Brain-derived Neurotrophic Factor Signaling in the Mesolimbic Dopamine System:

Social Defeat Stress-induced

Cross-sensitization to Psychostimulants and Escalation of Cocaine Intake

by

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ABSTRACT

Intermittent social defeat stress induces cross-sensitization to psychostimulants and escalation of drug self-administration. These behaviors could result from the stress-induced neuroadaptation in the mesocorticolimbic dopamine circuit. Brain-derived neurotrophic factor (BDNF) in the ventral tegmental area (VTA) is persistently elevated after social defeat stress, and may contribute to the stress-induced neuroadaptation in the mesocorticolimbic dopamine circuit. BDNF modulates synaptic plasticity, and facilitates stress- and drug-induced neuroadaptations in the mesocorticolimbic system. The present research examined the role of mesolimbic BDNF signaling in social defeat stress-induced cross-sensitization to psychostimulants and the escalation of cocaine self-administration in rats. We measured drug taking behavior with the acquisition, progressive ratio, and binge paradigms during self-administration. With BDNF overexpression in the ventral tegmental area (VTA), single social defeat stress-induced cross-sensitization to amphetamine (AMPH) was significantly potentiated. VTA-BDNF overexpression also facilitates acquisition of cocaine self-administration, and a positive correlation between the level of VTA BDNF and drug intake during 12 hour binge was observed. We also found significant increase of DeltaFosB expression in the nucleus accumbens (NAc), the projection area of the VTA, in rats received intra-VTA BDNF overexpression. We therefore examined whether BDNF signaling in the NAc is important for social defeat stress-induced cross-sensitization by knockdown of the receptor of BDNF (neurotrophin tyrosine kinase receptor type 2, TrkB) there. NAc TrkB knockdown prevented social defeat stress-induced cross-sensitization to psychostimulant. Also social defeat stress-induced increase of DeltaFosB in the NAc was prevented by TrkB knockdown. Several

other factors up-regulated by stress, such as the GluA1 subunit of Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and BDNF in the VTA were also prevented. We conclude that BDNF signaling in the VTA increases social defeat stress-induced vulnerability to psychostimulants, manifested as potentiated cross-sensitization/sensitization to AMPH and escalation of cocaine self-administration. Also BDNF signaling in the NAc is necessary for the stress-induced neuroadaptation and behavioral sensitization to psychostimulants. Therefore, TrkB in the NAc could be a therapeutic target to prevent stress-induced vulnerability to drugs of abuse in the future. DeltaFosB in the NAc shell could be a neural substrate underlying persistent cross-sensitization and augmented cocaine self-administration induced by social defeat stress.

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INTRODUCTION AND BACKGROUND

Overview of Addiction

Epidemiology of addiction

Addiction is a chronic, often relapsing brain disease that causes compulsive drug seeking and intake, despite harmful consequences. The Diagnostic and Statistical Manual of Mental Disorders (DSM) IV defines psychostimulants (such as amphetamine and cocaine) abuse as the failure to fulfill major obligations, use despite physical hazard, recurrent legal problems, or recurrent social and interpersonal problems (American Psychiatric Association., 2000). Drug addiction is one of the top three public health concerns in the United States, and it is the most costly neuropsychiatric disorder (G. R. Uhl & Grow, 2004). Substance abuse and addiction have an economic impact of $524 billion per year in the U.S, due to addiction related health care, loss of productivity, crime and drug enforcement (United Nations Office on Drugs and Crime, 2007 and Office of National Drug Control Policy, 2004). Based on World Drug Report (2007), the annual prevalence of illicit drug abuse is about 4.8% of the world population. The National Survey on Drug Use and Health (NSDUH) estimates that currently there are at least 20.4 million drug abusers in the U.S, and 1.9 million among them are cocaine users, and the number is still increasing. Although tremendous resources have been devoted to cut down the consumption of addictive drugs, such efforts are unsuccessful in general. The crux of this problem is the lack of effective therapy to counter addiction's powerful disruptive effects on human brain, due to the poor understanding on the neural mechanism of addiction.

Addictive drugs are taken initially to achieve the pleasant feeling or to alleviate the negative feelings caused by aversive life experience, but with repeated drug use, the brain reward system is usurped and changed by addictive drugs, and drugs become more important than other goals important for survival, and all activities are directed towards obtaining and consuming drugs (S. E. Hyman, Malenka, & Nestler, 2006). In according to DSM-IV, the diagnosis of substance dependence should be made in the presence of three or more of following behavioral manifestations: tolerance or increased amounts of the substance to achieve desired effect; withdrawal symptoms; persistent desire or unsuccessful efforts to control substance use; use of substances regardless of persistent physical or psychological problem and the reduction of social, occupational or recreational activities. Whereas the diagnostic criterion of substance abuse are as following: frequent use of substances in situation in which it is physically hazardous (such as driving); failure to fulfill major obligations at work, school, home due to substance use; continued use regardless of frequent social or interpersonal, or even legal problems (American Psychiatric Association., 2000). Such criteria of substance dependence and abuse apply to psychostimulants, although psychostimulants use does not lead to clinically significant withdrawal syndrome, as opiate drugs and alcohol (Miller, Summers, & Gold, 1993).

As a pathological process, pathogenesis of addiction encompasses three stages: 1) substance abuse out of control regardless of adverse consequences; 2) development of the tolerance to the effect of the substance upon repeated exposure, leading to the requirement of larger doses to achieve the desired effect; and 3) physical and psychological symptoms of distress, discomfort, or impairment upon reduction or

cessation of substance use, which is also well-known as the "withdrawal syndrome". The withdrawal syndrome is commonly found in abusers of non-psychostimulants, such as opiates, ethanol, nicotine and so on, but not obvious in abusers of psychostimulants, such as cocaine and amphetamine. The uncontrolled drug use regardless of aversive consequences and the persistent risk of relapse even after years of abstinence are the most harmful aspects of addiction. The current study is primarily focused on psychostimulant sensitization and compulsive abuse.

Pharmacology of psychostimulants and pathological effects

Psychostimulants, including amphetamine (AMPH) and cocaine, are highly addictive substances inducing psychomotor activation by direct increasing extra neuronal dopamine levels through different mechanisms.

AMPH is one member of a group of compounds referred as amphetamines, including AMPH, methamphetamine and methylenedioxymethamphetamine. Due to its structural similarity with dopamine, AMPH is a substrate for the dopamine transporter (DAT). Extraneuronal AMPH binds with DAT and is transported into the cytosol. It competitively inhibits DA uptake. Due to its lipophilicity, when in high extraneuronal concentration, AMPH also can diffuse directly into the cells without the aid of DAT (Gulaboski et al., 2007). In the cytoplasm, AMPH increases the intracellular binding sites of DAT for dopamine and induces the reverse transport of dopamine mediated by DAT (Sulzer et al., 1995), through the exchange of extracellular amphetamine with intracellular dopamine (S. R. Jones, Joseph, Barak, Caron, & Wightman, 1999). Intracellular AMPH also interferes with the vesicular monoamine transporter 2 (VMAT-2), impairs active transport of the monoamines into synaptic vesicles. Furthermore,

AMPH can diffuse into the vesicle and induces the release of vesicular dopamine to the cytosol (Sulzer, Sonders, Poulsen, & Galli, 2005), further increases the dopamine concentration in the cytosol. Cytosolic dopamine is then released to the extracellular space via reverse transport by the DAT, leading to an increase in extracellular dopamine. Ultimately, AMPH induced abnormal DA release can saturate DA receptors and eventually lead to depletion of intracellular DA stores (Schmitz, Lee, Schmauss, Gonon, & Sulzer, 2001).

Compared with the effects of AMPH, cocaine directly inhibits the DAT (J. M. Brown, Hanson, & Fleckenstein, 2001), prevents the reuptake of dopamine into the nerve terminals, resulting in increased levels of dopamine in the synaptic cleft. Although both AMPH and cocaine have effects on the monoamine transporters noradrenaline transporter and serotonin transporter (SERT) (Gatley, Gifford, Volkow, & Fowler, 1998), their reinforcing effects are largely dependent on their action on dopaminergic neurotransmission (M C Ritz & Kuhar, 1989; Mary C. Ritz, Lamb, Goldberg, & Kuhar, 1987). It is important to note that the potency of cocaine on SERT is much higher than amphetamine (Han & Gu, 2006).

Several neurological impairments are present in psychostimulants abusers. Some cocaine abusers suffer from seizures, epilepsy (Koppel, Samkoff, & Daras, 1996), cerebral ischaemia, cerebral hemorrhages, cerebral atrophy, cognitive impairment, and mood and movement disorders (Rodnitzky & Keyser, 1992). Clinical studies discovered that psychostimulant addicts exhibit a higher rate of occurrence of depressive disorders, attention deficit disorder, and other psychopathologies (Clure et al., 1999) than normal people. Furthermore, degraded performance on response inhibition tasks (Goldstein,

Volkow, Wang, Fowler, & Rajaram, 2001) and working memory tasks (Hester & Garavan, 2004) were found commonly in chronic abusers of psychostimulants, suggesting dysfunction in prefrontal cortex induced by chronic psychostimulants abuse. Brain imaging studies are consistent with the clinical data: decreased structural integrity in prefrontal white matter (Lim, Choi, Pomara, Wolkin, & Rotrosen, 2002) and gray matter density in frontal and temporal cortical regions (Franklin et al., 2002) were broadly found in chronic cocaine addicts.

Besides the pathological effect on the central nervous system, chronic psychostimulants abuse also causes cardiovascular and respiratory complications. Cocaine increases the level of circulating catecholamines in the central nervous system, resulting in arterial vasoconstriction. Cocaine also increases myocardial oxygen demand, leading to myocardial ischemia, and tachycardia. Cocaine inhibits the reuptake of catecholamines at adrenergic nerve terminals in the peripheral cardiovascular system and inhibits baroreceptors, ultimately induces myocardial toxicity (Perper & Van Thiel, 1992a). The cardiovascular complications associated with cocaine abuse range from episodic arrhythmias to myocardial infarction, strokes, and cardiomyopathy, which are very common and dangerous.

Upper respiratory and pulmonary complications are also common in addicts who snort cocaine or smoke “crack”, ranging from ischemia, necrosis, infections of the nasal mucosa, pulmonary hemorrhages/barotrauma/infection, asthma, and persistent gas-exchange abnormalities (Perper & Van Thiel, 1992b). If compulsive consumption of psychostimulants can be curbed, the consequent pathological complications and

impairment to nervous system would not take place. Thus the better understanding on the molecular mechanism underlying compulsive drug taking and craving is in urgent need.

Hypothetical models for a mechanism of drug dependence

Opponent processes theory

Several theories have been raised trying to explain the mechanism of addiction. Opponent processes theory is a well-established psychological version of the pleasure/withdrawal view of addiction. Based on this theory, the pathogenesis of drug dependence is composed of an initial rewarding process and a following negative (opponent) process, and a negative process serves to restore the homeostasis and bring the drug stimulated brain rewarding system back to normal state. The subjective experience of drug taking is dependent on the summation of active process and the negative process, as depicted in Figure 1. Following the repeated drug use, the effect of active process remains unchanged but the negative process is strengthened and elongated, the manifestation will be the tolerance to drug euphoria and the unpleasant withdrawal syndrome.

Koob and colleagues suggested the neural mechanism of opponent processes theory. In their theory, drug exposure activates mesolimbic dopamine circuitry, and increases dopamine neurotransmission in the NAc, which mediates the reinforcing effect of acute drug exposure. Upon repeated exposure to drug, tolerance will develop in the mesolimbic dopamine circuit. After the cessation of drug use, mesolimbic dopamine levels drop to the level below the baseline for a long-period of time therefore causing the negative effect during withdrawal. Escape from the negative withdrawal effect drives the compulsive drug taking/seeking behavior in addicts. This mechanism is also defined as

"negative reinforcement" in contrast to the "positive reinforcement" experienced during the initial stage of addiction. Repeated drug exposure also activates the HPA axis, resulting in an increase of corticosteroids and stress response, further exacerbating the negative state during drug withdrawal. However, withdrawal symptoms are maximal within several days after the cessation of drug use, but the drug craving and sensitization are persistent and can be further enhanced following long periods of drug abstinence (J. W. Grimm, Hope, Wise, & Shaham, 2001). Thus the opponent processes theory cannot sufficiently explain all the essential aspects addiction, especially the long-lasting characteristic of drug craving, sensitization and life-long risk of relapse after the negative process has decayed away.

Incentive sensitization theory

Because compulsive drug taking behavior and persistent craving cannot be fully explained by simple avoiding negative state during withdrawal, Robinson and Berridge raised the incentive-sensitization theory of addiction. This theory suggests that repeated drug exposure induces long-lasting neural sensitization in mesolimbic-related systems, which assigns excessive incentive salience toward drug and related cues, leading to compulsive drug seeking, intake and relapse (T. E. Robinson & Berridge, 1993, 2000). This theory also suggests the distinction between drug "liking" and "wanting"; sensitized brain reward circuits attribute incentive salience to reward-related stimuli and cause excessive "wanting" for the reward regardless of whether it is "liked" or not (Berridge & Robinson, 1998). When sensitized incentive salience is attributed to drug cues in addicts, it can trigger compulsive drug pursuit.

In human addiction studies, many researchers take subjective 'high' and euphoria as their primary dependent measures for sensitization; however, accumulating evidence suggests that drug-induced neural sensitization, such as sensitized DA transmission, is more closely related to incentive salience attributed to drug, rather than drug-induced euphoria. This is demonstrated most obvious from the observation that stimulant drug abusers develop a tolerance to the euphorigenic effects of the drug, but still show enhanced drug-seeking behavior (Pontieri, Mainero, La Riccia, Passarelli, & Orzi, 1995; Pontieri, Tanda, & Di Chiara, 1995). In other words, drugs of abuse sensitize neural mechanisms involved in incentive valence assigned to drug (wanting), rather than the drug-induced euphoria (liking).

Psychostimulants-induced Sensitization

Overview on sensitization

Sensitization is defined as the augmented locomotor response to drug(psychomotor sensitization) and the potentiated incentive salience (incentive sensitization) attributed to drug or drug-related cues upon repeated drug exposure, a key process of addiction (T. E. Robinson & Berridge, 1993). Both psychomotor sensitization and incentive sensitization are mediated by sensitization of the mesocorticolimbic dopamine circuit (T. E. Robinson & Berridge, 1993). Neural sensitization in the mesocorticolimbic dopamine circuit encompasses neurotransmission strength changes on circuit-level, and neural physiological changes on neuronal level, which will be further discussed in the following chapters. A simplified illustration of effects of a variety of drugs of abuse, including psychostimulants on mesocorticolimbic circuit can be found in Figure 2.

Psychomotor sensitization is augmented behavioral response to drugs or drugs related cues, manifested in many types of behaviors response, such as locomotor activity, stereotypical head movements, arousal, and exploration. The manifestation of psychomotor sensitization is dependent on the dose of drug administered. For example, at moderate dose, psychostimulants primarily induced locomotor activity, but at a higher dose, qualitative changes took place in behavior, and motor stereotypies take place and compete with locomotion. The incentive sensitization is the excessive incentive salience attributed to drug and drug related cues, and manifested in compulsive drug taking/seeking behavior. Despite the widespread use of locomotor sensitization as the behavioral model to study the neuroadaptations underlying sensitization, cocaine self-administration, which can measure reinforcing effect of drugs and the motivation to acquire drugs, has greater face validity as a model to study the neuroadaptation underlying the motivational changes during compulsive drug taking/seeking behaviors.

It is important to note that treatment inducing psychomotor sensitization also facilitates subsequent drug taking behavior during cocaine self-administration with extended drug access (C. R. Ferrario & Robinson, 2007). Vice versa, extended access to cocaine during self-administration induces locomotor sensitization to cocaine one month after the cessation of self-administration (Carrie R. Ferrario et al., 2005). These data suggest that the neural changes mediating psychomotor sensitization are important for the transition from casual to compulsive drug use, and imply that incentive sensitization and psychomotor activation share some common neural substrates.

There is also criticism about the validity of sensitization behavioral models based on the lack of evidence showing behavioral sensitization in humans. Indeed, several

studies have shown that behavioral and neural sensitization in humans in response to drugs of abuse (Di Chiara, 2002; Strakowski, Sax, Setters, & Keck, 1996), although in humans the demonstration of behavioral sensitization is not in the form of locomotor activity. Strakowski and colleagues found that repeated amphetamine (0.25mg/kg) injection induces sensitization in human subjects, demonstrated as enhanced activity/energy level, heart rate, amount of speech and eye-blink rates (Strakowski et al., 1996). Therefore, these data suggest that behavior sensitization model in rodents is a valid model to study the neuroadaptation underlying repeated psychostimulants challenge in humans.

Neural substrates and mechanism underlying sensitization

The mesoaccumbens dopamine (DA) system is the primary neural substrate underlying the behavioral sensitization to psychostimulants. To further determine the site of action of drugs during the induction of sensitization, AMPH or morphine has been microinfused directly into the different regions of mesolimbic dopamine circuit, such as the VTA, where the dopamine neurons located, or the NAc, the terminal field of dopamine neurons. Repeated infusion of drugs into the NAc only induced acute locomotor response, but no sensitization observed upon repeated administration. In contrast, both psychostimulants and opioids induce sensitized response through actions in the VTA (Joyce & Iversen, 1979; Paul Vezina & Stewart, 1990), and animals received intra-VTA microinjection of AMPH exhibited behavioral sensitization to systemic AMPH injection (P W Kalivas & Weber, 1988). Thus VTA is required for the induction of behavioral sensitization to repeated psychostimulants. Whereas microinfusion of psychostimulant into the NAc can maintain the expression of sensitization, suggesting

that NAc is the region responsible for the expression of sensitization. To further characterize the mechanism of the action of dopamine in the VTA during the induction of sensitization, selective dopamine type 1 (D1) and type 2 (D2) receptor antagonists were microinfused into the VTA. Intra-VTA infusion of D1 receptor, but not D2 receptor antagonist can prevent repeated AMPH or morphine injection induced sensitization (Stewart & Vezina, 1989; P. Vezina & Stewart, 1989). These data suggest that D1R activation in the VTA is necessary for the sensitizing effect of drugs of abuse.

Besides mesoaccumben DA system, other structures in the mesocorticolimbic system are involved in the induction of sensitization. Medial prefrontal cortex originating glutamatergic transmission to the VTA is also necessary for the induction of sensitization to repeated psychostimulants (Cador, Bjijou, Cailhol, & Stinus, 1999). It was found that infusion of glutamate into the VTA enhances dopamine transmission in the NAc and induces motor stimulating effect (P. W. Kalivas, Duffy, & Barrow, 1989).

Based on the aforementioned evidence, psychostimulants induce DA released somatodendritically in the VTA, which can act on D1 receptors located primarily on afferents to the VTA, such as GABAergic projections from the NAc and glutamatergic pyramidal neurons from the PFC, thus lead to the sensitization to drugs of abuse.

Primary Neural Substrate of Drugs of Abuse: Mesocorticolimbic Dopamine Circuit

Role of dopamine in mesocorticolimbic dopamine circuit

The mesocorticolimbic dopamine circuit is composed of the VTA, where the cell bodies of dopaminergic neurons located, and its projection areas, including the NAc, amygdala (AMY), hippocampus and the prefrontal cortex (PFC). The mesocorticolimbic dopamine circuit is the major substrate of reward and reinforcement for natural rewards.

