) were inserted through the guide cannula, directly above the VTA.

To prevent phosphorylation of AKT, this study utilized a dual AKT/mTORC (mammalian target of rapamycin complex) inhibitor (10 µM; NVP-BEZ235, #10565, Cayman Chemicals, Ann Arbor, MI). Although this particular AKT inhibitor has not been previously used in the brain, its effects have been well validated in other tissue cultures and with systemic administration (Maira et al., 2008, Serra et al., 2008, Chapuis et al., 2010, Mukherjee et al., 2012). Additionally, this drug is currently undergoing clinical trials at the Novartis Pharmaceutical Corporation. While more traditional pAKT inhibitors act to block phosphorylation at Thr308 phosphorylation site, AKT is also phosphorylated at Ser473, and it is this site that has been implicated in the full catalytic activity of pAKT (Bellacosa et al., 1998, Scheid and Woodgett, 2001, Iwanami et al., 2009). By contrast, the inhibitor used in this study blocks the induction of pAKT by preventing both PI3K-

induced phosphorylation at Thr308, and mTORC2-mediated phosphorylation at Ser473 (Maira et al., 2008). Unlike traditional inhibitors, such as LY294002, which interacts with various classes of PI3Ks and other unrelated targets (Gharbi et al., 2007), NVP-BEZ235 is specific for Class 1 PI3Ks and mTOR signaling, but does not does not target other protein kinases (Maira et al., 2008).

Using sonification at 4ºC, the AKT inhibitor (NVP-BEZ235) was dissolved in saline to make a 20 µM stock that was aliquoted and stored at -20ºC. Prior to each set of infusions, a fresh solution of 10 µM inhibitor was prepared from stock and kept on ice. Intra-VTA infusions of either 1µl inhibitor or 0.5 µl saline were administered at a rate of 0.175 µl/min, after which subjects were returned to their home cages for 1 h to allow the inhibitor to take full effect (Maira et al., 2008). In pilot testing, when given concurrently with systemic amphetamine (2.0 mg/kp, i.p.), chromogen immunohistochemistry showed that this intra-VTA dose of inhibitor significantly reduced AKT phosphorylation 24 h later (Fig. 4.2B-D). Subjects were given 7 days to recover before the start of intermittent social stress or handling procedures (Fig. 4.1B). At the conclusions of the study, subjects were perfused and the accuracy of infusions was verified with nissl stain (Fig. 4.2A).

4.4 Behavioral assessments

4.4.1 Intermittent social defeat stress and handling procedures

In both experiments, social defeat stress was induced by a short confrontation between an aggressive resident and an experimental intruder rat (see Chapter 1, Fig. 1.1), as described previously (Tidey and Miczek, 1996, Nikulina et al., 2004, Nikulina et al., 2012). After the female was removed, an experimental subject was placed inside the resident's home cage within the confines of a protective metal mesh cage (15x25x15 cm^3). After 5 min the protective cage was removed, allowing for the resident to attack. Attacks persisted until subjects engaged in submissive supine posture for at least 4 sec, after which the experimental rat was placed back in the protective cage and exposed to threat from the resident for an additional 20 min. Intermittent social stress procedures were administered every third day for 10 days (Fig. 4.1). At each

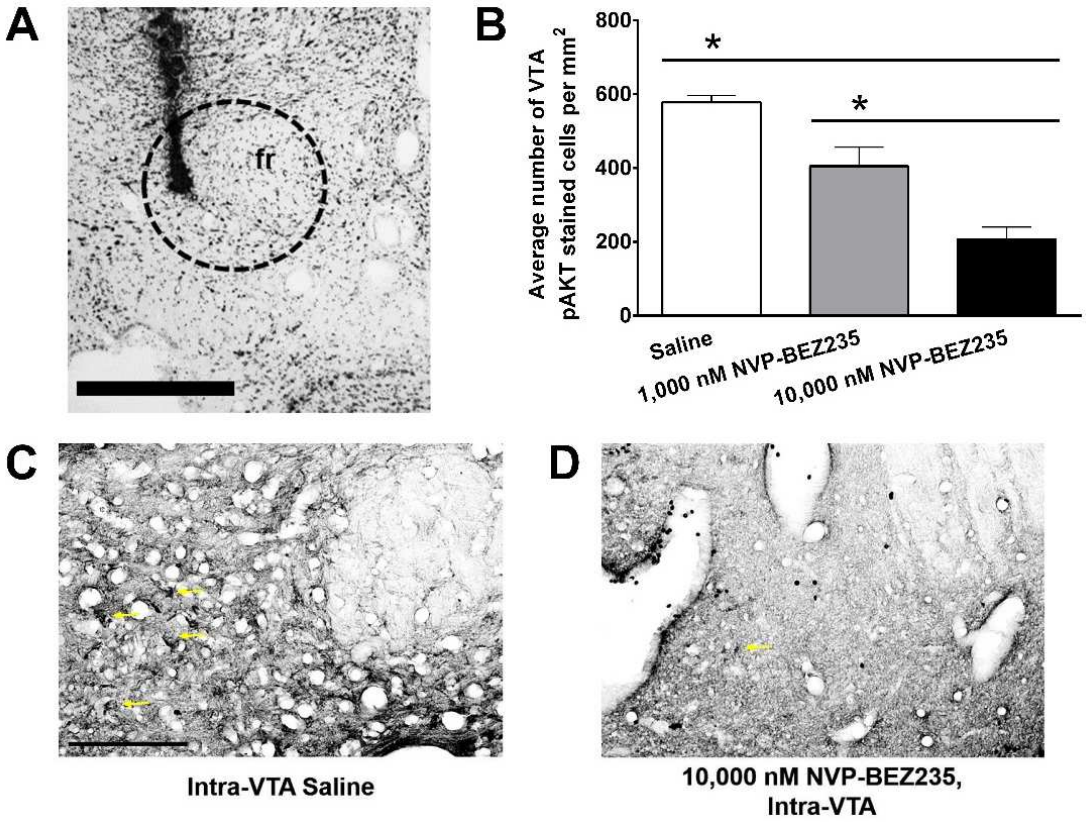

Figure 4.2. Cannula Infusions Were Accurate to the VTA and NVP-BEZ235 Effectively Inhibited Local AKT Phosphorylation. (A) Representative black and white image of nissl stained tissue showing cannula placement above the left VTA (fr: fasciculus retroflexus; scale bar = 500 µm). (B) Compared to saline infusions, a lower intra-VTA dose of 1,000 nM of NVP-BEZ235, a dual PI3K/mTORC inhibitor of pAKT, produced a small but significant decrease in pAKT expression (* indicates $p < 0.04$). By contrast a higher dose of 10,000 nM was far more effective, significantly reducing the number of pAKT labeled cells compared to both saline ($p < 0.001$) and 1,000 nM of inhibitor conditions (* indicates $p < 0.03$). (C and D) Representative images of pAKT expression in the VTA 24 h after either infusion of saline (C) or 10,000 nM of NVP-BEZ235 pAKT inhibitor (D), arrows indicated labeled cells (Scale bar = 100 µm). Given how effectively intra-VTA infusion of 10,000 nM of NVP-BEZ235 inhibited local pAKT expression, this dose was used throughout subsequent studies.

corresponding time point, rats in the control groups were handled for approximately 2.5 min. Defeat or handling procedures were carried out 4x in 10 days (every 3rd day), and at the end of each episode of defeat or handling, all rats were returned to their home cages.

4.4.2 Amphetamine challenge

Using a low dose of d-amphetamine, two separate challenges were administered as described in (Nikulina et al., 2004, Nikulina et al., 2012) to respectively test for the induction and expression of social stress-induced cross-sensitization to amphetamine (Fig. 4.1B). To test whether inhibition of pAKT during social stress prevented the *induction* of cross-sensitization to amphetamine, the first challenge was conducted without intra-VTA infusions of pAKT inhibitor. To determine whether inhibition of pAKT is sufficient to prevent the *expression* of social stress-induced cross-sensitization to amphetamine, before the start of the second challenge, stressed subjects that previously showed cross-sensitization were given intra-VTA infusions of pAKT inhibitor.

For two days prior to the first amphetamine challenge, rats were injected with vehicle (0.9% sterile saline; 1.0 ml/kg, i.p.), and were acclimated to the procedure room for 1 h in their home cages. On the day of the challenge locomotor activity was recorded at 10 min intervals using video tracking software (Videotrack, Viewpoint Life Sciences; Montreal, Canada). Locomotor activity was detected as the number of and distance travelled during locomotion (>10 cm) across 170 min consisting of 3 phases: Baseline, Saline, and Amphetamine. Baseline data were recorded for 30 min, after which a saline injection (1.0 ml/kg, i.p.) was given and locomotor activity was recorded for 60 min. Finally, rats received an injection of d-amphetamine sulfate (1.0 mg/kg, i.p.; Sigma-Aldrich; St. Louis, MO), and locomotor data were recorded for 80 min. Video tracking and data collection were paused during the administration of saline and amphetamine injections. Rather than stereotypical behaviors, this dose of amphetamine has been shown to primarily induce large ambulatory movements (Geyer et al., 1987). In order to quantify

amphetamine sensitization, ambulatory movements (> 10 cm) were measured in terms of the number of movements initiated and the distance travelled (cm) during such movements.

4.5 Tissue Preparation and Immunohistochemistry

4.5.1 Perfusion

Rats from both experiments were anesthetized with sodium pentobarbital (100 mg/kg, i.p.; Euthasol, Virbac Co., St. Louis, MO) and perfused transcardially with 4% paraformaldehyde as described in (Fanous et al., 2010, Johnston et al., 2015). Brains were then removed, post-fixed for 1.5 hours at 4°C, and then placed in graded sucrose solutions. Frozen brain tissue was sectioned (20 μm) and serial VTA sections were mounted onto slides.

4.5.2 Verification of Infusion Sites

In experiment 1, one analogous set of slides were used to verify the infusions of lentiviral constructs according to viral expression of GFP (data not shown). This efficacy of these lentiviral constructs has been previously validated and are known to produce a persistent depletion of MORs that is specific to the VTA (Johnston et al., 2015). In experiment 2, slides were processed for histology using Nissl stain, and then cannula placement was verified with light microscopy (Fig. 4.2A). In general, both lentiviral infusions and cannula placement were accurate and limited to the VTA.

4.5.3 Fluorescent Immunohistochemistry

Fluorescent immunohistochemistry was performed to quantify pAKT double labeling with either glutamic acid decarboxylase 65/67 (GAD 65/67) or tyrosine hydroxylase (TH) monoclonal antisera. Immunohistochemistry for pAKT and GAD 65/67 were done in the absence of detergent to prevent GAD 65/67 from leaving cells. Briefly, blocking solution (10% normal goat serum/0.5M KPBS, with 0.4% Triton X-100 for pAKT/TH labeling) was applied to sections for 1 h at room temperature, then the respective pair of primary antibodies were diluted in appropriate blocking solution and simultaneously applied for 48 h at 4°C (pAKT Ser473 1:500 dilution, #4060, Cell

Signaling Technology, Beverly, MA; GAD 65/67 1:250 dilution, M018-3, MBL International Corporation, Woburn, MA; TH 1:500 dilution, SC-7837, Santa Cruz). Sections were then incubated at room temperature for 2.5 h with fluorescent labelled secondary antibodies diluted to 1:1000. Specifically, pAKT was labeled with an Alexa 350 secondary rabbit antibody, while GAD 65/67 and TH were respectively labeled with an Alexa 660 secondary mouse antibody (Alexa 350-R, A-21068; Alexa 660-M, A-21055; Life Technologies, Grand Island, NY). To prolong fluorescent labeling, slides were cover slipped with Prolong® Gold Antifade Mountant (#P36934, Life Technologies).

Tissue sections were imaged using a Zeiss Axiophot microscope and digitalized using a black and white digital camera. Black and white images of each channel were then re-colored and merged together using ImageJ (National Institutes of Health, USA, http://imagej.nih.gov/ij). Although pAKT was labeled with a fluorescent Alexa 350 secondary (blue), it was pseudo-colored green in order to better visualize cases of double labeling with cells also stained with Alexa 660 secondary (pseudo-colored red). In order to maintain high resolution while conducting modified stereological counts of single and double labeled cells, 4 separate images of each coronal VTA section were taken at 40x magnification and then merged into one image showing immunolabeling throughout that entire section.

4.6 Modified Stereological Cell Counts

Immunolabeled cells were quantified using ImageJ (National Institutes of Health, USA, http://imagej.nih.gov/ij) and analyses were conducted using the modified stereology counting procedure described in Nikulina et al. (2012), and Johnston et al. (2015). Briefly, a grid of squares (0.0075 mm^2) was overlaid on both hemispheres of 2 sections from each subject. Immunolabelled cells were counted in half the grid squares, the precise squares being randomly determined. Cells positive for immunolabeling were counted such that cells crossing the bottom or right lines of each square were included, while cells crossing the top or left lines of the square were excluded from analysis. For each subject, estimates of total labeling density (mm^2) were calculated by averaging the bilateral counts of labeled cell profiles across sections, and then dividing the total

number of cell profiles by the total area assessed. In pilot analyses of chromogen staining, immunolabeling was performed as described in Johnston et al. (2015). For fluorescent analyses, both single and double labelled cells were counted. Single labeled labelled cells were indicated by either a green (pAKT) or red (GAD 65/67 or TH) color, while double labelled cells (green + red) were indicated by an orange/yellow color product.

4.7 Statistical Analyses

The results of each measure are expressed as mean ± standard error (SEM) and a *p* value ≤ 0.05 was considered to be significant. All statistical analyses were run using SPSS software, version 18 (SPSS Inc., Chicago, IL), and Tukey's HSD was used for all *post hoc* comparison. Data from subjects were excluded only in the case of error during video tracking: no statistical outliers were excluded. Due to issues with cannula integrity over time, some additional subjects were excluded from the amphetamine challenge(s) and maintained under normal housing conditions to collect long-term weight data and for verification of cannula sites.

4.7.1 Immunohistochemical Analyses

In experiment 1, the results of immunohistochemical cell counts were analyzed using separate Oneway ANOVAs of single and double labeled cells, significant main effects were followed by *post hoc* comparisons. No subjects were excluded and both pAKT/TH and pAKT/GAD 65/67 data were analyzed from 21 subjects. A violation of homogeneity was corrected for with Welch's test in both analyses of single labeled pAKT expression. In order to compare the results of pAKT co-localization with GAD 65/67 and TH, the total number of pAKT double labeled cells was calculated (Total = # of pAKT/GAD 65/67 + # of pAKT/TH cells) and then the proportion of pAKT in GAD 65/67 and TH cells were respectively calculated (proportion pAKT in TH or GAD 65/67 = # double labeled cells / Total), the resulting estimates of co-localization.

4.7.2 Weight Gain Data

In experiment 2, the initial weight obtained at the start of social stress procedures was used to normalize all subsequent data (n = 28) to weight gained from that time onward; no subjects were excluded from this analysis. Oneway ANOVA were run to assess for differences in weight at each time point, and significant main effects were analyzed for *post hoc* comparisons.

4.7.3 Locomotor Activity

In the second experiment, two amphetamine challenges were administered and amphetamine-induced changes in locomotor activity were analyzed using separate multivariate analysis of variance (MANOVA) for the mean number and distance (cm) travelled during ambulatory movements. In order to overcome violations of sphericity in the output of repeated measures ANOVA, MANOVA was used to analyze the number and distance of ambulatory movements exhibited throughout the amphetamine challenge. Significant multivariate effects were followed by univariate analyses to determine which time points produced significant group differences, significant univariate effects were further analyzed for *post hoc* comparisons. Given that immunohistochemical analyses of social stress-induced AKT phosphorylation in the VTA demonstrated that AKT is only phosphorylated after stress (Fig. 4.3 and 4.6), and because there were no differences in weight gain of Saline-Handled and Inhibitor-Handled rats (Fig. 4.7), these groups were pooled into one Control Handled group. In the first challenge to test for the induction of stress-induced amphetamine sensitization, data were analyzed from 23 subjects for both dependent measures: Control Handled = 10, Saline-Stressed = 5, Inhibitor-Stressed = 8. Since the second challenge was administered to investigate whether intra-VTA inhibition of AKT phosphorylation is sufficient to block the expression of stress-induced cross-sensitization, the Inhibitor-Stressed group was excluded (n = 8).

RESULTS

Experiment 1: Effects of Social Stress and MOR Knockdown on pAKT Expression in the VTA

There was a significant main effect of experimental group on the number of VTA cells in which pAKT co-localization with GAD 65/67 (n = 21, $F_{3,17} = 44.02$, $p < 0.001$). Specifically, GFP-Stressed rats had significantly more VTA cells double labeled with both pAKT and GAD 65/67 than either GFP-Handled, shMOR-Handled, or shMOR-Stressed rats ($p < 0.001$, Fig. 4.3A and 4.4). Co-localization of pAKT with GAD 65/67 did not significantly differ between GFP-Handled, shMOR-Handled or shMOR-Stressed groups ($p > 0.98$). In terms of single labeling, there was no significant main effect was detected for the number of cells positive for only pAKT (corrected with Welch's test; n = 21, $F_{3,8.69} = 2.57$, $p > 0.12$). By contrast, there was a significant main effect for the numbers of single labeled GAD 65/67 cells (n = 21, $F_{3,17} = 23.13$, $p < 0.001$). While this main effect was significant, *post hoc* tests revealed that it was limited to the GFP-Stressed group, which had significantly fewer single labeled GAD 65/67 cells ($p < 0.001$). Although GFP-Stressed rats had fewer single labeled GABA neurons, this difference is consistent with and proportional to the increased number of double labeled GAD 65/67 cells. Therefore while statistically significant, this decrease in single labeled GAD 65/67 cells does not reflect any methodological or functional significance and will not be discussed further.

The results of pAKT double labeling with TH were opposite to that observed with GAD 65/67. Specifically, sections processed for pAKT and TH labeling showed a significant main effect in the number of cells single labeled pAKT (corrected with Welch's test; n = 21, $F_{3,9.29} = 30.08$, $p < 0.001$, Fig. 4.3B and 4.5), and pAKT double labeled with TH (n = 21, $F_{3,17} = 7.097$, $p = 0.003$). There were no differences in the number of single labeled TH detected in the VTA (n = 21, $F_{3,17} = 0.97$, $p > 0.42$). In terms of pAKT that did not double label with TH, the GFP-Stressed group had significantly higher levels of single labeled pAKT compared to all other experimental groups ($p < 0.001$). There was also a tendency for GFP-Stressed rats to have lower levels of pAKT that co-localized with TH, with this effect being significant compared to shMOR-Stressed rats ($p = 0.001$).

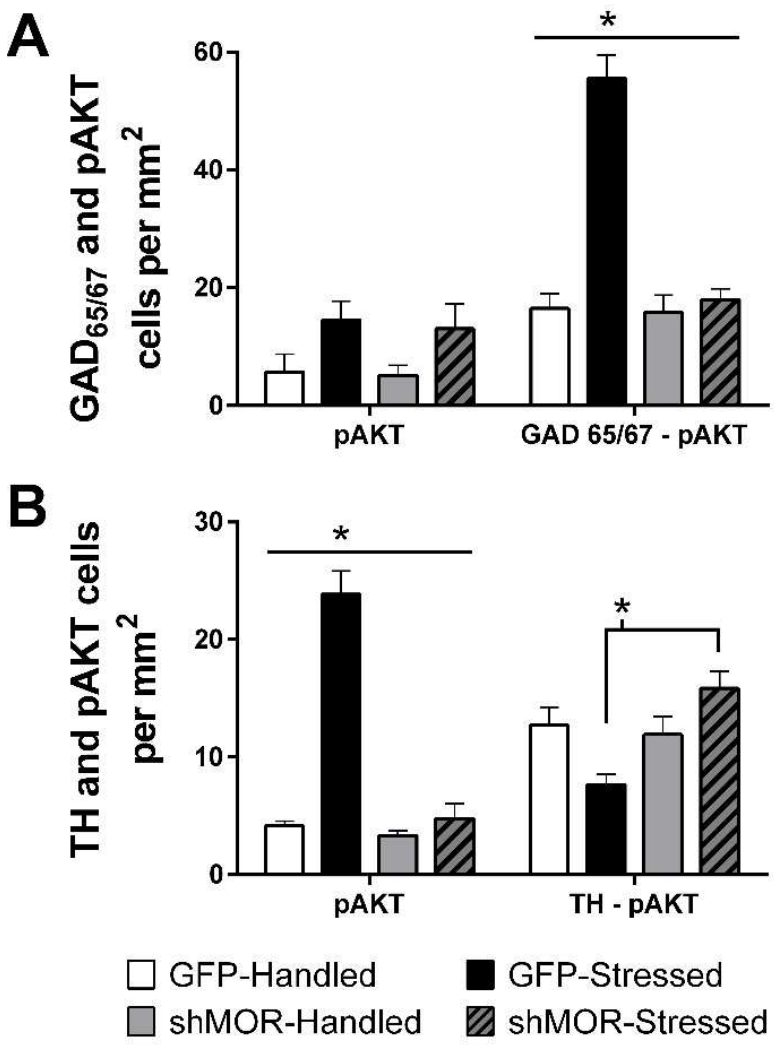

Figure 4.3. Intermittent Social Stress Significantly Increased AKT Phosphorylation Downstream of MOR Expression in VTA GABA, but Not DA Neurons. (A) Compared to all other groups, GFP-Stressed rats exhibited significantly more co-localization of pAKT in GAD 65/67 positive cells (* indicates $p < 0.001$). Co-localization of pAKT with GAD 65/67 did not significantly differ between GFP-Handled, shMOR-Handled or shMOR-Stressed groups ($p > 0.98$), and there was no effect of single labeled pAKT ($p > 0.12$). (B) Compared to all other groups, GFP-Stressed rats had significantly more VTA cells that were single labeled for pAKT (* indicates $p < 0.001$). GFP-Stressed rats also tended to have fewer cells double labeled for pAKT and TH, with this effect being significant compared to shMOR-Stressed rats (* indicates $p = 0.001$), but not GFP-Handled ($p = 0.09$) or shMOR-Handled subjects ($p = 0.144$). The shMOR-Stressed group did not significantly differ from either Handled groups in terms of either single labeled pAKT ($p > 0.87$) or pAKT co-localized with TH cells ($p > 0.19$).

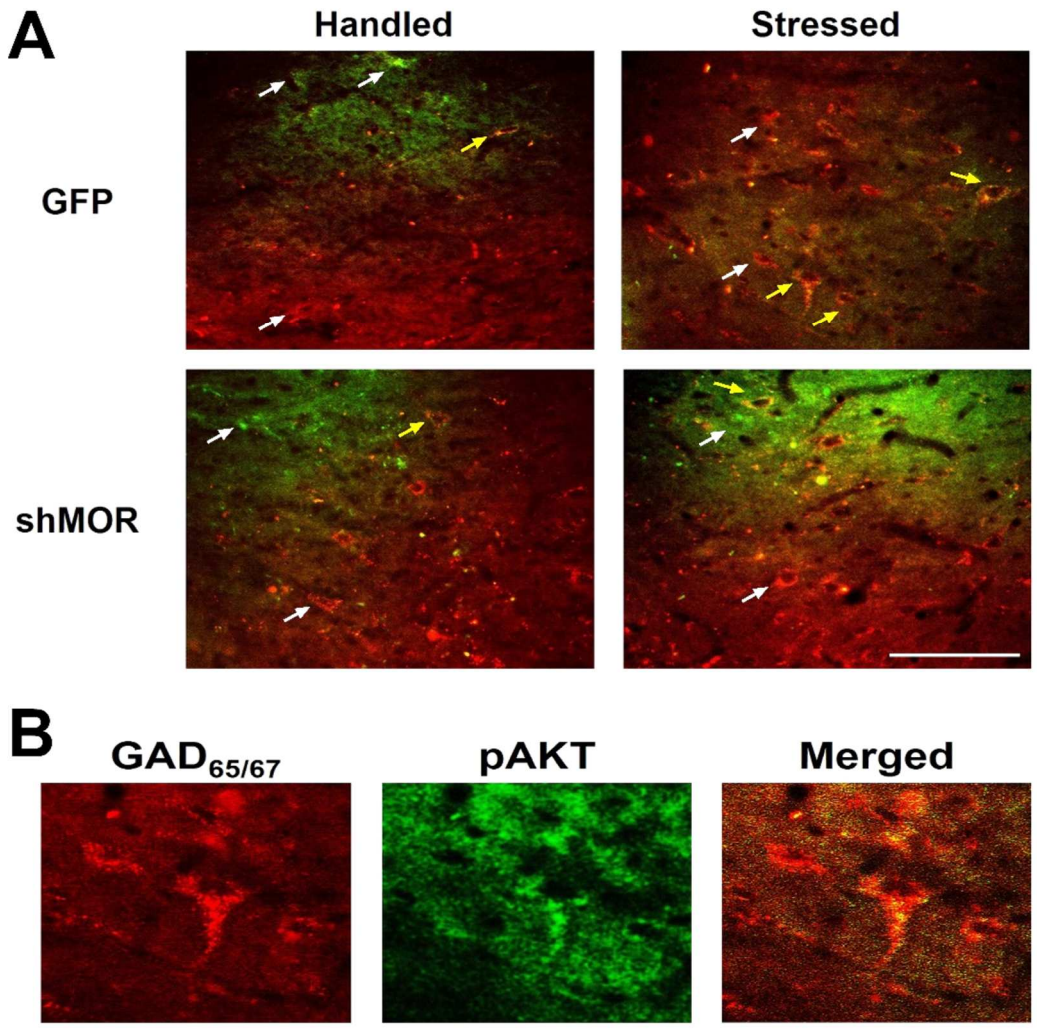

Figure 4.4. VTA MOR Knockdown Prevented the Induction of pAKT Labeling in VTA GABA Cells by Social Stress. (A) Representative images of pAKT (green) and GAD 65/67 (red) labeling in anterior VTA sections. Single labeled cells are indicated by white arrows, while yellow arrows indicate double labelled cells colored with an orange-yellow color product (Scale bar = 100 μm). (B) Colored single channel images of the immuno-labeling seen in the above representative GFP-Stressed image (close up of bottom center of image).