The role of DA in mesocorticolimbic system has been over-simplified, which is usually taken as neurotransmitter used in "brain reward system". The word "reward" is used too broadly therefore it might not be the most precise word to describe the main function of mesocorticolimbic dopamine system. Most importantly, the term "reward" evokes the concept of pleasure or hedonia in readers, which greatly obfuscates the discussion of the function of mesocorticolimbic dopamine system. The "brain reward system" also can be activated by aversive stimuli, shown from enormous literature (Abercrombie, Keefe, DiFrischia, & Zigmond, 1989; Carlson, Herrick, Baird, & Glick, 1987; Cenci, Kalen, Mandel, & Bjorklund, 1992). The sensitization effect of many types of stress on mesocorticolimbic dopamine circuit will be discussed in detail in the following chapters.

It is important to note that the consumption of the rewarding material does not induce the firing of DA neurons, but the cue predicting the possible rewarding stimuli does (Schultz, Dayan, & Montague, 1997). Trained rats exposed to a conditioned stimulus signaling that lever pressing would result in sucrose delivery was accompanied by an elevation of DA in the NAc, however, the actual presentation and consumption of reinforce were not (H. D. Brown, McCutcheon, Cone, Ragozzino, & Roitman, 2011; Roitman, Stuber, Phillips, Wightman, & Carelli, 2004).

Also extensive data suggest that DA transmission does not affect reinforcing stimuli-induced hedonia or fundamental motivation, but affects critical features of reinforcing stimuli-directed instrumental behavior, such as the sensitivity to the work requirements in effort-related tasks, or learning component of operant task (Denk et al., 2005; Salamone & Correa, 2012). The DA depletion or antagonism in mesolimbic circuit does not affect the appetitive taste reactivity to natural reward, but affect the instrumental

behavior, such as food seeking behavior, reinforcement learning (Baldo, Sadeghian, Basso, & Kelley, 2002; Koob, Riley, Smith, & Robbins, 1978; Salamone, Mahan, & Rogers, 1993), suggesting the distinction between instrumental versus consummatory behavior (seeking with taking). Therefore, the function of DA in mesocorticolimbic system is more specific and complicated than "neurotransmitter mediating reward", and requires further investigation.

Bromberg-Martin brought up a theory suggests that separated DA populations in mesocorticolimbic circuit encode motivation value, motivational salience, and alerting signal triggered by unexpected sensory inputs (Bromberg-Martin, Matsumoto, & Hikosaka, 2010). DA neurons are activated and exhibit phasic firing by reward greater than expectation or cue predicting the increase in reward value (Bromberg-Martin et al., 2010). In contrast, most DA neurons are depressed and show phasic pause in response to aversive stimuli or cue predicting the decrease of reward value (Brischoux, Chakraborty, Brierley, & Ungless, 2009). Phasic burst firing and pause in VTA DA neurons cause different pattern of dopamine release in the terminal regions (Grace, 2000). Optogenetic stimulation of VTA DA neurons in a phasic burst pattern, but not tonic pattern, induces condition place preference, suggested that phasic firing of VTA DA neurons is rewarding or reward predictive (H.-C. Tsai et al., 2009). In contrast, negative reward prediction errors induce phasic pause in VTA DA neurons, leading to the termination of behavior or the avoidance. Interestingly, when the reward is exactly the same as predicted, DA neurons show no response (Schultz et al., 1997).

Major components and network of mesocorticolimbic dopamine circuit

Ventral tegmental area

The VTA contains a mixture of DA neurons (~65%), GABAergic neurons (~30%), and glutamatergic neurons (~5%) (Fields, Hjelmstad, Margolis, & Nicola, 2007; Margolis, Lock, Hjelmstad, & Fields, 2006; Swanson, 1982), some evidence also indicates that some VTA DA neurons co-release glutamate (Chuhma et al., 2004; Tecuapetla et al., 2010; Yamaguchi, Sheen, & Morales, 2007). The data showed that VTA DA neurons in mice can elicit excitatory postsynaptic responses in projection neurons of the NAc (Tecuapetla et al., 2010). Also in situ hybridization results show that mRNA of vesicular glutamate transporter type 2, a transporter protein necessary for the package and secretion of glutamate, is expressed in VTA DA neurons (Yamaguchi et al., 2007), suggesting that some DA neurons can also release glutamate as neurotransmitter. The cyto-architecture of the VTA is not well-defined and is determined largely by adjacent structures. VTA DA neurons are heterogeneous in anatomical location and connectivity, electrophysiological properties and molecular features (Grace & Bunney, 1984; Stephan Lammel et al., 2008; S. Lammel, Ion, Roeper, & Malenka, 2011; Schultz, 2007). Based on the firing pattern, VTA dopamine neurons can be categorized into conventional slow-firing dopamine neurons, and unconventional fast-firing dopamine neurons with small DAT/TH mRNA expression ratios (Grace & Bunney, 1984; Schultz, 2007). Based on classical histofluorescence labeling and neural tracing studies, DA neurons of the VTA project to limbic and cortical areas along mesolimbic and mesocortical pathways (Bjorklund & Dunnett, 2007; German & Manaye, 1993; Marin, Herrero, Vyas, & Puelles, 2005). However, with the rapid advance in neural tracing techniques and functional studies, this has long been recognized as an oversimplification. The DA projections originated from the VTA demonstrate target-specific

neurophysiological characteristics. The conventional slow-firing DA neurons project to the lateral shell of the NAc and the dorsolateral striatum, and the unconventional fast-firing DA neurons selectively project to PFC and NAc core and medial shell as well as to basolateral AMY (Bjorklund & Dunnett, 2007; Ikemoto, 2007; Stephan Lammel et al., 2008).

With the rapid advance in neurotracing techniques and cell-type specific photo-stimulation, characterization of the functional connectivity of VTA dopaminergic neurons becomes possible. VTA dopaminergic neurons receive both excitatory and inhibitory regulation from a complex of brain regions. Lammel and colleagues recently showed that lateral VTA-dopamine neurons, which project to the NAc lateral shell, receive glutamatergic projection from laterodorsal tegmentum, whereas GABAergic neurons in the medial VTA, which inhibit the VTA dopaminergic neurons projecting to the medial prefrontal cortex, received excitatory input from lateral habenula glutamatergic neurons (S. Lammel et al., 2012).

It was shown that activation of VTA GABAergic neurons is aversive and inhibition of GABAergic neurons is rewarding, and animals will work actively to self-administer the inhibition of VTA GABAergic neurons (Jennings et al., 2013). Also, the bed nucleus of the stria terminalis (BNST) send excitatory glutamatergic and inhibitory GABAergic inputs to VTA GABAergic neurons (Kudo et al., 2012). BNST glutamatergic neurons were activated whereas BNST GABAergic neurons were suppressed by aversive foot shock (Jennings et al., 2013). Also activation of BNST glutamatergic projection to the VTA is aversive whereas activation of BNST GABAergic projection in the VTA is rewarding (Jennings et al., 2013).

Nucleus accumbens

The NAc integrates reward-related information conveyed by dopaminergic and GABAergic inputs from the midbrain with glutamatergic inputs from the forebrain. The NAc is composed of two anatomically distinct regions, core and shell (Jongen-Relo, Voorn, & Groenewegen, 1994; Zahm, 2000), which received different afferent projections and have different projection targets (Heimer, Zahm, Churchill, Kalivas, & Wohltmann, 1991). Under this neuroanatomical frame work, the complexity of NAc is further layered by multiple neuronal cell types. Approximate 95% of neurons in the NAc are medium spiny projection neurons (MSNs), which use GABA (Gamma-aminobutyric acid) as the main neurotransmitter, and expressing either D1-like receptors (D1 and D5) or D2-like receptors (D2, D3, and D4). D1 receptors are coupled with G_s/G_{olf}, the activation of which will stimulate adenylyl cyclase to produce cyclic AMP (cAMP), an intracellular secondary messenger. cAMP can activate the cAMP-dependent protein kinase (PKA), which will lead to the phosphorylation of downstream enzymes and transcription factors involved in synaptic plasticity. D2 receptors are coupled to G_i/G_o, and tend to inhibit adenylyl cyclase and induce an inwardly rectifying K^+ current, which will lead to the hypopolarization of the neuron. Importantly, activation of D2 auto-receptors on the dopamine terminals will inhibit release of dopamine (Berhow, Hiroi, & Nestler, 1996). Based on radioligand binding studies of dopamine receptors in striatum, D1 receptors have lower affinity for dopamine compared with D2 receptors (Creese, Sibley, Hamblin, & Leff, 1983), thus will only be activated when synaptic DA level is high. In contrast, D2 receptors have high affinity for dopamine, thus are very sensitive to the reduction of synaptic DA level. MSNs can express D1R or D2R predominantly, or

both D1R and D2R, and these different subtypes of MSNs in the NAc exert balanced but antagonistic influences on reward-related behaviors. There is evidence suggesting that D2-MSNs in the NAc exert an inhibitory influence on brain rewarding circuitry. Activation of MSNs expressing D2 receptors (D2R-MSNs) reduces the reinforcing effect of drugs of abuse and suppresses the drug seeking behavior, and the inhibition of D2R-MSNs leads to enhanced motivation for cocaine (Bock et al., 2013). In contrast to the effect of D2-MSNs activation, activation of D1R-MSN has the opposite effect, which leads to the increased rewarding effect of cocaine shown by conditioned place preference. Such opposite actions of D1 and D2-MSNs is very similar to the cell-type specific role of brain-derived neurotrophic factor (BDNF) in this area (Lobo et al., 2010), and role BDNF-TrkB signaling in the NAc in sensitization to drugs of abuse will be discussed in the following chapters.

Neurons in the striatum, including the NAc, also have distinct functions in motor activity, which are D1 or D2 receptor-specific and neural circuit-specific. The D1 expressing striatal neurons, which project to the basal ganglia (direct pathway), can facilitate body movement; the D2 expressing striatum neurons, which project to the globus pallidus (indirect pathway), can inhibit body movements. Due to the difference in binding affinity with dopamine, D1R and D2R respond to different pattern of dopamine release distinctively. DA neurons phasic burst leads to heightened DA level, which primarily activate D1R expressing neurons in direct pathway, and stimulate locomotor function to actively pursue the reward. In contrast, negative reward prediction error induces pause of VTA DA neuron firing that will produce low DA, and D2R expressing

neurons are more susceptible to such change and will be inhibited, thus lead to the suppression of movement via indirect pathway.

These MSNs are supplemented by sparsely distributed cholinergic interneurons and GABAergic interneurons. Recently, more evidence suggests that cholinergic interneurons in the NAc, which constitute only 3% of the neuronal population, provide cholinergic tone for the whole area and play an important role in reward/aversion discrimination (M. T. Brown et al., 2012) and modulation of dopamine signaling related with the reinforcing effect of cocaine (Mark, Shabani, Dobbs, & Hansen, 2011).

Prefrontal cortex

In rodents, the prefrontal cortex (PFC), composed of anterior cingulate (ACG), prelimbic (PrL), and infralimbic (IL) cortices, is a major component of the mesocorticolimbic circuit. It receives extensive dopaminergic projections from the VTA. The PFC is a critical region mediating goal-directed behaviors and impulse control. The major projection neurons in the ACG, PrL and IL cortices are pyramidal neurons, which send glutamatergic projection to the NAc and the VTA. Cortical inputs to the NAc (arising in the prefrontal, anterior cingulate cortices) modulate accumbal function and its subsequent inhibitory outputs to the VTA, which can modulate excitability of VTA DA neurons and the strength of dopamine transmission in the brain rewarding circuit. The loss of control over drug intake is partially due to the disruption of the normal function of prefrontal cortex (PFC), which are the regulation of mesolimbic reward pathway and higher-order executive function (Goldstein & Volkow, 2011).

Based on positron emission tomography (PET) and functional MRI (fMRI) brain imaging studies in humans, multiple sub-regions of the PFC (ACG, the orbitofrontal, and

dorsal lateral prefrontal cortices) showed significant differences in activity between addicts and healthy control subjects during tasks involving working memory, decision making, inhibitory control, emotion and motivation, and cue reactivity and drug administration (Bolla et al., 2004; Ersche et al., 2005; Goldstein et al., 2009). Also decreased basal activity in the PFC was found during drug withdrawal (Goldstein & Volkow, 2002) and a significant increase after exposure to drug-related cues (Childress et al., 1999).

Mesocorticolimbic circuit in response to psychostimulants and stress

The function of the mesocorticolimbic dopamine circuit during the induction and expression of sensitization had been investigated extensively in the previous several decades, and the VTA has been identified as the action site of psychostimulants during the induction of sensitization (Paul Vezina & Stewart, 1990). Microinjection of AMPH into the VTA, but not the NAc, can induce the behavioral sensitization (P W Kalivas & Weber, 1988) and a sensitized dopaminergic response in the NAc to peripheral psychostimulants (Paul Vezina, 1993), suggested that the VTA is necessary for the induction of sensitization. In contrast, 6-hydroxydopamine-induced (6-OHDA) destruction of DA neurons in the VTA disrupts cocaine self-administration (Roberts & Koob, 1982), and prevents AMPH/cocaine induced behavioral sensitization (Kelly, Seviour, & Iversen, 1975). Destruction of dopaminergic terminals in the NAc also disrupts cocaine and amphetamine self-administration (Roberts, Koob, Klonoff, & Fibiger, 1980). The evidence above suggests that VTA DA neurons innervating the NAc are necessary for compulsive drug taking behavior.

Besides drugs of abuse, stress also can activate the mesocorticolimbic dopamine system. Interestingly, mild/acute stress preferentially activates mesocortical dopamine transmission and increases extracellular DA levels in the prefrontal cortex (Thierry et al, 1976). In contrast, higher intensities of stress reduce dopamine levels in the prefrontal cortex but increases dopamine transmission in the mesolimbic circuit (Prasad, Sorg, Ulibarri, & Kalivas, 1995; Sorg & Kalivas, 1991). This hetero-regulation of mesocorticolimbic dopamine transmission by stress suggests that different subpopulation of dopamine cells in the VTA are differentially affected by different intensities of stress. The stress induced neuroadaptation in the mesocorticolimbic circuit will be discussed in more detail in the coming chapters.

Stress

Overview on physiological effects of stress

Any external threat, real or perceived, which can alter homeostasis, is defined as "stress"(Rivier, 1990). Stress takes place in conditions where an environmental demand exceeds the natural regulatory capacity of an organism, in particular situations that include unpredictability and uncontrollability. The hypothalamus is the principal target of stress, which will transduce stress induced neural impulses into hormonal secretion (Brodish & Redgate, 1973). Stress induced excitatory inputs first converge on neurons located in the paraventricular nuclei of the hypothalamus (PVN), which will release corticotrophin-releasing factor (CRF)(M. R. Brown, Koob, & Rivier, 1990). CRF is released into hypothalamo-hypophyseal portal vessels, and is carried a short distance to the anterior pituitary. In response to CRF, the pituitary gland secrets a hormone, known as adrenocorticotrophic hormone (ACTH) into blood. ACTH is carried a long distance to

the adrenal cortex. In the adrenal cortex, ACTH promotes the synthesis and secretion of corticosteroids. In rat, the major corticosteroid is corticosterone, and in humans it is cortisol. Corticosteroid (alternative name: glucocorticoid) can stimulate glucose metabolism (gluconeogenesis), facilitate breakdown of fats (lipolysis), and reduce glucose uptake (Baxter & Rousseau, 1979). All these effects of corticosteroid lead to increased plasma glucose, which provides readily metabolizable fuel for the reaction to an emergency.

Viewing from a holistic fashion, the response to stressors can be beneficial: it helps animals be ready to adapt to the environment and can lead to a positive psychological state in some particular situation. HPA-axis stimulation, indexed by the level of corticosteroids in response to different types of stimuli in animals is shown in Figure 3.

However, prolonged exposure to stress or too intensive stressful stimulation could cause adaptation failure, wherein the organism reaches a phase of exhaustion and adverse consequences will follow. Depression and post-traumatic stress disorders are common pathologies induced by chronic stressful experience. Chronic stress also impairs the immune system by reducing lymphocytes levels in the circulation. There is a high density of steroid-receptors located on lymphocytes, and stress induced elevation of corticosteroids leads to excessive activation of those receptors and causes the removal or even the destruction of lymphocytes, resulting in the immune-suppressive effects (Craddock, 1978).

Predictability and controllability determine the efficacy of a particular stressor (Toates, 1995). The aversive effect of the stressor can be greatly alleviated if the stressor

is predictable. For example, when a sound cue preceded the onset of electrical shock, the effect of the shock, indicated by stomach ulceration, is significantly reduced compared with un-signaled shock (Guile & McCutcheon, 1984). Also the elevation of corticosterone level is greater following unpredictable stress than predictable stress (Weiss, 1970). When the stressor is signaled by a cue, the cue acquires a fear-eliciting capacity, and the anticipation induces higher corticosteroids than the confrontation of the stressor (A. Z. Arthur, 1987). In the absence of the cue, the subjects can relax. However, if the stressor is unpredictable, then the whole environment acquires a fear-eliciting capacity and the subject can never escapes from the stressful condition (Toates, 1995). The aversive effect of stress can be ameliorated if subjects are able to exert control to terminate stress or avoid it. For example, rhesus monkeys that had control over the stressor, a high-intensity noise, showed normal levels of plasma corticosteroid. In contrast, the yoked monkeys, who were exposed to the same amount of stressful stimulation, showed significantly elevated levels of corticosteroid (Hanson, Larson, & Snowdon, 1976).

Effect of stress on dopamine transmission in mesocorticolimbic circuit

Exposure to various forms of stress can result in a metabolic activation of mesocorticolimbic dopamine system. For example, extracellular DA concentration in the NAc are increased in response to electric shock, tail pinch, physical restraint, and social defeat (Gresch, Sved, Zigmond, & Finlay, 1994; Imperato, Angelucci, Casolini, Zocchi, & Puglisi-Allegra, 1992; Tidey & Miczek, 1996). The magnitude and specificity of the dopaminergic response to stress depends on the intensity, the duration, and the intermittency of stress (A. Y. Deutch, Clark, & Roth, 1990; Thierry, Tassin, Blanc, &

Glowinski, 1976). As noted earlier, mild stress selectively influences the metabolic activation of the dopaminergic innervation onto the prefrontal cortical areas, whereas increases in either the intensity or duration of footshock stress increases DA utilization in the NAc as well as in the PFC (Dunn, 1988; Roth, Tam, Ida, Yang, & Deutch, 1988). These data suggest that the pattern of stress-induced DA neurons activation is differentiated according to different stressful states.

The mesocortical DA projection arises primarily from the ventral tegmental area (VTA) and terminates mainly on pyramidal neurons in deep layers V and VI of medial PFC (mPFC) (Bjorklund et al., 1978; Emson and Koob, 1978; Berger et al., 1991; Carr et al., 1999). Many of the PFC neurons project to the VTA and the NAc, and modulate the mesolimbic DA system (Sesack and Pickel, 1992; Carr and Sesack, 2000). Interestingly, the depletion of mesocortical dopaminergic innervation results in enhanced responsiveness of the mesolimbic dopamine circuit to stress (A. Y. Deutch et al., 1990), suggesting a homeostasis compensatory mechanism in mesocorticolimbic DA circuit.