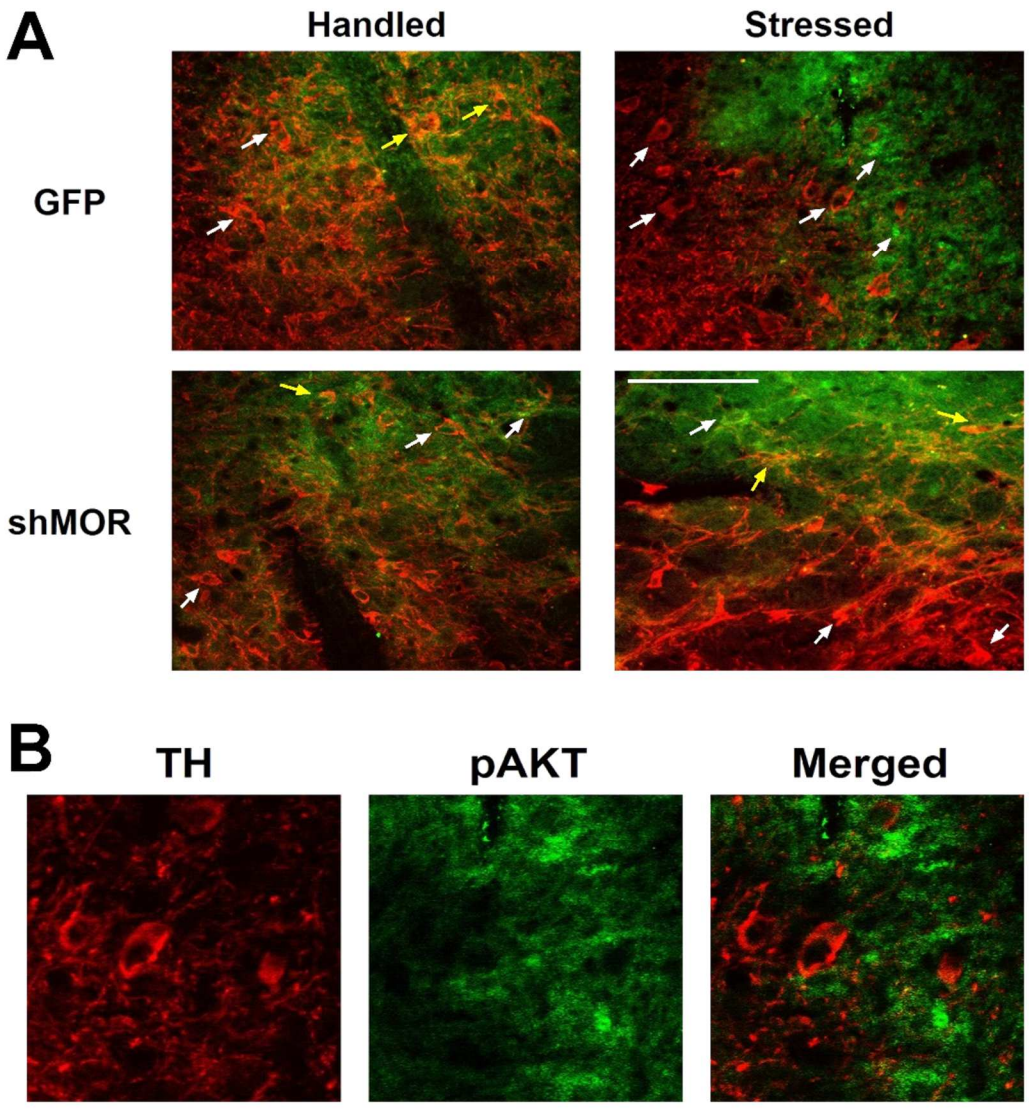

Figure 4.5. Social stress did not increase co-localization of pAKT with DA neurons in the VTA. (A) Representative images of pAKT (green) and TH (red) labeling in the anterior VTA. Single labeled cells are indicated by white arrows, while yellow arrows indicate double labelled cells colored with an orange-yellow color product (Scale bar = 100 μm). (B) Colored single channel images of the immuno-labeling seen in the above representative GFP-Stressed image (close up of center of image).

GFP-Handled, shMOR-Handled and shMOR-Stressed rats had no significant differences in either single labeled pAKT ($p > 0.87$) or pAKT co-localization with TH cells ($p > 0.19$).

When the proportion of double labeled pAKT that co-localized with either TH or GAD 65/67 are compared side by side (Fig. 4.6), it is clear that these analyses show that social stress increases the proportion of VTA AKT phosphorylation that occurs in GABA cells, but not DA cells, and that this change in expression is mediated by VTA MORs.

Experiment 2: Effect of Intra-VTA Inhibition of pAKT on Intermittent Social Stress-Induced Deficit of Weight gain

Weight gain data (n = 28) revealed a significant main effect both during social stress exposure ($F_{3,27} = 3.73$, $p < 0.03$), and 23 days after the last episode of stress ($F_{3,27} = 7.87$, $p = 0.001$). *Post hoc* comparisons from weight data collected during stress and handling procedures showed that the Saline-Stressed group had a tendency to gain less weight than Saline-Handled ($p = 0.048$) or Inhibitor-Handled ($p = 0.053$; Fig. 4.7). By contrast, rats in the Inhibitor-Stressed group did not significantly differ from Saline-Handled, Saline-Stressed, or Inhibitor-Handled rats ($p > 0.20$). Twenty-three days after the final episode of stress or handling, the Saline-Stressed group had significantly lower weights than both Saline- and Inhibitor-Handled groups, but also rats in the Inhibitor-Stressed group ($p < 0.03$). Conversely, no significant differences existed between the Inhibitor-Stressed group and either Saline- or Inhibitor-Handled groups ($p > 0.60$). These data show that the long-term social stress-induced weight gain deficits are mediated by AKT phosphorylation in the VTA, and that inhibition of AKT phosphorylation is not sufficient to alter weight gain in the absence of stress.

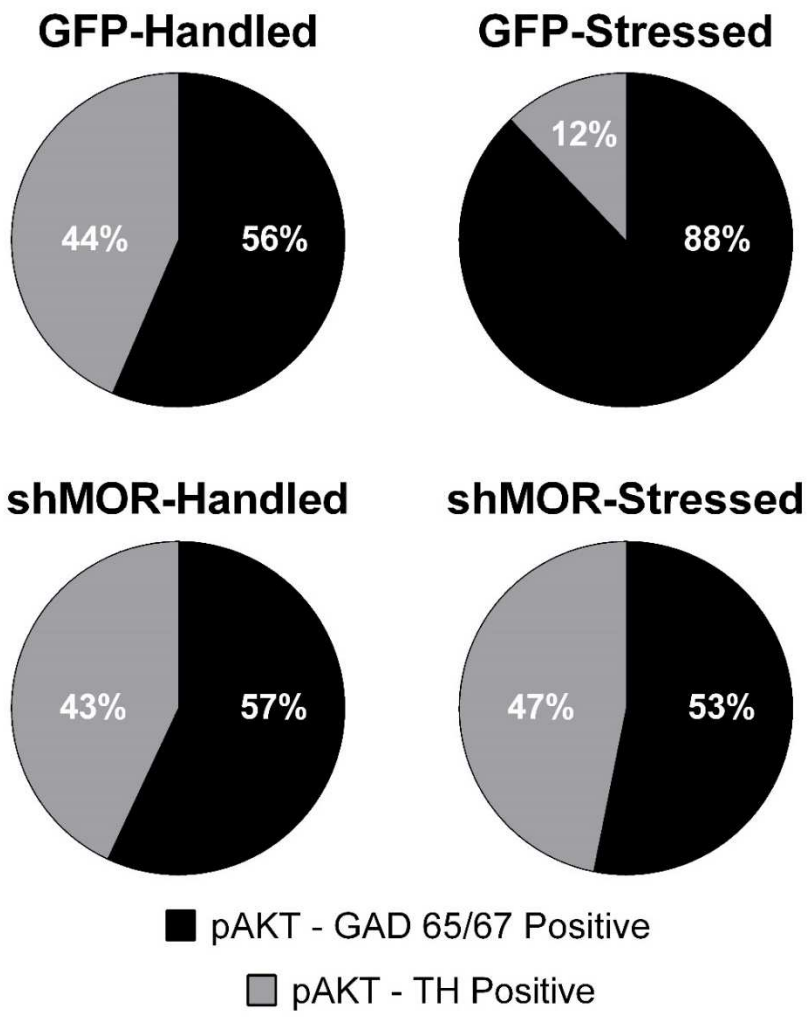

Figure 4.6. Knockdown of VTA MORs Prevented the Proportional Increase of pAKT Expression in VTA GABA Neurons That Was Observed After Social Stress. While in control conditions pAKT co-localization was generally evenly distributed between VTA GAD 65/67 and TH positive cells in the VTA, much more double labeled pAKT was found in VTA GAD 65/67 neurons after stress (88% in GFP-Stressed rats). Knockdown of VTA MORs prevent this shift in the expression of double labeled pAKT, maintain a roughly even distribution of double labeled pAKT cells.

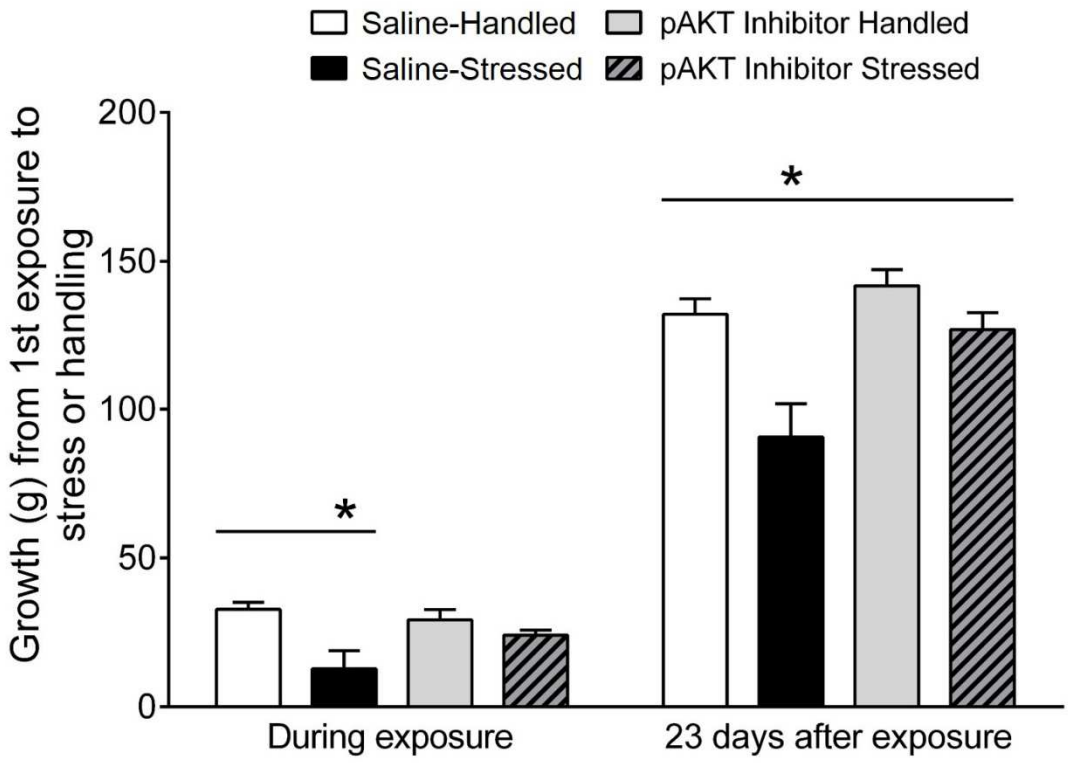

Figure 4.7. Inhibition of VTA AKT Phosphorylation During Stress Prevented the Development of Long-Term Weight Gain Deficits. During stress and handling procedures, Saline-Stressed rats (n = 9) began to exhibit a weight gain deficit, showing significantly less weight gain than Saline-Handled rats (n = 4; * indicates $p < 0.05$), however this effect was not significant compared to Inhibitor-Handled (n = 7, $p = 0.053$) or Inhibitor-Stressed groups (n = 8, $p > .20$). Twenty-three days after the last infusion of inhibitor and episode of stress, Saline-Stressed rats exhibited a significant reduction in weight gain compared to all other groups (* indicates $p < 0.03$). Neither Inhibitor-Handled or Inhibitor-Stressed rats differed from Saline-Handled rats at either time point ($p > 0.60$).

Effect of Intra-VTA pAKT Inhibition on the Induction of Intermittent Social Stress-Induced Cross-Sensitization

There were significant main effects of experimental group on the number of ambulatory movements (n = 23, *Wilks' λ* = 0.196, $F_{16,26}$ = 2.05, p = 0.05, η^2 = 0.56, *observed power* = 0.85) and distance travelled during ambulatory movements(n = 23, *Wilks' λ* = 0.114, $F_{16,26}$ = 3.20, p = 0.004, η^2 = 0.663, *observed power* = 0.98) across all time points. After amphetamine injection, both the number and distance of movements significantly differed at all but the last time point ($p \leq$ 0.05, see Table 1 for details). *Post hoc* tests showed that compared to Control Handled rats, the Saline-Stressed group made significantly more movements from 100 – 160 min ($p < 0.04$; Fig. 4.8A), and also significantly differed from Inhibitor-Stressed rats at 160 min ($p < 0.005$). Inhibitor-Stressed rats did not significantly differ from Handled Controls at any time point ($p > 0.05$). Additionally, immediately after amphetamine injection, subjects in the Saline-Stressed group travelled significantly farther than either Handled Controls ($p < 0.001$) or Inhibitor-Stressed rats ($p < 0.005$) at 100 min. during 100 – 150 min (Fig. 4.8B). In contrast, from 110 – 150 min, the Saline-Stressed group only traveled significantly further than Handled Controls ($p < 0.05$), the Inhibitor-Stressed group did not differ from Control Handled or Saline-Stressed rats during this time period ($p > 0.05$). Combined, these data suggest that inhibition of VTA AKT phosphorylation during intermittent social stress is not sufficient to prevent the induction of social stress-induced cross-sensitization to amphetamine.

Effect of acute intra-VTA pAKT inhibition on the expression of intermittent social stress-induced cross-sensitization

To test whether inhibition of VTA AKT phosphorylation is sufficient to block the expression of stress-induced amphetamine sensitization. One week later Saline-Stressed rats that had previously exhibited cross-sensitization (Fig. 4.8) were given intra-VTA infusions of inhibitor (Saline-Stressed+Inhibitor), and then a second amphetamine challenge was

Table 1. Details on Univariate Differences Found During Analysis of the Induction of Social Stress-Induced Cross-Sensitization to Amphetamine

Time after amphetamine injection	Number of Movements	Distance Traveled (cm)
10 min (bin 100)	($F_{2,20} = 3.98$, $p = 0.035$)	($F_{2,20} = 9.60$, $p = 0.001$)
20 min (bin 110)	($F_{2,20} = 4.50$, $p = 0.024$)	($F_{2,20} = 5.85$, $p = 0.01$)
30 min (bin 120)	($F_{2,20} = 5.01$, $p = 0.017$)	($F_{2,20} = 7.69$, $p = 0.003$)
40 min (bin 130)	($F_{2,20} = 7.61$, $p = 0.003$)	($F_{2,20} = 5.66$, $p = 0.012$)
50 min (bin 140)	($F_{2,20} = 8.44$, $p = 0.002$)	($F_{2,20} = 8.77$, $p = 0.002$)
60 min (bin 150)	($F_{2,20} = 4.22$, $p = 0.03$)	($F_{2,20} = 3.81$, $p = 0.04$)
70 min (bin 160)	($F_{2,20} = 4.36$, $p = 0.027$)	n.s.
80 min (bin 170)	n.s.	n.s.

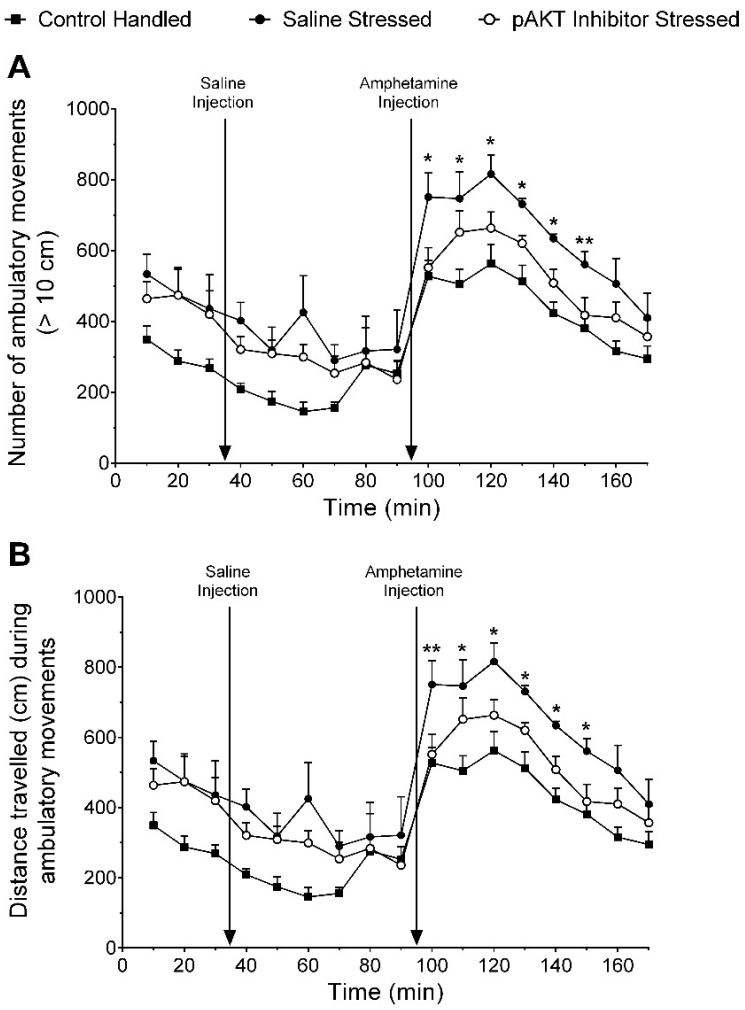

Figure 4.8. Inhibition of pAKT in the VTA During Stress Attenuated the Induction of Cross-Sensitization to Amphetamine. Vertical arrows denote when saline or amphetamine injections were administered, during which data collection and video tracking were paused. (A) After amphetamine injection, Saline-Stressed rats (n = 5) made significantly more locomotor movements from 100 – 150 min ($p < 03$) than subjects in the Control Handled (n = 10) but not Inhibitor-Stressed (n = 8) group. At 160 min, the Saline-Stressed group exhibited significantly more locomotor activity than either Control Handled or Inhibitor-Stressed groups (* indicates $p < 0.04$). Inhibitor-Stressed rats did not significantly differ from Saline-Stressed rats at any time point ($p > 0.05$). (B) Immediately after amphetamine injection, at 100 min, Saline-Stressed rats traveled significantly further than subjects in either Control Handled or Inhibitor-Stressed groups (** indicates $p < 0.005$). From 110 – 150 min, Saline-Stressed rats traveled significantly farther than Control Handled rats (* indicates $p < 0.05$), but not Inhibitor-Stressed rats ($p > 0.05$). Inhibitor-Stressed rats did not significantly differ from Control Handled rats at any time point ($p > 0.05$).

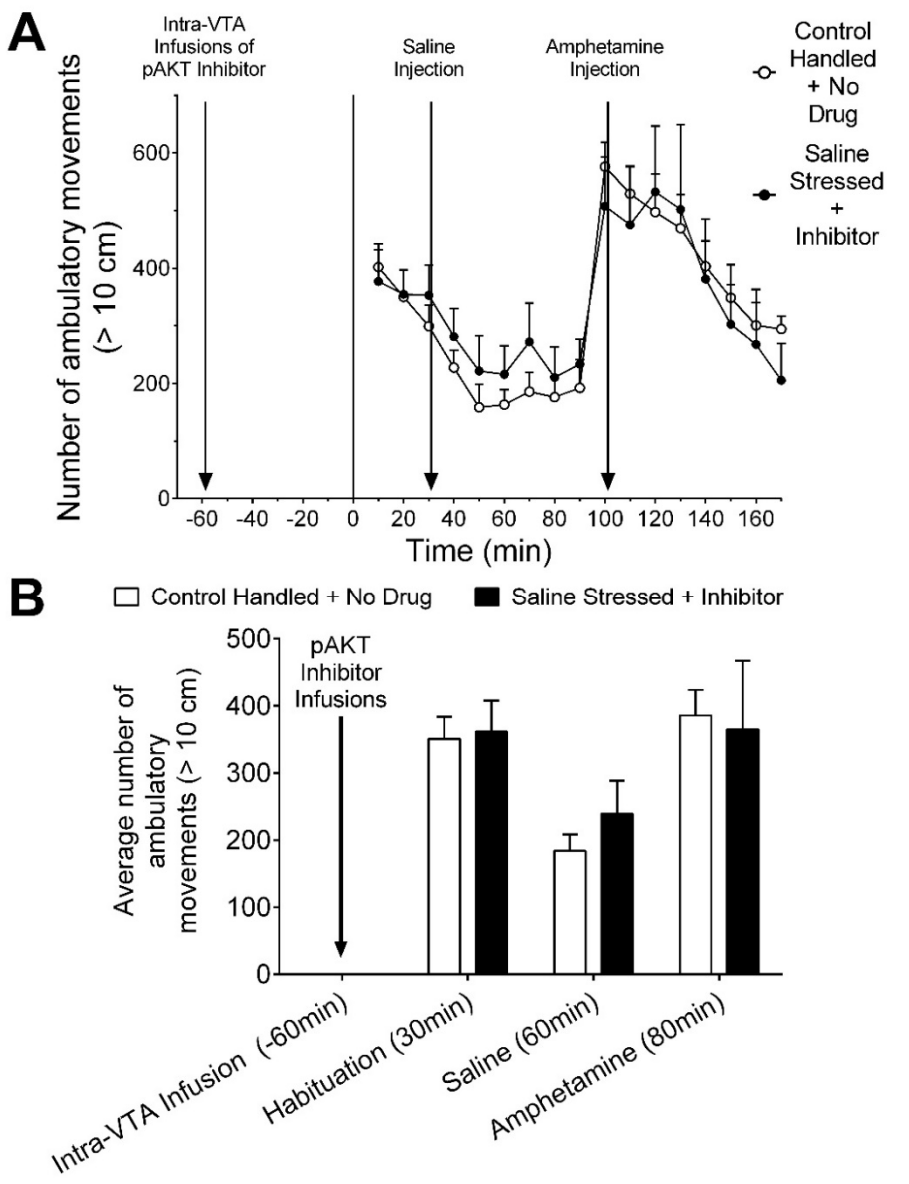

Figure 4.9. Acute Intra-VTA Inhibition of AKT Phosphorylation Blocked the Expression of Social Stress-Induced Cross-Sensitization to Amphetamine. (A) Number of locomotor movements made throughout the amphetamine challenge. (B) Average number of movements made during each phase of the amphetamine challenge. Vertical arrows indicate infusion of inhibitor, as well as injections of saline and amphetamine. Compared to Control Handled rats (n = 8), an effect of stress was no longer observed on amphetamine-induced locomotion in Saline-Stressed rats that received intra-VTA infusions of pAKT inhibitor (Saline-Stressed+Inhibitor; n = 7, $p > 0.10$).

administered to these and Control Handled rats. In general, there was no overall effect of group on either number of (n = 15, *Wilks' λ* = 0.203, $F_{13,1}$ = 2.94, $p > 0.10$; Fig 4.9) or distance travelled during (data not shown; n = 15, *Wilks' λ* = 0.223, $F_{13,1}$ = 2.62, $p > 0.12$) amphetamine-induced ambulatory movements. Given that rats in the Saline-Stressed+Inhibitor group had previously exhibited significant stress-induced cross-sensitization (Fig. 4.8), these data suggest that acute intra-VTA inhibition of AKT phosphorylation is sufficient to block the expression of amphetamine cross-sensitization.

DISCUSSION

Taken together, experiments 1 and 2 show that intermittent social defeat stress increases AKT phosphorylation in VTA GABA cells, and that this increase in pAKT is involved in the expression of stress-induced cross-sensitization to amphetamine and long-term weight gain deficits. Specifically, in experiment 1, VTA pAKT double labeled significantly more with GAD 65/67 than TH positive cells after social stress, and this effect was dependent on the expression of VTA MORs. In experiment 2, inhibition of VTA AKT phosphorylation during stress was sufficient to prevent the development of a long-term social stress-induced weight gain deficit, as well as the expression of amphetamine cross-sensitization.

Intermittent Social Stress Increases AKT Phosphorylation in VTA GABA Cells, but Not DA

While previous studies have reported a regional decrease in VTA expression of pAKT after continuous social stress (Krishnan et al., 2008), the present study found that intermittent social stress significantly increased AKT phosphorylation in a subset of VTA neurons. Specifically, analysis of pAKT expression revealed a three-fold stress-induced increase of AKT phosphorylation in cells that also expressed GAD 65/67, an immunohistochemical marker for GABA neurons. Correspondingly, social stress significantly reduced the co-expression of pAKT in DA, or TH positive cells, and increased the expression of single labeled pAKT. Although Krishnan et al. (2008) previously used Western blots to show that pAKT expression is reduced in the VTA after social continuous stress, by using homogenates, this finding is heavily biased towards DA

neurons, which make up the majority of cells in the VTA (see review of Nikulina et al., 2014). Thus the significant decrease in pAKT co-localization with TH that was observed in this study is consistent with the Western blot findings of Krishnan et al. (2008), which showed that continuous social stress reduced the expression of pAKT in the VTA. While at face value the current finding of increased expression of pAKT in VTA GABA neurons appears inconsistent with the results of (Krishnan et al., 2008), it important to note that GABA neurons comprise a small percentage of VTA cells, therefore it is unlikely that the regional Western blots used in their study were capable of detecting changes in this subset of neurons. However by using a fluorescent immunohistochemistry, a more sensitive technique to determine cell-type specific effects, the present study was able to detect a significant increase of pAKT in VTA GABA cells.