Stress selectively increases dopamine metabolism in the VTA but not in the substantia nigra (Ariel Y. Deutch, Tam, & Roth, 1985), and these data suggest that cell bodies of origin of the mesocorticolimbic dopaminergic system are activated by stress in contrast to those DA neurons innervating the striatum. Indeed, stress-induced increases of dopamine transmission and metabolism were observed in the NAc, but not the dorsal striatum (Roth et al., 1988). Therefore the mesolimbic DA neurons appear to be more responsive to stress than the striatal DA system. Stress induced increase of DA transmission inhibits pyramidal neurons in the PFC (Bunney & Aghajanian, 1976). Also the inhibitory effect of DA on PFC neurons appears to be more potent in the deep cortical

layers in which the neurons projecting to the striatum are located (Sesack, Deutch, Roth, & Bunney, 1989). These data suggest that stress selectively activate dopamine transmission and metabolism in mesocorticolimbic circuit.

Animal models in stress research and the advantage of social defeat stress

Many stress models have been employed in animals to study the neuroadaptation, physiological and behavioral changes induced by stress. All these experimental stressors can be categorized into two types: the exposure to an aversive stimulation or the deprivation of a condition which is necessary for homeostasis (L. Lu, Shepard, Hall, & Shaham, 2003). To name a few, electrical foot shock, restraint stress, and social defeat belong to the first type of stressor, and food/water deprivation, maternal deprivation, social isolation belong to the second type of stressor. Also as mentioned above, the controllability and predictability determines the efficacy of stress in general. It is important to note that the effect of stress on behavior is stressor-specific, schedule-specific, and intensity-specific. For example, exposure to the same stressor, but in the different intermittency and duration, leads to qualitatively different effects on behaviors and neurochemistry (Miczek, Nikulina, Shimamoto, & Covington, 2011).

The stress model used in this dissertation, social defeat stress, is a naturalistic intraspecific agonistic encounter. The defeat experience after a brief episode of agonistic interaction cannot be habituated upon repeated exposure due to its uncontrollability and unpredictability. Because social conflict and aggression behavior is a critical facet of natural behavior, it has high face validity in replicating environment change and personal defeat experienced in humans, and serves as a biologically relevant mean of examining

stress induced vulnerability to drugs of abuse. Social defeat stress is the stress model used in the present study.

During the social defeat stress, a rise of corticosteroid levels in plasma is found in both winner and loser rats following conflict. However, the rise was higher and more durable in losers than winners. After the corticosteroid levels had returned to baseline, the confrontation with a non-aggressive conspecific induced sensitized HPA axis response and significantly higher corticosteroids level only in defeated rats (Schuurman, 1981). Importantly, the intermittent social defeat stress model and the continuous subordination model use the same stressor, intraspecific agonistic encounter, but with different duration and intermittency, have totally opposite effect on behaviors. The qualitative difference between these two models leads to the activation of distinct brain circuits and behavioral outputs. Intermittent social defeat stress will activate VTA-accumbens-PFC-amygdala circuit and induce sensitization to stimulant drugs, whereas the continuous subordination will activate raphe-PFC-hippocampus circuit and lead to anhedonia-like effects and depression (Miczek, Yap, & Covington, 2008). In contrast to intermittent social defeat stress, continuous social subordination leads to suppressed locomotor response to cocaine challenge, and reduced DA transmission in the NAc and VTA BDNF expression (Miczek et al., 2011). In the present studies, we used acute or intermittent social defeat stress models, which will sensitize animals to psychostimulants without causing any depressive effect.

It is of great importance to investigate the neural mechanisms underlying the social defeat stress induced cross-sensitization to drugs of abuse, which will shed light into potential therapy to prevent stress induced vulnerability to addiction.

Cross-sensitization Between Psychostimulants and Stress

Although the initial drug-taking decision is voluntary for most people, the transition from the leisure drug using to compulsive drug taking varies among individuals. The predisposition to develop addiction to drugs of abuse depends on the intrinsic nervous system and can be heavily affected by stimulation from the environment, such as stress. Stress is an etiological factor in the pathogenesis of addiction, which can increase the likelihood to develop drug abuse in vulnerable individuals. Stress from the society contributes, at least partially, to the soaring number of drug abusers nowadays, presumably due to its effect on compulsive drug taking and relapse (Sinha, 2008).

Cross sensitization is the augmented response to a specific type of drug of abuse induced by some stimuli other than drug. Similar to the sensitization developed during repeated drug exposure, exposure to stress also can induce sensitized response to abused substances, which engender higher vulnerability to develop addiction. Stress induced vulnerability to addiction is a phenomenon found broadly in humans, and has been recapitulated by laboratory animal models (Antelman, Eichler, Black, & Kocan, 1980; J. J. Yap & K. A. Miczek, 2008). Many forms of stressors were found to be able to augment locomotor activity in response to drugs of abuse in animals (Antelman et al., 1980; Piazza, Deminiere, le Moal, & Simon, 1990; Tidey & Miczek, 1997).

Stress sensitizes the mesocorticolimbic dopamine circuit

Enhanced DA function in the mesocorticolimbic system, especially in the NAc, is the common neural substrate underlying the sensitization to drugs of abuse (Vanderschuren & Kalivas, 2000) and stress-induced cross-sensitization. For example, forced swim stress can increase the sensitivity of VTA dopamine neurons to excitatory

inputs, and this acute stress induced synaptic strength in VTA dopamine neurons can be prevented by glucocorticoid receptor antagonist (Saal, Dong, Bonci, & Malenka, 2003). Also acute restraint stress leads to increased extracellular dopamine levels in the NAc and cross-sensitization to cocaine (Garcia-Keller et al., 2013). Foot shock stress can increase dopamine levels in the VTA of cocaine experienced rats but not drug-naïve rats, and such an effect can be prevented by intra-VTA infusion of CRF-receptor 2 antagonist (Wise & Morales, 2010). Social defeat stress induces similar dopamine response in the NAc and motor activity in relative to AMPH challenge (Miczek, Mutschler, van Erp, Blank, & McInerney, 1999).

Extensive evidence suggests that stress induced increase of dopamine transmission or the sensitivity of dopamine neurons is mediated, at least partially, by the increase of stress hormones. A study by the Wise group (B. Wang et al., 2005) found that foot shock induces elevation of glutamate, dopamine and CRF release in the VTA of cocaine-experienced rats, accompanied with stress-induced reinstatement to cocaine. Such stress induced neurochemical changes and reinstatement can be recapitulated by intra-VTA infusion of CRF, or be prevented by either CRF antagonist or inotropic glutamate receptor antagonist, kynurenic acid (B. Wang et al., 2005). Taken together, foot shock stress induces the release of CRF in the VTA, which can bind with CRF receptors on the presynaptic terminals and trigger glutamate release from these terminals in the VTA. The elevation of glutamate release in the VTA activates local dopamine neurons and leads to the increased release of dopamine, which induces vigorous reinstatement. Wang and colleagues further showed that such stress-induced neurochemical changes and reinstatement are mediated by CRF2-receptor (B. Wang, You,

Rice, & Wise, 2007). Direct infusion of CRF into the VTA increase the sensitivity of dopamine neurons to glutamatergic input through the potentiation of N-methyl-D-aspartate receptor (NMDAR)-mediated synaptic transmission in VTA dopamine neurons (Ungless et al., 2003). The potentiation of NMDAR-mediated synaptic transmission leads to increased neural activation and Ca^{2+} influx, which can stimulate many downstream targets and activity induced gene transcription, such as BDNF. The role of BDNF in stress-induced neuroadaptation will be discussed in the following chapters.

The role of the HPA axis and stress hormones in cross-sensitization

Both stress and psychostimulants administration enhance dopamine transmission from the VTA to the limbic forebrain and cortex (P. W. Kalivas & Stewart, 1991; Tidey & Miczek, 1997) and can activate the HPA axis leading to elevated ACTH and corticosteroids (Borowsky & Kuhn, 1991; Natelson, Tapp, Adamus, Mittler, & Levin, 1981). There are two common substrates in the central nervous system affected by stress and drugs of abuse: the mesocorticolimbic dopamine circuit and the hypothalamo-pituitary-adrenal (HPA) axis. Therefore, "between system cross-sensitization" was not restrictive to the changes in the mesocorticolimbic dopamine circuit induced by stress; drugs of abuse induce sensitization in both mesocorticolimbic dopamine circuit and brain stress system. Many kinds of abused substances such as alcohol, nicotine, psychostimulants, and opiates that stimulate mesocorticolimbic dopamine systems also activate brain stress systems (Baumann et al., 1995; Cinciripini et al., 1989; Cobb & Van Thiel, 1982). Greater behavioral reactivity and bodily responses to stress are found in cocaine addicts, reflected the sensitization in both the HPA axis and the autonomic nervous system (Abercrombie et al., 1989). Based on evidence from laboratory animals,

non-contingent acute cocaine administration increases plasma levels of ACTH and corticosteroids, which is mediated by the cocaine induced release of CRF from paraventricular nucleus (Goeders, 2002; Sarnyai, Shaham, & Heinrichs, 2001). Also clinical research showed that increased secretion of cortisol and ACTH was found in cocaine addicts after acute intravenous cocaine administration (Mello & Mendelson, 1997), and positive correlation between stress and craving for cocaine was found in cocaine dependent individuals (Brady et al., 2009). Thus these data suggest a reciprocal sensitization process between the brain stress system and the brain reward system which can be induced by exposure to stress or drugs of abuse.

However, elevation of stress hormone and dopamine elevation cannot fully explain the persistence of cross-sensitization behavior. There is a study showing that behavioral sensitization to drugs of abuse is independent of corticosterone levels (Nelson, Kleschen, & Zahniser, 2010). Elevation in dopamine transmission and corticosteroid circulation returned to baseline level in a short period of time, but the cross-sensitization can last several months. Thus, the enduring nature of cross-sensitization suggests the involvement of factors which are capable of inducing long-term neuroplasticity and recruiting transcription machinery. Such factors can translate the short-lasting fluctuation of neurotransmitters levels into the long-lasting neuroplasticity and gene expression changes. BDNF is one such candidate factor. Immediately after social defeat stress, psychostimulant challenge-induced stimulation of psychomotor response remained insignificant, and only became obvious several days after the defeat. It is important to note that BDNF expression in the VTA also remains unchanged immediately after social defeat stress, and becomes significantly elevated at least one week after defeat. This

similarity between the temporal pattern of cross-sensitization and VTA BDNF expression after social defeat stress suggests that the elevation of BDNF in the VTA may underlying the persistent cross-sensitization after intermittent social defeat stress (Fanous, Hammer, & Nikulina, 2010).

Role of Mesocorticolimbic BDNF in Stress- and Drug-induced Neuroplasticity

Overview of the structure of bdnf gene

Brain-derived neurotrophic factor (BDNF) was the second neurotrophin to be discovered and purified; it shows similar amino acid sequence and neurotrophic effect on cultured neurons relative to nerve growth factor (Barde, Edgar, & Thoenen, 1982).

Based on genomic analyses, a total of nine promoters have been identified in rodents and human bdnf gene, in the 5' untranslated region, and one protein coding 3' exon (Aid, Kazantseva, Piirsoo, Palm, & Timmusk, 2007; Pruunsild, Kazantseva, Aid, Palm, & Timmusk, 2007). Although alternative splicings produce multiple transcripts of BDNF, all of them share the same coding region. Among all 9 promoters found so far, promoter IV is sensitively regulated by neural activity, and it contains multiple calcium-response sequences and calcium-response elements (CRE) to which calcium responsive transcription factor (CaRF) and cAMP response-element binding protein (CREB) bind, respectively (Metsis, Timmusk, Arenas, & Persson, 1993). The complex BDNF gene structure leads to the production of distinctive BDNF transcripts through alternative splicing and polyadenylation, which allow brain region-specific, neural type-specific and neural activity-specific regulation of BDNF expression. BDNF transcript synthesis can be initiated from multiple distinctive promoters, and two types of BDNF transcripts can be produced, with either short or long 3' untranslated regions (3' UTR), due to the

distinctive polyadenylation at two alternative sites. The transcripts with short 3' UTR are restricted in the soma portion, only the transcripts with long 3' UTR can be localized in the distant dendrites (Lau et al., 2010). The long 3' UTR of the BDNF transcript is required for the development of dendritic spines and long-term potentiation (An et al., 2008).

Translation, processing and secretion of bdnf gene products

BDNF expression is tightly regulated by neural activity. Both endogenous synaptic activity in response to sensory input (Castren, Zafra, Thoenen, & Lindholm, 1992) or artificial stimulation which are capable of inducing LTP can lead to the expression of BDNF mRNAs (Patterson, Grover, Schwartzkroin, & Bothwell, 1992). BDNF is synthesized as pro-BDNF (32 kilodalton, KD) in the cell then proteolytically processed into mature BDNF (14 KD). In contrast with the neurotrophic effect of mature BDNF via binding with neurotrophin tyrosine kinase receptor type 2 (TrkB), pro-BDNF binds with $P75^{NTR}$ and induces neuronal apoptosis (Teng et al., 2005). In addition, pro-BDNF can be processed in the extracellular space by tissue plasminogen activator/plasmin (Pang et al., 2004). In layer II/III of cortical slices, θ-burst stimulation induced BDNF synthesis and secretion in its pro-form. Released pro-BDNF can be rapidly internalized in perineuronal astrocytes, thereby restricting the availability of BDNF at extraneuronal space (Bergami et al., 2008). After internalization in astrocites, BDNF undergoes a recycling process, endowing astrocytes with the ability to resecrete BDNF upon stimulation. Bedsides internalization of BDNF, astrocytes can sequester mature BDNF secretion from neuronal terminals, as a protective mechanism under stress (Crish et al., 2013). It was recently reported that cultured hippocampal neurons primarily

store and secrete mature BDNF during neural activity (Matsumoto et al., 2008). However, the cellular location and mechanism of the processing of pro-BDNF into mature BDNF remain elusive.

Several transcription factors can regulate transcription of bdnf gene in response to neural activity. In general, neurotransmitter binding and membrane depolarization can initiate Ca^{2+} influx through voltage-gated Ca^{2+} channels, N-methyl-D-aspartate receptors (NMDARs), or from the inner stores. As a secondary messenger, Ca^{2+} can activate intracellular signaling pathways which transduce the signal to the nucleus, leading to the activation of transcription factors, such as CREB and CaRF. Both CREB and CaRF can activate the expression of BDNF (X. Tao, Finkbeiner, Arnold, Shaywitz, & Greenberg, 1998; Xu Tao, West, Chen, Corfas, & Greenberg, 2002). Another transcription factor activated by Ca^{2+}, methyl-CpG binding protein 2 (MeCP2), also can regulate BDNF gene expression (W. G. Chen et al., 2003). MeCP2 is a transcriptional repressor of the bdnf gene and well known for its role in the pathology of Rett syndrome (Chahrour & Zoghbi, 2007). It selectively binds to the promoter of exon IV of bdnf gene, recruits histone deacetylase which can cause DNA methylation in this area, resulting in transcriptional repression (P. L. Jones et al., 1998). The promoter region of BDNF exon IV contains specific binding sites for both MeCP2 and CREB, and both are phosphorylated in the presence of neuronal activity-induced Ca^{2+} elevation. Upon phosphorylation, phosphorylated CREB (pCREB) translocates into the nucleus and associates with CRE elements in the exon IV promoter of bdnf, thus leading to the transcriptional activation. In contrast, phosphorylation of MeCP2 in the MeCP2/HDAC/Sin3A complex will lead to

the dissociation of the complex from bdnf gene (W. G. Chen et al., 2003), thus leading to the removal of transcriptional repression on the bdnf gene.

Because bdnf mRNA can be transported to dendrites and translated there in response to local stimulation, BDNF can be synthesized both in the soma and the dendrites. High potassium depolarization induces elevation of BDNF mRNA levels in dendrites of cultured hippocampal neurons. This increase of BDNF mRNA is independent of new RNA synthesis occurring in the soma, but is neural activity-dependent, and requires the presence of extracellular Ca^{2+} (Tongiorgi, Righi, & Cattaneo, 1997). Neural activity-induced BDNF expression can be detected in dendrites even when transport of BDNF from soma to dendrites is inhibited by nocodazole. However, such dendritic BDNF expression can be abolished by the protein synthesis inhibitor, cycloheximide (Tongiorgi et al., 1997). These data suggest that bdnf mRNA can be transported to dendrites and translated into protein there in response to the local neural activation.

Release of BDNF and intracellular signaling cascade activated by BDNF, and reuptake of BDNF

Due to the essential role of BDNF in synaptic plasticity, the neuroplasticity in the mesocorticolimbic circuit can be modulated by the level of BDNF signaling. The release of BDNF is mainly Ca^{2+} dependent. Voltage-gated Ca^{2+} channels, NMDA receptors and intracellular stores are the major sources of intracellular Ca^{2+}, which induces the exocytosis of BDNF-containing vesicles (Kolarow, Brigadski, & Lessmann, 2007; Kuczewski, Porcher, & Gaiarsa, 2010). BDNF has the high affinity for the TrkB receptor. The binding of BDNF with TrkB induces the dimerization of TrkB receptors and the

autophosphorylation at tyrosine sites of the receptors (Minichiello, 2009). Phosphorylation on the tyrosine sites on the intracellular domain of TrkB provide the docking sites for adapter molecules, such as Src homology 2 domain containing adaptor protein (Shc). The adaptor proteins link the activated receptor to other enzymes, culminating in the activation of at least three signaling cascades: 1) the microtubule-associated protein kinase (MAPK) pathway, which is involved in neuronal differentiation and neurite outgrowth; 2) the phosphatidyl inositol-3 kinase (PI3K)-Akt (serine threonine kinase) pathway, which enables cell survival; and, 3) the phospholipase Cγ (PLC gamma)-Ca^{2+} pathway, which mediates synaptic plasticity-induced Ca^{2+} release from intracellular stores. An illustration of BDNF-TrkB intracellular signaling pathways can be found in Figure 4.

It is important to note that BDNF is a critical modulator of activity-induced synaptic plasticity (LTP and LTD). BDNF potentiates the efficacy of excitatory synapses (E. S. Levine, Dreyfus, Black, & Plummer, 1995) but depresses the efficacy of GABAergic synapses (Bolton, Pittman, & Lo, 2000; Tanaka, Saito, & Matsuki, 1997). Therefore, elevated BDNF in the VTA may enhance the excitability of DA neurons there.

BDNF signaling in response to psychostimulants.

Acute exposure to psychostimulants induces the short-lasting increase of BDNF mRNA and protein in the PFC and NAc (Fumagalli, Di Pasquale, Caffino, Racagni, & Riva, 2007; Le Foll, Diaz, & Sokoloff, 2005; Saylor & McGinty, 2008), which might be mediated by cocaine-induced phosphorylation on MeCP2 and CREB. A progressive increase of BDNF expression levels with time was also observed in several regions of the mesolimbic circuit (VTA, NAc, and amygdala) up to 90 days after the cessation of

cocaine self-administration, accompanied by intensified cue-induced reinstatement and increased risk for relapse (Jeffrey W. Grimm et al., 2003), which is defined as "incubation of drug craving".

Many studies showed that BDNF can enhance behavioral response and sensitivity to reinforcing effect of drugs of abuse. The infusion of BDNF protein into the VTA or the NAc can induce the sensitization and reinstatement to drug or drug-related cues (Bahi, Boyer, Chandrasekar, & Dreyer, 2008; Graham et al., 2007; Guillin et al., 2001; Horger et al., 1999; L. Lu, Dempsey, Liu, Bossert, & Shaham, 2004; Vargas-Perez et al., 2009). In contrast, reduction of BDNF-TrkB signaling in the NAc can prevent social defeat stress induced cross-sensitization to AMPH (our unpublished data) and the psychomotor sensitization to meth-AMPH (Narita, Aoki, Takagi, Yajima, & Suzuki, 2003).

In contrast, BDNF in the PFC showed a different expression pattern in response to drugs of abuse compared with BDNF in the NAc. Twenty-two h after the cocaine self-administration, a reduction of BDNF mRNA levels was observed in the PFC, but such reduction is no longer observable 21 days later (McGinty, Whitfield, & Berglind, 2010). Also, infusion of BDNF into the PFC after cocaine self-administration attenuates relapse and cue/cocaine prime-induced reinstatement of cocaine-seeking after extinction (Berglind et al., 2007). These data suggest the brain region-specific effect of BDNF in the mesocorticolimbic circuit during sensitization and reinstatement to drugs of abuse.

BDNF expression in response to social defeat stress.

As mentioned above, different duration of social defeat stress exposure results in the distinct neurochemical and behavioral outputs. The VTA BDNF expression is also affected by the duration of social defeat stress exposure. In response to intermittent social

defeat stress, VTA BDNF is increased, whereas chronic social defeat stress (continuous subordination) leads to the reduction of VTA BDNF. Also long-term behavioral sensitization to psychostimulant and facilitated acquisition of drug self-administration are found in rats exposed to intermittent social defeat stress (4 defeats in 10 days) (Miczek et al., 2011). In contrast, continuous social defeat stress (5 weeks) induces blunted sensitization to psychostimulant and acquisition of drug self-administration (Miczek et al., 2011). Continuous social subordination-induced depression and anhedonia may contribute to the blunted response to psychostimulants and reduced reinforcing effect of psychostimulants during self-administration. The level of BDNF expression in the VTA may determine the behavioral output, either the sensitization to psychostimulants or the depression/anhedonia.

A large preclinical literature shows that stress reduces BDNF expression in the hippocampus, whereas chronic antidepressants treatment upregulates BDNF signaling (Duman & Monteggia, 2006). A variety of acute (single stress) and chronic (7 to 21 days) stress paradigms, including social defeat stress (Pizarro et al., 2004), can decrease the expression of BDNF in the hippocampus, which can be reversed by chronic treatment with antidepressants (Duman & Monteggia, 2006; Roceri, Hendriks, Racagni, Ellenbroek, & Riva, 2002; Ueyama et al., 1997; Xu et al., 2004). More importantly, direct infusion of BDNF into the hippocampus induces antidepressant effects (Shirayama, Chen, Nakagawa, Russell, & Duman, 2002). In contrast to the antidepressant effect of BDNF in the hippocampus, BDNF exerts a potent pro-depressant effect in the mesolimbic circuit (Eisch et al., 2003), and increases susceptibility to social defeat stress (Vaishnav

Krishnan et al., 2008). Taken together, these data underscore crucial region-specific action of BDNF during depression.

The effect of social defeat stress on BDNF expression is also age dependent. Social defeat stress induces robust elevation of BDNF mRNA in hippocampus in adolescence, but not in adulthood (Coppens et al., 2011). The coping style during agonistic confrontation also affects the level of BDNF expression. A significant increase of BDNF mRNA in the NAc was found in reactive coping defeated rats (>65% time spent on offensive behavior) than proactive coping defeated rats (<15% time spent on offensive behavior) and control handled rats (Coppens et al., 2011).

BDNF gene polymorphism-induced propensity for drug abuse in humans

Recently a single-nucleotide polymorphism (SNP) in the human BDNF gene was found, which causes a valine-to-methionine change at position 66 (Val66Met) in the prodomain of BDNF. Val66Met impairs sorting of BDNF into secretory granules and activity-induced BDNF secretion (Z. Y. Chen et al., 2004; Egan et al., 2003). Thus, the 66Val allele homozygous is associated with higher BDNF secretion in response to neuronal stimulation compared with the 66Met allele homozygous and Met/Val heterozygous. Higher BDNF 66Val homozygote frequency in people with drug addiction was found compared with normal control subjects (Cheng et al., 2005; S. J. Tsai, 2007), and more sensitized response to AMPH was reported from BDNF 66Val carriers (Flanagin, Cook, & de Wit, 2006). These data suggest that higher activity-induced BDNF transmission positively correlates with higher vulnerability and physical response to drugs abuse.

Transcriptional Mechanism of DeltaFosB in response to Chronic Exposure of Drug or Stress

General background: stability of FosB family transcription factors

The acute administration of almost all drugs of abuse induces expression of members of the Fos family transcription factors, also called immediately early genes, in the NAc. Those Fos family members can couple with Jun family proteins to form the AP-1 complex of transcription factors, which can bind to AP-1 sites (consensus sequence: TGAC/GTCA) in promoter areas of genes and regulate their expression. However, during chronic exposure to drugs of abuse, transcription of all other members of Fos family but DeltaFosB develop tolerance (Renthal et al., 2008), showing reduced levels of expression compared to the level after acute drug exposure. In contrast, the truncated isoform DeltaFosB continues to be produced and accumulates to considerable level due to its unique stability.

DeltaFosB and FosB are encoded by the same fosb gene, and shared the same fosb pre-mRNA transcript, which contained 5 exons. The alternative splicing of fosb pre-mRNA leads to fosb and Deltafosb mRNAs. There is an intron retention (Intron IV) in the middle of exon 4 and 5, and during the excision of this 140 nucleotide Intron IV, one nucleotide frameshift takes place and a stop codon (TGA) is generated, leading to the premature truncation of the protein, which is DeltaFosB. In contrast, processing without excision of Intron IV produces the full length fosb mRNA containing all 5 exons (Figure 5). Therefore, fosb mRNA contains all 5 exons of the mRNA, but Deltafosb mRNA only contains exons 1 to 4.

There is no difference between the half-lives of mRNAs of DeltaFosB and FosB (Alibhai, Green, Potashkin, & Nestler, 2007). Also both fosB and DeltafosB mRNAs are induced in the striatum by acute administration of psychostimulants or stress. In contrast, DeltaFosB protein showed unique stability due to two mechanisms. First, DeltaFosB lacks the C-terminal 101 amino acids sequence, which contains two degrons domains targeted by ubiquitin-mediated degradation (Carle et al., 2007). Second, DeltaFosB can be phosphorylated at the N-terminus by casein kinase 2 or other unknown kinases, conferring extra stability (Ulery, Rudenko, & Nestler, 2006). Figure 6 shows the amino-acid sequence of FosB and DeltaFosB proteins.

Mesolimbic DeltaFosB expression induced by stress or drugs of abuse

DeltaFosB is one molecule with a very long half-life induced after chronic exposure to drugs of abuse (Hope et al., 1994), which could be responsible for the persistence of addictive state after the cessation of the drug exposure. DeltaFosB in the NAc is induced by chronic exposure to almost all kinds of drugs of abuse, such as opiates (Nye & Nestler, 1996), cocaine (Hope et al., 1994), amphetamine, methamphetamine (McDaid, Graham, & Napier, 2006), nicotine (Pich et al., 1997), ethanol, phencyclidine, and cannabinoids (Perrotti et al., 2008).

A growing body of evidence suggests that DeltaFosB expression in the NAc regulates the sensitivity to drugs of abuse. Overexpression of DeltaFosB in dynorphin-expressing neuron in the NAc and striatum, the neural population in which DeltaFosB induction takes place during chronic drugs exposure, can induce sensitization to cocaine in drug naïve rats (Kelz et al., 1999). In another study, overexpression of DeltaFosB specifically in the NAc was found to be sufficient to increase the sensitivity to the

rewarding effect of morphine (Zachariou et al., 2006). In contrast, overexpression of DeltaC-Jun, a dominant negative form of C-Jun, which can antagonize the action of all Fos family proteins, reduces the rewarding effect of cocaine and prevents some of the neurochemical alterations induced by chronic cocaine exposure in the mesocorticolimbic circuit (Peakman et al., 2003). Thus DeltaFosB in the NAc is critical for the long-term neurochemical changes and increased sensitivity to numerous kinds of drugs of abuse.

Besides chronic exposure to drugs of abuse, chronic stress exposure also induces DeltaFosB accumulation in terminal regions of the mesocorticolimbic circuit, especially in the NAc (Nikulina, Arrillaga-Romany, Miczek, & Hammer, 2008; Perrotti et al., 2004). It was also found that DeltaFosB accumulation in the NAc can enhance the resilience to social defeat stress (V. Vialou et al., 2010), which suggests that the induction of DeltaFosB works as a defensive mechanism in the mesocorticolimbic circuit against the aversive effects of chronic stress.

Chronic exposure to drugs of abuse or unpredictable stress leads to DeltaFosB expression in different types of neurons in the NAc: drugs of abuse induce DeltaFosB expression in D1-NAc neurons, while stress induces DeltaFosB in both D1- and D2-like receptor-expressing NAc neurons (Perrotti et al., 2004).

Potential interaction between DeltaFosB and BDNF signaling pathway

DeltaFosB mediates function through the regulation of many downstream genes leading to the long-term neuroplasticity in the brain (McClung et al., 2004). VTA BDNF overexpression per se induces DeltaFosB accumulation in the PFC and NAc, the major terminal regions of the mesocorticolimbic circuit. Also increased colocalization of BDNF and DeltaFosB in the NAc, prelimbic (PrL) cortex and medial amygdala was found in

rats exposed to intermittent social defeat stress (Nikulina, Lacagnina, Fanous, Wang, & Hammer, 2012). A representative image of BDNF/ DeltaFosB double labeling in the NAc can be found in the Figure 7.

It is important to note that MSNs, the major neuronal population in the NAc produce very low levels of BDNF. Therefore the strong immunoreactivity of BDNF found here (Figure 7) suggests that considerable level of BDNF is received and retrogradely transported to the soma of MSNs in the NAc. BDNF is known to be retrogradely transported from nerve terminals to cell body regions after binding to the TrkB receptor (DiStefano et al., 1992). Therefore, BDNF innmunoreactivity in MSNs could represent the level of BDNF signaling received, internalized, and retrogradely transported to the some in these neurons. The increased colocalization of BDNF and DeltaFosB after intermittent social defeat stress implicates a potential interaction between BDNF signaling and DeltaFosB expression in these neurons. In addition, overexpression of BDNF in the VTA induces significant increase of DeltaFosB in the NAc shell (J. Wang et al., 2013), suggesting that enhanced BDNF projections from the VTA to the NAc may be responsible for accumulation of DeltaFosB in the NAc shell. However, there are no biochemical data about the direct interaction between BDNF signaling cascades and DeltaFosB expression. Based on the literature, VTA BDNF may prime the transcriptional potential of fosb gene by stimulation of the TrkB receptor - extracellular signal-regulated kinase (ERK) - cAMP response element binding protein (CREB) signaling pathway (J. S. Arthur et al., 2004; Lonze & Ginty, 2002). Activated CREB recruits CREB binding protein (CBP) to elevate histone H4 acetylation in the promoter region of the fosb gene (A. A. Levine et al., 2005), which increases the accessibility of

fosb promoter and up-regulates transcription. Whether there are epigenetic changes on the histone modifications around the promoter region of the fosb gene induced by BDNF signaling is the topic of future study. The investigation of this potential interaction and the underlying molecular mechanism will shed light into the persistence of intermittent social defeat stress induced cross-sensitization to psychostimulants.

Chapter 2

BDNF OVEREXPRESSION IN THE VTA PROLONGS SOCIAL DEFEAT STRESS-INDUCED CROSS-SENSITIZATION TO AMPHETAMINE AND INCREASES DELTAFOSB EXPRESSION IN MESOCORTICOLIMBIC REGIONS OF RATS

Abstract

Social defeat stress induces persistent cross-sensitization to psychostimulants, but the molecular mechanisms underlying the development of cross-sensitization remain unclear. One candidate is brain-derived neurotrophic factor (BDNF). The present research examined whether ventral tegmental area (VTA) BDNF overexpression would prolong the time-course of cross-sensitization after a single social defeat stress, which normally produces transient cross-sensitization lasting less than one week. DeltaFosB, a classic molecular marker of addiction, was also measured in mesocorticolimbic terminal regions. Separate groups of intact male Sprague-Dawley rats underwent a single episode of social defeat stress or control handling, followed by amphetamine challenge 3 or 14 days later. AMPH cross-sensitization was apparent 3 but not 14 days after stress. Intra-VTA infusion of adeno-associated viral (AAV-BDNF) vector resulted in a two-fold increase of BDNF level in comparison with the group receiving the control virus (AAV-GFP), which lasted at least 45 days. Additionally, overexpression of BDNF in the VTA alone increased DeltaFosB in the nucleus accumbens (NAc) and prefrontal cortex. Fourteen days after viral infusions, a separate group of rats underwent a single social defeat stress or control handling and were challenged with amphetamine (AMPH) 14 and 24 days after stress. AAV-BDNF rats exposed to stress showed prolonged cross-

sensitization and facilitated sensitization to the second drug challenge. Immunohistochemistry showed that the combination of virally enhanced VTA BDNF, stress, and AMPH resulted in increased DeltaFosB in the NAc shell compared to other groups. Thus, elevation of VTA BDNF prolongs cross-sensitization, facilitates sensitization, and increases DeltaFosB in mesocorticolimbic terminal regions. As such, elevated VTA BDNF may be a risk factor for drug sensitivity.

Introduction

Cross-sensitization is defined as the augmented behavioral response to a drug challenge produced by intermittent exposure to an agent other than the challenge drug, such as stress or another drug (Antelman et al., 1980; Stewart & Badiani, 1993). In humans, stressful life events increase vulnerability to drugs of abuse, particularly psychomotor stimulants (Sinha, 2008). Likewise, it has been demonstrated in animal models that various forms of stress can increase sensitivity and vulnerability to drugs of abuse (de Jong, Wasilewski, van der Vegt, Buwalda, & Koolhaas, 2005; Miczek, Covington, Nikulina, & Hammer, 2004; Nikulina, Covington, Ganschow, Hammer, & Miczek, 2004; Piazza et al., 1990; Terry E. Robinson, Angus, & Becker, 1985).

The mesocorticolimbic circuitry is comprised of the ventral tegmental area (VTA), where dopamine neurons are located, and its projection areas: the prefrontal cortex (PFC), nucleus accumbens (NAc), hippocampus and amygdala (Swanson, 1982). Both stress and psychostimulants activate the mesocorticolimbic dopamine system and increase dopamine release in VTA terminal regions (Di Chiara & Imperato, 1988; Tidey & Miczek, 1996). Although aversive social events such as social stress can increase vulnerability to the development of drug sensitization, not all individuals exposed to

social stress become sensitized to drugs of abuse. These differences may result from individual genetic differences.

One candidate gene for such individual differences is brain-derived neurotrophic factor (BDNF). Genetic addiction studies in humans have determined that a polymorphism in the bdnf gene is associated with vulnerability for polysubstance abuse (George R. Uhl, Liu, Walther, Hess, & Naiman, 2001). In particular, the presence of the BDNF 66Val allele, which confers greater activity-dependent secretion of BDNF compared to the BDNF 66Met allele (Egan et al., 2003), is associated with greater vulnerability to drug addiction (Cheng et al., 2005; Flanagin et al., 2006; S. J. Tsai, 2007). This finding, coupled with the fact that BDNF plays an important role in survival of dopamine neurons (Baquet, Bickford, & Jones, 2005), activity-dependent neural plasticity (Cohen-Cory, Kidane, Shirkey, & Marshak, 2010), and learning and memory (Bekinschtein et al., 2008), places BDNF in a good position to influence response to both stress or drugs of abuse.

Several lines of research support a role for VTA BDNF in stress- and drug-related behavior. In rodents, intermittent social defeat stress elevates BDNF expression in the VTA (Fanous et al., 2010) and produces lasting cross-sensitization to amphetamine (AMPH) (Nikulina et al, 2004). Furthermore, infusion of BDNF into the VTA increases locomotor response to cocaine (Pierce & Bari, 2001), and facilitates drug sensitization (Horger et al., 1999). In contrast, depletion of BDNF in the VTA prevents the social aversion induced by chronic social defeat stress and increases general social interaction (Berton et al., 2006; Fanous, Terwilliger, Hammer, & Nikulina, 2011). As VTA BDNF is involved in modulating responses to both social defeat stress and psychostimulants, VTA

BDNF may play a role in sensitivity to psychostimulants after social defeat stress. The present study examined the association between enhanced VTA BDNF and increased vulnerability to cross-sensitization and sensitization to AMPH in the presence of social defeat stress.

Another factor that may affect stress-induced sensitization to psychostimulants is DeltaFosB, a truncated form of the FosB transcription factor. DeltaFosB is considered a molecular marker for chronic stimulation of reward circuitry, stress-induced neuroplasticity and sensitization to psychostimulants. DeltaFosB, barely detectable immediately after acute stimuli, accumulates to considerable levels after repeated social defeat stress or repeated drug administration, and persists due to its stability (Nestler, Kelz, & Chen, 1999; Nikulina et al., 2008; Perrotti et al., 2004). DeltaFosB regulates the expression of many neuroplasticity-related genes in reward circuitry after chronic drug exposure (McClung et al., 2004), and increased DeltaFosB in reward circuitry enhances the sensitivity to psychostimulants such as cocaine (Kelz et al., 1999). In the current study, we characterized DeltaFosB in VTA terminal regions as a molecular marker for long term sensitization related to neuroadaptive changes.

Here we over-expressed BDNF in the VTA using an adeno-associated virus (AAV) vector to examine effects on AMPH sensitization in two non-sensitizing behavioral manipulations: 1) long-term cross-sensitization after a single episode of social defeat stress and 2) sensitization after non-sensitizing repeated dosing regimen of AMPH injections (Segal & Mandell, 1974). We chose a single social defeat paradigm because single social defeat stress-induced effects are transient (de Jong, Wasilewski, et al., 2005; Miczek, Nikulina, Kream, Carter, & Espejo, 1999) in comparison with intermittent social

defeat stress that result in long-lasting cross-sensitization (H. E. Covington, 3rd & Miczek, 2001; Fanous et al., 2010; Nikulina et al., 2004). We also investigated whether a single social defeat stress exposure in the presence of elevated VTA BDNF could induce long-lasting DeltaFosB expression in mesocorticolimbic projection regions.

Material and Methods

Animals

Subjects were male Sprague-Dawley rats (Charles River Laboratories, Hollister, CA). Rats weighed 225–250 g upon arrival and were acclimated to laboratory conditions for one week prior to the start of experiments. All subjects were maintained under a reverse light/dark cycle (12 h: 12 h, lights off at 0900 h) with unlimited access to food (Purina Rodent Diet, Brentwood, MO) and water. After the acclimation period, rats underwent stereotaxic surgery, and were single housed for the remainder of study in standard plastic cages (25×50×20 cm). Male Long-Evans rats (Charles River Laboratories), termed "residents," were pair-housed with females in larger (37×50×20 cm) cages. Residents were screened for aggressive behavior as described previously (Nikulina et al., 2004). All experimental procedures were approved by the Arizona State University Institutional Animal Care and Use Committee and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). All efforts were used to minimize suffering and to limit the number of animals used.