Taken together, these data suggest that intermittent social stress-induced increases in AKT phosphorylation do not occur in DA, and are instead limited to VTA GABA neurons. Moreover, while Western blots are an accurate measure of degree of protein expression at a regional level, it is important to remember that they are not capable of detecting cell-type specific changes. In the case of intracellular signaling cascades that are activity-dependent, such as phosphorylation of AKT, and also likely dependent on cell type, Western blots may not be the best measure of changes at the cellular level, due to their regional limitations. Thus the present finding that social stress increased AKT phosphorylation in VTA GABA cells, is not likely to be detected by a Western blot. Moreover, given that the current study did not detect an increase in pAKT expression in DA neurons, the present findings do not directly conflict with the results of Western blot analyses showing decreased pAKT in the VTA after stress.

Knockdown of VTA MORs Prevents Social Stress-Induced Increases in AKT Phosphorylation in VTA GABA Cells

In addition to finding that intermittent social stress increases the co-localization of pAKT with GAD 65/67, this study also determined that this effect is dependent on the expression of MORs in the VTA. Specifically, lentiviral-mediated knockdown of VTA MORs prevented increases in AKT phosphorylation in GABA cells, without altering pAKT co-expression in TH-positive cells.

That phosphorylation of AKT in the VTA is dependent on the local expression of MORs is consistent with a study showing that delta-opioid receptors activate pAKT (Margolis et al., 2011), which given the high degree of homology among opioid receptor types (Waldhoer et al., 2004, Zollner and Stein, 2007, Pradhan et al., 2012), suggests that pAKT may be a common downstream target of all opioid receptors. Stress significantly increased the proportion of pAKT found in GABA cells, such that 88% of double labeled pAKT was found in GABA cells, while only 22% of double labeled pAKT co-localized with DA. By contrast subjects in either GFP-Handled, shMOR-Handled, and shMOR-Stressed groups exhibited similar levels of pAKT co-localization in cells positive for markers of either GABA (53-57%) or DA (43-47%). There was no effect of shMOR lentivirus alone on pAKT expression in the VTA, shMOR-Handled rats did not significantly differ from GFP-Handled controls. Taken together, these data suggest that intermittent social stress increases AKT phosphorylation in VTA GABA cells downstream of stress-induced upregulation of VTA MORs. However, it is important to note that AKT phosphorylation can be induced by receptors other than MORs, in particular pAKT is a downstream target of BDNF's receptor, tropomyosin-related kinase B (TrkB) (TrkB; see reviews of Park and Poo, 2013, Nikulina et al., 2014). Although it is possible that for AKT to be phosphorylated independent of MOR activity, and while it was recently reported that a small percentage of VTA MORs are also found on VTA DA neurons (Margolis et al., 2014), the current study demonstrated that the effect of stress on pAKT expression is limited to GABA neurons and preventable by knockdown of MORs. Taken together, these results provide evidence that social stress predominantly acts to increase MOR expression in VTA GABA cells, the upregulation of which mediates increased phosphorylation of AKT.

Intra-VTA Inhibition of AKT Phosphorylation Prevents Social Stress-Induced Weight Gain Deficits

Consistent with previous reports (Meerlo et al., 1996, Pulliam et al., 2010, Fanous et al., 2011, Venzala et al., 2012, Johnston et al., 2015), intermittent social stress reduced weight gain both during and 23 days after social stress exposure. Intra-VTA inhibition of pAKT during stress attenuated this stress-induced weight gain deficit during stress, and fully rescued the long-term

deficit observed 23 days later. This finding is particularly interesting, as the effect of VTA inhibition of pAKT was greatest 23 days after stress and infusions of inhibitor, suggesting that inhibition of pAKT alone does not acutely alter weight gain. However it was previously shown that knockdown of VTA MORs also rescues this stress-induced weight gain deficit (Johnston et al., 2015), so is possible that AKT phosphorylation converges with other MOR-activated intracellular signaling cascades, such as MAPK/ERK, to help mediate stress-induced weight gain deficits. Given that stress-induced increase of pAKT is limited to GABA neurons and dependent on local MOR expression, the present finding that inhibition of VTA AKT phosphorylation during stress attenuated and promoted recovery from the social stress-induced deficit in weight gain is consistent with the crucial function of VTA MOR activity in this deficit (Johnston et al., 2015). Taken together, these studies suggest that social stress-induced decreases in weight gain are mediated by MOR – pAKT signaling in VTA GABA neurons.

The Induction of Stress-Induced Amphetamine Cross-Sensitization is Attenuated by Intra-VTA Inhibition of pAKT

Compared to the Control Handled group, subjects that received infusions of saline during stress exhibited significantly more locomotor activity in a low dose amphetamine challenge, confirming prior reports that intermittent social stress induces amphetamine cross-sensitization 10 days after the last stress episode (Covington and Miczek, 2001, Nikulina et al., 2012, Johnston et al., 2015). In contrast, compared to Control Handled and Saline-Stressed groups, subjects that had received intra-VTA infusions of pAKT inhibitor during stress did not significantly differ in their response to amphetamine. Thus, while inhibition of pAKT in the VTA during stress is not sufficient to prevent the induction of social stress-induced cross-sensitization, it did attenuate this effect. That inhibition of pAKT did not fully prevent the induction of stress-induced cross-sensitization to amphetamine is consistent with a previous study in which intracerebroventricular inhibition of pAKT, via its activator PI3K, blocked the expression, but not the induction of sensitization to cocaine (Izzo et al., 2002). Despite this, it was previously reported that lentivirus-mediated knockdown of VTA MORs is sufficient to prevent cross-sensitization to amphetamine (Johnston et

al., 2015). Since AKT phosphorylation occurs in VTA GABA neurons downstream of MOR upregulation (Fig. 4.3 – 4.6), intra-VTA inhibition of pAKT was expected to mirror the effect of VTA MOR knockdown and prevent the induction of cross-sensitization. It is surprising that inhibition of VTA AKT phosphorylation replicated the rescue of VTA MOR knockdown on weight gain, but not the induction of the induction of cross-sensitization.

This discrepancy may be attributed to differences in the methods employed in this study and that of Johnston et al. (2015). Specifically, Johnston et al. (2015) used a lentiviral construct to induced persistent depletion of VTA MORs, meaning that this viral effect was continuous throughout the experiment. By contrast, in the current study, AKT phosphorylation was only inhibited on days where subjects were stressed or handled, and since this happened every third day and the inhibitor is metabolized within 24 h, this means that AKT phosphorylation may have increased in-between episodes of social stress. Although this could not be verified, due to having conducted a reversal that altered pAKT expression in previously Saline-Stressed rats, this theory fits with the attenuation of cross-sensitization observed in Inhibitor-Stressed rats. Thus, it is possible that intra-VTA inhibition of pAKT would have significantly prevented stress-induced cross-sensitization if the drug had been administered daily, rather than intermittently, during social stress or handling procedures.

The Expression of Social Stress-Induced Cross-Sensitization to Amphetamine is Prevented by Co-Administration of Intra-VTA pAKT Inhibitor

After the first amphetamine challenge verified the induction of social stress-induced cross-sensitization in previously Saline-Stressed rats, these rats were given infusions of pAKT inhibitor (Saline-Stressed+Inhibitor) and put through a second amphetamine challenge to access for the expression of cross-sensitization. The results showed that intra-VTA inhibition of AKT phosphorylation was sufficient to block the expression of social stress-induced cross-sensitization. Specifically, after intra-VTA infusion of pAKT inhibitor, and compared to Control Handled subjects, rats that had previously showed cross-sensitization no longer displayed significant differences in amphetamine-induced locomotor activity. This finding is consistent with

previous reports that inhibition of pAKT signaling, either systemic or intracerebroventricular, is sufficient to block the expression of cocaine sensitization (Izzo et al., 2002, Wu et al., 2011), and extends upon them in suggesting that the VTA is a crucial site of action for pAKT-mediated expression of psychostimulant sensitization.

Concluding Remarks

In summary, experiment 1 found that intermittent social stress significantly increased the expression of pAKT in VTA GABA, but not DA neurons. Experiment 2 demonstrated that inhibition of VTA AKT phosphorylation is sufficient to prevent social stress-induced deficits in weight gain, as well as the *expression* of amphetamine cross-sensitization. That intra-VTA inhibition of pAKT failed to prevent the *induction* of amphetamine cross-sensitization may reflect specificity in the functional role of VTA AKT phosphorylation, or may have been due to the temporal pattern of inhibitor administration. Thus while VTA pAKT is crucial for vulnerability to psychomotor stimulant drugs, future studies will be necessary to clarify the role of VTA pAKT in the induction of stress-induced cross-sensitization.

In conclusion, social stress increased MOR – pAKT signaling in VTA GABA cells, and this increase of pAKT is necessary for the expression of stress-induced cross-sensitization to amphetamine and weight gain deficits. Given that inhibition of VTA AKT phosphorylation alone did not affect normal amphetamine-induced locomotor activity and that the effect on weight gain persisted in the absence of inhibitor, VTA pAKT may represent a novel target for the therapeutic intervention of substance abuse and stress disorders.

CHAPTER 5: GENERAL DISCUSSION

Summary of Major Results

The aim of the current study was to investigate whether VTA MOR signaling serves a functional role in social stress-induced vulnerability to psychomotor stimulant drugs. These experiments utilized the intermittent model of social defeat stress, which is well known to induce cross-sensitization to psychostimulant and opioid drugs (see reviews of Miczek et al., 2008, Miczek et al., 2011l, Nikulina et al., 2014). To investigate the role of social stress-induced MOR upregulation in the VTA, lentiviral constructs were used to manipulate local MOR levels, either non-manipulated or knocked-down, and then subjects put through social defeat stress procedures. The experiment in chapter 1 determined whether VTA MORs are necessary for the behavioral consequences of social stress. In chapter 2, immunohistochemical analyses of the mesocorticolimbic circuit were conducted at a time when cross-sensitization is known to be present. Finally, separate experiments were conducted in chapter 3 to investigate whether social stress induces AKT phosphorylation downstream of MOR upregulation in the VTA, and whether intra-VTA inhibition of AKT phosphorylation is sufficient to mimic the behavioral effects of VTA MOR knockdown.

In chapter 2, VTA MOR expression was knocked down prior to stress, and after stress or control handling procedures, subjects were assessed for social stress-induced cross-sensitization to amphetamine, as well as for social avoidance and deficits in weight gain. Knockdown of VTA MORs was sufficient to prevent social stress-induced social avoidance and long-term deficits in weight gain, without effecting normal weight gain or social interaction. Moreover, knockdown of VTA MORs prevented social stress-induced cross-sensitization, without affecting normal amphetamine-induced locomotion. Mesolimbic BDNF – TrkB receptor expression is also enhanced after social stress and also serves to mediate social stress-induced cross-sensitization (Fanous et al., 2010, Nikulina et al., 2012, Wang et al., 2013, Wang et al., 2014). Given that social stress-induced upregulation of VTA MORs occurs prior to increases in VTA BDNF

expression, it was hypothesized that knockdown of VTA MORs would prevent social stress-induced changes related to mesolimbic BDNF signaling.

In chapter 3, at the time that cross-sensitization was studied in chapter 2, knockdown of VTA MOR was found to attenuate social stress-induced changes in protein expression in the mesolimbic pathway. Specifically, VTA MOR expression was necessary for social stress-induced increases of VTA BDNF and its receptor TrkB in the NAc, additionally, expression of delta FosB was attenuated in the NAc. Thus, while mesolimbic BDNF – TrkB expression mediates social stress-induced cross-sensitization to amphetamine and the expression of delta FosB in the NAc (Wang et al., 2013, Wang et al., 2014), these effects are mediated by upstream increases in VTA MOR activity. A schematic representation of the mesolimbic and behavioral effects of stress and VTA MOR knockdown can be seen in Figure 5.1. Given that in the VTA, MORs predominantly have inhibitory effects on GABA neurons, it is possible that social stress-induced MOR upregulation increases activity of the receptors intracellular signaling cascades. Specifically, it was hypothesized that social stress would increase the phosphorylation of AKT, a downstream target of MORs, in VTA GABA neurons, and this increase would have functional implications for social stress-induced behaviors.

In chapter 4, fluorescent immunohistochemical analyses were conducted to examine social stress-induced changes in pAKT co-localization with VTA GABA and DA neurons, and whether these changes are a result of VTA MOR expression. Although GABA neurons represent a minor sub-population of VTA neurons, social stress significantly increased the co-localization of pAKT in GABA neurons, an effect which was prevented by knockdown of VTA MORs. Correspondingly, social stress significantly reduced the co-localization of pAKT with VTA DA neurons. Moreover, in these sections processed for pAKT and a marker of DA, social stress increased the expression of single labeled pAKT, which is consistent with its co-expression in VTA GABA cells. Taken together, these data are evidence that social stress augments pAKT

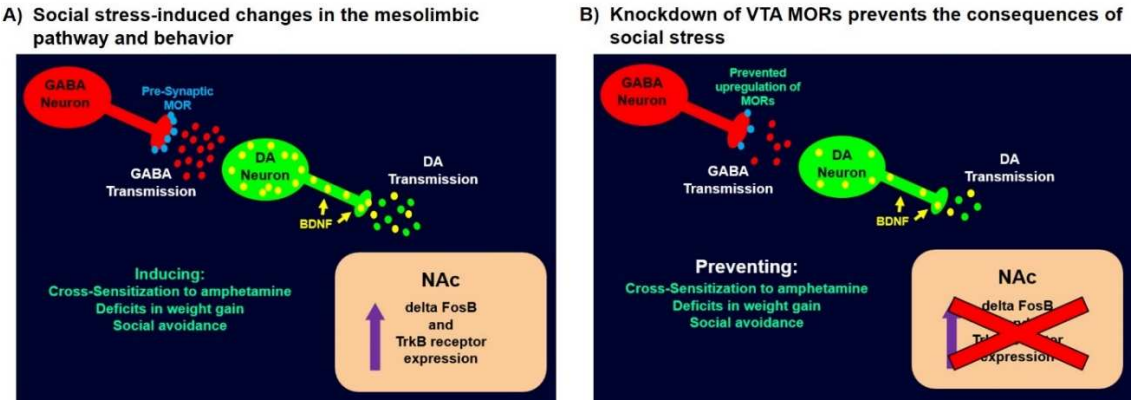

Figure 5.1. Social Stress-Induced Changes in the Mesolimbic Pathway That Have Been Related to Cross-Sensitization are Prevented by VTA MOR Knockdown. (A) Social stress increases VTA BDNF expression and signaling with its TrkB receptor and expression of delta FosB in the NAc (Fanous et al., 2010, Nikulina et al., 2012, Wang et al., 2013, Wang et al., 2014). Additionally, intermittent social stress increases VTA DA activity (Tidey and Miczek, 1996, Miczek et al., 2011a) and the expression of VTA MORs (Nikulina et al., 1999, Nikulina et al., 2005, Nikulina et al., 2008), which are implicated in the control of VTA DA neurotransmission (Johnson and North, 1992). These changes have all been related to social stress-induced weight gain deficits, social avoidance, and cross-sensitization to psychostimulants (Fanous et al., 2010, Fanous et al., 2011, Wang et al., 2013, Wang et al., 2014, Johnston et al., 2015). (B) Stimulation of MORs on a sub-population of VTA GABA neurons results in disinhibition of local DA neurons (Johnson and North, 1992). Consistent with this, knockdown of VTA MORs prevents social stress-induced increase of mesolimbic BDNF – TrkB receptor expression, and attenuates the expression of NAc delta FosB (see Chapter 3). Similar to the role of mesolimbic BDNF – TrkB receptor expression, VTA MOR expression is also necessary for the behavioral consequences of social stress (Chapter 2; see also Johnston et al., 2015). Red circle: GABA neurotransmitter; green circle: DA neurotransmitter; yellow circle: BDNF; blue circle: MOR; number of red or green circles indicate degree of neurotransmission.

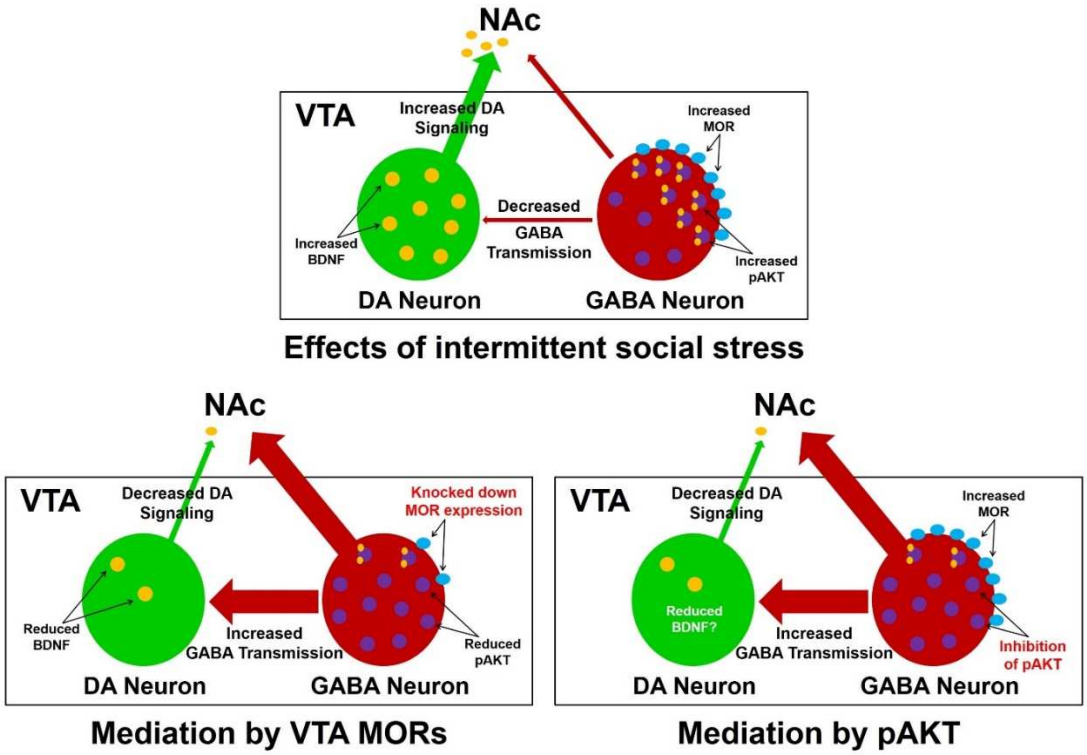

Figure 5.2. Schematic Illustration of the Effects of Local MOR Knockdown or Inhibition of AKT Phosphorylation on Social Stress-Induced Changes in the VTA. While the effects of MOR knockdown on the mesolimbic pathway have been largely confirmed (Chapters 2-4; see also Johnston et al., 2015), the mediation by AKT phosphorylation is theoretical and based on pAKT's being a downstream target of MORs and its involvement in the behavioral outcomes of stress. Large green circle: DA neuron; large red circle GABA neuron; size of projecting arrows indicate respective degrees of DA and GABA neurotransmission; small yellow circles: BDNF; small blue circles: MORs; purple circles: AKT; pairs of smallest orange circles: phospho-groups on AKT (pAKT). Manipulations of the VTA (bottom row) are indicated by red text.

expression in the VTA, such that AKT phosphorylation is increased downstream of VTA MOR upregulation (Fig. 5.2).

Given that AKT phosphorylation is limited to GABA neurons and requires the expression of VTA MORs, it was investigated whether inhibition of AKT phosphorylation during stress is sufficient to the induction of amphetamine cross-sensitization and weight gain deficits. In fact, intra-VTA inhibition of pAKT during stress, prevented the development a long-term weight gain deficit, well after the last episode of stress or inhibitor infusion. In contrast, inhibition of AKT phosphorylation during stress only attenuated the induction of cross-sensitization to amphetamine. Since a previous study had indicated that inhibition of pAKT is sufficient to block the expression, but not the induction of drug-induced psychostimulant sensitization (Izzo et al., 2002), a reversal was conducted to determine whether acute intra-VTA inhibition of pAKT was sufficient to block the expression of stress-induced cross-sensitization. In rats that had previously showed a sensitized response to amphetamine, acute inhibition of VTA AKT phosphorylation blocked the expression of cross-sensitization. Although intra-VTA inhibition of pAKT rescued the stress-induced vulnerability to amphetamine, it did not augment the normal locomotor-activating effect of amphetamine, suggesting that inhibition of VTA AKT phosphorylation does not produce unnatural augmentation of VTA DA transmission. Taken together, these results suggest that inhibition of AKT phosphorylation may represent a novel therapeutic target for the intervention stress-induced disorders and the treatment of substance abuse.

Expression of Excitatory and Inhibitory MORs in the VTA: Implications for Stress-linduced Increase of VTA MOR Activity

While stimulation of MORs is generally thought to have an inhibitory effect, a recent study illustrated that MORs can be either inhibitory or excitatory, and that either receptor sub-type can be found on both VTA GABA and DA neurons (Margolis et al., 2014). Given that the current experiments were conducted prior to this report, the overlying assumption throughout these studies was that social stress upregulates VTA MORs on GABA neurons. Although VTA MOR activation can have a variety of effects, depending on inhibitory vs. excitatory function and

neuronal type, it is possible to parse out the net effect of social stress-induced increases in MOR expression. While some VTA MORs are excitatory, the majority are inhibitory, thus at any time there is a greater likelihood that MOR stimulation will produce an inhibitory effect, regardless of neuronal type (Margolis et al., 2014). Social defeat stress is known to increase mesolimbic DA transmission (Tidey and Miczek, 1996, Miczek et al., 2011a) and produce cross-sensitization to both opioid and psychostimulant drugs that affect mesolimbic DA transmission (see reviews of Miczek et al., 2008, Miczek et al., 2011l, Nikulina et al., 2014). If the majority of VTA MORs upregulated by social stress were either inhibitory on DA neurons, or excitatory on GABA interneurons, then the net effect should be decreased VTA DA transmission, which is inconsistent with social defeat stress-induced increase of DA transmission. In contrast, the increased net effect of increased excitatory MORs on DA neurons, or inhibitory MORs on GABA neurons would be consistent with increased VTA DA transmission. While it is possible that social stress increases the expression of excitatory MORs on VTA DA neurons, the effect of such upregulation is likely to be minimal, as excitatory MORs on DA comprise a very small proportion of VTA MORs (Margolis et al., 2014). This is supported by the data in chapter 4, which illustrate that AKT phosphorylation is increased in VTA GABA neurons downstream of MOR upregulation. Although any MOR is capable of phosphorylating AKT, since there was no effect of VTA MOR knockdown on pAKT expression in DA neurons, it is likely that social stress predominantly upregulates inhibitory MORs on VTA GABA neurons.

Indirect Mediation of VTA DA Transmission by Local GABA Neurons: A Role for MOR – pAKT Signaling

The inhibitory effects of VTA MORs are generally associated with increased GIRK activity, which functions to increase K^+ conductance to reduce neurotransmission (Johnson and North, 1992, Bergevin et al., 2002). When MORs are stimulated on a sub-population of VTA GABA neurons, they have been shown to inhibit GABA release, thereby disinhibiting local DA neurons (Johnson and North, 1992). Given that social defeat stress is associated with increased VTA DA release (Tidey and Miczek, 1996, Miczek et al., 2011a), it is likely that social stress

decreases local GABA release in the VTA to disinhibit neighboring DA neurons and increase DA release in the NAc (Fig. 5.2). While future studies utilizing electrophysiology will be necessary to verify this, this theory is supported by a report of increased VTA GABA release onto local DA neurons and reduced cocaine self-administration in MOR knockout mice (Mathon et al., 2005). Based on this finding, it is likely that knockdown of VTA MORs prevented cross-sensitization to amphetamine by either increasing or maintaining tonic levels of local GABA release in the VTA. Although GIRKS are one downstream target of MORs that result in inhibition, phosphorylation of AKT has been shown to increase the insertion of $GABA_AR$ into the cell membrane (Wang et al., 2003). Given that the data in chapter 4 show that social stress increases pAKT in VTA GABA neurons, and that VTA AKT phosphorylation serves a functional role in the behavioral outcomes of social stress, it is possible that $GABA_AR$ expression is increased in GABA neurons downstream social stress-induced increase of MOR – pAKT signaling. If social stress increases the expression of $GABA_ARs$ on VTA GABA neurons, one would expect this to further inhibit GABA release and disinhibit local DA neurons.

Methodological Considerations and Future Studies

Although knockdown of VTA MORs prevented the social stress-induced cross-sensitization to amphetamine, intra-VTA inhibition of pAKT during stress did not, even though it acutely blocked the expression of cross-sensitization (Table 2). While this effect of pAKT inhibition on the expression, but not the induction of cross-sensitization has also been observed with drug-induced cocaine-sensitization and may in fact be valid, there are several methodological considerations that must be noted. First, knockdown of VTA MORs was accomplished using lentivirus-mediated interference, which means that the effect of knockdown persisted throughout the entire study. Given that the effect of VTA MOR knockdown persisted through testing, it is difficult to say whether knockdown of VTA MORs prevented the induction or the expression of cross-sensitization. In contrast to the persistent effect of lentivirus-mediated knockdown, inhibition of AKT phosphorylation was accomplished with a drug that was administered prior to each episode of stress. As a drug, the pAKT inhibitor is metabolized within

Table 2. Comparison of the Effect of VTA MOR Knockdown Versus Intra-VTA Inhibition of AKT Phosphorylation During Stress

Time after amphetamine injection	Number of Movements	Distance Traveled (cm)
Lentivirus vs. Drug Duration	Persistent	Metabolized - Intermittent
Stress-Induced Weight Gain Deficits	Prevented	Prevented
Expression of Amphetamine Cross-Sensitization	Blocked	Blocked

24 h, meaning that while stress-induced AKT phosphorylation was inhibited each day that stress procedures were administered, VTA AKT phosphorylation may have increased on subsequent days. Based on these considerations, it is possible that if the experiment in chapter 2 were repeated using intra-VTA infusions of a MOR antagonist, it would mimic the differential effect of intra-VTA inhibition of pAKT on the induction, versus the expression of cross-sensitization. Conversely, it is also possible that if VTA pAKT inhibition were accomplished using a viral construct, it would replicated the effect of VTA MOR knockdown on the expression of cross-sensitization. While both VTA MOR expression and AKT phosphorylation are critical for the behavioral effects of social defeat stress, additional studies will be needed to determine whether MOR – pAKT signaling plays a differential role in the induction, rather than the expression, of stress-induced cross-sensitization to psychomotor stimulant drugs.