Viral vector

Pseudotyped AAV2/10 vectors were used in all experiments. The AAV2/10 rep/cap plasmid provided the AAV2 replicase and AAV10 capsid genes (Gao et al., 2002), while adenoviral helper functions were supplied by the pHelper plasmid

(Stratagene, La Jolla, CA). The AAV plasmids contain a transgene cassette, which consists of the cytomegalovirus (CMV) promoter, and either the gene for rat BDNF fused to enhanced green fluorescent protein or GFP alone (GFP gift from R. Klein), followed by a combined intron/polyadenylation signal derived from SV40. These elements are flanked by two AAV2 inverted terminal repeats. A standard triple transfection protocol was used to generate the helper-free pseudotyped AAV2/10 vectors as previously described (Xiao, Li, & Samulski, 1998). Briefly, the three plasmids were co-transfected into HEK-293 cells (Stratagene, La Jolla, CA) via calcium phosphate precipitation. Cells were harvested 48 h later, resuspended in DMEM, freeze-thawed with dry ice-ethanol slurry three times and centrifuged to produce a clarified cell lysate. The resulting viral stocks produced either GFP (AAV-GFP, as control virus) or BDNF-GFP (AAV-BDNF) upon infection, and were stored at -80°C prior to use.

Intracranial viral infusion

Rats were anesthetized with isofluorane and positioned in a stereotaxic frame (Leica Angle Two; Richmond, IL). Two holes were drilled at stereotaxic coordinates (AP −5.1 mm; ML ±2.15 mm from the bregma) on the surface of skull, and Hamilton syringes (Model 7105 KH; Reno, NV) with 24 gauge injector tips were lowered through the holes bilaterally at a 10° angle until needle tips reached the VTA (AP −5.1 mm; ML ±0.6 mm; DV −8.8 mm from the bregma; Paxinos and Watson, 2007); 0.5 µl of virus was bilaterally infused into each side of the VTA for 10 min at a speed of 0.05 µl/min. After infusion, the syringes remained in the infusion sites for 5 min to prevent retrace of virus.

Confirmation of viral efficacy

To confirm viral efficacy in enhancing VTA BDNF at the relevant time-points for the behavioral experiments, we examined VTA BDNF expression by BDNF immunohistochemistry 14 and 45 days after viral infusions. Rats received AAV-GFP or AAV-BDNF. One cohort of rats was used only to measure VTA BDNF expression 14 days after viral infusions. Measurement of VTA BDNF expression 45 days after viral infusions was performed in tissue from the rats used in Experiment 2 below.

All placements of infusion sites were confirmed based on observation of GFP expression in serially-collected brain sections. The cases in which infusion sites were inaccurate were excluded from further assessment.

Experimental design

A timeline of the experiments is presented in Figure 8. Based on the finding that VTA BDNF is elevated from two weeks to at least 45 days after viral infusions (described in Results below), we waited at least two weeks after the viral infusion to commence any experimental procedures.

Experiment 1: Time-course of a single social defeat stress-induced cross-sensitization

Rats were randomly assigned to 4 groups, exposed to either a single social defeat stress or handling procedure, then received d-amphetamine sulfate (1.0 mg/kg, i.p.; Sigma-Aldrich; St. Louis, MO) either 3 days (n = 6 each) or 14 days (n = 4 and 5, respectively) after defeat or the handling procedure (Figure 8A). Following AMPH challenge, locomotor activity was tracked and assessed.

Experiment 2: Molecular alterations induced by VTA BDNF overexpression

Rats received bilateral intra-VTA infusion of either AAV-GFP (n= 4) or AAV-BDNF (n = 7). Two weeks after the surgery, rats were handled, followed two weeks later by 0.9% saline (1.0 ml/kg, i.p.) injection. Brain tissue was collected and processed 45 days after viral infusion surgery (Figure 8B). BDNF in the VTA and DeltaFosB expression in the PFC and NAc were examined.

Experiment 3: Effect of single social defeat stress and VTA BDNF overexpression on behavioral sensitization to AMPH and molecular alterations

Rats were randomly assigned to four groups based on 2 experimental factors: virus (AAV-GFP vs. AAV-BDNF) and behavioral treatment (handling vs. single social defeat). The groups were: AAV-GFP-handled (n = 7), AAV-GFP-stressed (n = 7), AAV-BDNF-handled (n = 8), and AAV-BDNF-stressed (n = 7). The stressed groups underwent a single social defeat stress exposure two weeks after the surgery, at which time the handled groups were handled, followed by two AMPH (1.0 mg/kg, i.p.) challenge injections 14 and 24 days after stress. To examine whether prolonged cross-sensitization was induced by VTA BDNF overexpression in rats exposed to single social defeat stress, the first AMPH challenge was given two weeks after defeat or handling. Three rats were excluded from this study due an artifact during locomotor tracking. The second AMPH challenge was given 10 days later (24 days after defeat stress; one rat was excluded due to an artifact during tracking). All animals were euthanized at day 45 after surgery, whereupon brain tissue was collected and processed (Figure 8C). Besides locomotor activity, BDNF single labeling and BDNF/TH double labeling in the VTA, and DeltaFosB expression in the PFC and NAc were examined. The sample numbers of

different immunohistochemical procedures varied due to the exclusion of samples with occasional heterogeneity of section quality.

Social defeat stress

Social defeat stress consisted of a short aggressive confrontation between an aggressive resident rat and an experimental intruder rat as described in detail previously (Nikulina et al., 2004). After removing the female from the resident's cage, an experimental intruder rat was placed into the home cage of a resident male rat under a stainless steel protective cage (15×25×15 cm) for 5 min, then the protective cage was removed, and the resident displayed aggressive behavior; "defeat" occurred when the intruder exhibited a supine posture for at least 4 s. Aggressive interactions were 2–5 min in duration. The protective cage was then replaced for an additional 15 min exposure, after which the intruder was returned to its home cage. Control rats were handled and weighed on the days their counterparts were stressed and weighed.

Locomotor tracking

For two consecutive days immediately before AMPH challenge, rats were given a 0.9% saline injection (1.0 ml/kg, i.p.) in their home cages. Rats inside their home cages were placed into the locomotion tracking arena for 1 h to acclimate to the testing environment and injection procedure. On the drug challenge day, rats were placed into the locomotion tracking arena within their home cages, habituated to the testing environment for 30 min during which locomotion was tracked, injected with saline followed by tracking for 40 min, then challenged with AMPH followed by tracking for 70 min. Peak locomotor activity in response to AMPH was achieved 10 – 50 min after challenge. Locomotor activity was assessed using Videotrack (Viewpoint Life Sciences;

Montreal, Canada); total distance travelled in the form of large ambulatory movements (greater than 10 cm) was measured in 10 min bins. The dosage of AMPH used herein mainly induces large ambulatory movements (Geyer, Russo, Segal, & Kuczenski, 1987), so total distance of such movements can be used as an indicator of the extent of psychomotor sensitization in response to AMPH.

Perfusion and tissue processing

Forty-five days after surgery, all rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 10 ml of 10% heparin in 0.1 M phosphate-buffered saline (pH 7.4) followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were post-fixed for 1.5 h in the same fixative at 4°C, soaked in 12.5% sucrose, then in 25% sucrose in 0.1 M PBS at 4°C until saturated, then stored at 4°C prior to sectioning. Brains were sectioned at 20 µm in a cryostat at −22°C, and thaw-mounted onto glass slides (Superfrost Plus; Fisher; Waltham, MA). Sections were collected from +3.2 to +2.8 mm from bregma for the PFC, +1.8 to +0.8 mm from bregma for the NAc, and −4.8 to −5.6 mm from bregma for the VTA (Paxinos, 2007).

Immunohistochemistry

Sections were washed in 0.05 M potassium phosphate-buffered saline (KPBS), then blocked for 1 h in 10% normal donkey serum and 0.4% Triton X-100 in 0.05 M KPBS. Sections were then incubated with primary antibody: BDNF (AB1779SP, 1:3,000 dilution; Millipore; Temecula, CA) or FosB (SC-48, 1:10,000 dilution; Santa Cruz Biotechnology, Inc.**;** Santa Cruz**,** CA). The FosB antibody used here targets the N terminal of FosB protein contained in both FosB and DeltaFosB. However, based on the

timeline of the present research, FosB-like labeling would primarily capture accumulation of DeltaFosB because FosB expression is transient and only DeltaFosB persists after stimulation (Perrotti et al., 2004). Following incubation with primary antibody for 48 h at 4°C, slides were washed in 0.05 M KPBS and incubated for 1 h in biotin-conjugated goat anti-rabbit serum (1:200 dilution in blocking solution, Vectastain ABC kit; Vector Laboratories; Burlingame, CA). After washing in 0.05 M KPBS, sections were incubated with avidin–biotin–peroxidase complex (ABC kit, Vector Laboratories) for 1 h, then washed again and developed using DAB chromogen with nickel (DAB Peroxidase Substrate kit, Vector Laboratories). After dehydration in graded concentrations of ethanol and xylene, coverslips were applied.

To examine the cellular localization of BDNF expression in the VTA of animals that showed persistent cross-sensitization, we performed fluorescent double labeling of BDNF and tyrosine hydroxylase (TH). Primary antibodies, rabbit anti-BDNF (AB1779SP, 1:500 dilution; Millipore; Temecula, CA) and mouse anti-TH (SC-7837, 1:500 dilution, Santa Cruz Biotechnology; Santa Cruz, CA), were applied simultaneously and incubated for 48 h at 4°C. After incubation with biotinylated goat anti-rabbit IgG (1:200 dilution, Vector Laboratories) for 1 h, Alexa Fluor 488 Anti-Rabbit conjugated streptavidin and Alexa Fluor 548 Goat Anti-Mouse (1:500 dilution, Invitrogen, San Diego, CA) were applied for 2 h. After washing with 0.05 M KPBS, coverslips were applied with ProLong Gold Antifade Reagent with DAPI (Invitrogen, San Diego, CA).

Image analysis

Tissue sections were examined for the presence of chromogen or fluorophore reaction products using a Zeiss Axioskop microscope with 20× or 40× objectives;

selected areas were captured and digitized using a color digital video camera (MBF Biosciences; Williston, VT) or AxioCam MR (Zeiss) interfaced to the microscope. A cell profile was considered labeled if its pixel intensity was more than 2 standard deviations greater than the background, as calculated by Stereo Investigator software (MBF Biosciences). Fluorescent-labeled cells were counted manually, first in single channels for single labeling, then in merged channels for double labeling. At least three adjacent sections were analyzed for each brain region per animal, and labeling density was calculated by dividing the estimated total number of labeled profiles by the total area analyzed.

Statistics

In experiment 1, one-way ANOVA (between subjects factor: handling vs. stress) was used to analyze locomotor activity during the peak locomotor response period (10 – 50 min after AMPH injection). In experiment 2, one-way ANOVA was used to analyze the number of BDNF or DeltaFosB-labeled cells in saline-treated animals (between subjects factor: AAV-BDNF vs. AAV-GFP). In experiment 3, locomotor activity and immunohistochemical data were analyzed by two-way ANOVA (between subjects factors: viral vector [AAV-BDNF vs. AAV-GFP] and behavioral treatment [handling vs. stress]). Three-way mixed ANOVA with repeated measures (two between subjects factors: viral vector [AAV-BDNF vs. AAV-GFP] and behavioral treatment [handling vs. stress]; one within subjects factor: number of AMPH injections [first vs. second]) was used to analyze locomotor activity in response to the first and the second AMPH challenge. In a few cases, an optical artifact produced an unrecoverable error in locomotor tracking. Therefore, several cases were excluded due to the presence of this

artifact. To assure that the same cases were assessed repeatedly across both challenge sessions by three-way ANOVA with repeated measures, samples with such artifact in either of the two AMPH challenges were excluded. Thus, the final number of cases assessed using three-way mixed ANOVA with repeated measures is n = 6 per group.

T-test was used for planned comparisons between AAV-BDNF-stressed and all other groups when significant interaction was not present, and post-hoc Tukey test was used in the presence of significant interaction. All data are reported as mean ± SEM. Results were considered significant if $p \leq 0.05$.

Results

Experiment 1

Time-course of single social stress-induced cross-sensitization

To measure the time-course of single social defeat stress-induced cross-sensitization in naive rats, locomotor response to AMPH challenge was evaluated in adult male Sprague-Dawley rats 3 or 14 days after exposure to a single social defeat or control handling. Rats exposed to single social defeat 3 days earlier exhibited significantly more total locomotor activity after AMPH than rats handled 3 days earlier (measured 10 - 50 min after AMPH challenge; $F_{(1, 11)} = 7.170$, $p = 0.023$; Figure 9A), reflecting the presence of cross-sensitization. No difference in total locomotor activity was observed between stressed and handled rats in a separate cohort that were challenged with AMPH 14 days after exposure to single social defeat stress or handling ($F_{(1, 7)} = 0.230$, $p = 0.646$; Figure 9B). This indicates that cross-sensitization after a single defeat is short-lasting and detectable 3 days, but not 14 days later.

Experiment 2

Confirmation of viral efficacy

The viral infusion site was visualized using GFP as an indicator of transfection (Figure 10A). Based on GFP expression from serially collected sections, we observed that transfected neurons in the VTA extend approximately 300μm in diameter on the rostral-caudal axis, and 200 μm in diameter on dorsal-ventral and medial-lateral axes around the infusion site. Expression of viral product at forty-five days after infusion is consistent with the observation that AAV-mediated transgene expression persists 1-3 months in neurons after transfection (Klein et al, 1998). To compare increase in VTA BDNF after AAV-BDNF across time-points, VTA BDNF expression was normalized to that of the AAV-GFP control group at each respective time-point (Figure 10C). Fourteen and 45 days after viral infusion, we observed two fold increase of BDNF in rats receiving AAV-BDNF compared to those receiving AAV-GFP: 14 days $F_{(1,7)} = 11.105$, $p = 0.013$; Figure 9C; 45 days $F_{(1, 9)} = 7.312$, $p = 0.027$; Figure 10B, C, D). At both 14 and 45 days, the volume of infection was approximately 25% of the rostral VTA (bregma -4.8 to -5.6) around the infusion site (bregma -5.1).

DeltaFosB expression after BDNF overexpression in the VTA of drug-naïve rats

VTA BDNF overexpression alone increased DeltaFosB labeling in anterior cingulate (ACG; $F_{(1, 6)} = 9.880$, $p = 0.020$), prelimbic (PrL; $F_{(1, 6)} = 12.930$, $p = 0.007$) and infralimbic (IL; $F_{(1, 6)} = 15.620$, $p = 0.008$) regions of the PFC (Figure 11A, C), and in the NAc core ($F_{(1,8)} = 16.108$, $p = 0.043$) and shell ($F_{(1,8)} = 11.978$, $p = 0.011$; Figure 11B, C) compared to the AAV-GFP-saline rats.

Experiment 3

Social defeat stress-induced cross-sensitization and sensitization to AMPH

To determine whether elevated VTA BDNF prolonged single social defeat stress-induced cross-sensitization, rats receiving AAV-GFP control virus or AAV-BDNF were exposed to a single episode of stress or handling followed by AMPH (1.0 mg/kg) 14 days later. The absence of stress-induced cross-sensitization to AMPH 14 days later (Experiment 1) was confirmed in rats which received AAV-GFP (one-way ANOVA, $F_{(1, 12)} = 1.873$, $p = 0.198$). By contrast, AAV-BDNF-stressed rats showed significantly greater locomotor activity in response to the first AMPH injection compared to AAV-BDNF-handled rats (one-way ANOVA, $F_{(1, 12)} = 4.730$, $p = 0.050$). Two-way ANOVA showed significant main effects of stress ($F_{(1, 23)} = 6.228$, $p = 0.020$) and AAV-BDNF ($F_{(1, 23)} = 4.464$, $p = 0.046$), but the interaction between the two factors was not significant ($F_{(1, 23)} = 0.277$, $p = 0.603$; Figure 12A). Ten days after the first AMPH challenge, a second AMPH challenge was given, and a significant main effect of AAV-BDNF ($F_{(1,25)} = 8.284$, $p = 0.008$) and interaction between stress and AAV-BDNF on locomotor activity was observed ($F_{(1,25)} = 5.950$, $p = 0.022$), but we observed no main effect of stress ($F_{(1,25)} = 1.587$, $p = 0.219$; Figure 12B). Additionally, AAV-BDNF-stressed rats showed significantly greater locomotor activity than AAV-GFP-handled, AAV-GFP-stressed and AAV-BDNF-handled groups in response to the second AMPH injection (post-hoc multiple comparisons: $p = 0.008$, $p = 0.001$ and $p = 0.014$, respectively; Figure 12C).

To examine locomotor response to a non-sensitizing AMPH dose regimen in rats with a history of stress exposure and enhanced VTA BDNF, we compared locomotor activity in response to first and second AMPH challenges within each group (Figure 12C).

Analysis of the amplitude of variation of locomotor activity between the first and the second AMPH challenge was achieved using three-way ANOVA with repeated measures. The data showed significant interaction of AAV-BDNF and number of AMPH injections ($F_{(1,22)} = 4.503$, $p = 0.045$). Also, a significant interaction among stress, AAV-BDNF, and number of AMPH injections ($F_{(1,22)} = 5.463$, $p = 0.029$) was observed. Furthermore, AAV-BDNF-stressed rats showed significantly augmented locomotor response to the second AMPH injection compared to the first AMPH injection (post-hoc multiple comparisons: $p = 0.031$) indicating facilitated sensitization after repeated dosing. Although a weak increasing trend was present in AAV-GFP-handled rats in response to multiple AMPH injections, this effect was not statistically significant ($p = 0.09$). No significant changes in locomotion after AMPH was observed in AAV-GFP-stressed or AAV-BDNF-handled rats (post-hoc multiple comparisons: $p = 0.418$, $p = 0.241$, respectively, Figure 12C).

BDNF expression in VTA dopaminergic neurons

To characterize the distribution and phenotype of BDNF-expressing neurons in the VTA, double-label fluorescent immunohistochemistry was performed on tissue from Experiment 3. Two-way ANOVA of the total number of BDNF expressing neurons in the rostral VTA also indicated a significant main effect of AAV-BDNF ($F_{(1, 22)} = 41.381$, $p = 0.001$) and an interaction between AAV-BDNF and stress approaching significance ($F_{(1, 22)} = 4.055$, $p = 0.056$). Also, AAV-BDNF-stressed rats showed higher BDNF labeling across the entire rostral VTA compared to all other groups (Figure 13A; post-hoc comparisons: $p \leq 0.05$ for all comparisons)

Furthermore, two-way ANOVA showed significant main effect of AAV-BDNF on BDNF/TH double-labeled cells in the rostral VTA ($F_{(1, 22)} = 14.298$, $p = 0.001$); and significantly more BDNF/TH double-labeled cells were present in the rostral VTA in AAV-BDNF-stressed rats than in AAV-GFP-handled or stressed rats (t-test: $p = 0.018$ and $p = 0.001$, respectively; Figure 13B, C).