The finding that inhibition of AKT phosphorylation in the VTA during stress is sufficient to prevent stress-induced weight gain deficits 23 days after the last infusion of inhibitor, suggests that VTA AKT phosphorylation confers susceptibility to intermittent social defeat stress in rats. By contrast, a previous study showed there is a regional decrease of VTA pAKT after continuous social defeat stress in mice (Krishnan et al., 2008). Additionally, when mice were classified as susceptible or resilient after defeat, they found that resilient mice exhibited greater regional levels of pAKT in the VTA. Although this seems to contradict the current findings regarding stress and VTA AKT phosphorylation, it important to note several distinctions between these studies. The study by Krishnan et al. (2008) used regional Western blot analyses which are not sensitive to

changes in specific sub-populations of cells, such as MOR containing GABA neurons. Thus it is possible that despite their finding of a regional decrease of VTA pAKT after continuous social stress, that the mice in the study of Krishnan et al. (2008) may have also exhibited an increase of pAKT in a sub-population of VTA GABA neurons. In support of this, the current fluorescent immunohistochemical results showed that intermittent social stress tended to decrease pAKT expression in VTA DA neurons, and given that DA makes up the majority of VTA neurons, it is possible that Western blot analyses of the VTA after intermittent social defeat stress would also show a regional decrease of pAKT.

It is also important to note that the study of Krishnan et al. (2008) utilized mice, while the present studies utilized rats. In rats, continuous and intermittent social defeat stress produce opposite effects on responses to psychomotor stimulants and DA release in the NAc (Miczek et al., 2011a). Moreover, in mice continuous social defeat stress produces an increase in VTA BDNF (Berton et al., 2006, Krishnan et al., 2008), while in rats, VTA BDNF is increased after intermittent, but decreased after continuous social defeat stress (Miczek et al., 2011a). Given that social stress has different effects on VTA BDNF expression in rats and mice, it also possible that the continuous social stress-induced decrease in VTA pAKT is specific to mouse models of social stress. Future studies will be needed to directly compare whether intermittent social stress-induced increase of VTA MOR – pAKT signaling also mediates the effects of continuous social defeat stress in rats.

Concluding Remarks

The studies described in chapters 2-4 show that intermittent social stress increases MOR – pAKT signaling in the VTA, and that the expression of VTA MORs and pAKT are crucial for stress-induced cross-sensitization to psychostimulant drugs of abuse. In addition to mediating the behavioral consequences of social stress, knockdown of VTA MORs prevented social stress-induced increases in mesolimbic BDNF – TrkB signaling, as well as attenuating the expression of delta FosB in the NAc. While the behavioral effects of intra-VTA inhibition of pAKT were similar to the preventative effects of MOR knockdown, future studies will be necessary to determine

whether inhibition of VTA pAKT also mediates social stress-induced changes in the mesolimbic pathway. Taken together, these studies suggest that increased MOR – pAKT signaling in the VTA may underlie susceptibility to psychomotor stimulants, and that interruption of this signaling may have beneficial effects for the therapeutic intervention of stress-induced disorders and substance abuse.

REFERENCES

Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJ, Frech M, Cron P, Cohen P, Lucocq JM, Hemmings BA (1997) Role of translocation in the activation and function of protein kinase B. J Biol Chem 272:31515-31524.

Badiani A, Belin D, Epstein D, Calu D, Shaham Y (2011) Opiate versus psychostimulant addiction: the differences do matter. Nat Rev Neurosci 12:685-700.

Badiani A, Leone P, Noel MB, Stewart J (1995) Ventral tegmental area opioid mechanisms and modulation of ingestive behavior. Brain Research 670:264-276.

Bahi A, Boyer F, Chandrasekar V, Dreyer JL (2008) Role of accumbens BDNF and TrkB in cocaine-induced psychomotor sensitization, conditioned-place preference, and reinstatement in rats. Psychopharmacology (Berl) 199:169-182.

Bellacosa A, Chan TO, Ahmed NN, Datta K, Malstrom S, Stokoe D, McCormick F, Feng J, Tsichlis P (1998) Akt activation by growth factors is a multiple-step process: the role of the PH domain. Oncogene 17:313-325.

Bergevin A, Girardot D, Bourque MJ, Trudeau LE (2002) Presynaptic mu-opioid receptors regulate a late step of the secretory process in rat ventral tegmental area GABAergic neurons. Neuropharmacology 42:1065-1078.

Berglind WJ, See RE, Fuchs RA, Ghee SM, Whitfield TW, Jr., Miller SW, McGinty JF (2007) A BDNF infusion into the medial prefrontal cortex suppresses cocaine seeking in rats. The European Journal of Neuroscience 26:757-766.

Berglind WJ, Whitfield TW, Jr., LaLumiere RT, Kalivas PW, McGinty JF (2009) A single intra-PFC infusion of BDNF prevents cocaine-induced alterations in extracellular glutamate within the nucleus accumbens. The Journal of Neuroscience : The official journal of the Society for Neuroscience 29:3715-3719.

Berton O, McClung CA, Dileone RJ, Krishnan V, Renthal W, Russo SJ, Graham D, Tsankova NM, Bolanos CA, Rios M, Monteggia LM, Self DW, Nestler EJ (2006) Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. Science 311:864-868.

Bocklisch C, Pascoli V, Wong JC, House DR, Yvon C, de Roo M, Tan KR, Luscher C (2013) Cocaine disinhibits dopamine neurons by potentiation of GABA transmission in the ventral tegmental area. Science 341:1521-1525.

Bolanos CA, Nestler EJ (2004) Neurotrophic mechanisms in drug addiction. Neuromolecular Med 5:69-83.

Brenhouse HC, Stellar JR (2006) c-Fos and deltaFosB expression are differentially altered in distinct subregions of the nucleus accumbens shell in cocaine-sensitized rats. Neuroscience 137:773-780.

Brewer DD, Catalano RF, Haggerty K, Gainey RR, Fleming CB (1998) A meta-analysis of predictors of continued drug use during and after treatment for opiate addiction. Addiction 93:73-92.

Burford NT, Wang D, Sadee W (2000) G-protein coupling of mu-opioid receptors (OP3): elevated basal signalling activity. The Biochemical Journal 348 Pt 3:531-537.

Carle TL, Ohnishi YN, Ohnishi YH, Alibhai IN, Wilkinson MB, Kumar A, Nestler EJ (2007) Proteasome-dependent and -independent mechanisms for FosB destabilization: identification of FosB degron domains and implications for DeltaFosB stability. The European Journal of Neuroscience 25:3009-3019.

Cates HM, Thibault M, Pfau M, Heller E, Eagle A, Gajewski P, Bagot R, Colangelo C, Abbott T, Rudenko G, Neve R, Nestler EJ, Robison AJ (2014) Threonine 149 phosphorylation enhances DeltaFosB transcriptional activity to control psychomotor responses to cocaine. The Journal of Neuroscience : The official journal of the Society for Neuroscience 34:11461-11469.

Chakrabarti S, Regec A, Gintzler AR (2005) Chronic morphine acts via a protein kinase Cgamma-G(beta)-adenylyl cyclase complex to augment phosphorylation of G(beta) and G(betagamma) stimulatory adenylyl cyclase signaling. Brain Research: Molecular Brain Research 138:94-103.

Chapuis N, Tamburini J, Green AS, Vignon C, Bardet V, Neyret A, Pannetier M, Willems L, Park S, Macone A, Maira SM, Ifrah N, Dreyfus F, Herault O, Lacombe C, Mayeux P, Bouscary D (2010) Dual inhibition of PI3K and mTORC1/2 signaling by NVP-BEZ235 as a new therapeutic strategy for acute myeloid leukemia. Clinical Cancer Research : An official journal of the American Association for Cancer Research 16:5424-5435.

Chartoff EH, Connery HS (2014) It's MORe exciting than mu: crosstalk between mu opioid receptors and glutamatergic transmission in the mesolimbic dopamine system. Frontiers in Pharmacology 5:116.

Chefer VI, Denoroy L, Zapata A, Shippenberg TS (2009) Mu opioid receptor modulation of somatodendritic dopamine overflow: GABAergic and glutamatergic mechanisms. Eur J Neurosci 30:272-278.

Chen J, Nye HE, Kelz MB, Hiroi N, Nakabeppu Y, Hope BT, Nestler EJ (1995) Regulation of delta FosB and FosB-like proteins by electroconvulsive seizure and cocaine treatments. Mol Pharmacol 48:880-889.

Chong ZZ, Li F, Maiese K (2005) Activating Akt and the brain's resources to drive cellular survival and prevent inflammatory injury. Histology and Histopathology 20:299-315.

Chu NN, Zuo YF, Meng L, Lee DY, Han JS, Cui CL (2007) Peripheral electrical stimulation reversed the cell size reduction and increased BDNF level in the ventral tegmental area in chronic morphine-treated rats. Brain Res 1182:90-98.

Clark MJ, Harrison C, Zhong H, Neubig RR, Traynor JR (2003) Endogenous RGS protein action modulates mu-opioid signaling through Galphao. Effects on adenylyl cyclase, extracellular signal-regulated kinases, and intracellular calcium pathways. J Biol Chem 278:9418-9425.

Cleck JN, Blendy JA (2008) Making a bad thing worse: adverse effects of stress on drug addiction. The Journal of Clinical Investigation 118:454-461.

Connor M, Christie MD (1999) Opioid receptor signalling mechanisms. Clinical and Experimental Pharmacology and Physiology 26:493-499.

Corominas M, Roncero C, Ribases M, Castells X, Casas M (2007) Brain-derived neurotrophic factor and its intracellular signaling pathways in cocaine addiction. Neuropsychobiology 55:2-13.

Council NR (2011) Guide for the Care and Use of Laboratory Animals: Eighth Edition: The National Academies Press.

Covington HE, 3rd, Miczek KA (2001) Repeated social-defeat stress, cocaine or morphine. Effects on behavioral sensitization and intravenous cocaine self-administration "binges". Psychopharmacology 158:388-398.

Covington HE, 3rd, Miczek KA (2005) Intense cocaine self-administration after episodic social defeat stress, but not after aggressive behavior: dissociation from corticosterone activation. Psychopharmacology 183:331-340.

Crooks KR, Kleven DT, Rodriguiz RM, Wetsel WC, McNamara JO (2010) TrkB signaling is required for behavioral sensitization and conditioned place preference induced by a single injection of cocaine. Neuropharmacology 58:1067-1077.

Cruz FC, Quadros IM, Hogenelst K, Planeta CS, Miczek KA (2011) Social defeat stress in rats: escalation of cocaine and "speedball" binge self-administration, but not heroin. Psychopharmacology 215:165-175.

Dacher M, Nugent FS (2011) Opiates and plasticity. Neuropharmacology 61:1088-1096.

Dobrazanski P, Noguchi T, Kovary K, Rizzo CA, Lazo PS, Bravo R (1991) Both products of the fosB gene, FosB and its short form, FosB/SF, are transcriptional activators in fibroblasts. Mol Cell Biol 11:5470-5478.

Du K, Tsichlis PN (2005) Regulation of the Akt kinase by interacting proteins. Oncogene 24:7401-7409.

DuMars LA, Rodger LD, Kalivas PW (1988) Behavioral cross-sensitization between cocaine and enkephalin in the A10 dopamine region. Behavioural Brain Research 27:87-91.

Fanous S, Hammer RP, Jr., Nikulina EM (2010) Short- and long-term effects of intermittent social defeat stress on brain-derived neurotrophic factor expression in mesocorticolimbic brain regions. Neuroscience 167:598-607.

Fanous S, Terwilliger EF, Hammer RP, Jr., Nikulina EM (2011) Viral depletion of VTA BDNF in rats modulates social behavior, consequences of intermittent social defeat stress, and long-term weight regulation. Neuroscience Letters 502:192-196.

Fields HL, Hjelmstad GO, Margolis EB, Nicola SM (2007) Ventral tegmental area neurons in learned appetitive behavior and positive reinforcement. Annual Review of Neuroscience 30:289-316.

Fields HL, Margolis EB (2015) Understanding opioid reward. Trends in Neurosciences.

Francis TC, Chandra R, Friend DM, Finkel E, Dayrit G, Miranda J, Brooks JM, Iniguez SD, O'Donnell P, Kravitz A, Lobo MK (2015) Nucleus accumbens medium spiny neuron subtypes mediate depression-related outcomes to social defeat stress. Biological Psychiatry 77:212-222.

Franke TF (2008) PI3K/Akt: getting it right matters. Oncogene 27:6473-6488.

Fumagalli F, Caffino L, Racagni G, Riva MA (2009) Repeated stress prevents cocaine-induced activation of BDNF signaling in rat prefrontal cortex. European Neuropsychopharmacology : The journal of the European College of Neuropsychopharmacology 19:402-408.

Fumagalli F, Moro F, Caffino L, Orru A, Cassina C, Giannotti G, Di Clemente A, Racagni G, Riva MA, Cervo L (2013) Region-specific effects on BDNF expression after contingent or non-contingent cocaine i.v. self-administration in rats. Int J Neuropsychopharmacol 16:913-918.

Gall CM, Gold SJ, Isackson PJ, Seroogy KB (1992) Brain-derived neurotrophic factor and neurotrophin-3 mRNAs are expressed in ventral midbrain regions containing dopaminergic neurons. Mol Cell Neurosci 3:56-63.

Garcia-Perez D, Laorden ML, Milanes MV, Nunez C (2012) Glucocorticoids regulation of FosB/DeltaFosB expression induced by chronic opiate exposure in the brain stress system. PloS One 7:e50264.

Garzon M, Pickel VM (2002) Ultrastructural localization of enkephalin and mu-opioid receptors in the rat ventral tegmental area. Neuroscience 114:461-474.

Gether U (2000) Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocrine Reviews 21:90-113.

Geyer MA, Russo PV, Segal DS, Kuczenski R (1987) Effects of apomorphine and amphetamine on patterns of locomotor and investigatory behavior in rats. Pharmacology Biochemistry and Behavior 28:393-399.

Gharbi SI, Zvelebil MJ, Shuttleworth SJ, Hancox T, Saghir N, Timms JF, Waterfield MD (2007) Exploring the specificity of the PI3K family inhibitor LY294002. The Biochemical Journal 404:15-21.

Ghitza UE, Zhai H, Wu P, Airavaara M, Shaham Y, Lu L (2010) Role of BDNF and GDNF in drug reward and relapse: a review. Neurosci Biobehav Rev 35:157-171.

Graham DL, Krishnan V, Larson EB, Graham A, Edwards S, Bachtell RK, Simmons D, Gent LM, Berton O, Bolanos CA, DiLeone RJ, Parada LF, Nestler EJ, Self DW (2009) Tropomyosin-related kinase B in the mesolimbic dopamine system: region-specific effects on cocaine reward. Biological Psychiatry 65:696-701.

Grimm JW, Lu L, Hayashi T, Hope BT, Su T-P, Shaham Y (2003) Time-Dependent Increases in Brain-Derived Neurotrophic Factor Protein Levels within the Mesolimbic Dopamine System after Withdrawal from Cocaine: Implications for Incubation of Cocaine Craving. The Journal of Neuroscience 23:742-747.

Han W, Hata H, Imbe H, Liu QR, Takamatsu Y, Koizumi M, Murphy NP, Senba E, Uhl GR, Sora I, Ikeda K (2006) Increased body weight in mice lacking mu-opioid receptors. Neuroreport 17:941-944.

Hayashida S, Oka T, Mera T, Tsuji S (2010) Repeated social defeat stress induces chronic hyperthermia in rats. Physiology & Behavior 101:124-131.

Heller EA, Cates HM, Pena CJ, Sun H, Shao N, Feng J, Golden SA, Herman JP, Walsh JJ, Mazei-Robison M, Ferguson D, Knight S, Gerber MA, Nievera C, Han MH, Russo SJ, Tamminga CS, Neve RL, Shen L, Zhang HS, Zhang F, Nestler EJ (2014) Locus-specific epigenetic remodeling controls addiction- and depression-related behaviors. Nature Neuroscience 17:1720-1727.

Herkenham M, Pert C (1982) Light microscopic localization of brain opiate receptors: a general autoradiographic method which preserves tissue quality. The Journal of Neuroscience 2:1129-1149.

Hiroi N, Brown JR, Haile CN, Ye H, Greenberg ME, Nestler EJ (1997) FosB mutant mice: loss of chronic cocaine induction of Fos-related proteins and heightened sensitivity to cocaine's psychomotor and rewarding effects. Proceedings of the National Academy of Sciences of the United States of America 94:10397-10402.

Hope BT, Nye HE, Kelz MB, Self DW, Iadarola MJ, Nakabeppu Y, Duman RS, Nestler EJ (1994) Induction of a long-lasting AP-1 complex composed of altered Fos-like proteins in brain by chronic cocaine and other chronic treatments. Neuron 13:1235-1244.

Horger BA, Iyasere CA, Berhow MT, Messer CJ, Nestler EJ, Taylor JR (1999) Enhancement of locomotor activity and conditioned reward to cocaine by brain-derived neurotrophic factor. J Neurosci 19:4110-4122.

Iglesias M, Segura MF, Comella JX, Olmos G (2003) Mu-opioid receptor activation prevents apoptosis following serum withdrawal in differentiated SH-SY5Y cells and cortical neurons via phosphatidylinositol 3-kinase. Neuropharmacology 44:482-492.

Iniguez SD, Warren BL, Neve RL, Nestler EJ, Russo SJ, Bolanos-Guzman CA (2008) Insulin receptor substrate-2 in the ventral tegmental area regulates behavioral responses to cocaine. Behavioral Neuroscience 122:1172-1177.

Iwanami A, Cloughesy TF, Mischel PS (2009) Striking the balance between PTEN and PDK1: it all depends on the cell context. Genes & Development 23:1699-1704.

Izzo E, Martin-Fardon R, Koob GF, Weiss F, Sanna PP (2002) Neural plasticity and addiction: PI3-kinase and cocaine behavioral sensitization. Nature euroscience 5:1263-1264.

Johnson SW, North RA (1992) Opioids excite dopamine neurons by hyperpolarization of local interneurons. The Journal of Neuroscience : The official journal of the Society for Neuroscience 12:483-488.

Johnston CE, Herschel DJ, Lasek AW, Hammer RP, Jr., Nikulina EM (2015) Knockdown of ventral tegmental area mu-opioid receptors in rats prevents effects of social defeat stress: implications for amphetamine cross-sensitization, social avoidance, weight regulation and expression of brain-derived neurotrophic factor. Neuropharmacology 89:325-334.

Joyce EM, Iversen SD (1979) The effect of morphine applied locally to mesencephalic dopamine cell bodies on spontaneous motor activity in the rat. Neuroscience Letters 14:207-212.

Kalivas PW, O'Brien C (2008) Drug addiction as a pathology of staged neuroplasticity. Neuropsychopharmacology : Official publication of the American College of Neuropsychopharmacology 33:166-180.

Kalivas PW, Stewart J (1991) Dopamine transmission in the initiation and expression of drug- and stress-induced sensitization of motor activity. Brain Research: Brain Research Reviews 16:223-244.

Kaplan GB, Leite-Morris KA, Fan W, Young AJ, Guy MD (2011) Opiate sensitization induces FosB/DeltaFosB expression in prefrontal cortical, striatal and amygdala brain regions. PloS One 6:e23574.

Kelz MB, Nestler EJ (2000) deltaFosB: a molecular switch underlying long-term neural plasticity. Curr Opin Neurol 13:715-720.

Komatsu H, Ohara A, Sasaki K, Abe H, Hattori H, Hall FS, Uhl GR, Sora I (2011) Decreased response to social defeat stress in mu-opioid-receptor knockout mice. Pharmacology, Biochemistry, and Behavior 99:676-682.

Koo JW, Mazei-Robison MS, Chaudhury D, Juarez B, LaPlant Q, Ferguson D, Feng J, Sun H, Scobie KN, Damez-Werno D, Crumiller M, Ohnishi YN, Ohnishi YH, Mouzon E, Dietz DM, Lobo MK, Neve RL, Russo SJ, Han M-H, Nestler EJ (2012) BDNF Is a Negative Modulator of Morphine Action. Science 338:124-128.

Koyama S, Appel SB (2006) A-type K+ current of dopamine and GABA neurons in the ventral tegmental area. Journal of Neurophysiology 96:544-554.

Kreek MJ, Nielsen DA, Butelman ER, LaForge KS (2005) Genetic influences on impulsivity, risk taking, stress responsivity and vulnerability to drug abuse and addiction. Nature Neuroscience 8:1450-1457.

Krishnan V, Han MH, Graham DL, Berton O, Renthal W, Russo SJ, Laplant Q, Graham A, Lutter M, Lagace DC, Ghose S, Reister R, Tannous P, Green TA, Neve RL, Chakravarty S, Kumar A, Eisch AJ, Self DW, Lee FS, Tamminga CA, Cooper DC, Gershenfeld HK, Nestler EJ (2007) Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. Cell 131:391-404.

Krishnan V, Han MH, Mazei-Robison M, Iniguez SD, Ables JL, Vialou V, Berton O, Ghose S, Covington HE, 3rd, Wiley MD, Henderson RP, Neve RL, Eisch AJ, Tamminga CA, Russo SJ, Bolanos CA, Nestler EJ (2008) AKT signaling within the ventral tegmental area regulates cellular and behavioral responses to stressful stimuli. Biological Psychiatry 64:691-700.

Larson EB, Akkentli F, Edwards S, Graham DL, Simmons DL, Alibhai IN, Nestler EJ, Self DW (2010) Striatal regulation of DeltaFosB, FosB, and cFos during cocaine self-administration and withdrawal. Journal of Neurochemistry 115:112-122.

Lasek AW, Janak PH, He L, Whistler JL, Heberlein U (2007) Downregulation of mu opioid receptor by RNA interference in the ventral tegmental area reduces ethanol consumption in mice. Genes, Brain, and Behavior 6:728-735.

Laviolette SR, Gallegos RA, Henriksen SJ, van der Kooy D (2004) Opiate state controls bi-directional reward signaling via GABAA receptors in the ventral tegmental area. Nat Neurosci 7:160-169.

Law PY, Loh HH (1999) Regulation of opioid receptor activities. The Journal of Pharmacology and Experimental Therapeutics 289:607-624.

Lenoir M, Guillem K, Koob GF, Ahmed SH (2012) Drug specificity in extended access cocaine and heroin self-administration. Addiction Biology 17:964-976.

Lipton JO, Sahin M (2014) The neurology of mTOR. Neuron 84:275-291.

Liu H, Li H, Guo L, Li M, Li C, Wang S, Jiang W, Liu X, McNutt MA, Li G (2010) Mechanisms involved in phosphatidylinositol 3-kinase pathway mediated up-regulation of the mu opioid receptor in lymphocytes. Biochemical Pharmacology 79:516-523.

Liu P, Cheng H, Roberts TM, Zhao JJ (2009) Targeting the phosphoinositide 3-kinase pathway in cancer. Nature Reviews Drug Discovery 8:627-644.

Lobo MK, Zaman S, Damez-Werno DM, Koo JW, Bagot RC, DiNieri JA, Nugent A, Finkel E, Chaudhury D, Chandra R, Riberio E, Rabkin J, Mouzon E, Cachope R, Cheer JF, Han MH, Dietz DM, Self DW, Hurd YL, Vialou V, Nestler EJ (2013) DeltaFosB induction in striatal medium spiny neuron subtypes in response to chronic pharmacological, emotional, and optogenetic stimuli. The Journal of Neuroscience : The official journal of the Society for Neuroscience 33:18381-18395.

Lopez A, Salome L (2009) Membrane functional organisation and dynamic of mu-opioid receptors. Cellular and Molecular Life Sciences : CMLS 66:2093-2108.

Lu L, Dempsey J (2004) Cocaine seeking over extended withdrawal periods in rats: time dependent increases of responding induced by heroin priming over the first 3 months. Psychopharmacology (Berl) 176:109-114.

Lu L, Dempsey J, Liu SY, Bossert JM, Shaham Y (2004) A single infusion of brain-derived neurotrophic factor into the ventral tegmental area induces long-lasting potentiation of cocaine seeking after withdrawal. J Neurosci 24:1604-1611.

MacDonald AF, Billington CJ, Levine AS (2004) Alterations in food intake by opioid and dopamine signaling pathways between the ventral tegmental area and the shell of the nucleus accumbens. Brain Research 1018:78-85.

Magendzo K, Bustos G (2003) Expression of amphetamine-induced behavioral sensitization after short- and long-term withdrawal periods: participation of mu- and delta-opioid receptors. Neuropsychopharmacology : Official publication of the American College of Neuropsychopharmacology 28:468-477.

Maira SM, Stauffer F, Brueggen J, Furet P, Schnell C, Fritsch C, Brachmann S, Chene P, De Pover A, Schoemaker K, Fabbro D, Gabriel D, Simonen M, Murphy L, Finan P, Sellers W, Garcia-Echeverria C (2008) Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent in vivo antitumor activity. Molecular Cancer Therapeutics 7:1851-1863.

Manning BD, Cantley LC (2007) AKT/PKB signaling: navigating downstream. Cell 129:1261-1274.

Mansour A, Fox CA, Thompson RC, Akil H, Watson SJ (1994) mu-Opioid receptor mRNA expression in the rat CNS: comparison to mu-receptor binding. Brain Res 643:245-265.

Marcinkiewcz CA, Green MK, Devine DP, Duarte P, Vierck CJ, Yezierski RP (2009) Social defeat stress potentiates thermal sensitivity in operant models of pain processing. Brain Res 1251:112-120.

Margolis EB, Hjelmstad GO, Fujita W, Fields HL (2014) Direct bidirectional mu-opioid control of midbrain dopamine neurons. The Journal of Neuroscience : The official journal of the Society for Neuroscience 34:14707-14716.

Margolis EB, Mitchell JM, Hjelmstad GO, Fields HL (2011) A novel opioid receptor-mediated enhancement of GABAA receptor function induced by stress in ventral tegmental area neurons. The Journal of Physiology 589:4229-4242.

Marinelli M, Piazza PV (2002) Interaction between glucocorticoid hormones, stress and psychostimulant drugs. The European Journal of Neuroscience 16:387-394.

Mashayekhi FJ, Rasti M, Rahvar M, Mokarram P, Namavar MR, Owji AA (2012) Expression levels of the BDNF gene and histone modifications around its promoters in the ventral tegmental area and locus ceruleus of rats during forced abstinence from morphine. Neurochemical Research 37:1517-1523.

Mathon DS, Lesscher HM, Gerrits MA, Kamal A, Pintar JE, Schuller AG, Spruijt BM, Burbach JP, Smidt MP, van Ree JM, Ramakers GM (2005) Increased gabaergic input to ventral tegmental area dopaminergic neurons associated with decreased cocaine reinforcement in mu-opioid receptor knockout mice. Neuroscience 130:359-367.

Mazei-Robison MS, Koo JW, Friedman AK, Lansink CS, Robison AJ, Vinish M, Krishnan V, Kim S, Siuta MA, Galli A, Niswender KD, Appasani R, Horvath MC, Neve RL, Worley PF, Snyder SH, Hurd YL, Cheer JF, Han MH, Russo SJ, Nestler EJ (2011) Role for mTOR signaling and neuronal activity in morphine-induced adaptations in ventral tegmental area dopamine neurons. Neuron 72:977-990.

Meerlo P, Overkamp GJ, Daan S, Van Den Hoofdakker RH, Koolhaas JM (1996) Changes in behaviour and body weight following a single or double social defeat in rats. Stress 1:21-32.

Meier R, Alessi DR, Cron P, Andjelkovic M, Hemmings BA (1997) Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase Bbeta. J Biol Chem 272:30491-30497.

Miczek KA, Nikulina EM, Shimamoto A, Covington HE, 3rd (2011a) Escalated or suppressed cocaine reward, tegmental BDNF, and accumbal dopamine caused by episodic versus continuous social stress in rats. The Journal of Neuroscience : The official journal of the Society for Neuroscience 31:9848-9857.

Miczek KA, Nikulina EM, Takahashi A, Covington HE, 3rd, Yap JJ, Boyson CO, Shimamoto A, de Almeida RM (2011l) Gene expression in aminergic and peptidergic cells during aggression and defeat: relevance to violence, depression and drug abuse. Behavior Genetics 41:787-802.

Miczek KA, Yap JJ, Covington HE, 3rd (2008) Social stress, therapeutics and drug abuse: preclinical models of escalated and depressed intake. Pharmacology & Therapeutics 120:102-128.

Morales M, Pickel VM (2012) Insights to drug addiction derived from ultrastructural views of the mesocorticolimbic system. Annals of the New York Academy of Sciences 1248:71-88.

Morales M, Root DH (2014) Glutamate neurons within the midbrain dopamine regions. Neuroscience 282C:60-68.

Morgan JI, Curran T (1995) Immediate-early genes: ten years on. Trends in Neurosciences 18:66-67.

Mukherjee B, Tomimatsu N, Amancherla K, Camacho CV, Pichamoorthy N, Burma S (2012) The dual PI3K/mTOR inhibitor NVP-BEZ235 is a potent inhibitor of ATM- and DNA-PKCs-mediated DNA damage responses. Neoplasia 14:34-43.

Muller DL, Unterwald EM (2004) In vivo regulation of extracellular signal-regulated protein kinase (ERK) and protein kinase B (Akt) phosphorylation by acute and chronic morphine. The Journal of Pharmacology and Experimental Therapeutics 310:774-782.

Murphy CA, Russig H, Pezze MA, Ferger B, Feldon J (2003) Amphetamine withdrawal modulates FosB expression in mesolimbic dopaminergic target nuclei: effects of different schedules of administration. Neuropharmacology 44:926-939.

Nair-Roberts RG, Chatelain-Badie SD, Benson E, White-Cooper H, Bolam JP, Ungless MA (2008) Stereological estimates of dopaminergic, GABAergic and glutamatergic neurons in the ventral tegmental area, substantia nigra and retrorubral field in the rat. Neuroscience 152:1024-1031.

Nathan PJ, Bullmore ET (2009) From taste hedonics to motivational drive: central mu-opioid receptors and binge-eating behaviour. The International Journal of Neuropsychopharmacology: Official scientific journal of the Collegium Internationale Neuropsychopharmacologicum 12:995-1008.

Nestler EJ (2008) Review. Transcriptional mechanisms of addiction: role of DeltaFosB. Philosophical transactions of the Royal Society of London Series B, Biological sciences 363:3245-3255.

Nestler EJ (2013) Cellular basis of memory for addiction. Dialogues Clin Neurosci 15:431-443.

Nestler EJ (2014) FosB: A transcriptional regulator of stress and antidepressant responses. European Journal of Pharmacology.

Nestler EJ, Barrot M, Self DW (2001) DeltaFosB: a sustained molecular switch for addiction. Proceedings of the National Academy of Sciences of the United States of America 98:11042-11046.

NIDA (2012) Drug Facts: Understanding Drug Abuse and Addiction. pp 1-4 Washington D.C.: National Institutes of Health, National Institute on Drug Abuse.

Nikulina EM, Arrillaga-Romany I, Miczek KA, Hammer RP, Jr. (2008) Long-lasting alteration in mesocorticolimbic structures after repeated social defeat stress in rats: time course of mu-opioid receptor mRNA and FosB/DeltaFosB immunoreactivity. The European Journal of Neuroscience 27:2272-2284.

Nikulina EM, Covington HE, 3rd, Ganschow L, Hammer RP, Jr., Miczek KA (2004) Long-term behavioral and neuronal cross-sensitization to amphetamine induced by repeated brief

social defeat stress: Fos in the ventral tegmental area and amygdala. Neuroscience 123:857-865.

Nikulina EM, Hammer RP, Jr., Miczek KA, Kream RM (1999) Social defeat stress increases expression of mu-opioid receptor mRNA in rat ventral tegmental area. Neuroreport 10:3015-3019.

Nikulina EM, Johnston CE, Wang J, Hammer RP, Jr. (2014) Neurotrophins in the ventral tegmental area: Role in social stress, mood disorders and drug abuse. Neuroscience 282C:122-138.

Nikulina EM, Lacagnina MJ, Fanous S, Wang J, Hammer RP, Jr. (2012) Intermittent social defeat stress enhances mesocorticolimbic DeltaFosB/BDNF co-expression and persistently activates corticotegmental neurons: implication for vulnerability to psychostimulants. Neuroscience 212:38-48.

Nikulina EM, Miczek KA, Hammer RP, Jr. (2005) Prolonged effects of repeated social defeat stress on mRNA expression and function of mu-opioid receptors in the ventral tegmental area of rats. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 30:1096-1103.

Numan S, Lane-Ladd SB, Zhang L, Lundgren KH, Russell DS, Seroogy KB, Nestler EJ (1998) Differential regulation of neurotrophin and trk receptor mRNAs in catecholaminergic nuclei during chronic opiate treatment and withdrawal. J Neurosci 18:10700-10708.

Nunez C, Martin F, Foldes A, Luisa Laorden M, Kovacs KJ, Victoria Milanes M (2010) Induction of FosB/DeltaFosB in the brain stress system-related structures during morphine dependence and withdrawal. Journal of Neurochemistry 114:475-487.

Oades RD, Halliday GM (1987) Ventral tegmental (A10) system: neurobiology. 1. Anatomy and connectivity. Brain Res 434:117-165.

Ohnishi YN, Ohnishi YH, Vialou V, Mouzon E, LaPlant Q, Nishi A, Nestler EJ (2015) Functional role of the N-terminal domain of DeltaFosB in response to stress and drugs of abuse. Neuroscience 284:165-170.

Okkenhaug K (2013) Signaling by the phosphoinositide 3-kinase family in immune cells. Annual Review of Immunology 31:675-704.

Park H, Poo MM (2013) Neurotrophin regulation of neural circuit development and function. Nat Rev Neurosci 14:7-23.

Pasternak GW (2014) Opioids and their receptors: Are we there yet? Neuropharmacology 76 Pt B:198-203.

Paulson PE, Camp DM, Robinson TE (1991) Time course of transient behavioral depression and persistent behavioral sensitization in relation to regional brain monoamine concentrations during amphetamine withdrawal in rats. Psychopharmacology 103:480-492.

Paxinos G, Watson C (2007) The Rat Brain in Stereotaxic Coordinates. Amsterdam, The Netherlands: Elsevier Science.

Pearce LR, Komander D, Alessi DR (2010) The nuts and bolts of AGC protein kinases. Nature Reviews: Molecular Cell Biology 11:9-22.

Perrotti LI, Hadeishi Y, Ulery PG, Barrot M, Monteggia L, Duman RS, Nestler EJ (2004) Induction of deltaFosB in reward-related brain structures after chronic stress. The Journal of Neuroscience : The official journal of the Society for Neuroscience 24:10594-10602.

Piazza PV, Le Moal ML (1996) Pathophysiological basis of vulnerability to drug abuse: role of an interaction between stress, glucocorticoids, and dopaminergic neurons. Annual Review of Pharmacology and Toxicology 36:359-378.

Pierce RC, Kumaresan V (2006) The mesolimbic dopamine system: the final common pathway for the reinforcing effect of drugs of abuse? Neuroscience and Biobehavioral Reviews 30:215-238.

Pierce RC, Pierce-Bancroft AF, Prasad BM (1999) Neurotrophin-3 contributes to the initiation of behavioral sensitization to cocaine by activating the Ras/Mitogen-activated protein kinase signal transduction cascade. J Neurosci 19:8685-8695.

Pitchers KK, Coppens CM, Beloate LN, Fuller J, Van S, Frohmader KS, Laviolette SR, Lehman MN, Coolen LM (2014) Endogenous opioid-induced neuroplasticity of dopaminergic neurons in the ventral tegmental area influences natural and opiate reward. J Neurosci 34:8825-8836.

Polakiewicz RD, Schieferl SM, Gingras AC, Sonenberg N, Comb MJ (1998) mu-Opioid receptor activates signaling pathways implicated in cell survival and translational control. The Journal of Biological Chemistry 273:23534-23541.

Pradhan AA, Smith ML, Kieffer BL, Evans CJ (2012) Ligand-directed signalling within the opioid receptor family. British Journal of Pharmacology 167:960-969.

Pulliam JV, Dawaghreh AM, Alema-Mensah E, Plotsky PM (2010) Social defeat stress produces prolonged alterations in acoustic startle and body weight gain in male Long Evans rats. Journal of Psychiatric Research 44:106-111.

Razzoli M, Carboni L, Arban R (2009) Alterations of behavioral and endocrinological reactivity induced by 3 brief social defeats in rats: relevance to human psychopathology. Psychoneuroendocrinology 34:1405-1416.

Ren X, Lutfy K, Mangubat M, Ferrini MG, Lee ML, Liu Y, Friedman TC (2013) Alterations in phosphorylated CREB expression in different brain regions following short- and long-term morphine exposure: relationship to food intake. Journal of Obesity 2013:764742.

Robison AJ, Vialou V, Mazei-Robison M, Feng J, Kourrich S, Collins M, Wee S, Koob G, Turecki G, Neve R, Thomas M, Nestler EJ (2013) Behavioral and structural responses to chronic cocaine require a feedforward loop involving DeltaFosB and calcium/calmodulin-dependent protein kinase II in the nucleus accumbens shell. The Journal of Neuroscience: The official journal of the Society for Neuroscience 33:4295-4307.

Rodgers RJ (1995) Neuropharmacological aspects of adaptive pain inhibition in murine “victims” of aggression. Aggressive Behavior 21:29-39.

Russo SJ, Bolanos CA, Theobald DE, DeCarolis NA, Renthal W, Kumar A, Winstanley CA, Renthal NE, Wiley MD, Self DW, Russell DS, Neve RL, Eisch AJ, Nestler EJ (2007) IRS2-Akt pathway in midbrain dopamine neurons regulates behavioral and cellular responses to opiates. Nature Neuroscience 10:93-99.

Saidak Z, Blake-Palmer K, Hay DL, Northup JK, Glass M (2006) Differential activation of G-proteins by mu-opioid receptor agonists. British Journal of Pharmacology 147:671-680.

SAMHSA (2014) Results from the 2013 National Survey on Drug Use and Health: Summary of National Findings. pp 1-170 Rockville, MD, USA: Substance Abuse and Mental Health Services Administration.

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307:1098-1101.

Scheid MP, Woodgett JR (2001) PKB/AKT: functional insights from genetic models. Nature Reviews: Molecular Cell Biology 2:760-768.

Segall MA, Margules DL (1989) Central mediation of naloxone-induced anorexia in the ventral tegmental area. Behavioral Neuroscience 103:857-864.

Seroogy KB, Lundgren KH, Tran TM, Guthrie KM, Isackson PJ, Gall CM (1994) Dopaminergic neurons in rat ventral midbrain express brain-derived neurotrophic factor and neurotrophin-3 mRNAs. The Journal of Comparative Neurology 342:321-334.

Serra V, Markman B, Scaltriti M, Eichhorn PJ, Valero V, Guzman M, Botero ML, Llonch E, Atzori F, Di Cosimo S, Maira M, Garcia-Echeverria C, Parra JL, Arribas J, Baselga J (2008) NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. Cancer Research 68:8022-8030.

Sesack SR, Grace AA (2010) Cortico-Basal Ganglia reward network: microcircuitry. Neuropsychopharmacology : Official publication of the American College of Neuropsychopharmacology 35:27-47.

Sesack SR, Pickel VM (1995) Ultrastructural relationships between terminals immunoreactive for enkephalin, GABA, or both transmitters in the rat ventral tegmental area. Brain Res 672:261-275.

Shank EJ, Seitz PK, Bubar MJ, Stutz SJ, Cunningham KA (2007) Selective ablation of GABA neurons in the ventral tegmental area increases spontaneous locomotor activity. Behavioral Neuroscience 121:1224-1233.

Siegfried B, Frischknecht HR, Nunes de Souza RL (1990) An ethological model for the study of activation and interaction of pain, memory and defensive systems in the attacked mouse. Role of endogenous opioids. Neuroscience and Biobehavioral Reviews 14:481-490.

Sinha R (2001) How does stress increase risk of drug abuse and relapse? Psychopharmacology 158:343-359.

Sinha R (2008) Chronic stress, drug use, and vulnerability to addiction. Annals of the New York Academy of Sciences 1141:105-130.

Sinha R (2011) New findings on biological factors predicting addiction relapse vulnerability. Curr Psychiatry Rep 13:398-405.

Sinha R, Catapano D, O'Malley S (1999) Stress-induced craving and stress response in cocaine dependent individuals. Psychopharmacology 142:343-351.

Sinha R, Fuse T, Aubin LR, O'Malley SS (2000) Psychological stress, drug-related cues and cocaine craving. Psychopharmacology 152:140-148.

Solecki W, Krowka T, Kubik J, Kaczmarek L, Przewlocki R (2008) Increased analgesic tolerance to acute morphine in fosB knock-out mice: a gender study. Pharmacol Biochem Behav 90:512-516.

Staal SP (1987) Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proceedings of the National Academy of Sciences of the United States of America 84:5034-5037.

Staal SP, Hartley JW, Rowe WP (1977) Isolation of transforming murine leukemia viruses from mice with a high incidence of spontaneous lymphoma. Proceedings of the National Academy of Sciences of the United States of America 74:3065-3067.

Steffensen SC, Stobbs SH, Colago EE, Lee RS, Koob GF, Gallegos RA, Henriksen SJ (2006) Contingent and non-contingent effects of heroin on mu-opioid receptor-containing ventral tegmental area GABA neurons. Experimental Neurology 202:139-151.

Stephens L, Anderson K, Stokoe D, Erdjument-Bromage H, Painter GF, Holmes AB, Gaffney PR, Reese CB, McCormick F, Tempst P, Coadwell J, Hawkins PT (1998) Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. Science 279:710-714.

Swanson LW (1982) The projections of the ventral tegmental area and adjacent regions: a combined fluorescent retrograde tracer and immunofluorescence study in the rat. Brain Res Bull 9:321-353.

Teskey GC, Kavaliers M (1991) Modifications of social conflict-induced analgesic and activity responses in male mice receiving chronic opioid agonist and antagonist treatments. Pharmacology, Biochemistry, and Behavior 38:485-493.

Thomas MJ, Kalivas PW, Shaham Y (2008) Neuroplasticity in the mesolimbic dopamine system and cocaine addiction. British Journal of Pharmacology 154:327-342.

Tidey JW, Miczek KA (1996) Social defeat stress selectively alters mesocorticolimbic dopamine release: an in vivo microdialysis study. Brain Res 721:140-149.

Ting AKR, van der Kooy D (2012) The neurobiology of opiate motivation. Cold Spring Harbor Perspectives in Medicine 2.

Ting AKR, Vargas-Perez H, Mabey JK, Shin SI, Steffensen SC, van der Kooy D (2013) Ventral tegmental area GABA neurons and opiate motivation. Psychopharmacology 227:697-709.

Trescot AM, Datta S, Lee M, Hansen H (2008) Opioid pharmacology. Pain Physician 11:S133-153.

Trigo JM, Martin-Garcia E, Berrendero F, Robledo P, Maldonado R (2010) The endogenous opioid system: a common substrate in drug addiction. Drug and Alcohol Dependence 108:183-194.

Ulery PG, Rudenko G, Nestler EJ (2006) Regulation of DeltaFosB stability by phosphorylation. The Journal of Neuroscience: The official journal of the Society for Neuroscience 26:5131-5142.

UNODC (2014) World Drug Report 2014. pp 1-197: United Nations Office on Drugs and Crime.

USDOJ (2011) The Economic Impact of Illicit Drug Use on American Society, 2011. pp 1-108 Washington D.C., USA: Department of Justice, National Drug Intelligence Center.

van der Poel AM, Miczek KA (1991) Long ultrasonic calls in male rats following mating, defeat and aversive stimulation: Frequency modulation and bout structure. Behavior 119:137-142.

Van Ree JM, Niesink RJ, Van Wolfswinkel L, Ramsey NF, Kornet MM, Van Furth WR, Vanderschuren LJ, Gerrits MA, Van den Berg CL (2000) Endogenous opioids and reward. European Journal of Pharmacology 405:89-101.

van Zessen R, Phillips JL, Budygin EA, Stuber GD (2012) Activation of VTA GABA neurons disrupts reward consumption. Neuron 73:1184-1194.

Vander Haar E, Lee SI, Bandhakavi S, Griffin TJ, Kim DH (2007) Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nature Cell Biology 9:316-323.

Vanderschuren LJ, Kalivas PW (2000) Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: A critical review of preclinical studies. Psychopharmacology 151:99-120.

Vanderschuren LJ, Niesink RJ, Van Ree JM (1997) The neurobiology of social play behavior in rats. Neuroscience and Biobehavioral Reviews 21:309-326.

Vanderschuren LJ, Stein EA, Wiegant VM, Van Ree JM (1995) Social isolation and social interaction alter regional brain opioid receptor binding in rats. European Neuropsychopharmacology: The journal of the European College of Neuropsychopharmacology 5:119-127.

Vargas-Perez H, Bahi A, Bufalino MR, Ting AKR, Maal-Bared G, Lam J, Fahmy A, Clarke L, Blanchard JK, Larsen BR, Steffensen S, Dreyer JL, van der Kooy D (2014) BDNF signaling in the VTA links the drug-dependent state to drug withdrawal aversions. The Journal of Neuroscience: The official journal of the Society for Neuroscience 34:7899-7909.

Vargas-Perez H, Ting AKR, van der Kooy D (2009a) Different neural systems mediate morphine reward and its spontaneous withdrawal aversion. The European Journal of Neuroscience 29:2029-2034.

Vargas-Perez H, Ting AKR, Walton CH, Hansen DM, Razavi R, Clarke L, Bufalino MR, Allison DW, Steffensen SC, van der Kooy D (2009b) Ventral tegmental area BDNF induces an opiate-dependent-like reward state in naive rats. Science 324:1732-1734.

Venzala E, García-García AL, Elizalde N, Delagrange P, Tordera RM (2012) Chronic social defeat stress model: behavioral features, antidepressant action, and interaction with biological risk factors. Psychopharmacology 224:313-325.

Vezina P, Giovino AA, Wise RA, Stewart J (1989) Environment-specific cross-sensitization between the locomotor activating effects of morphine and amphetamine. Pharmacology, Biochemistry, and Behavior 32:581-584.

Vezina P, Kalivas PW, Stewart J (1987) Sensitization occurs to the locomotor effects of morphine and the specific μ opioid receptor agonist, DAGO, administered repeatedly to the ventral tegmental area but not to the nucleus accumbens. Brain Research 417:51-58.

Vezina P, Stewart J (1989) The effect of dopamine receptor blockade on the development of sensitization to the locomotor activating effects of amphetamine and morphine. Brain Res 499:108-120.

Vialou V, Feng J, Robison AJ, Ku SM, Ferguson D, Scobie KN, Mazei-Robison MS, Mouzon E, Nestler EJ (2012) Serum response factor and cAMP response element binding protein are both required for cocaine induction of DeltaFosB. The Journal of Neuroscience: The official journal of the Society for Neuroscience 32:7577-7584.

Vivian JA, Miczek KA (1999) Interactions between social stress and morphine in the periaqueductal gray: effects on affective vocal and reflexive pain responses in rats. Psychopharmacology 146:153-161.

Waldhoer M, Bartlett SE, Whistler JL (2004) Opioid receptors. Annual Review of Biochemistry 73:953-990.

Walsh JJ, Friedman AK, Sun H, Heller EA, Ku SM, Juarez B, Burnham VL, Mazei-Robison MS, Ferguson D, Golden SA, Koo JW, Chaudhury D, Christoffel DJ, Pomeranz L, Friedman JM, Russo SJ, Nestler EJ, Han MH (2014) Stress and CRF gate neural activation of BDNF in the mesolimbic reward pathway. Nature Neuroscience 17:27-29.

Walsh JJ, Han MH (2014) The heterogeneity of ventral tegmental area neurons: Projection functions in a mood-related context. Neuroscience 282C:101-108.

Wang J, Bina RW, Wingard JC, Terwilliger EF, Hammer RP, Jr., Nikulina EM (2014) Knockdown of tropomyosin-related kinase B receptor expression in the nucleus accumbens shell prevents intermittent social defeat stress-induced cross-sensitization to amphetamine in rats. The European Journal of Neuroscience 39:1009-1017.

Wang J, Fanous S, Terwilliger EF, Bass CE, Hammer RP, Jr., Nikulina EM (2013) BDNF Overexpression in the Ventral Tegmental Area Prolongs Social Defeat Stress-induced Cross-Sensitization to Amphetamine and Increases DeltaFosB Expression in Mesocorticolimbic Regions of Rats. Neuropsychopharmacology: Official publication of the American College of Neuropsychopharmacology 38:2286-2296.

Wang Q, Liu L, Pei L, Ju W, Ahmadian G, Lu J, Wang Y, Liu F, Wang YT (2003) Control of synaptic strength, a novel function of Akt. Neuron 38:915-928.

Wang Z, Bilsky EJ, Porreca F, Sadee W (1994) Constitutive mu opioid receptor activation as a regulatory mechanism underlying narcotic tolerance and dependence. Life Sciences 54:PL339-350.

Williams JL, Worland PD, Smith MG (1990) Defeat-induced hypoalgesia in the rat: effects of conditioned odors, naltrexone, and extinction. Journal of Experimental Psychology: Animal Behavior Processes 16:345-357.

Williams JT, Christie MJ, Manzoni O (2001) Cellular and synaptic adaptations mediating opioid dependence. Physiological Reviews 81:299-343.

Winstanley CA, Green TA, Theobald DE, Renthal W, LaPlant Q, DiLeone RJ, Chakravarty S, Nestler EJ (2009) DeltaFosB induction in orbitofrontal cortex potentiates locomotor sensitization despite attenuating the cognitive dysfunction caused by cocaine. Pharmacology, Biochemistry, and Behavior 93:278-284.

Wu J, McCallum SE, Glick SD, Huang Y (2011) Inhibition of the mammalian target of rapamycin pathway by rapamycin blocks cocaine-induced locomotor sensitization. Neuroscience 172:104-109.

Xia Y, Driscoll JR, Wilbrecht L, Margolis EB, Fields HL, Hjelmstad GO (2011) Nucleus accumbens medium spiny neurons target non-dopaminergic neurons in the ventral tegmental area. The Journal of Neuroscience: The official journal of the Society for Neuroscience 31:7811-7816.

Xu J, Tian W, Ma X, Guo J, Shi Q, Jin Y, Xi J, Xu Z (2011) The molecular mechanism underlying morphine-induced Akt activation: roles of protein phosphatases and reactive oxygen species. Cell Biochemistry and Biophysics 61:303-311.

Yamaguchi T, Sheen W, Morales M (2007) Glutamatergic neurons are present in the rat ventral tegmental area. The European Journal of Neuroscience 25:106-118.

Yap JJ, Chartoff EH, Holly EN, Potter DN, Carlezon WA, Jr., Miczek KA (2014) Social defeat stress-induced sensitization and escalated cocaine self-administration: the role of ERK signaling in the rat ventral tegmental area. Psychopharmacology.