DeltaFosB in the mesocorticolimbic projection areas

Consistent with the effect of VTA BDNF overexpression on DeltaFosB expression in PrL and ACG cortices in drug naïve rats (experiment 2), two-way ANOVA showed a significant main effect of AAV-BDNF on DeltaFosB expression in both the ACG and PrL cortices in AMPH treated rats (ACG: $F_{(1, 21)} = 6.837$, $p = 0.016$; PrL: $F_{(1, 21)} = 4.469$, $p = 0.047$; Table 1). In IL cortex, a main effect of stress was observed ($F_{(1,21)} = 4.941$, $p = 0.037$).

Among all brain regions examined, the greatest induction of DeltaFosB labeling was present in the NAc shell of AAV-BDNF-stressed animals (post-hoc multiple comparisons: $p \leq 0.01$ for all comparisons, Figure 14A, B), where we observed significant main effect of stress ($F_{(1,23)} = 26.100$, $p = 0.001$) and AAV-BDNF ($F_{(1,23)} = 39.670$, $p = 0.001$) estimated by two-way ANOVA. Also, significant interaction between these two factors ($F_{(1,23)} = 10.010$, $p = 0.004$) on DeltaFosB expression was found in NAc shell. In contrast, no difference in DeltaFosB cell counts was observed in the NAc core after AMPH challenge in any group (t-test: $p > 0.05$ for all comparisons; two-way ANOVA: behavioral treatment $F_{(1, 22)} = 0.110$, $p = 0.743$; virus: $F_{(1, 22)} = 0.187$, $p = 0.743$; behavioral treatment × virus: $F_{(1, 22)} = 1.244$, $p = 0.277$; Table 1).

Discussion

The present results demonstrate that virus-mediated enhancement of VTA BDNF expression both extends the time-course of single social defeat stress induced cross-sensitization to AMPH and facilitates sensitization to a non-sensitizing dosing regimen of AMPH in rats exposed to a single episode of social defeat stress. We also show that enhancement of VTA BDNF alone is sufficient to increase DeltaFosB expression in projection areas of the VTA.

Our behavioral results are consistent with previous findings (de Jong, Wasilewski, et al., 2005) that single social defeat-induced cross-sensitization to AMPH is transient, apparent at 3 days but not at 14 days after a single exposure to social defeat. It is important to note that handled rats single housed for 14 days showed higher locomotor response to AMPH than did handled rats single housed for three days. The increased locomotor response to AMPH in drug-naive rats handled/stressed 14 days earlier is likely due to the longer period of single-housing, a form of social isolation which can elevate rewarding effects of psychostimulants (Deroche, Piazza, Le Moal, & Simon, 1994) and can amplify the physiological effect of social defeat (de Jong, van der Vegt, Buwalda, & Koolhaas, 2005). In the present study, handled/stressed rats received control virus and non-viral treated rats that were single housed for two weeks showed similar locomotor responses to AMPH. This suggests that single housing induces an up-ward shift of locomotor activity to AMPH, which is consistent across experiments.

In particular, single social defeat stress-induced cross-sensitization to AMPH was not observed 14 days after stress in non-viral treated rats, or in rats that received the control GFP virus. The fact that cross-sensitization to AMPH was observed 14 days after

single social defeat stress in the presence of enhanced VTA BDNF suggests that BDNF prolonged single stress-induced cross-sensitization. However, such an effect should be interpreted with caution because persistent VTA BDNF elevation also might alter single social stress-induced cross-sensitization at early time points. This possibility remains to be investigated. Cross-sensitization and sensitization share the common molecular mechanism of enhanced dopamine transmission in mesolimbic circuitry (Imperato, Puglisi-Allegra, Casolini, Zocchi, & Angelucci, 1989; P. W. Kalivas & Stewart, 1991), and both behaviors represent increased responsiveness of mesolimbic dopaminergic neurons to drugs of abuse. In addition, intermittent social defeat stress sensitizes NAc dopamine response to cocaine administration (Miczek et al., 2011). Our data show that AAV-mediated VTA BDNF is enhanced in DA neurons, which may directly increase DA release (Blochl & Sirrenberg, 1996) and metabolism (Altar et al., 1992). As such, the increased number of BDNF expressing dopamine neurons in the rostral VTA could enhance mesocorticolimbic dopamine neurotransmission, contributing to the prolonged cross-sensitization and the sensitization to AMPH we observed. Our viral infusion resulted in increased VTA BDNF in a physiological range similar to the level of VTA BDNF in rats exposed to intermittent social defeat stress (Fanous et al., 2010), so it is possible that VTA BDNF overexpression induced neuroadaptive changes similar to those induced by intermittent social defeat stress to result in sensitized drug response.

The ability of VTA BDNF to directly modulate sensitization is consistent with the finding that infusions of BDNF into the VTA enhance cocaine sensitization (Horger et al., 1999). Increased endogenous BDNF signaling in the VTA leads to sensitized synaptic response in VTA dopamine neurons after cocaine withdrawal (Pu, Liu, & Poo, 2006).

BDNF in the VTA may act to titrate the effect of social defeat stress on activating mesolimbic neurochemistry by rendering this circuit more vulnerable to stress-induced activation. This enhanced vulnerability might then cause the VTA to be more sensitive to stress or multiple drug challenges, leading to facilitated sensitization. One mechanism for facilitated sensitization may be through amphetamine-induced enhancement of calcium signaling, which has been associated with the development of sensitization and produces increased BDNF in regions such as the cortex and hippocampus (Brodish & Redgate, 1973; M. R. Brown et al., 1990; Herman et al., 1989). Calcium signaling specifically in the VTA is important in the development of psychostimulant sensitization (Herman et al., 1989). The presence of increased VTA BDNF may therefore facilitate prolonged synaptic plasticity in the face of a stimulus that produces calcium release (but whose magnitude would not normally result in such prolonged plasticity, such as a single administration of amphetamine). In this way, enhanced VTA BDNF may be considered a possible "risk factor" for development of neuronal and behavioral drug sensitization, which may interact with factors such as stress to further increase "risk." This agrees with the role mesolimbic BDNF has been hypothesized to play in addictive behavior (Baxter & Rousseau, 1979).

Intermittent social defeat stress resulted in prolonged BDNF elevation in the VTA (Fanous et al., 2010) and DeltaFosB accumulation in the projection areas of the VTA (Nikulina et al., 2008). In the present study, we found that VTA BDNF overexpression alone was sufficient to induce DeltaFosB accumulation in the NAc and PFC. This finding indicates that persistent elevation of VTA BDNF leads to DeltaFosB accumulation,

whose mechanism may be similar to DeltaFosB elevation observed after repeated social defeat stress.

DeltaFosB accumulation in the NAc could represent a homeostatic mechanism to balance increased VTA BDNF associated with social defeat, as DeltaFosB in the NAc can enhance the resilience of animals to the aversive effects of chronic social defeat stress (Vincent Vialou et al., 2010). The effect of a single episode of social defeat stress alone is too transient to induce considerable DeltaFosB accumulation in the NAc shell in rats receiving AAV-GFP. However, in the presence of VTA BDNF overexpression, a single defeat stress-induced neuroadaptation may persist, and transcriptional activation of DeltaFosB may be prolonged in the NAc shell. The mechanism underlying increased DeltaFosB accumulation in terminal regions resulting from VTA BDNF overexpression requires further investigation.

As discussed above, VTA BDNF overexpression significantly increased DeltaFosB expression in the PFC and the NAc of handled drug-naïve rats. However, repeated AMPH challenges to AAV-BDNF- stressed rats induced a significant increase of DeltaFosB expression in the NAc shell, but not in the NAc core and PFC, and this effect was region-specific. The explanation for this effect on DeltaFosB expression is likely the differential responsiveness of different regions to stress- and drug-related stimuli. This is supported by data demonstrating different anatomical connections and biochemical characteristics of the NAc core and shell (Zahm, 2000), and as such the subregions may mediate distinct functions related to motor and motivation (Craddock, 1978). Furthermore, recent research indicates regionally specific effects of BDNF signaling in NAc core and shell in drug seeking (X. Li et al., 2013). Also DeltaFosB

modulates synaptic plasticity and reward-related behaviors in a sub-region and cell-type specific manner in the NAc core and shell (Grueter, Robison, Neve, Nestler, & Malenka, 2013). Although the involvement of PFC in the sensitization to AMPH is well identified (Fanous et al., 2012), no further DeltaFosB increase was observed in AAV-BDNF-stressed rats after AMPH injections in the PFC. It is possible that DeltaFosB in PFC is not susceptible to AMPH in rats with BDNF overexpression in the VTA, at least at the time-point we observed. Therefore, DeltaFosB expression may vary in different mesocorticolimbic regions in response to the same stimulation (drugs and stress), which may underlie why the current data reflect a distinct trend of increased DeltaFosB expression only in NAc shell.

In conclusion, rats with higher VTA BDNF levels developed prolonged AMPH cross-sensitization and sensitization to non-sensitizing dosing regimen of AMPH after social defeat stress. This was associated with increased DeltaFosB expression in the NAc shell. The present findings suggest that the elevated VTA BDNF could be a risk factor that amplifies vulnerability for drug abuse triggered by socially aversive stimuli.

Chapter 3

KNOCKDOWN OF TRKB RECEPTOR EXPRESSION IN THE NAC SHELL PREVENTS INTERMITTENT SOCIAL DEFEAT STRESS-INDUCED CROSS-SENSITIZATION TO AMPHETAMINE IN RATS

Abstract

The nucleus accumbens (NAc) is a critical region for the rewarding effects of drugs of abuse. Brain-derived neurotrophic factor (BDNF) can facilitate stress- and drug-induced neuroadaptation in the mesocorticolimbic system. BDNF-containing afferents to the NAc originate from the ventral tegmental area (VTA) and the prefrontal cortex; BDNF release activates neurotrophin tyrosine kinase receptor type 2 (TrkB). In this study, we examined the necessity of BDNF-TrkB signaling in NAc shell during social defeat stress-induced cross-sensitization to amphetamine. Adeno-associated virus expressing short hairpin RNA directed against TrkB (AAV-shTrkB) was infused bilaterally into the NAc shell to knockdown TrkB, while AAV-GFP was used as the control virus. Rats were exposed to intermittent social defeat stress or handling procedures; amphetamine challenge was given ten days after the last defeat and locomotor activity was measured. Stressed rats that received control virus showed cross-sensitization to amphetamine compared to the handled rats. In contrast, NAc TrkB knockdown prevented social defeat stress-induced cross-sensitization. TrkB knockdown in the NAc was found to reduce phospho-extracellular signal-regulated kinase (ERK) expression in this region. Together with this, NAc TrkB knockdown also prevented stress-induced elevation of BDNF and GluA1 subunit of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)

receptor in the VTA and as well as DeltaFosB expression in the NAc. These findings indicate that NAc BDNF-TrkB signaling is required for social defeat stress-induced cross-sensitization. NAc TrkB-BDNF signaling also appears to be involved in the regulation of GluA1 in the VTA, in addition to NAc DeltaFosB accumulation that could trigger cross-sensitization after social defeat stress.

Introduction

Stress augments drug-induced mesolimbic dopamine transmission and locomotor activity (Sorg & Kalivas, 1991; Jasmine J. Yap & Klaus A. Miczek, 2008), known as cross-sensitization. Among all types of stressors, social defeat has face validity to replicate aversive social interaction and personal failure in humans (Koolhaas, Meerlo, de Boer, Strubbe, & Bohus, 1997), and induces long-lasting cross-sensitization to psychostimulants (H. E. Covington, 3rd & Miczek, 2001; Nikulina et al., 2004). After intermittent social defeat stress, elevation of brain-derived neurotrophic factor (BDNF) expression in the ventral tegmental area (VTA) was observed with a time course similar to that of cross-sensitization to psychostimulants (Fanous et al., 2010), and enhanced VTA BDNF signaling was found to potentiate cross-sensitization to AMPH (J. Wang et al., 2013). In the mesolimbic circuit, BDNF is synthesized in VTA dopamine neurons and transported anterogradely to the nucleus accumbens (NAc; (Altar et al., 1997)), the brain region mediating addictive effects of drugs of abuse. Enhanced BDNF signaling in the NAc facilitates the development of sensitization to cocaine (Horger et al., 1999). Also, NAc TrkB overexpression enhances sensitization to cocaine, which can be prevented by overexpression of the dominant negative form of TrkB (Bahi et al., 2008; Crooks, Kleven, Rodriguiz, Wetsel, & McNamara, 2010). BDNF-induced activation of TrkB receptors

triggers several downstream signaling pathways (Minichiello, 2009), among which the extracellular signal-regulated protein kinase (ERK) pathway in the NAc was found to be involved in sensitization to psychostimulants (Kim, Shin, Yoon, & Kim, 2011; Mattson et al., 2005).

Social defeat stress-induced cross-sensitization to psychostimulants is concomitant with increased expression of the GluA1 subunit of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor in the VTA (H. E. Covington, 3rd, Tropea, Rajadhyaksha, Kosofsky, & Miczek, 2008). In contrast, AMPA receptor blockade in the VTA prevents sensitization to psychostimulants (Y. Li, Vartanian, White, Xue, & Wolf, 1997). Therefore elevation of GluA1 expression in the VTA may contribute to the stress induced cross-sensitization.

DeltaFosB, a truncated variant of FosB, is induced in the NAc by chronic exposure to many drugs of abuse (Nestler, 2008). After repeated social defeat stress, DeltaFosB expression and its co-localization with BDNF were increased in the NAc (Nikulina et al., 2004; Perrotti et al., 2004). BDNF immunoreactivity in this region could be exogenous BDNF from afferents to the NAc. Also, elevated DeltaFosB in the NAc shell accompanies augmentation of the stress induced cross-sensitization induced by VTA BDNF overexpression (J. Wang et al., 2013). Furthermore, NAc DeltaFosB overexpression induces sensitization to cocaine (Kelz et al., 1999), possibly through regulation of genes involved in drug-induced neuroplasticity (McClung & Nestler, 2003). Thus, the presence of DeltaFosB in the NAc can be used as a marker for enhanced sensitivity to the rewarding effect of drugs of abuse.

The present study investigated the effect of NAc BDNF signaling in social defeat stress-induced cross-sensitization. We used adeno-associated virus-mediated gene transfer to deplete the TrkB level in the NAc by overexpression of short hairpin RNA directed against TrkB. To identify the factors associated with social defeat stress-induced cross-sensitization and potentially affected by BDNF signaling in mesolimbic circuit, such as BDNF, GluA1 in the VTA and phospho-ERK1/2, DeltaFosB in the NAc, were also measured to further characterize the molecular substrates of social defeat stress-induced cross-sensitization.

Material and Methods

Animals

Subjects were male Sprague Dawley rats (Charles River Laboratories, Hollister, CA, USA), weighing 225–250 g upon arrival, which were single-housed in 25×50×20 cm cages under a reverse light/dark cycle (12 h: 12 h, lights off at 0900 h) with unlimited access to food (Purina Rodent Diet, Brentwood, MO, USA) and water. Male Long-Evans rats (Charles River), termed "residents," were pair-housed with females in 37×50×20 cm cages. Residents were screened for aggressive behavior as described previously (Nikulina et al., 2004). All experimental procedures were approved by the Arizona State University Institutional Animal Care and Use Committee, and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). All efforts were used to minimize suffering and to limit the number of animals used.

Viral vector

Pseudotyped AAV2/10 vectors were used in all experiments. An AAV2/10 rep/cap plasmid from the U Penn Vector Core facility provided the AAV2 replicase and

AAV10 capsid genes (Gao et al., 2002), while adenoviral helper functions were supplied by the pHelper plasmid (Stratagene, La Jolla, CA). The vector plasmids contained either of two different shRNA under control of a murine U6 promoter. The shRNA target sequences were as follows: TGGAGTTGACTATGAGACAAA and CCACGGATGTTGCTGACCAAA. Each AAV vector containing a shRNA also included an upstream eGFP indicator gene cassette flanked by a viral CMV-IE promoter and an SV40 intron/polyA sequence. A mixture of viral vectors containing the two shRNA was used to achieve maximum trkb mRNA knockdown. A similarly packaged and otherwise isogenic AAV–eGFP plasmid with no shRNA was used as a control virus. A standard triple transfection protocol was used to generate the helper-free pseudotyped AAV2/10 vectors, as previously described (Xiao et al., 1998). Briefly, the three plasmids were co-transfected into HEK-293 cells (Stratagene, La Jolla, CA) via calcium phosphate precipitation. Cells were harvested 48 h later, resuspended in DMEM, freeze-thawed with a dry ice-ethanol slurry three times and centrifuged at 13,000 rpm for 10 min to produce a clarified cell lysate. The resulting viral stocks were stored at -80°C prior to use.

Intracranial viral infusion

After one week of acclimation to the environment, rats were anesthetized with isoflurane and positioned in a stereotaxic frame (Leica Angle Two; Richmond, IL). Burr holes were drilled bilaterally at stereotaxic coordinates: AP: +2.0 mm; ML: ±1.1 mm from the bregma (Paxinos G & 2007), and a Hamilton syringes with 24 gauge flat injector tip (Hamilton; Reno, NV) were lowered through the holes to reach the NAc shell (DV: −7.8 mm); 0.5 µl of purified virus) was infused into the NAc shell bilaterally at a speed of 0.05 µl/min. After infusion, the syringes remained at the infusion site for 5 min

to prevent retrograde filling of the virus along the infusion tract.. AAV-10-mediated transgene overexpression achieves maximum expression in 1-2 weeks, and lasts at least three month after transfection (Klein et al., 1998). This vector does not possess the sequence encoding capsid proteins, so it cannot disseminate beyond the transfection site. Behavioral treatments began two weeks after surgery to allow complete recovery and viral expression.

Social defeat stress

Social defeat stress consisted of a short exposure to aggressive behavior of a resident rat, described in detail previously (Nikulina et al., 2012). Each resident rat was group housed with one female rat for at least two weeks before beginning the experiment. After removing the female, the experimental intruder rat was placed into the resident's home cage under a stainless steel mesh protective cage (15×25×15 cm) for 5 min. The protective cage was then removed, and the resident displayed aggressive behavior toward the intruder for 2-5 min until "defeat" occurred, demonstrated by the intruder exhibiting a supine posture for at least 4 s. Further physical contact was then prevented by placing the intruder under the protective cage for an additional 15 min before returning to its home cage. Control rats were handled and weighed on the days their counterparts were defeated and weighed.

Validation of AAV-shTrkB induced TrkB knockdown

One week after arrival, rats received intra-NAc shell viral infusion of AAV-GFP or AAV-shTrkB (n = 6). After surgery, rats were single housed for 35 days without any behavioral treatment, and then perfused transcardially to examine the efficacy of AAV-

shTrkB-mediated TrkB knockdown by immunohistochemistry. A time line for validation of viral effects is presented in Figure 15. A.

Experimental design

Rats were randomly separated into four groups based on two factors: viral vector (AAV-GFP vs. AAV-shTrkB) and behavioral treatment (handling vs. intermittent social defeat stress). Therefore, the groups were: AAV-GFP-handled (n = 7), AAV-GFP-stressed (n = 8), AAV-shTrkB-handled (n = 8), and AAV-shTrkB-stressed (n = 8). Following surgical recovery, rats were exposed to intermittent social defeat stress four times in ten days or were handled according to the same schedule. Ten days after the last defeat, locomotor activity was assessed in all rats after saline or AMPH injection. Three rats were excluded from this study due to the occasional presence of video artifacts during tracking. All rats were euthanized one week later, 41 days after viral infusion surgery, as shown on the time line in Figure 16.A. In subsequent processing of brain tissue, sample number varied slightly across different procedures and different brain regions due to the exclusion of samples with artifacts during tissue collection or processing.