Zachariou V, Bolanos CA, Selley DE, Theobald D, Cassidy MP, Kelz MB, Shaw-Lutchman T, Berton O, Sim-Selley LJ, Dileone RJ, Kumar A, Nestler EJ (2006) An essential role for DeltaFosB in the nucleus accumbens in morphine action. Nature Neuroscience 9:205-211.

Zadina JE, Hackler L, Ge LJ, Kastin AJ (1997) A potent and selective endogenous agonist for the mu-opiate receptor. Nature 386:499-502.

Zhang H, Torregrossa MM, Jutkiewicz EM, Shi YG, Rice KC, Woods JH, Watson SJ, Ko MC (2006) Endogenous opioids upregulate brain-derived neurotrophic factor mRNA through delta- and mu-opioid receptors independent of antidepressant-like effects. The European Journal of Neuroscience 23:984-994.

Zhang Y, Landthaler M, Schlussman SD, Yuferov V, Ho A, Tuschl T, Kreek MJ (2009) Mu opioid receptor knockdown in the substantia nigra/ventral tegmental area by synthetic small interfering RNA blocks the rewarding and locomotor effects of heroin. Neuroscience 158:474-483.

Zhou L, Hammer RP, Jr. (1995) Gonadal steroid hormones upregulate medial preoptic μ-opioid receptors in the rat. European Journal of Pharmacology 278:271-274.

Zollner C, Stein C (2007) Opioids. Handbook of Experimental Pharmacology 31-63.

APPENDIX A

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APPENDIX A:

KNOCKDOWN OF VENTRAL TEGMENTAL AREA MU-OPIOID RECEPTORS IN RATS PREVENTS EFFECTS OF SOCIAL DEFEAT STRESS: IMPLICATIONS FOR AMPHETAMINE CROSS-SENSITIZATION, SOCIAL AVOIDANCE, WEIGHT REGULATION, AND EXPRESSION OF BRAIN-DERIVED NEUROTROPHIC FACTOR

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Abstract:

Social defeat stress causes social avoidance and long-lasting cross-sensitization to psychostimulants, both of which are associated with increased brain-derived neurotrophic factor (BDNF) expression in the ventral tegmental area (VTA). Moreover, social stress upregulates VTA mu-opioid receptor (MOR) mRNA. In the VTA, MOR activation inhibits GABA neurons to disinhibit VTA dopamine neurons, thus providing a role for VTA MORs in the regulation of psychostimulant sensitization. The present study determined the effect of lentivirus-mediated MOR knockdown in the VTA on the consequences of intermittent social defeat stress, a salient and profound stressor in humans and rodents. Social stress exposure induced social avoidance and attenuated weight gain in animals with non-manipulated VTA MORs, but both these effects were prevented by VTA MOR knockdown. Rats with non-manipulated VTA MOR expression exhibited cross-sensitization to amphetamine challenge (1.0 mg/kg, i.p.), evidenced by a significant augmentation of locomotion. By contrast, knockdown of VTA MORs prevented stress-induced cross-sensitization without blunting the locomotor-activating effects of amphetamine. At the time point corresponding to amphetamine challenge, immunohistochemical analysis was performed to examine the effect of stress on VTA BDNF expression. Prior stress exposure increased VTA BDNF expression in rats with non-manipulated VTA MOR expression, while VTA MOR knockdown prevented stress-induced expression of VTA BDNF. Taken together, these results suggest that upregulation of VTA MOR is necessary for the behavioral and biochemical changes induced by social defeat stress. Elucidating VTA MOR regulation of stress effects on the mesolimbic system may provide new therapeutic targets for treating stress-induced vulnerability to substance abuse.

Key Words:

mu-opioid receptor, social defeat stress, ventral tegmental area, cross-sensitization, brain-derived neurotrophic factor, amphetamine

Highlights:

- VTA MORs are necessary for social stress-induced weight gain and behavior deficits
- VTA MOR knockdown prevented cross-sensitization to amphetamine after social defeat
- VTA MOR knockdown prevented social stress-induced increase of VTA BDNF

Abbreviations:

- [^{3}H]DAMGO - tritiated [D-Ala^{2},*N*-MePhe^{4},Gly-ol^{5}] enkephalin
- BDNF – brain-derived neurotrophic factor
- CREB – cAMP responsive binding element protein
- DA – dopamine
- fr – fasciculus retroflexus
- GABA – gamma-aminobutyric acid
- GFP – green fluorescent protein
- MOR – mu-opioid receptor
- ml – medial lemniscus
- MT – medial terminal nucleus of the accessory optic system
- pCREB – phosphorylated cAMP responsive binding element protein
- shMOR – short hairpin mu-opioid receptor lentiviral construct
- SNc – substantia nigra pars compacta
- SNr – substantia nigra pars reticulata
- VTA – ventral tegmental area

1. Introduction

In humans, stress is one variable that influences the transition from recreational drug use to abuse, and it has been correlated with increased risk of substance abuse and relapse (Sinha, 2001, 2008, 2011). Rodent studies have shown that repeated social defeat stress exposure consistently produces social avoidance (Berton et al., 2006, Krishnan et al., 2007, Razzoli et al., 2009, Fanous et al., 2011b, Komatsu et al., 2011) and augments the effect of psychomotor stimulants, a phenomena known as 'cross-sensitization' (Covington and Miczek, 2001, Nikulina et al., 2004, Nikulina et al., 2012). Genetic mu-opioid receptor (MOR) knockout mice do not exhibit social avoidance following continuous social defeat (Komatsu et al., 2011), suggesting that MORs play a critical role in stress-induced changes in long-term neuroplasticity. In fact, even acute social defeat stress has been shown to rapidly upregulate MOR mRNA expression in the ventral tegmental area (VTA; Nikulina et al., 1999), while repeated social stress exposure increases VTA MOR mRNA expression for up to 14 days after the last episode (Nikulina et al., 2008). In the VTA MORs are expressed by gamma-aminobutyric acid (GABA) neurons (Sesack and Pickel, 1995, Garzon and Pickel, 2002), which are hyperpolarized in response to MOR stimulation, thus disinhibiting local dopamine (DA) transmission and facilitating response to drugs of abuse (Johnson and North, 1992, Bergevin et al., 2002, Vargas-Perez et al., 2009b, Dacher and Nugent, 2011). Rats exposed to repeated social defeat stress, then challenged with an intra-VTA infusion of a MOR-specific agonist exhibited sensitized locomotor activity (Nikulina et al., 2005, Nikulina et al., 2008). This VTA opiate-induced sensitized locomotor activity was present at the same time point that social stress-induced cross-sensitization to psychomotor stimulants was observed (Covington and Miczek, 2001, Nikulina et al., 2004, Nikulina et al., 2012). Taken together, these findings indicate that increased VTA MOR expression might play a role in social stress-induced psychostimulant sensitization. Consistent with this view, MOR knockout mice exhibit reduced cocaine self-administration and increased VTA GABA transmission (Mathon et al., 2005). Furthermore, the expression of amphetamine sensitization is associated with persistent VTA MOR upregulation, and can be blocked by a treatment with MOR antagonist (Magendzo and Bustos, 2003, Trigo et al., 2010).

Increased expression of brain-derived neurotrophic factor (BDNF) in the VTA is frequently observed as a consequence of psychostimulant administration (Horger et al., 1999, Grimm et al., 2003, Bolanos and Nestler, 2004, Corominas et al., 2007, Thomas et al., 2008). Studies with morphine have shown that an interaction exists between VTA BDNF and MORs (Chu et al., 2007, Vargas-Perez et al., 2009b, Koo et al., 2012). Additionally, increased VTA BDNF expression has been implicated as a long-term mediator of social stress-induced cross-sensitization (Nikulina et al., 2012), and in the VTA this increase persists for at least 2 weeks after the last social stress exposure (Berton et al., 2006, Fanous et al., 2010, Nikulina et al., 2012). In particular, overexpression of VTA BDNF was observed to exacerbate social stress-induced cross-sensitization to amphetamine (Wang et al., 2013), while viral deletion of VTA BDNF prevented social stress-induced social avoidance (Berton et al., 2006, Krishnan et al., 2007, Fanous et al., 2011b). Although VTA MOR mRNA expression rapidly increases following social stress exposure (Nikulina et al., 2005, Nikulina et al., 2008), VTA BDNF expression is affected more slowly (Fanous et al., 2010). Based on the modulatory relationship that exists between VTA MORs and BDNF (Chu et al., 2007, Vargas-Perez et al., 2009b, Koo et al., 2012), it is possible that intermittent social defeat stress-induced increases of VTA BDNF are related to MOR upregulation in this brain region.

Although research has implicated VTA MORs in drug sensitization and social behaviors (Van Ree et al., 2000, Miczek et al., 2011b, Lutz and Kieffer, 2013a, b, Pitchers et al., 2014), it is unknown whether upregulation of VTA MORs causes the behavioral and biological effects of social defeat stress exposure. To address this question, the present study used lentivirus-mediated gene transfer and RNA interference to knockdown MORs in the VTA, and then assessed social stress-induced cross-sensitization to amphetamine and BDNF expression in the VTA. Given that social avoidance is altered in MOR knockout mice after continuous social stress (Komatsu et al., 2011), we also examined the effect of VTA MOR knockdown on social avoidance after stress exposure. Finally, the effect of VTA MOR knockdown on stress-induced deficits of weight gain was examined.

2. Experimental Methods

2.1 Subjects

Experimental animals were male Sprague-Dawley rats (N = 71; Charles River Laboratories, Hollister, CA) weighing 200-250 g on arrival. Three days before social stress exposure, subjects were individually housed in standard plastic cages (25x50x20 cm^3). Twelve additional age-matched Sprague Dawley rats were group-housed 3 per cage and served solely as novel stimulus subjects during the social approach and avoidance test. Male Long-Evans rats (weighing 550-700 g), termed 'residents', were pair-housed with a tubal-ligated female in large plastic cages (37x50x20 cm^3). All rats were maintained on a 12-12 reverse light-dark cycle (lights out at 0900 h) with free access to food (Purina Rodent Diet, Brentwood, MO) and water. Residents were previously screened for aggressive behavior and were used to induce social defeat stress in experimental "intruder" rats. All experimental procedures were approved by the Institutional Animal Care and Use Committees at the Arizona State University and the University of Arizona. All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), and every effort was made to minimize pain and suffering, as well as the number of animals used.

2.2 Experimental Design

2.2.1 General Procedure

Upon arrival, experimental rats were habituated to laboratory conditions for 7 days before surgery to manipulate regional MOR level. Rats were randomly assigned to one of four experimental conditions: Non-Manipulated MOR+Handled, Non-Manipulated MOR+Stressed, MOR Knockdown+Handled, MOR Knockdown+Stressed. Three experiments were conducted in parallel (Fig 1A); one group of subjects (n = 25) received an amphetamine challenge 10 days after the last episode of intermittent social stress or handling to study the effects of VTA MOR knockdown on social stress-induced cross-sensitization. Seven days later, VTA tissue from this group of subjects was flash frozen for radioligand binding to verify the efficacy of MOR knockdown. A second group of drug-naïve subjects (n = 21) were perfused at the same time point

after stress or handling to quantify VTA BDNF expression. Social approach and avoidance testing was performed two days after termination of social stress or handling procedures in both these groups. The third group of rats (n = 25) were weighed prior to each episode of intermittent social stress and handling, and again 10 days later to investigate the influence of VTA MOR knockdown on social stress-induced deficits in weight gain.

2.2.2 Bilateral VTA infusion of lentiviral constructs

Rats assigned to control viral groups received infusions of lentivirus that expresses green fluorescent protein (GFP) and a short hairpin RNA (shRNA) that does not target any known rat gene, while rats assigned to VTA MOR knockdown groups received a lentivirus that expresses GFP and a shRNA that targets MOR (shMOR) for RNA interference. Lentiviral constructs were prepared as previously described (Lasek et al., 2007). The shMOR lentivirus reduces VTA MOR expression by 88-97% (Lasek et al., 2007). Therefore, the viral titre was diluted by 50% with cold sterile saline to reduce the efficacy. After random assignment to GFP or shMOR knockdown conditions, rats were anesthetized using isoflurane and positioned in a stereotaxic frame (Leica Angle Two; Richmond, IL). The appropriate lentiviral construct (1.0 µl each) was infused bilaterally into the VTA (AP -5.15, ML ±2.15, DV -8.7, Tilt 10°; Paxinos and Watson, 2007) at a flow rate of 0.1 µl/min, and allowed to diffuse for 10 min before withdrawal of the syringe (Hamilton; Model 7105 KH; 24 gauge tip; Reno, NV) . The accuracy of each infusion was later verified using localization of GFP expression. Subjects were given 7 days to recover before the start of intermittent social stress or handling procedures (Fig. 1A).

2.2.3 Intermittent social defeat stress and handling procedures

Social defeat stress was induced by a short confrontation between an aggressive resident and an experimental intruder rat, as previously described (Tidey and Miczek, 1996, Nikulina et al., 2004, Nikulina et al., 2012). After removing the female from the resident's home cage, an experimental rat was placed inside the resident's home cage for 5 min within the confines of a protective metal cage (15x25x15 cm^3). The protective cage was then removed,

allowing the resident to attack the experimental intruder rat until it displayed supine posture for at least 4 sec. Once submissive posture was exhibited, the experimental rat was placed back in the protective cage and exposed to threat from the resident for an additional 20 min before being returned to its own home cage. Intermittent social stress procedures were administered every third day for 10 days (Fig. 1A). At each corresponding time point, rats in the control groups were handled for approximately 2.5 min and then returned to their home cages.

2.3 Behavioral assessments

2.3.1 Social interaction

The social approach and avoidance test was conducted in a large plastic container (58x38x41 cm^3) equipped with a lightweight containment cage. Experimental rats were habituated to the empty test chamber for 5 min, then reintroduced when a novel stimulus rat was within the containment cage (Fig. 1B). The behavior of experimental rat was recorded for 5 min using TopScan (Clever Systems Inc.; Reston, VA). The software divided the chamber into virtual zones: Interaction, which comprised the area surrounding the containment cage, and Avoidance, which comprised the two corners opposite the containment cage (Fig. 1B; arena adapted from Berton et al., 2006). The number of respective entries into the avoidance and interaction zones was recorded, as was the distance (cm) moved in each zone.

2.3.2 Amphetamine challenge

A low dose d-amphetamine challenge was administered to test for social stress-induced cross-sensitization (Nikulina et al., 2004, Nikulina et al., 2012). For two days prior to the challenge, rats were injected with vehicle (0.9% sterile saline; 1.0 ml/kg, i.p.), and were acclimated in their home cage to the procedure room for 1 h. On the day of the challenge, rats were moved in their home cage to the procedure room, and locomotor activity was recorded at 10 min intervals using video tracking software (Videotrack, Viewpoint Life Sciences; Montreal, Canada). Locomotor activity was detected as the number of and distance travelled during movements (>10 cm) across 170 min consisting of 3 phases: Baseline, Saline, and

Amphetamine. Baseline data were recorded for 30 min, after which a saline injection (1.0 ml/kg, i.p.) was given and locomotor activity was recorded for 60 min. Finally, rats received an injection of d-amphetamine sulfate (1.0 mg/kg, i.p.; Sigma-Aldrich; St. Louis, MO), and locomotor data were recorded for 80 min. Video tracking and data collection were paused during the administration of saline and amphetamine injections. Rather than stereotypical behaviors, this dose of amphetamine has been shown to primarily induce large ambulatory movements (Geyer et al., 1987). In order to quantify amphetamine sensitization, ambulatory movements (> 10 cm) were measured in terms of the number of movements initiated and the distance travelled (cm) during such movements.

2.4 Tissue harvesting

2.4.1 Fresh frozen VTA tissue for radioligand binding

Rats were anesthetized with isoflurane, and their brains were rapidly removed and frozen in -35°C 2-methylbutane for 15 sec, then stored at -80°C prior to sectioning. On a cryostat, serial 20 µm sections through the VTA were collected (from AP -4.8 to -5.5; Paxinos and Watson, 2007) for radioligand binding and localization of GFP expression. Sections were thaw-mounted onto glass microslides (Superfrost Plus; Fisher Scientific; Waltham, MA), dried in a vacuum chamber at 4°C, and stored at -80°C prior to processing. Separate slides were used to verify the accuracy and distribution of lentiviral infusions based on fluorescent detection of GFP expression.

2.4.2 Perfused VTA tissue for BDNF immunohistochemistry

As previously described (Fanous et al., 2010, Fanous et al., 2011a, Fanous et al., 2011b), rats were anesthetized with sodium pentobarbital (100 mg/kg, i.p.; Euthasol, Virbac Co., St. Louis, MO) and perfused transcardially with 4% paraformaldehyde. Brains were then removed, post-fixed for 1.5 hours at 4°C, and placed in graded sucrose solutions. Frozen brain tissue was sectioned on a sliding microtome (20 µm) and serial VTA sections were mounted onto slides from 0.05 M phosphate buffer (pH = 7.4). Adjacent slides from each brain were processed for either BDNF immunohistochemistry or fluorescent localization of GFP expression.

2.5 [^{3}H]DAMGO autoradiography

2.5.1 Radioligand binding

Fresh frozen brain sections were used to verify shMOR knockdown in the VTA using tritiated [D-Ala^2,*N*-MePhe^4,Gly-ol^5] enkephalin ([^{3}H]DAMGO; NIDA Drug Supply Program; Bethesda, MD), as described by Zhou and Hammer (1995). Briefly, slides were placed in pre-incubation solution (15 mM Tris HCl, 150 mM NaCl, 1.0 mg/ml BSA) for 30 min at 4°C, then were incubated in 10 nM [^{3}H]DAMGO solution (50 mM Tris buffer, 3.0 mM Mn acetate, 1.0 mg/ml BSA) with or without the addition of naloxone (10 μM; NIDA Drug Supply Program) for 60 min at 22°C. Slides were washed with a 50 mM Tris buffer at 4°C, then dried and exposed on Kodak BIO Max MR X-ray film (Carestream; Sigma-Aldrich; St. Louis, MO) for 10 weeks at room temperature. Sections incubated in 1000-fold excess unlabeled naloxone were utilized to determine non-specific binding in subsequent autoradiography.

2.5.2 Autoradiography analysis

Autoradiograpy film was developed and scanned at high resolution. In order to determine whether the shMOR viral construct infected regions outside of the VTA, the substantia nigra pars compacta (SNc) was chosen as a control region due to its close proximity to the VTA, and because social stress does not affect MOR expression in substantia nigra regions (Nikulina et al., 1999, Nikulina et al., 2005). The SNc, not to be confused with the medial terminal nucleus accessory optic tract (MT), contains a higher density of MOR labeling than either the substantia nigra pars reticulata (SNr) or VTA (Herkenham and Pert, 1982). Using this difference in expression, the SNc could be clearly demarcated on scans of autoradiographs by measuring the area directly above the SNr, lateral to the MT, and ventrolateral to the medial lemniscus. Optical densities for these regions were measured bilaterally in 2-3 sections using ImageJ (National Institutes of Health, USA, http://imagej.nih.gov/ij), and then converted to μCi/g using calibrated [^{3}H] radiostandards (ART-123, ARC Inc.; St. Louis, MO) co-exposed with sections. For each subject, bilateral measurements were averaged across sections to yield total ligand binding in the VTA and SNc, respectively.

2.6 BDNF immunohistochemistry and quantification

2.6.1 Immunohistochemistry

Immunohistochemistry was performed using BDNF polyclonal antisera as described previously (Fanous et al., 2010). Briefly, blocking solution (10% normal goat serum/0.5M KPBS/0.4% Triton X-100) was applied to sections for 1 h at room temperature, then the primary antibody diluted in blocking solution (1:1000 dilution; SP1779, Millipore/Chemicon; Temecula, CA) was applied for 48 hr at 4°C. Sections were then incubated for 1 h with a biotinylated rabbit secondary antibody, treated with avidin/biotin complex solution for 45 min (Vectastain Elite ABC Kit; Vector Laboratories; Burlingame, CA), and developed using a diaminobenzidine (DAB) peroxidase substrate kit with nickel intensification (Vector Laboratories).

2.6.2 Modified stereological cell counts

Tissue sections were imaged using a Zeiss Axioskop with a 20x objective, and digitalized using a color digital camera. Immunolabeled cells were quantified using Stereo Investigator software (MBF Biosciences; Williston, VT), and the analysis was conducted using the modified stereology counting procedure described in Fanous et al. (2011a) and Nikulina et al. (2012). Briefly, a grid of 48 squares (0.0075 mm^2) was overlaid on each of 2-3 VTA sections from each subject. Immunolabeled cells were counted in half the grid squares, the precise squares being randomly determined. Cells exhibiting a black-blue reaction product indicative of immunolabeling were counted such that cells crossing the bottom or right lines of each square were included, while cells crossing the top or left lines of the square were excluded from analysis. For each subject, estimates of total labeling density (mm^2) were calculated by averaging the bilateral counts of labeled cell profiles across sections, and then dividing the total number of cell profiles by the total area assessed (0.18 mm^2).

2.7 Statistical analyses

The results of each measure are expressed as mean ± standard error (SEM) and a *p* value ≤ 0.05 was considered to be significant. All statistical analyses were run using SPSS

software, version 18 (SPSS Inc., Chicago, IL), and Tukey's HSD was considered the preferred *post hoc* test across experiments. An exception was made in the case of the amphetamine challenge, where Fisher's LSD was used because violations of sphericity necessitated our use of a more conservative test of the main effects. Data from subjects were excluded only in the case of error during video tracking or loss of data due to damaged tissue sections: no statistical outliers were excluded. The locomotor and social approach and avoidance assays relied on automated video tracking systems, requiring that the animals be housed in black bedding to block light from reflecting off the cage bottom. However in some instances, rats exposed the cage floor while moving, causing illumination artifacts that necessitated the removal of individual bin data due to inaccurate tracking. In addition, damage to tissue sections during processing sometimes precluded data collection from brain regions. More specifically in the locomotor and social approach and avoidance assays, which relied on automated video tracking systems, individual bin data were removed in those instances where reflection artifacts prevented accurate tracking. For analyses of mounted tissue sections, the sample size of each group was also reduced in cases where tissue was damaged in the course of processing.

2.7.1 Weight gain data

The initial weight obtained at the start of social stress procedures was used to normalize all subsequent data (n = 25) to weight gained from that time onward; no subjects were excluded from the analysis. A one-way analysis of variance (ANOVA) was performed to assess differences in weight at each time point, and all significant main effects were analyzed using Tukey's test for *post hoc* comparisons among the means.

2.7.2 Social interaction

Social approach and avoidance data were analyzed in terms of the number of entries to, and the distance travelled (cm) within the interaction and avoidance zones (Fig. 1B). Where illumination artifacts interfered with tracking, data were lost in a zone-specific manner. For example, avoidance zone entry data were analyzed from 40 subjects because illumination

artifacts resulted in the exclusion of subjects from the following groups: GFP-Handled: 1; GFP-Stressed: 3; shMOR-Handled: 2. For distance travelled in the avoidance zone, an additional tracking error which occurred after a subject entered the zone further reduced the number of analyzed subjects to 35; subjects were excluded from the following groups: GFP-Handled: 2; GFP-Stressed: 1; shMOR-Handled: 5; shMOR-Stressed: 3. Illumination artifacts and tracking error reduced the number of subjects in the interaction zone to 37; subjects were excluded from the following groups: GFP-Stressed: 3; shMOR-Handled: 4; shMOR-Stressed: 2. A one-way ANOVA was run on data pertaining to each zone and any significant main effects were followed by an analysis of *post hoc* comparisons with Tukey's test.

2.7.3 Locomotor activity

Locomotor data were analyzed using separate multivariate analysis of variance (MANOVA) for the mean number and distance (cm) travelled during ambulatory movements. In order to overcome violations of sphericity in the output of repeated measures ANOVA, MANOVA was used to analyze the number and distance of ambulatory movements exhibited throughout the amphetamine challenge. Significant multivariate effects were followed by univariate analyses to determine which time points produced significant group differences. Significant univariate effects were further analyzed for *post hoc* comparisons using Fisher's Least Significant Difference (LSD) test. Data were analyzed from 21 subjects for both dependent measures of ambulatory movements. Some subjects' data were excluded from analysis due to the presence of illumination artifacts that interfered with tracking: GFP-Stressed: 2; shMOR-Handled: 1; shMOR-Stressed: 1.

2.7.4 MOR binding and BDNF expression

The results of radioligand binding with [^{3}H]DAMGO in the VTA and SNc, as well as the results of BDNF immunohistochemistry in the VTA were analyzed using separate one-way ANOVAs, and where necessary, significant main effects were followed by *post hoc* comparisons with Tukey's test. In the case of VTA [^{3}H]DAMGO results, a violation of homogeneity was corrected for with Welch's F test. Sample sizes were reduced after the target region was

damaged during processing for BDNF immunohistochemistry in 2 subjects from the shMOR-Handled group, and during [^{3}H]DAMGO binding for 1 shMOR treated subject. Consequently, BDNF data were analyzed from 19 subjects, while receptor autoradiography was analyzed from 25 subjects in the VTA and 24 subjects in the SNc.

3. Results

3.1 Verification of MOR Knockdown Using [^{3}H]DAMGO Autoradiography

Fluorescent detection of virally expressed GFP revealed that lentiviral infusions were specific to the VTA (Fig. 2A), and GFP was not detected in either SN region (data not shown). While lentiviral constructs were infused at AP -5.15, GFP expression indicated infusions to the target site varied by ±0.1 mm, and that the average spread of GFP was within AP -4.8 to -5.5 and Lateral 0.4 mm to 1.4 mm (Fig. 2B). Quantitative in vitro autoradiography with [^{3}H]DAMGO was used to determine the functionality of VTA MORs after lentivirus-mediated knockdown. Compared to the control GFP lentiviral construct, the subjects infused with the shMOR construct showed reduced [^{3}H]DAMGO binding (Fig. 2C). One-way ANOVA revealed that this effect was significant in the VTA (n = 25, $F_{1,20.13} = 102.46$, $p < 0.0001$), but not the SNc (n = 24, $F_{1,22} = 1.63$, $p > 0.22$; Fig. 2D). Thus, our surgeries were accurate and bilateral shMOR knockdown selectively reduced VTA MOR binding density.

3.2 Effect of VTA MOR knockdown on intermittent social stress-induced deficit of weight gain

Weight gain data (n = 25) revealed a significant main effect during social stress exposure ($F_{3,21} = 10.15$, $p < 0.0003$; Fig. 3), and 10 days after the last stress episode ($F_{3,21} = 9.46$, $p < 0.0004$). *Post hoc* comparisons at this time point show that the GFP-Stressed group experienced less weight gain than either the GFP-Handled or shMOR-Handled groups ($p < 0.006$), while the shMOR-Stressed group only differed from the shMOR-Handled group ($p < 0.02$). Ten days after the final episode of social stress, the GFP-Stressed group showed significantly lower body weights compared not only to GFP-Handled and shMOR-Handled groups ($p \leq 0.006$), but also the shMOR-Stressed group ($p < 0.05$). These data suggest that social stress significantly reduces

body weight, and that while VTA MOR knockdown attenuated this effect during social stress exposure, it rescued this effect 10 days after termination of stress.

3.3 Effect of VTA MOR knockdown on intermittent social stress-induced social avoidance

The social approach and avoidance test revealed a main effect of experimental group on number of entries to the avoidance zone (n = 40, $F_{3,36}$ = 5.89, p = 0.002), with significantly more entries by GFP-Stressed rats compared to both GFP-Handled (p < 0.005) and shMOR-Stressed (p < 0.004) groups (Fig. 4A). Similarly, there was a significant main effect of experimental group on the distance traveled in the avoidance zone (n = 35, $F_{3,31}$ = 4.77, p = 0.008), with significantly more activity in the GFP-Stressed group than the GFP-Handled (p = 0.011), shMOR-Handled (p < 0.05), or shMOR-Stressed (p < 0.05) groups. There was no main effect of experimental group on the number of entries to the interaction zone (n = 37, $F_{3,26}$ = 1.14, p = 0.351; Fig. 4C). These data suggest that prior social stress exposure significantly increases social avoidance, and local VTA depletion of MOR prevents social stress-induced social avoidance without significantly altering social interaction.

3.4 Effect of VTA MOR knockdown on intermittent social stress-induced cross-sensitization

There were significant main effects of experimental group on the number of ambulatory movements (n = 21, *Wilks' λ* = 3.78×10^{-7}, $F_{51.0,17}$ = 10.57, p = 0.019, η^2 = 0.993, *observed power* = 0.87) and distance travelled during ambulatory movements(n = 21, *Wilks' λ* = 1.26×10^{-6}, $F_{51.0,17}$ = 7.03, p = 0.039, η^2 = 0.989, *observed power* = 0.87) across all time points. The number of movements differed significantly only, at 30 ($F_{3,17}$ = 3.66, p = 0.034), 40 ($F_{3,17}$ = 3.36, p = 0.043), and 50 ($F_{3,17}$ = 4.46, p = 0.017) min after amphetamine injection, but there were no differences across groups before or after saline injection (p > 0.05 at all other time points). *Post hoc* testing (Fig. 5A) showed that the GFP-Stressed group exhibited significantly greater number of movements compared to GFP-Handled (p < 0.005) and both shMOR-Handled and -Stressed groups (p < 0.05) at 30 min after amphetamine injection, compared to GFP-Handled (p < 0.01) 40

min post-amphetamine, and compared to GFP-Handled ($p < 0.002$) and both shMOR-Handled and -Stressed groups ($p < 0.03$) 50 min after amphetamine.

Similarly, distance travelled exhibited significant main effects only 20 ($F_{3,17} = 3.51$, $p = 0.038$), 30 ($F_{3,17} = 6.83$, $p = 0.003$), and 40 ($F_{3,17} = 4.86$, $p = 0.013$) min after amphetamine injection. *Post hoc* analyses (Fig. 5B) showed that the GFP-Stressed group moved a significantly greater distance compared to the GFP-Handled, shMOR-Handled, and shMOR-Stressed groups ($p < 0.02$) 20 min after amphetamine injection, compared to GFP-Handled and both shMOR groups ($p < 0.002$) 30 min post-injection, and compared to the GFP-Handled and both shMOR groups ($p < 0.03$) groups 40 min post-amphetamine. Thus, the GFP-Stressed group showed social stress-induced cross-sensitization following amphetamine challenge, but the shMOR-Stressed group did not.

3.5 Effect of VTA MOR knockdown on VTA BDNF after intermittent social stress exposure

There was a significant main effect of experimental group on VTA BDNF expression (n = 19, $F_{3,15} = 30.17$, $p < 0.0001$), such that the GFP-Stressed group had significantly greater VTA BDNF expression compared to GFP-Handled, shMOR-Handled, and shMOR-Stressed groups ($p < 0.0001$, Fig. 6). There were no significant differences between GFP-Handled and either of the shMOR groups, regardless of stress treatment ($p > 0.15$). Thus, social stress-induced increase of VTA BDNF expression is blocked by knockdown of MORs in the VTA.

4. Discussion

Our data show that lentivirus-mediated overexpression of shMOR successfully reduced MOR binding activity in the VTA, and that the affected region was limited to the VTA. Furthermore, we show that intermittent social stress induction of VTA MORs is required for various behavioral and biological changes. For example, we observed that lentivirus-mediated knockdown of VTA MORs blocks intermittent social stress-induced social avoidance, cross-sensitization to amphetamine, and deficit of weight gain, as well as the augmented VTA BDNF expression which normally persists 1-4 weeks after stress exposure.

4.1 VTA MOR upregulation is necessary for intermittent social stress-induced weight gain deficits

Exposure to social stress attenuated weight gain both during and 10 days after social stress exposure, which is consistent with previous findings (Meerlo et al., 1996, Pulliam et al., 2010, Fanous et al., 2011b, Venzala et al., 2012). VTA MOR knockdown rescued the deficit of weight gain 10 days after the last episode of stress, but not during stress exposure. That knockdown of VTA MORs attenuated and promoted recovery from social stress-induced weight gain deficit is consistent with a report of increased body weight in MOR *knockout* mice (Han et al., 2006). Another study using the same lentiviral construct in the VTA (Lasek et al., 2007) also showed no significant effect on weight, indicating that VTA MOR knockdown is not sufficient to alter weight gain in the absence of social stress.

The role of MORs in the regulation of food intake and weight gain is complex, making it difficult to separate MOR effects on food palatability, food intake, and a more general increase of hedonic value. Pharmacological stimulation of MORs has frequently been associated with increased hedonic value of food and drug stimuli (Badiani et al., 1995, Nathan and Bullmore, 2009), while MOR antagonism has been associated with decreased consumption of highly palatable food (Segall and Margules, 1989), as well as decreased sensitivity to natural reward (Pitchers et al., 2014). Stimulation of VTA MORs has been found to facilitate food consumption in a dopamine D_1 receptor-dependent manner (Badiani et al., 1995, MacDonald et al., 2004), while antagonism reduced consumption of palatable foods (Segall and Margules, 1989). Based on this, one might expect that VTA MOR knockdown would further reduce weight gain by altering feeding behaviors. By contrast, our data show that VTA MOR knockdown rescues the stress-induced deficit in weight gain without affecting normal weight gain.

If knockdown of VTA MORs rescued the stress-induced reduction of weight gain by attenuating the psychological effects of stress, one might expect to see signs of increased reward or hedonic value in the amphetamine challenge or social approach and avoidance test. However, compared to GFP-Handled rats, subjects in the shMOR-Handled group did not show increased, or impaired response to amphetamine, or differ in social interaction. That subjects with VTA MOR knockdown, regardless of stress treatment, did not exhibit significant differences in weight gain

compared to control GFP-Handled subjects, suggests that the rescue of weight gain is likely due to the prevention of downstream stress-induced changes in the mesolimbic circuit. In support of this idea, stress-induced increase of VTA BDNF expression was prevented by VTA MOR knockdown, and BDNF expression in the VTA is necessary for the stress-induced deficit of weight gain (Fanous et al., 2011b).

The current study did not measure food consumption, so we cannot ascertain whether altered food intake contributed to the weight gained after stress with or without VTA MOR knockdown. However, if the stress-induced deficit of weight gain were related to VTA MOR-mediated changes in food intake, one would expect both Handled- and Stressed-shMOR knockdown groups to show significant differences in weight gain compared to GFP-Handled rats, which was not the case. There is some evidence to suggest that that MOR activity can alter weight gain without producing deficits in food consumption. In particular, daily morphine injection for 8 days had no effect on weight gain or food intake, while a parallel group of subjects that received escalating doses of morphine exhibited reduced weight gain without significant any significant effect on food consumption (Ren et al., 2013). In the same study, injections of escalating doses of morphine led to activation of cAMP responsive binding element protein (pCREB) in the VTA, implicating this region in MOR-mediated reduction of weight gain, but not food intake. Based on this, it is possible that escalating endogenous mu-opioid activity in the VTA underlies the weight gain deficit seen after social stress.

4.2 Upregulation of VTA MORs is necessary for intermittent social stress-induced social avoidance

Rodents with non-manipulated VTA MORs and a history of social stress engaged in significantly more social avoidance (Berton et al., 2006, Fanous et al., 2010, Komatsu et al., 2011). However, MOR knockout mice do not show social avoidance after continuous social stress (Komatsu et al., 2011), just as our knockdown of VTA MORs prevented intermittent social stress-induced social avoidance. MORs have been implicated in the rewarding components of social behavior, while MOR antagonists are associated with reduced social play (Vanderschuren et al.,

1997) and experience-induced facilitation of sexual behavior (Pitchers et al., 2014), allowing for the possibility that VTA MOR knockdown might alter normal social interaction. However, our data reveal that VTA MOR knockdown in handled rats did not alter any measures of social interaction, suggesting that VTA MORs affect social behavior only upon the impact of stress exposure.

Previous research has also indicated that social history alone (isolation vs. social housing) or in conjunction with a social interaction test has a profound effect on MOR expression (Vanderschuren et al., 1995). Specifically, long-term social isolation increased MOR binding density in the VTA, while an acute social interaction reduced VTA MOR binding. Taken together, it is possible that positive and negative social situations alter VTA MOR expression, respectively decreasing or increasing VTA MOR activity.

4.3 Knockdown of VTA MORs prevents intermittent social stress-induced cross-sensitization

Stressed rats with non-manipulated VTA MORs exhibited significantly greater locomotor activity after a low dose amphetamine challenge, confirming prior reports that intermittent social stress induces amphetamine cross-sensitization 10 days after the last stress episode (Covington and Miczek, 2001, Nikulina et al., 2012). By contrast, knockdown of VTA MORs prevented social stress-induced cross-sensitization without blocking amphetamine-induced locomotion. VTA MORs are presynaptically expressed by GABA neurons (Sesack and Pickel, 1995, Garzon and Pickel, 2002), and when activated, reduce GABAergic inhibition of VTA DA neurons (Johnson and North, 1992, Bergevin et al., 2002, Vargas-Perez et al., 2009b, Trigo et al., 2010, Dacher and Nugent, 2011) and facilitate response to psychomotor stimulants . Thus, if stimulation of MORs in the VTA indirectly increases VTA DA activity by reducing GABA transmission, then it is likely that knockdown of VTA MORs increases GABA release. In fact, MOR knockout mice showed enhanced VTA GABA release onto local DA neurons, resulting in reduced cocaine self-administration (Mathon et al., 2005).

We observed that knockdown of VTA MORs did not block psychomotor activation following amphetamine challenge, even though treatment with a MOR antagonist has been shown to abolish amphetamine responses (Magendzo and Bustos, 2003). This suggests that

knockdown of VTA MORs does not produce unnatural alterations of mesolimbic tone. Our results reveal that VTA MOR upregulation is necessary for intermittent social stress-induced cross-sensitization to amphetamine. As such, in the VTA social stressors may function to increase endogenous MOR activity on GABA neurons, thus reducing the GABAergic inhibition of local DA neurons and facilitating behavioral sensitization to psychostimulant drugs.

4.4 VTA MORs are necessary for induction of VTA BDNF by intermittent social stress

The two-fold increase of VTA BDNF expression which we observed in the VTA is consistent with previous reports (Berton et al., 2006, Fanous et al., 2010, Nikulina et al., 2012). More importantly, we found that knockdown of VTA MORs prevents induction of VTA BDNF by social stress exposure. That knockdown of VTA MORs blocks social stress-induced increase of BDNF labeling suggests that VTA BDNF induction after social stress exposure is dependent on local MOR upregulation. In fact, increased MOR activity in hippocampus also induces local BDNF mRNA (Zhang et al., 2006). While others have suggested that VTA BDNF modulates the function of local MORs (Vargas-Perez et al., 2009b, Koo et al., 2012), we show herein that VTA MORs can regulate the local expression of BDNF. These reciprocal findings may be attributed to differences between exogenous opiate and endogenous opioid functions, as well by differences in the VTA input systems recruited by exposure to morphine and social stress.

Although VTA BDNF is predominantly thought to be found in DA neurons (Gall et al., 1992, Seroogy et al., 1994), it is possible that MORs may control the transmission of VTA GABA neurons to indirectly produce subsequent changes in local DA neurons. Specifically, if MOR activity on GABA neurons increases the excitability of local DA neurons (Mathon et al., 2005), then the subsequent reduction in VTA GABAergic tone allows for MORs to affect BDNF expression in VTA DA neurons, potentially by altering the intracellular signaling that regulates BDNF expression in DA, for example mitogen-activated protein kinase/extracellular regulated kinase (MAPK/ERK) or cAMP responsive binding element protein (CREB; Shieh and Ghosh, 1999, Lu et al., 2006, Covington et al., 2011, Russo and Nestler, 2013).

Previous studies have indicated that VTA BDNF is crucial for social stress-induced social avoidance (Berton et al., 2006, Fanous et al., 2011b) and cross-sensitization to amphetamine (Wang et al., 2013), and have shown that VTA MOR upregulation occurs early in the cross-sensitization process (Nikulina et al., 1999, Nikulina et al., 2005, Nikulina et al., 2008), prior to a persistent increase of VTA BDNF expression (Fanous et al., 2010). Thus it is likely that our VTA MOR knockdown prevented social stress-induced alterations of behavior by preventing increased BDNF expression as a consequence of MOR-dependent increases in VTA GABAergic tone. In support of this, and consistent with the preventative behavioral effects of our VTA MOR knockdown, is the study by Mathon et al. (2005), in which genetic MOR knockout mice showed increased GABAergic input onto local VTA DA neurons and decreased cocaine reinforcement. Thus it is likely that social stress upregulates MOR expression on VTA GABA neurons to facilitate BDNF expression in local DA neurons, while VTA MOR knockdown may increase VTA GABAergic tone, preventing subsequent social stressor-induced changes in the region.

4.5 Concluding remarks

In summary, knockdown of MORs in the VTA prevents intermittent social stress-induced cross-sensitization to amphetamine, social avoidance, deficit of weight gain, and increase of VTA BDNF expression. In rats, continuous social stress suppresses cocaine reward and decreases VTA BDNF expression (Miczek et al., 2011a), however it is unknown whether continuous social stress alters VTA MOR expression. It is possible that continuous social stress reduces cocaine reward and VTA BDNF expression as a function of downregulated VTA MOR expression, which would suggest that VTA MORs may mediate a switch between the sensitizing effects seen with intermittent social stress and the suppressed cocaine reward observed after continuous social stress.

The nucleus accumbens (NAc), with reciprocal projections to the VTA, has also been identified as a brain region crucial for the effects of stress, drugs of abuse, and food intake/palatability. Since VTA MOR knockdown likely functioned in the present study to block stress-induced increase of dopaminergic tone, this manipulation also might prevent stress-

induced changes in the NAc. Future studies are needed to determine whether VTA MOR knockdown alters stress-induced changes in the NAc, and the importance of NAc neurotransmission for stress-induced effects associated with the VTA.

In conclusion, our results indicate that social stress exposure increases VTA MOR activity, potentially disinhibiting VTA dopaminergic tone to facilitate response to drugs of abuse. The present data suggest that upregulation of VTA MORs following social stress exposure may underlie vulnerability to psychostimulant drugs in some individuals, thereby providing a potential target for therapeutic intervention during abuse of these drugs.

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References

Badiani, A., Leone, P., Noel, M. B., Stewart, J., 1995. Ventral tegmental area opioid mechanisms and modulation of ingestive behavior. Brain Res 670, 264-276.

Bergevin, A., Girardot, D., Bourque, M. J., Trudeau, L. E., 2002. Presynaptic mu-opioid receptors regulate a late step of the secretory process in rat ventral tegmental area GABAergic neurons. Neuropharmacology 42, 1065-1078.

Berton, O., McClung, C. A., Dileone, R. J., Krishnan, V., Renthal, W., Russo, S. J., Graham, D., Tsankova, N. M., Bolanos, C. A., Rios, M., Monteggia, L. M., Self, D. W., Nestler, E. J., 2006. Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. Science 311, 864-868.

Bolanos, C. A., Nestler, E. J., 2004. Neurotrophic mechanisms in drug addiction. Neuromolecular Med 5, 69-83.

Chu, N. N., Zuo, Y. F., Meng, L., Lee, D. Y., Han, J. S., Cui, C. L., 2007. Peripheral electrical stimulation reversed the cell size reduction and increased BDNF level in the ventral tegmental area in chronic morphine-treated rats. Brain Res 1182, 90-98.

Corominas, M., Roncero, C., Ribases, M., Castells, X., Casas, M., 2007. Brain-derived neurotrophic factor and its intracellular signaling pathways in cocaine addiction. Neuropsychobiology 55, 2-13.

Council, N. R., 2011. Guide for the Care and Use of Laboratory Animals: Eighth Edition. The National Academies Press.

Covington, H. E., 3rd, Maze, I., Sun, H., Bomze, Howard M., DeMaio, Kristine D., Wu, Emma Y., Dietz, David M., Lobo, Mary K., Ghose, S., Mouzon, E., Neve, Rachael L., Tamminga, Carol A., Nestler, Eric J., 2011. A role for repressive histone methylation in cocaine-induced vulnerability to stress. Neuron 71, 656-670.

Covington, H. E., 3rd, Miczek, K. A., 2001. Repeated social-defeat stress, cocaine or morphine. Effects on behavioral sensitization and intravenous cocaine self-administration "binges". Psychopharmacology (Berl) 158, 388-398.

Dacher, M., Nugent, F. S., 2011. Opiates and plasticity. Neuropharmacology 61, 1088-1096.

Fanous, S., Hammer, R. P., Jr., Nikulina, E. M., 2010. Short- and long-term effects of intermittent social defeat stress on brain-derived neurotrophic factor expression in mesocorticolimbic brain regions. Neuroscience 167, 598-607.

Fanous, S., Lacagnina, M. J., Nikulina, E. M., Hammer, R. P., Jr., 2011a. Sensitized activation of Fos and brain-derived neurotrophic factor in the medial prefrontal cortex and ventral tegmental area accompanies behavioral sensitization to amphetamine. Neuropharmacology 61, 558-564.

Fanous, S., Terwilliger, E. F., Hammer, R. P., Jr., Nikulina, E. M., 2011b. Viral depletion of VTA BDNF in rats modulates social behavior, consequences of intermittent social defeat stress, and long-term weight regulation. Neurosci Lett 502, 192-196.

Gall, C. M., Gold, S. J., Isackson, P. J., Seroogy, K. B., 1992. Brain-derived neurotrophic factor and neurotrophin-3 mRNAs are expressed in ventral midbrain regions containing dopaminergic neurons. Mol Cell Neurosci 3, 56-63.

Garzon, M., Pickel, V. M., 2002. Ultrastructural localization of enkephalin and mu-opioid receptors in the rat ventral tegmental area. Neuroscience 114, 461-474.

Geyer, M. A., Russo, P. V., Segal, D. S., Kuczenski, R., 1987. Effects of apomorphine and amphetamine on patterns of locomotor and investigatory behavior in rats. Pharmacology Biochemistry and Behavior 28, 393-399.

Grimm, J. W., Lu, L., Hayashi, T., Hope, B. T., Su, T.-P., Shaham, Y., 2003. Time-dependent increases in brain-derived neurotrophic factor protein levels within the mesolimbic dopamine system after withdrawal from cocaine: Implications for incubation of cocaine craving. The Journal of Neuroscience 23, 742-747.

Han, W., Hata, H., Imbe, H., Liu, Q. R., Takamatsu, Y., Koizumi, M., Murphy, N. P., Senba, E., Uhl, G. R., Sora, I., Ikeda, K., 2006. Increased body weight in mice lacking mu-opioid receptors. NeuroReport 17, 941-944.

Herkenham, M., Pert, C., 1982. Light microscopic localization of brain opiate receptors: a general autoradiographic method which preserves tissue quality. The Journal of Neuroscience 2, 1129-1149.

Horger, B. A., Iyasere, C. A., Berhow, M. T., Messer, C. J., Nestler, E. J., Taylor, J. R., 1999. Enhancement of locomotor activity and conditioned reward to cocaine by brain-derived neurotrophic factor. J Neurosci 19, 4110-4122.

Johnson, S. W., North, R. A., 1992. Opioids excite dopamine neurons by hyperpolarization of local interneurons. J Neurosci 12, 483-488.

Komatsu, H., Ohara, A., Sasaki, K., Abe, H., Hattori, H., Hall, F. S., Uhl, G. R., Sora, I., 2011. Decreased response to social defeat stress in mu-opioid-receptor knockout mice. Pharmacol Biochem Behav 99, 676-682.

Koo, J. W., Mazei-Robison, M. S., Chaudhury, D., Juarez, B., LaPlant, Q., Ferguson, D., Feng, J., Sun, H., Scobie, K. N., Damez-Werno, D., Crumiller, M., Ohnishi, Y. N., Ohnishi, Y. H., Mouzon, E., Dietz, D. M., Lobo, M. K., Neve, R. L., Russo, S. J., Han, M.-H., Nestler, E. J., 2012. BDNF Is a Negative Modulator of Morphine Action. Science 338, 124-128.

Krishnan, V., Han, M. H., Graham, D. L., Berton, O., Renthal, W., Russo, S. J., Laplant, Q., Graham, A., Lutter, M., Lagace, D. C., Ghose, S., Reister, R., Tannous, P., Green, T. A., Neve, R. L., Chakravarty, S., Kumar, A., Eisch, A. J., Self, D. W., Lee, F. S., Tamminga, C. A., Cooper, D. C., Gershenfeld, H. K., Nestler, E. J., 2007. Molecular adaptations underlying susceptibility and resistance to social defeat in brain reward regions. Cell 131, 391-404.

Lasek, A. W., Janak, P. H., He, L., Whistler, J. L., Heberlein, U., 2007. Downregulation of mu opioid receptor by RNA interference in the ventral tegmental area reduces ethanol consumption in mice. Genes Brain Behav 6, 728-735.

Lu, L., Koya, E., Zhai, H., Hope, B. T., Shaham, Y., 2006. Role of ERK in cocaine addiction. Trends Neurosci 29, 695-703.

Lutz, P. E., Kieffer, B. L., 2013a. The multiple facets of opioid receptor function: implications for addiction. Curr Opin Neurobiol 23, 473-479.

Lutz, P. E., Kieffer, B. L., 2013b. Opioid receptors: distinct roles in mood disorders. Trends Neurosci 36, 195-206.

MacDonald, A. F., Billington, C. J., Levine, A. S., 2004. Alterations in food intake by opioid and dopamine signaling pathways between the ventral tegmental area and the shell of the nucleus accumbens. Brain Res 1018, 78-85.

Magendzo, K., Bustos, G., 2003. Expression of amphetamine-induced behavioral sensitization after short- and long-term withdrawal periods: participation of mu- and delta-opioid receptors. Neuropsychopharmacology 28, 468-477.

Mathon, D. S., Lesscher, H. M., Gerrits, M. A., Kamal, A., Pintar, J. E., Schuller, A. G., Spruijt, B. M., Burbach, J. P., Smidt, M. P., van Ree, J. M., Ramakers, G. M., 2005. Increased gabaergic input to ventral tegmental area dopaminergic neurons associated with decreased cocaine reinforcement in mu-opioid receptor knockout mice. Neuroscience 130, 359-367.

Meerlo, P., Overkamp, G. J., Daan, S., Van Den Hoofdakker, R. H., Koolhaas, J. M., 1996. Changes in behaviour and body weight following a single or double social defeat in rats. Stress 1, 21-32.

Miczek, K. A., Nikulina, E. M., Shimamoto, A., Covington, H. E., 3rd, 2011a. Escalated or suppressed cocaine reward, tegmental BDNF, and accumbal dopamine caused by episodic versus continuous social stress in rats. J Neurosci 31, 9848-9857.

Miczek, K. A., Nikulina, E. M., Takahashi, A., Covington, H. E., 3rd, Yap, J. J., Boyson, C. O., Shimamoto, A., de Almeida, R. M., 2011b. Gene expression in aminergic and peptidergic cells during aggression and defeat: relevance to violence, depression and drug abuse. Behav Genet 41, 787-802.

Nathan, P. J., Bullmore, E. T., 2009. From taste hedonics to motivational drive: central mu-opioid receptors and binge-eating behaviour. Int J Neuropsychopharmacol 12, 995-1008.

Nikulina, E. M., Arrillaga-Romany, I., Miczek, K. A., Hammer, R. P., Jr., 2008. Long-lasting alteration in mesocorticolimbic structures after repeated social defeat stress in rats: time course of mu-opioid receptor mRNA and FosB/DeltaFosB immunoreactivity. Eur J Neurosci 27, 2272-2284.

Nikulina, E. M., Covington, H. E., 3rd, Ganschow, L., Hammer, R. P., Jr., Miczek, K. A., 2004. Long-term behavioral and neuronal cross-sensitization to amphetamine induced by repeated brief social defeat stress: Fos in the ventral tegmental area and amygdala. Neuroscience 123, 857-865.