Locomotor tracking

Ten days after the last social defeat or handling procedure, rats were placed into the locomotion tracking area in their home cages to acclimate to the novel environment for one hour per day for two consecutive days, and 0.9% saline injection (1.0 ml/kg, i.p.) was given to habituate rats to injection. On the third day, locomotor activity was measured using Videotrack (Viewpoint Life Sciences; Montreal, Quebec, Canada). Rats first acclimated to the testing environment for 30 min, during which baseline locomotor

activity was measured. Rats then received a saline injection (1.0 ml/kg, i.p) followed by locomotor tracking for 40 min, and AMPH (1.0 mg/kg, i.p.) challenge followed by locomotor tracking for 70 min. Based on prior research, the dose of AMPH used in the present study produces mainly large ambulatory movements (Geyer et al., 1987), so total distance of large ambulatory movements (greater than 10 cm) measured in 10 min bins was used as an indicator of the extent of behavioral sensitization in response to AMPH . Peak locomotor activity was achieved during the 10 to 50 min period after AMPH injection.

Perfusion and tissue processing

Rats used for validation of viral vector were deeply anesthetized with sodium pentobarbital (100 mg/kg) and perfused transcardially with 10 ml of 10% heparin in 0.1 M phosphate-buffered saline (pH 7.4), followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were post-fixed for 1.5 h in the same fixative at 4°C, soaked in 12.5% sucrose, then in 25% sucrose in 0.1 M PBS at 4°C until they sank to the bottom of the solution for each concentration. Coronal brain sections (20 μm) were collected in a -22°C cryostat, and thaw-mounted onto glass slides (Superfrost Plus; Fisher; Waltham, MA). NAc sections were collected from +1.8 to +0.8 mm from bregma.

Fresh frozen tissue collection

After locomotor sensitization assessment, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg) and decapitated; brains were removed and frozen for 15 sec in 2-methylbutane at -70°C. Brains were sectioned in a -22°C cryostat to reach the level of the NAc, where 2.0 mm diameter tissue punches were removed centered on the coordinates: AP: 1.8 mm; DV: -7.0 mm ; ML: 1.3 mm. Similarly,1.0 mm diameter tissue

punches were taken from the VTA centered on the coordinates: AP: -5.0 mm; DV: -8.8mm ; ML: 0.8mm (Paxinos G & 2007). The depth of each punch was limited to 1.0 mm. Brain tissue was lysed in 1×RIPA buffer (9806, Cell signaling Technology, Beverly, MA), with protease inhibitor (P8340, Sigma) and phosphatase inhibitor (P8340, Sigma), heated at 37°C for 20 min, then sonicated at 4°C for 1 sec, and centrifuged at 14,000 g for 10 min. The supernatant was collected, and total protein concentration was determined using a BCA protein assay kit (Pierce-Thermo Scientific, Waltham, MA).

Immunohistochemistry and image analysis

Sections on slides were washed extensively in 0.05 M potassium phosphate-buffered saline (KPBS), then blocked for 1 h in 10% normal goat serum and 0.4% Triton X-100 in 0.05 M KPBS. Sections were then incubated with primary antibody: TrkB (1:1,000; cat. #4606, Cell Signaling Technology, Beverly, MA) for 48 h. Slides were then washed in 0.05 M KPBS and incubated in biotin-conjugated goat anti-rabbit serum for 1 h (1:200 dilution in blocking solution, Vectastain ABC kit; Vector Laboratories; Burlingame, CA). After washing in 0.05 M KPBS, sections were incubated with avidin–biotin–peroxidase complex for 1 h, then washed again and developed using DAB chromogen with nickel intensification (DAB Peroxidase Substrate kit, Vector Laboratories). After dehydration in graded concentrations of ethanol and xylene, coverslips were applied.

Tissue sections were examined for the presence of chromogen reaction products using a Zeiss Axioskop microscope with 20× or 40× objectives; selected areas were captured and digitized using a color digital video camera (MBF Biosciences; Williston, VT) interfaced to the microscope. A cell profile was considered labeled if its pixel

intensity was more than 2 standard deviations greater than the background, as calculated by Stereo Investigator software (MBF Biosciences). At least three adjacent sections were analyzed for each brain region per animal, and labeling density was calculated by dividing the estimated total number of labeled profiles by the total area analyzed.

Western blotting

Samples (~30 μg of total protein/lysate) were loaded and electrophoresed on 4–20% gradient precast polyacrylamide gel (Bio-Rad, Hercules, CA) under reducing conditions, and proteins were transferred onto supported nitrocellulose membranes (0.2 μm pore size) for immunoblotting (Bio-Rad, Hercules, CA). Membranes were washed in 1× TBS-Tween 20 (pH 7.4) (TBS-T) 3 X 5 min, and blocked with 5% non-fat dry milk in TBS-T for 1 h at room temperature. Membranes were then incubated overnight at 4°C with antibodies to BDNF (1:1,000; #sc-546, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), FosB (1:1,000; #sc-48, Santa Cruz Biotechnology), TrkB (1:1,000; #4606, Cell Signaling Technology, Beverly, MA), ERK (1:1,000; #4695, Cell Signaling Technology), phospho-ERK (1:1,000; #4370S, Cell Signaling Technology), GluA1 (1:1,000; #MAB2263, Millipore; Temecula, CA) or β-actin (1:5,000; #A5441, Sigma). Extensive washing with TBS-T solution was accomplished at each step. Membranes were incubated for 120 min with IRDye®800 CW Goat anti-Mouse IgG1 (1:10,000; LI-COR, Lincoln, NE) for two hours, or with biotin-conjugated anti-rabbit or anti-mouse IgG for one hour (1:500; Vector, Burlingame, CA), then immersed in IRDye®680 streptavidin (1:5,000; Lincoln, NE) for one hour, depending on the antibody. The fluorescence signal bands were scanned using the Odyssey FC quantitative fluorescence imaging system (LI-COR, Lincoln, NE), and further analyzed using LI-COR Image Studio.

Data analysis

Viral efficacy was validated by counting of immunolabeled cells and analyzed with one-way ANOVA (viral vector as the between-subjects factor). Two-way ANOVA with viral vector and behavioral treatment as between-subjects factors was used to analyze locomotor response to AMPH during the peak locomotor response period, and the intensity of bands in western blots. To examine whether the significant main effect of stress on locomotor response to AMPH and on gene expression in groups received AAV-GFP were prevented by TrkB knockdown, planned contrast comparison was used (Keppel, 1991). All data are reported as mean ± SEM, and all significance levels were set at $p \leq 0.05$.

Results

AAV-shTrkB-induced TrkB knockdown in the NAc shell

We examined the efficacy of AAV-shTrkB induced TrkB knockdown in the NAc shell 35 days after viral infusion (Figure 15.A and B). TrkB expression level in the NAc shell was significantly reduced in rats that received AAV-shTrkB compared to rats that received AAV-GFP control virus, measured by IHC ($F_{(1, 11)} = 10.662$, $p=0.008$; Figure 15.C and D).

TrkB knockdown in the NAc shell prevents intermittent social defeat induced cross-sensitization to AMPH

AMPH challenge (1.0 mg/kg, i.p.) was given ten days after the last episode of intermittent social defeat stress. Baseline and saline-induced locomotor activity was similar among the groups. Significantly higher locomotor activity was found after AMPH in AAV-GFP-stressed rats in comparison with in AAV-GFP-handled rats and AAV-

shTrkB-stressed rats during the peak locomotion period (10 to 50 min after AMPH administration, planned comparison, $F_{(1,11)} = 4.64$, $p = 0.042$, $F_{(1,11)} = 10.04$, $p = 0.004$). In contrast, no difference in locomotor activity in response to AMPH challenge was found between AAV-shTrkB-stressed rats and AAV-shTrkB-handled rats ($F_{(1,12)} = 0.19$, $p = 0.664$). A significant main effect of the virus was also found during peak locomotion period (10 to 50 min after AMPH challenge) in two-way ANOVA ($F_{(1,23)} = 7.262$, $p = 0.013$; Figure 16.C). In addition, locomotor activity in AAV-GFP-stressed rats was significantly higher than in AAV-GFP-handled, AAV-shTrkB-handled, and AAV-shTrkB-stressed (t-test: $p = 0.029$, $p = 0.014$, $p = 0.012$ respectively; Figure 16.B)

TrkB knockdown reduces phospho-ERK in the NAc

Examined seventeen days after the last social defeat stress, neither intermittent social defeat stress nor TrkB knockdown had any significant effect on total ERK expression in the NAc (two-way ANOVA: stress: $F_{(1, 26)} = 0.274$, $p = 0.605$; TrkB knockdown: $F_{(1, 26)} = 3.219$, $p = 0.084$; stress × TrkB knockdown: $F_{(1, 26)} = 0.106$, $p = 0.747$; Figure 17.A). In contrast, phospho-ERK in the NAc was significantly reduced by NAc TrkB knockdown, as indicated by the significant main effect of AAV-shTrkB from two-way ANOVA ($F_{(1, 26)} = 12.851$, $p = 0.001$; Figure 17.B). However, no effect of stress on phospho-ERK was observed at this time point ($F_{(1, 26)} = 0.591$, $p = 0.449$).

TrkB knockdown in the NAc shell prevents intermittent social defeat induced increase of BDNF and GluA1 in the VTA

Intermittent social defeat stress resulted in a significant increase of BDNF expression in the rostral VTA of rats that received the control AAV-GFP virus, examined seventeen days after the last defeat ($F_{(1, 12)} = 10.52$, $p = 0.003$; Figure 18.A). In contrast,

with TrkB knockdown in the NAc shell, no difference on VTA BDNF expression was found between handled and stressed rats ($F_{(1, 13)} = 2.99$, $p = 0.096$). Also, a significant main effect of stress and a significant interaction between two factors on VTA BDNF expression were found (two-way ANOVA: stress: $F_{(1, 26)} = 5.03$, $p = 0.034$; stress × TrkB knockdown: $F_{(1, 26)} = 6.335$, $p = 0.018$), but no main effect of TrkB knockdown was observed ($F_{(1, 26)} = 0.01$, $p = 0.983$).

Significant main effects of stress and virus on VTA GluA1 expression were observed (two-way ANOVA, stress: $F_{(1, 26)} = 4.456$, $p = 0.045$; TrkB knockdown: $F_{(1, 26)} = 19.613$, $p = 0.001$; Figure 18.B). Intermittent social defeat stress-induced an increase of GluA1 subunit of AMPA receptor expression in the VTA of rats received control AAV-GFP virus ($F_{(1, 12)} = 6.80$, $p = 0.015$) and AAV-shTrkB-stressed rats ($F_{(1, 13)} = 20.45$, $p = 0.001$). In contrast, intermittent social defeat induced increase of GluA1 in the VTA was not found in rats with NAc shell TrkB knockdown ($F_{(1, 14)} = 3.42$, $p = 0.076$).

NAc shell TrkB knockdown reduces the intermittent social defeat stress-induced DeltaFosB expression in the NAc

Intermittent social defeat stress significantly increased DeltaFosB expression in the NAc of rats that received control AAV-GFP virus compared to AAV-GFP-handled rats ($F_{(1, 13)} = 6.60$, $p = 0.016$) and AAV-shTrKB-stressed rats ($F_{(1, 13)} = 18.09$, $p = 0.001$). However, no difference in DeltaFosB expression levels in the NAc was found between AAV-shTrkB-handled and AAV-shTrkB-stressed rats ($F_{(1, 13)} = 0.001$, $p = 0.948$). A significant main effect of stress and a significant interaction between two factors were found (two-way ANOVA, stress: $F_{(1,26)} = 8.725$, $p = 0.007$; TrkB knockdown:

$F_{(1,26)} = 0.601$, $p = 0.445$; stress × TrkB knockdown: $F_{(1,26)} = 8.166$, $p = 0.008$; Figure 18.C).

Discussion

The present study examined the necessity of BDNF-TrkB signaling in the NAc shell in social defeat stress-induced cross-sensitization to psychostimulants. By reducing the expression of TrkB in the NAc shell with AAV-shTrkB mediated knockdown, intermittent social defeat stress- induced cross-sensitization to AMPH was prevented. Of note, social defeat stress-induced key neurochemical changes in the mesolimbic circuit were also prevented and levels of activation of downstream of BDNF-TrkB signaling associated with cross-sensitization to psychostimulants were reduced in the NAc.

BDNF signaling in the NAc shell is necessary for the development of social defeat stress- induced cross-sensitization

Based on previous research, NAc is the pivotal brain nucleus mediating the rewarding effects of drugs of abuse and the expression of behavioral sensitization (Cador, Bjijou, & Stinus, 1995). Both stress and drugs of abuse increase dopamine transmission in the NAc (Garcia-Keller et al., 2013; Miczek, Mutschler, et al., 1999; Saal et al., 2003), which underlies the sensitized psychomotor response to the drugs of abuse. NAc is composed of shell and core, two anatomically and functionally distinctive subregions which have different afferent and efferent projections (Bahi et al., 2008; A. Y. Deutch & Cameron, 1992) and different functions in addiction related behaviors (Berton et al., 2006). Drugs of abuse preferentially increase dopamine levels in the shell, as opposed to its core counterpart (Pontieri, Tanda, et al., 1995; Tanda, Pontieri, & Di Chiara, 1997; Zocchi et al., 2003). The NAc shell is also more responsive to stress than the core in

terms of dopamine transmission (P. W. Kalivas & Duffy, 1995). Therefore, the NAc shell is the possible anatomical substrate underlying social defeat stress- induced cross-sensitization.

Long-lasting BDNF elevation was observed in the VTA following repeated social defeat stress, which is accompanied by persistent cross-sensitization to AMPH (Fanous et al., 2010; Nikulina et al., 2004). Overexpression of BDNF in the VTA engenders prolonged cross-sensitization and elevation of DeltaFosB in the NAc shell in stressed rats (J. Wang et al., 2013). These findings indicate that increased BDNF signaling in the mesolimbic circuit can potentiate stress induced cross-sensitization and that the NAc shell may be the one of the action sites of mesolimbic BDNF signaling during this potentiation. Our behavioral results demonstrate that BDNF signaling via TrkB receptors in the NAc shell is necessary for the development of cross-sensitization.

Stress-induced neurochemical alterations are prevented by TrkB knockdown in the NAc shell

Drugs of abuse can increase the levels of phospho-ERK in multiple regions of brain reward pathways (Berhow et al., 1996; Valjent, Pages, Herve, Girault, & Caboche, 2004), and ERK activation in various brain regions plays an important role in psychomotor sensitization and mediates the rewarding effects of drugs of abuse (Girault, Valjent, Caboche, & Herve, 2007; Lin Lu et al., 2005; L. Lu, Koya, Zhai, Hope, & Shaham, 2006). Therefore, an elevated level of phospho-ERK in the NAc may enhance the reinforcing and sensitizing effects of drugs of abuse. In addition, elevated ERK phosphorylation can be observed immediately after stress (Iniguez et al., 2010). It is possible that the elevated level of phospho-ERK in the NAc shell after social defeat stress

is critical for the development of cross-sensitization to psychostimulants and it is also possible that the persistent reduction of phospho-ERK in the NAc of rats that received TrkB knockdown could disrupt this same process. Also, the reduction of phospho-ERK in the NAc is a feature of resilience to social defeat stress (V. Krishnan et al., 2007). Therefore, rats with TrkB knockdown resulting in a reduction of phospho-ERK in the NAc could be more resilient to the aversive effects of social defeat stress and show attenuated stress-induced cross-sensitization. However, the molecular mechanism of the involvement of phospho-ERK in the NAc during the cross-sensitization, especially the downstream factors activated by phospho-ERK, remains to be characterized.

Elevation of BDNF in the VTA is one of the hallmark neurochemical changes that takes place after intermittent social defeat stress. However, the increase in BDNF is not obvious immediately after the stress exposures, but only becomes significant weeks after stress (Fanous et al., 2010). Together with results of the present study, these data suggest that TrkB-BDNF signaling onto the NAc shell involves stress-induced BDNF expression in the VTA.

Notably, BDNF can up-regulate the total cellular levels of AMPAR subunits GluA1–GluA4 (Caldeira et al., 2007). The AMPARs are tetramers comprised of four glutamate receptor subunits (GluA1-GluA4). GluA2 lacking AMPARs are Ca2+ permeable (CP-AMPAR), and therefore exhibit increased conductance and potentiated synaptic neurotransmission relative to GluA2 containing AMPARs (Burnashev, Monyer, Seeburg, & Sakmann, 1992). BDNF can enhance synaptic strength via the synaptic delivery of newly synthesized GluA1 subunits as CP-AMPARs (Fortin et al., 2012). The elevation of VTA GluA1 was involved in the sensitization to psychostimulants and

addiction and the level of homomeric GluA1 receptors was increased in the VTA of cocaine-sensitized rats (Carlezon & Nestler, 2002; P. W. Kalivas, 2009). Several lines of evidence show that stress exposure increases synthesis and membrane trafficking of AMPA-sensitive glutamate receptor subunits in dopaminergic neurons of the VTA, thus increasing the strength at excitatory synapses on dopamine neurons post-synaptically (H. E. Covington, 3rd et al., 2008; Fitzgerald, Ortiz, Hamedani, & Nestler, 1996). Therefore, we further examined GluA1 expression in the VTA, a target up-regulated by BDNF and involved in synaptic plasticity during psychostimulant/stress-induced sensitization. Our results showed that stress-induced increases of BDNF and GluA1 in the VTA are concomitant with stress-induced cross-sensitization to psychostimulants and that TrkB knockdown in the NAc shell significantly attenuated GluA1 expression. Considering the regulatory role of BDNF on GluA1 expression, our finding also suggests VTA BDNF might be the permissive factor required for GluA1 elevation in the VTA induced by social defeat stress.

The long-term effects of intermittent social defeat stress, in terms of cross-sensitization, suggest the involvement of long-lasting changes on gene expression. Repeated exposure to virtually all types of drugs of abuse and stressors induce DeltaFosB expression in the NAc (J. Chen, Kelz, Hope, Nakabeppu, & Nestler, 1997; Nestler, 2008; Nikulina et al., 2008; Perrotti et al., 2004), which progressively accumulates and persists for at least several weeks after the cessation of drug/stress exposure due to its unusual stability (Nestler, 2008). Overexpression of DeltaFosB in dynorphin-containing neurons within the NAc induces sensitized responses to drugs of abuse (Zachariou et al., 2006), and DeltaFosB in the NAc mediates the natural reward induced cross-sensitization to

AMPH (Pitchers et al., 2013). DeltaFosB can regulate many genes involved in chronic drug exposure induced neuroplasticity in the reward circuitry (McClung & Nestler, 2003). Stress-induced accumulation of DeltaFosB in the NAc could therefore change the transcription potential of many target genes, which may prime the brain reward circuit to the rewarding and sensitizing effects of drugs of abuse. Furthermore, higher co-localization of BDNF and DeltaFosB was found after intermittent social defeat stress in the projection areas of the mesocorticolimbic circuit, including the NAc (Nikulina et al., 2012), and VTA BDNF overexpression together with single social defeat stress specifically increased DeltaFosB in the NAc shell (J. Wang et al., 2013). In the current study, both elevation of BDNF in the VTA and accumulation of DeltaFosB expression in the NAc shell were prevented by TrkB knockdown in the NAc shell, which suggests that these two neurochemical changes in the mesolimbic circuit are gated by TrkB mediated signaling. Our finding also implicates a potential causative interaction between BDNF-TrkB signaling and expression of DeltaFosB in the NAc shell, which remains to be investigated.