Nikulina, E. M., Hammer, R. P., Jr., Miczek, K. A., Kream, R. M., 1999. Social defeat stress increases expression of mu-opioid receptor mRNA in rat ventral tegmental area. NeuroReport 10, 3015-3019.

Nikulina, E. M., Lacagnina, M. J., Fanous, S., Wang, J., Hammer, R. P., Jr., 2012. Intermittent social defeat stress enhances mesocorticolimbic DeltaFosB/BDNF co-expression and persistently activates corticotegmental neurons: implication for vulnerability to psychostimulants. Neuroscience 212, 38-48.

Nikulina, E. M., Miczek, K. A., Hammer, R. P., Jr., 2005. Prolonged effects of repeated social defeat stress on mRNA expression and function of mu-opioid receptors in the ventral tegmental area of rats. Neuropsychopharmacology 30, 1096-1103.

Paxinos, G., Watson, C., 2007. The Rat Brain in Stereotaxic Coordinates. Elsevier Science, Amsterdam, The Netherlands.

Pitchers, K. K., Coppens, C. M., Beloate, L. N., Fuller, J., Van, S., Frohmader, K. S., Laviolette, S. R., Lehman, M. N., Coolen, L. M., 2014. Endogenous opioid-induced neuroplasticity of dopaminergic neurons in the ventral tegmental area influences natural and opiate reward. J Neurosci 34, 8825-8836.

Pulliam, J. V., Dawaghreh, A. M., Alema-Mensah, E., Plotsky, P. M., 2010. Social defeat stress produces prolonged alterations in acoustic startle and body weight gain in male Long Evans rats. J Psychiatr Res 44, 106-111.

Razzoli, M., Carboni, L., Arban, R., 2009. Alterations of behavioral and endocrinological reactivity induced by 3 brief social defeats in rats: relevance to human psychopathology. Psychoneuroendocrinology 34, 1405-1416.

Ren, X., Lutfy, K., Mangubat, M., Ferrini, M. G., Lee, M. L., Liu, Y., Friedman, T. C., 2013. Alterations in phosphorylated CREB expression in different brain regions following short- and long-term morphine exposure: relationship to food intake. J Obes 2013, 764742.

Russo, S. J., Nestler, E. J., 2013. The brain reward circuitry in mood disorders. Nat Rev Neurosci 14, 609-625.

Segall, M. A., Margules, D. L., 1989. Central mediation of naloxone-induced anorexia in the ventral tegmental area. Behav Neurosci 103, 857-864.

Seroogy, K. B., Lundgren, K. H., Tran, T. M., Guthrie, K. M., Isackson, P. J., Gall, C. M., 1994. Dopaminergic neurons in rat ventral midbrain express brain-derived neurotrophic factor and neurotrophin-3 mRNAs. J Comp Neurol 342, 321-334.

Sesack, S. R., Pickel, V. M., 1995. Ultrastructural relationships between terminals immunoreactive for enkephalin, GABA, or both transmitters in the rat ventral tegmental area. Brain Res 672, 261-275.

Shieh, P. B., Ghosh, A., 1999. Molecular mechanisms underlying activity-dependent regulation of BDNF expression. Journal of Neurobiology 41, 127-134.

Sinha, R., 2001. How does stress increase risk of drug abuse and relapse? Psychopharmacology (Berl) 158, 343-359.

Sinha, R., 2008. Chronic stress, drug use, and vulnerability to addiction. Ann N Y Acad Sci 1141, 105-130.

Sinha, R., 2011. New findings on biological factors predicting addiction relapse vulnerability. Current Psychiatry Reports 13, 398-405.

Thomas, M. J., Kalivas, P. W., Shaham, Y., 2008. Neuroplasticity in the mesolimbic dopamine system and cocaine addiction. Br J Pharmacol 154, 327-342.

Tidey, J. W., Miczek, K. A., 1996. Social defeat stress selectively alters mesocorticolimbic dopamine release: an in vivo microdialysis study. Brain Res 721, 140-149.

Trigo, J. M., Martin-Garcia, E., Berrendero, F., Robledo, P., Maldonado, R., 2010. The endogenous opioid system: a common substrate in drug addiction. Drug Alcohol Depend 108, 183-194.

Van Ree, J. M., Niesink, R. J., Van Wolfswinkel, L., Ramsey, N. F., Kornet, M. M., Van Furth, W. R., Vanderschuren, L. J., Gerrits, M. A., Van den Berg, C. L., 2000. Endogenous opioids and reward. Eur J Pharmacol 405, 89-101.

Vanderschuren, L. J., Niesink, R. J., Van Ree, J. M., 1997. The neurobiology of social play behavior in rats. Neurosci Biobehav Rev 21, 309-326.

Vanderschuren, L. J., Stein, E. A., Wiegant, V. M., Van Ree, J. M., 1995. Social isolation and social interaction alter regional brain opioid receptor binding in rats. Eur Neuropsychopharmacol 5, 119-127.

Vargas-Perez, H., Ting, A. K. R., Walton, C. H., Hansen, D. M., Razavi, R., Clarke, L., Bufalino, M. R., Allison, D. W., Steffensen, S. C., van der Kooy, D., 2009. Ventral tegmental area BDNF induces an opiate-dependent-like reward state in naive rats. Science 324, 1732-1734.

Venzala, E., García-García, A. L., Elizalde, N., Delagrange, P., Tordera, R. M., 2012. Chronic social defeat stress model: behavioral features, antidepressant action, and interaction with biological risk factors. Psychopharmacology 224, 313-325.

Wang, J., Fanous, S., Terwilliger, E. F., Bass, C. E., Hammer, R. P., Jr., Nikulina, E. M., 2013. BDNF overexpression in the ventral tegmental area prolongs social defeat stress-induced cross-sensitization to amphetamine and increases deltaFosB expression in mesocorticolimbic regions of rats. Neuropsychopharmacology 38, 2286-2296.

Zhang, H., Torregrossa, M. M., Jutkiewicz, E. M., Shi, Y. G., Rice, K. C., Woods, J. H., Watson, S. J., Ko, M. C., 2006. Endogenous opioids upregulate brain-derived neurotrophic factor mRNA through delta- and mu-opioid receptors independent of antidepressant-like effects. Eur J Neurosci 23, 984-994.

Zhou, L., Hammer, R. P., Jr., 1995. Gonadal steroid hormones upregulate medial preoptic μ-opioid receptors in the rat. Eur J Pharmacol 278, 271-274.

Figure Captions

Figure 1. Timeline of experimental events and schematic of social approach and avoidance test procedure. (A) Rats were given 7 days to recover from surgery, and were then exposed to intermittent (4x in 10 days) social defeat or handling procedures. Two days after the last episode of defeat, all rats were given the social approach and avoidance test. Ten days after the last episode of defeat, one group received amphetamine challenge while a separate group was perfused to examine immunohistochemical changes at the time cross-sensitization is observed. Five days after receiving the amphetamine challenge, brains from the remaining rats were removed and processed for *in vitro* [^{3}H]DAMGO autoradiography to verify the location and efficacy of MOR knockdown. (B) All experimental subjects were assessed for social approach and avoidance using a procedure adapted from Berton et al. (2006). Left: Virtual arena dividing the chamber into 2 virtual zones: Interaction Zone (IZ), comprising of the 1019.35 cm^2 area immediately surrounding the containment cage, and Avoidance Zone (AZ), which comprised the two corners, combined 52.2 cm^2, opposite the containment cage. Right: Schematic of the timeline for the social approach and avoidance procedure.

Figure 2. [^{3}H]DAMGO autoradiography revealed that the shMOR construct, but not the scrambled GFP construct, significantly reduced MOR binding in the VTA, but not the SNc. (A) Left: Representative image of reporter GFP expression in infected VTA cells (fr: fasciculus retroflexus; scale bar = 100 μm). Right: Plate 37, modified from Paxinos and Watson (2007). (B) Parasagittal illustrations showing the extent of GFP expression across the VTA, drawn in green (lateral plates 0.40 - 1.4, modified from Paxinos and Watson, 2007). (C) Representative autoradiographs of [^{3}H]DAMGO binding in the VTA after infusion of either scrambled-GFP or shMOR lentiviral constructs (MT: medial terminal nucleus of the accessory optic system; scale bar = 500 μm). (D) shMOR lentiviral construct (n = 14) significantly (* - $p < 0.0001$) reduced MOR binding in the VTA compared to the scrambled-GFP construct (n = 11), without affecting MOR binding of either the GFP (n = 11) or shMOR (n = 14) groups in the adjacent SNc ($p > 0.22$).

Figure 3. Knockdown of VTA MORs prevents social stress-induced deficit of weight gain. While undergoing social stress or handling, GFP-Stressed rats (n = 5) exhibited significantly (** - $p < 0.05$) less weight gain than did GFP-Handled (n = 6) or shMOR-Handled (n = 7) rats. By contrast, shMOR-Stressed rats (n = 7) did not differ from GFP-Handled or -Stressed rats, showing significantly (α - $p < 0.05$) less weight gain than shMOR-Handled rats. Ten days after the last episode of exposure, GFP-Stressed rats had gained significantly (* - $p < 0.05$) less weight than all other groups.

Figure 4. Knockdown of VTA MORs prevents social stress-induced social avoidance. (A) GFP-Stressed rats (n = 7) made significantly (* - $p < 0.005$) more entries to the avoidance zones than did GFP-Handled (n = 7) or shMOR-Stressed rats (n = 14). (B) GFP-Stressed rats (n = 9) were significantly (*** - $p < 0.05$) more active in the avoidance zones than GFP-Handled (n = 7), shMOR-Handled (n = 8), or shMOR-Stressed (n = 11) rats. (C) GFP-Stressed rats (n = 7) showed a slight tendency to spend less time in the interaction zone, but there was no significant ($p > 0.3$) main effect compared to GFP-Handled (n = 9), shMOR-Handled (n = 9), or shMOR-Stressed (n = 12) groups.

Figure 5. Knockdown of VTA MORs prevents social stress-induced amphetamine cross-sensitization without affecting baseline activity. Multivariate analyses revealed that the only significant main effects occurred during the amphetamine phase of the assay. Data collection and video tracking were paused to administer saline and amphetamine, vertical arrows denote the time point when injection occurred. (A) GFP-Stressed rats (n = 4) exhibited significantly (*** - $p < 0.05$) more movements at 120 and 140 min compared to GFP-Handled (n = 5), shMOR-Handled (n = 6), and shMOR-Stressed (n = 6) rats, and differed significantly (* - $p < 0.02$) from GFP-Handled rats at 130 min. (B) GFP-Stressed rats travelled a significantly (*** - $p < 0.03$) greater distance at 110, 120, and 130 min compared to all other groups.

Figure 6. Knockdown of VTA MORs blocks social stress-induced increase of VTA BDNF expression. (A) Representative images of BDNF labeling in the VTA approximately AP -5.1 from bregma. More BDNF labeled cells (identified by arrows) are visible in the GFP-Stressed group than in any others. (Scale bar = 100 μm) (B) The GFP-Stressed group (n = 5) exhibited significantly (* - $p < 0.0001$) more VTA BDNF immunolabeling than GFP-Handled (n = 4), shMOR-Handled (n = 4), or shMOR-Stressed (n = 6) groups. Numbers of labelled cells did not significantly differ between GFP-Handled rats and either shMOR group ($p > 0.15$).

Figure 1

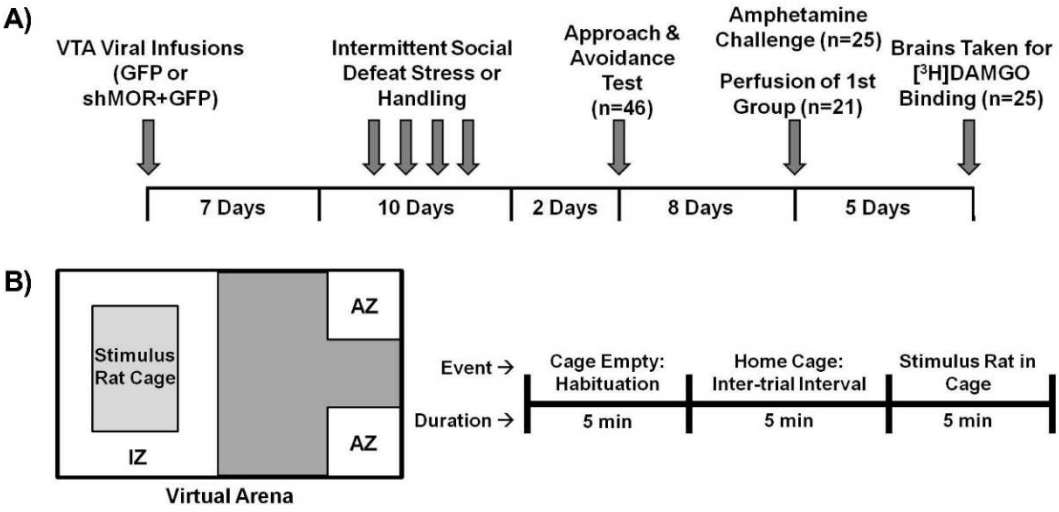


Figure 2

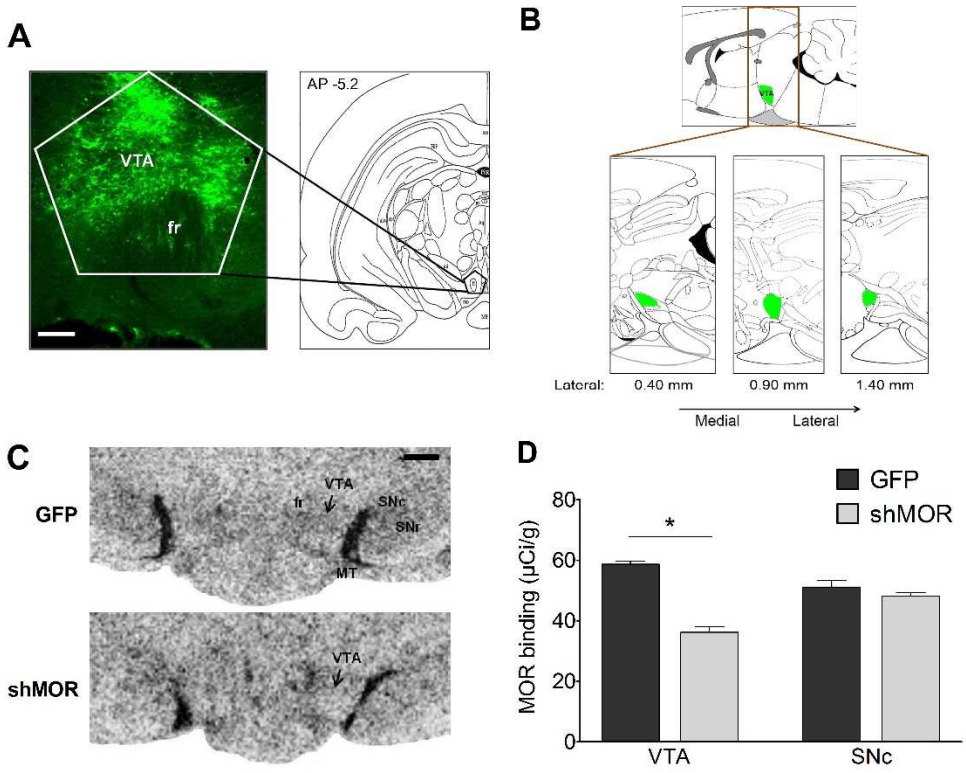


Figure 3

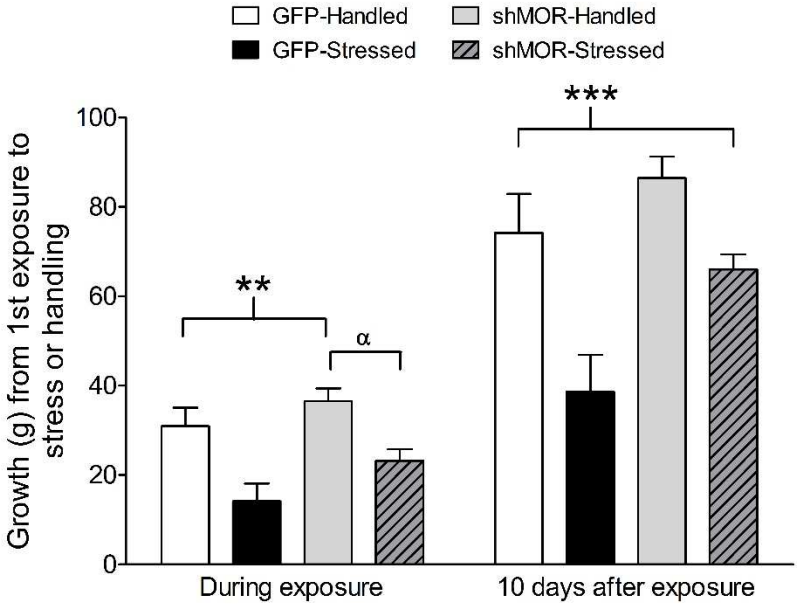


Figure 4

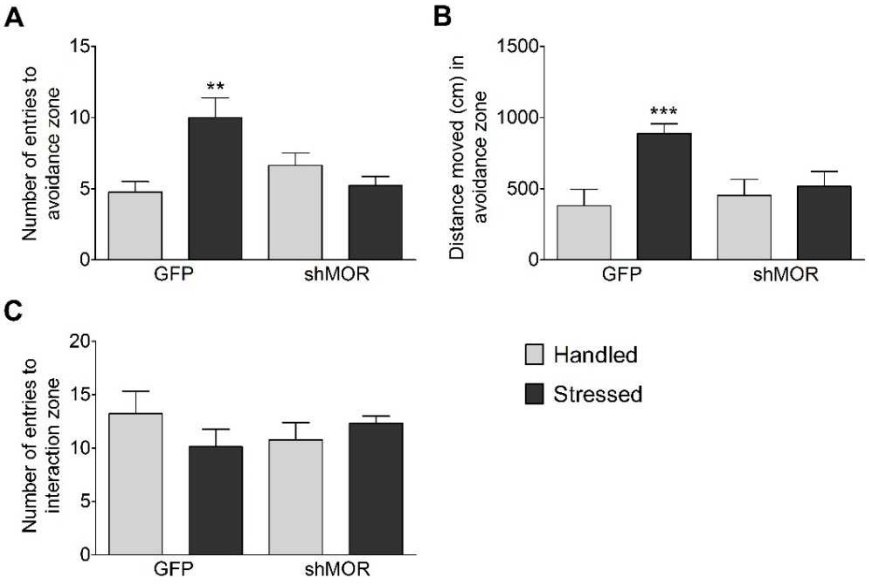


Figure 5

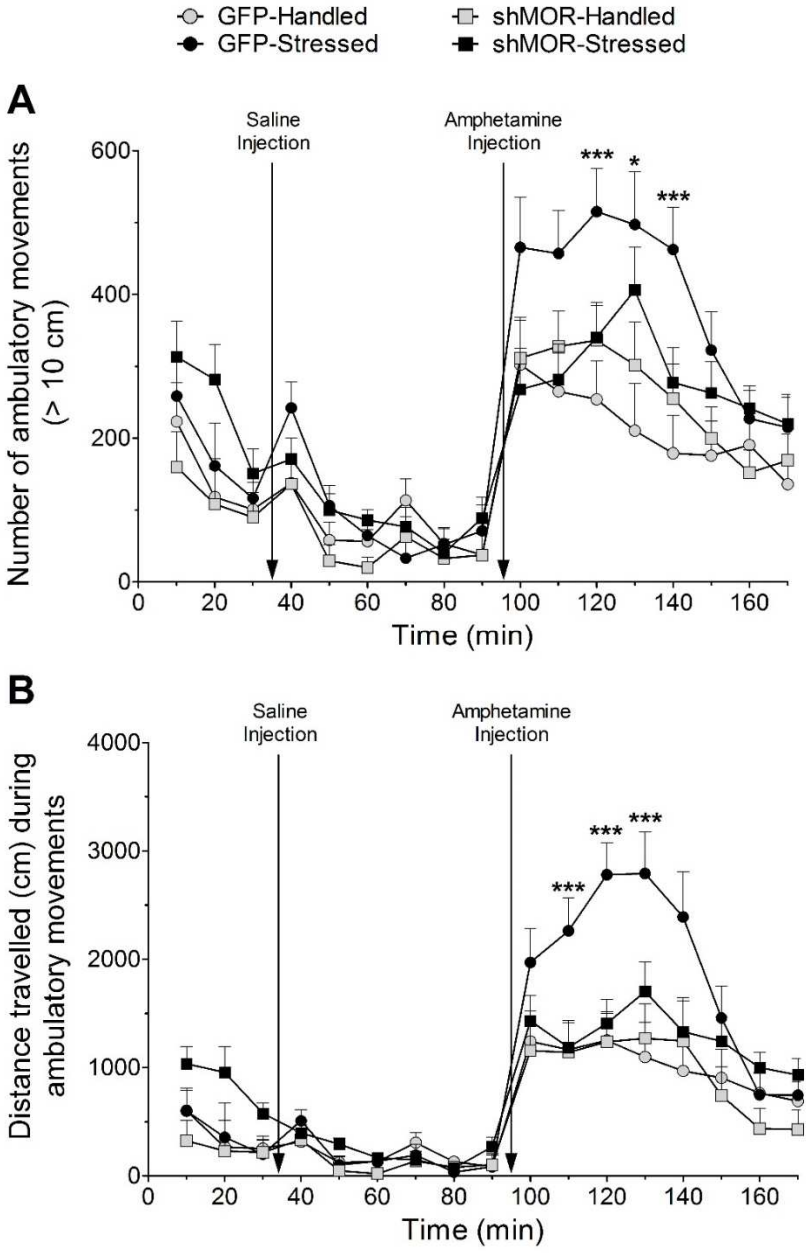
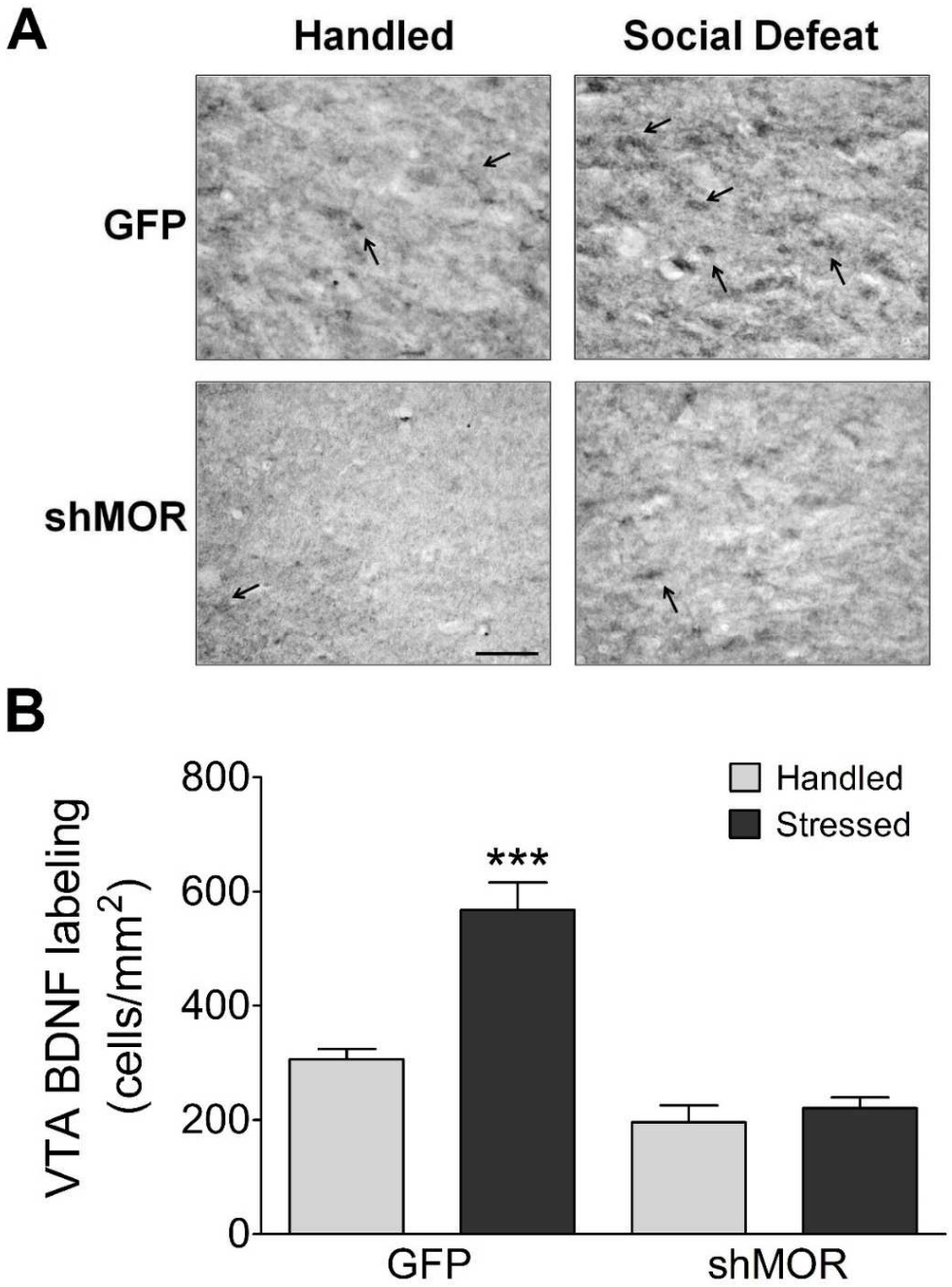

GFP-Handled
GFP-Stressed
shMOR-Handled
shMOR-Stressed
A
Saline Injection
Amphetamine Injection
Number of ambulatory movements (> 10 cm)
Time (min)
B
Distance travelled (cm) during ambulatory movements
Time (min)

Figure 6


A
Handled
Social Defeat
GFP
shMOR
B
VTA BDNF labeling
(cells/mm2)
800
600
400
200
0
***
Handled
Stressed
GFP
shMOR