In conclusion, BDNF-TrkB signaling in the NAc is critical for social defeat stress induced neuroadaptive changes that are necessary for cross-sensitization to psychostimulants. Phospho-ERK levels were significantly reduced by TrkB knockdown and the active form of ERK is a part of the intracellular pathways for BDNF, dopamine, and glutamate, all of which were found to be involved in the behavioral effect of psychostimulant action (L. Lu et al., 2006; Valjent et al., 2005). Therefore, BDNF-TrkB signaling onto the NAc shell might be a key factor that employs various signaling mechanisms and mediates cross-sensitization following social defeat stress. TrkB

receptors in the NAc-shell could be a novel therapeutic target for prevention of stress-induced vulnerability to drugs of abuse.

Chapter 4

BDNF OVEREXPRESSION IN THE VENTRAL TEGMENTAL AREA POTENTIATE EPISODIC SOCIAL DEFEAT STRESS-INDUCED ESCALATION OF COCAINE SELF-ADMINISTRATION

Abstract

Brain-derived neurotrophic factor (BDNF) is a neural growth factor which can enhance the survival and activity of dopaminergic neurons and is involved in synaptic plasticity. BDNF is elevated in several portions of the mesocorticolimbic circuit, such as the ventral tegmental area (VTA) after exposure to psychostimulants or intermittent social defeat stress, and can mediates sensitivity to cocaine. Intermittent exposure to social defeat stress escalates drug intake during self-administration in rats. We hypothesized that BDNF overexpression in the VTA will further potentiate stress-induced escalation of cocaine intake. Adult male Sprague-Dawley rats were implanted with intravenous jugular catheters and received bilateral infusion of an adeno-associated viral vector (AAV) containing cDNA of green fluorescent protein (AAV-GFP) or bdnf (AAV-BDNF) into the VTA. Rats were exposed to either intermittent social defeat stress (4 defeats over 10 days) or handling. One week after the last defeat, all rats (GFP-handled, GFP-stressed, BDNF-handled, BDNF-stressed) were trained to self-administer cocaine (0.75 mg/kg/0.1ml, i.v.) under fixed ratio (FR) schedules of reinforcement. After self-

administration (SA) stabilized on an FR5 schedule, rats were subjected to three progressive ratio (PR) sessions (0.375 mg/kg) on alternating days with maintenance sessions (0.75 mg/kg) on an FR5 schedule. The day after the last maintenance session, rats were given 12-h access to cocaine (0.375 mg/kg) on an FR5 schedule (12-h binge). Ten days after binge session, brains were removed and processed for immunohistochemistry. Facilitated acquisition of cocaine SA was found in BDNF-handled group compared to AAV-GFP-handled group. The BDNF-stressed group showed the highest level of drug intake during the 12-h binge and the greatest BDNF expression in the VTA. We also found a positive correlation between VTA BDNF level and drug intake during the 12-h binge. VTA BDNF overexpression significantly reduced BDNF in the prelimbic cortex (PrL) and prevented stress-induced BDNF increase in PrL. Also VTA BDNF overexpression resulted in greater DeltaFosB accumulation in both the shell and core of the NAc than did AAV-GFP after cocaine SA. Our results suggest that the level of BDNF in the VTA may titrate compulsive drug-taking behavior, and that higher VTA BDNF is a risk factor engendering greater vulnerability to cocaine abuse. Elevated BDNF expression in the VTA could induce persistent functional activation of the NAc, resulting in the accumulation of DeltaFosB. The reduction of BDNF expression in prelimbic cortex (PrL) might also contribute to escalating drug-taking behavior, possibly through decreased inhibitory output to areas involved in drug reinforcement and motivation.

Introduction

The propensity to develop compulsive patterns of drug taking varies in humans (Warner La, 1995), and the transition from recreational to compulsive drug use depends

on the vulnerability of the intrinsic nervous system and can be influenced by environmental stimuli, such as stress. Stress is an etiological factor which can increase likelihood of drug abuse in vulnerable individuals (Sinha, 2001). Among many stress models in laboratory animals, the social defeat stress model has great ethological value because it is natural and non-adaptable, and has a high degree of face validity when compares to the aversive consequences of peer competition and personal failure in humans. Rats exposed to intermittent social defeat stress exhibit facilitated acquisition of cocaine self-administration and enhanced motivation to self-administer cocaine (Miczek et al., 2004; Miczek & Mutschler, 1996), as well as behavioral sensitization to psychostimulants (Miczek, Nikulina, et al., 1999; Nikulina et al., 2004).

Repeated social defeat stress induces increase of brain-derived neurotrophic factor (BDNF) expression in the ventral tegmental area (VTA), a cell body region of dopaminergic neurons, which in turn has a potentiating effect on the sensitization to psychostimulants (J. Wang et al., 2013). Increased BDNF in the VTA is also associated with enhanced drug-seeking behavior that occurs during withdrawal from cocaine self-administration (Jeffrey W. Grimm et al., 2003). Further, administration of BDNF onto the VTA brain slices sensitize dopaminergic neurons to excitatory input (Pu et al., 2006). In contrast, heterozygous BDNF knock-out mice exhibit reduced behavioral response to the rewarding and psychomotor-activating effect of cocaine (Hall, Drgonova, Goeb, & Uhl, 2003). Considering the essential role of BDNF in synaptic plasticity (Kang & Schuman, 1995; Korte et al., 1995), enhanced BDNF in the VTA could induce neuroplasticity in mesocorticolimbic circuit leading to enhanced stress-induced vulnerability to drugs of abuse.

The VTA, nucleus accumbens (NAc), and prefrontal cortex (PFC) are the major nodes in the mesocorticolimbic circuit, which is the neural substrate of both drugs of abuse and stress (P. W. Kalivas & Stewart, 1991; Wise, 1998). BDNF can be synthesized from dopaminergic neurons in the VTA and glutamatergic neurons in the PFC and transported antereogradely to the NAc (Altar et al., 1997). Enhanced BDNF signaling in the VTA could cause neuroadaptation in these projected areas, and such changes may contribute to the increased susceptibility to the rewarding effect of drugs of abuse (Bolanos & Nestler, 2004). Previous evidence showed that VTA BDNF overexpression alone increases DeltaFosB, a truncated member of the Fos family of transcription factors, in both the PFC and NAc (J. Wang et al., 2013). Also increased co-localization of BDNF and DeltaFosB was found in the NAc and PFC of rats with elevated VTA BDNF levels after exposure to intermittent social defeat stress (Nikulina et al., 2012). DeltaFosB is induced in the NAc in response to chronic exposure of numerous drugs of abuse (Nestler, 2008). Elevation of DeltaFosB in the NAc induces stronger incentive salience attributed to cocaine (Colby, Whisler, Steffen, Nestler, & Self, 2003). In addition, overexpression of DeltaFosB in dynorphin expressing NAc neurons induces sensitization to cocaine (Kelz et al., 1999). Therefore elevated DeltaFosB, especially in the NAc, could be a molecular mechanism underlying persistent vulnerability to the addictive effects of cocaine found in stressed individuals.

In the current study, we investigated whether enhanced VTA BDNF signaling works as a risk factor inducing higher vulnerability to cocaine abuse and whether VTA BDNF can further enhance the potentiating effect of social defeat stress on compulsive cocaine taking behavior. To examine our hypotheses, self-administration paradigm was

used in which the drug intake during the acquisition, progressive ratio, and 12-h binge with unlimited access to cocaine were measured. DeltaFosB, a molecular switch of long-term addictive state, was also measured in the NAc of these rats. Also BDNF expression in the PFC was also measured as an index of reduced inhibitory control of compulsive drug use.

Material and Methods

Animals

Male Sprague-Dawley rats weighing 250-300 g were housed individually in a temperature-controlled colony room, with water and food available ad libitum, on a 14-h reversed light/dark cycle. Animal care and housing conditions were consistent with the Guide for Care and Use of Laboratory Animals (National Research Council, 1996). Surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee at Arizona State University. Rats were acclimated to handling for 7 days and weighed 310-400 g prior to surgery.

Surgery

Rats were administered an analgesic (buprenorphine, 0.05 mg/kg, s.c.) prior to induction of isoflurane anesthesia (2-3%; Abbott Laboratories, North Chicago, IL), vaporized in oxygen and delivered through a plastic nose cone. Catheters were constructed from Silastic tubing (10 cm length, 0.012 in inner diameter, 0.025 in outer diameter, Dow Corning, Midland, MI) connected to a 22-gauge nonferrous metal cannula encased within a plastic screw connector (Plastics One, Roanoke, VA). A miniature ball of aquarium sealant was affixed 2.7 cm from the free end of the catheter. A burrow was made subcutaneously from an incision on the neck to an incision across the skull, and the

catheter was pulled through the burrow. A small incision was made in the jugular vein, where the catheter was inserted and secured with sutures on both sides of the ball. Then Rats were positioned in a stereotaxic frame (David KOPF Instruments; Tujunga, California). Two holes were drilled at stereotaxic coordinates (AP −5.1 mm; ML ±2.15 mm from the bregma) on the surface of skull, and Hamilton syringes (Model 7105 KH; Reno, NV) with 24 gauge injector tips were lowered through the holes bilaterally at a 10° angle until needle tips reached the VTA (AP −5.1 mm; ML ±0.6 mm; DV −8.8 mm from the bregma; Paxinos and Watson, 2007); 0.5 µl of virus was bilaterally infused into each side of the VTA for 10 min at a speed of 0.05 µl/min. The microphotograph of the infusion site is presented in Figure 18.B. After infusion, the syringes remained in the infusion sites for 5 min to prevent retrace of virus.

After the intracranial viral infusion surgery, the cannula end of the catheter was then anchored to the skull using dental acrylic cement and four small anchor screws. The head and neck incisions were sutured and treated with a topical antibiotic and the rats were administered an anti-inflammatory (meloxicam; 1 mg/kg, s.c.). A flexible obturator made from Tygon tubing was fitted over the cannula to protect the catheter. Patency of the catheters was maintained throughout the experiment by daily flushing with 0.1 ml timentin (66.67 mg/mL; bioWORLD, Dublin, OH) in saline solution containing 70 units/mL heparin sodium. Catheter patency was tested periodically with 0.8 g methohexital sodium (Brevital, Sigma), a dose that produces rapid loss of muscle tone only when administered i.v. Following surgery, rats were left to recover for 7 days in their home cages and were handled and weighed daily.

Intermittent social defeat stress

One week after the surgery, rats were exposed to intermittent social defeat stress. Social defeat stress is consisted of a short exposure to aggressive behavior of a resident rat, described in detail previously (Nikulina et al., 2012). Each resident rat (long-evans rat, retired breeder) was group housed with one female rat for at least two weeks before assigning for social defeat experiment. After removing the female from the resident's cage, the experimental intruder rat was placed into the resident's home cage under a stainless steel mesh protective cage (15×25×15 cm) for the first 5 min. Then the protective cage was removed, and the resident displayed aggressive behavior. "Defeat" occurred when the intruder exhibited a supine posture for at least 4 s. Aggressive interactions were 2–5 min in duration. After the display of supine posture, further physical contact was prevented by putting the intruder under the protective cage for an additional 15 min before returning to its home cage. There are total 4 times exposure to social defeat stress in 10 days. Control rats were handled and weighed on the days their counterparts were defeated and weighed.

Apparatus of self-administration

One week after the last episode of social defeat stress, rats were put inside the self-administration chamber and started to receive training for cocaine self-administration. Training and testing were conducted in Plexiglas operant conditioning chambers (20 × 28 × 20 cm) equipped with a food pellet dispenser and a food well located between two retractable levers mounted on the front panel (Med Associates, St. Albans, VT). A stimulus light was mounted above one lever, a tone generator (500 Hz, 10 dB above background noise) was mounted on the side wall and a house light was mounted on the

rear wall opposite the levers. The lever below the stimulus light was designated as the active lever. Each conditioning chamber was housed within its own ventilated, sound-attenuating cabinet. An infusion pump containing a 10-ml syringe was located outside of the cabinet. Tygon tubing connected to the syringe was attached to a liquid swivel (Instech, Plymouth Meeting, PA) suspended above the operant conditioning chamber. The outlet of the swivel was fastened to the catheter via Tygon tubing that ran through a metal spring leash (Plastics One). The leash fastened onto the plastic screw of the cannula that was anchored on the animal's head.

Acquisition and maintenance

A timeline of the experiments is presented in Figure 19.A.

Following recovery from surgery, self-administration (SA) training was conducted during daily 2-h sessions that were signaled by the illumination of the stimulus light above the active lever. Rats were given the opportunity to self-administer cocaine (0.75 mg/kg/0.1 ml, i.v.) on a fixed ratio (FR) 1 schedule of reinforcement. Pressing the active lever was reinforced by an infusion followed by a 30-s timeout, during which the stimulus light was turned off and responses did not count toward the ratio requirement. Responses on the inactive lever were recorded, but did not result in any consequences. Each daily session terminated after the delivery of 15 infusions or 5 h of access. After the rat self-administered 15 cocaine infusions on 2 consecutive days, the response requirement was gradually increased from FR 2 to FR 5 over the next 3-5 days. The rats were maintained on a FR 5 schedule (15 infusions per day or 5 h access) for 5 consecutive days.

Progressive ratio

For the 6 days following acquisition and maintenance, self-administration according to a progressive ratio (PR) schedule of cocaine reinforcement (0.375 mg/kg/infusion) was studied in each rat every other day, with maintenance sessions (0.75mg/kg/infusion, FR 5, 15 infusions) to cocaine on alternating days. The algorithm for each successive cocaine infusion was derived from earlier work (Richardson & Roberts, 1996). The progression response requirement was as follows: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178… Rats had 1 h to successfully complete each ratio requirement. The last completed ratio, corresponding to the final infusion delivered, was defined as the breaking point.

12-h Binge session

After the final PR session, each rat was allowed one maintenance session of cocaine (0.75 mg/kg/infusion, FR 5, 15 infusions). The very next day, a prolonged access protocol was implemented starting at approximately 8:00 a.m. (i.e., 2 h after the start of the dark cycle). Each rat was allowed continuous access to cocaine (0.375 mg/kg/infusion, FR 5) during the entire 12-h session.

Reinstatement

Following 5 days of abstinence, rats were transported to the operant chambers for extinction and reinstatement test sessions conducted over four consecutive phases: (1) extinction, (2) cue reinstatement, (3) saline reinstatement, and (4) cocaine reinstatement. During extinction, the session was signaled by the illumination of the stimulus light, but lever presses had no consequences. After one hour, the cue reinstatement phase began by presentation of the stimulus light at the beginning of the session and active lever presses

resulted in termination of the light for a 30-s timeout on an FR 1 schedule of reinforcement to resemble the response-contingent presentation of the cue during self-administration training. Two hours later, the saline reinstatement phase began by administration of a priming injection of saline (IP) and cues were presented the same as during cue reinstatement. Following one hour, the cocaine reinstatement phase began by administration of a priming injection of cocaine (10 mg/kg, IP) and cues were presented the same as during cue reinstatement for a one hour session. The data of reinstatement is not conclusive, therefore not shown.

Perfusion and tissue processing

Ten days after binge session, all rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 10 ml of 10% heparin in 0.1 M phosphate-buffered saline (pH 7.4) followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were post-fixed for 1 h in the same fixative at 4°C, soaked in 15% sucrose, then in 30% sucrose in 0.1 M PBS at 4°C until saturated, then stored at 4°C prior to sectioning. Brains were sectioned at 20 µm in a cryostat at −22°C, and thaw-mounted onto glass slides (Superfrost Plus; Fisher; Waltham, MA). Sections were collected from +3.2 to +2.8 mm from bregma for the PFC, +1.8 to +0.8 mm from bregma for the NAc, and −4.8 to −5.6 mm from bregma for the VTA (Paxinos and Watson, 2007).

Immunohistochemistry

Sections were washed in 0.05 M potassium phosphate-buffered saline (KPBS), then blocked for 1 h in 10% normal donkey serum and 0.4% Triton X-100 in 0.05 M KPBS. Sections were then incubated with primary antibody: BDNF (AB1779SP, 1:3,000

dilution; Millipore; Temecula, CA) or FosB (SC-48, 1:5,000 dilution; Santa Cruz Biotechnology, Inc.; Santa Cruz, CA). The FosB antibody used here targets the N terminal of FosB protein contained in both FosB and DeltaFosB. However, based on the timeline of the present research, FosB-like labeling would primarily capture accumulation of DeltaFosB because FosB expression is transient and only DeltaFosB persists after stimulation (Perrotti et al, 2004). Following incubation with primary antibody for 48 h at 4°C, slides were washed in 0.05 M KPBS and incubated for 1 h in biotin-conjugated goat anti-rabbit serum (1:200 dilution in blocking solution, Vectastain ABC kit; Vector Laboratories; Burlingame, CA). After washing in 0.05 M KPBS, sections were incubated with avidin–biotin–peroxidase complex (ABC kit, Vector Laboratories) for 1 h, then washed again and developed using DAB chromogen with nickel (DAB Peroxidase Substrate kit, Vector Laboratories). After dehydration in graded concentrations of ethanol and xylene, coverslips were applied.

To examine the cellular localization of BDNF expression in the VTA of animals that showed persistent cross-sensitization, we performed fluorescent double labeling of BDNF and tyrosine hydroxylase (TH). Primary antibodies, rabbit anti-BDNF (AB1779SP, 1:500 dilution; Millipore; Temecula, CA) and mouse anti-TH (SC-7837, 1:500 dilution, Santa Cruz Biotechnology; Santa Cruz, CA), were applied simultaneously and incubated for 48 h at 4°C. After incubation with biotinylated goat anti-rabbit IgG (1:200 dilution, Vector Laboratories) for 1 h, Alexa Fluor 488 Anti-Rabbit conjugated streptavidin and Alexa Fluor 548 Goat Anti-Mouse (1:500 dilution, Invitrogen, San Diego, CA) were applied for 2 h. After washing with 0.05 M KPBS, coverslips were applied with ProLong Gold Antifade Reagent with DAPI (Invitrogen, San Diego, CA). Not all samples were

processed for fluorescent double labeling, making the sample number different from other IHC labeling. For fluorescence double labeling, sample numbers are as follow: n = 7 for AAV-GFP-handled, n = 13 for AAV-GFP-stressed, n = 8 for AAV-GFP-handled and n = 15 for AAV-BDNF-stressed.

Image analysis

Tissue sections were examined for the presence of chromogen or fluorophore reaction products using a Zeiss Axioskop microscope; selected areas were captured and digitized using a color digital video camera (MBF Biosciences; Williston, VT) or AxioCam MR (Zeiss) interfaced to the microscope. A cell profile was considered labeled if its pixel intensity was more than 2 standard deviations greater than the background, as calculated by Stereo Investigator software (MBF Biosciences). Fluorescent-labeled cells were counted manually, first in single channels for single labeling, then in merged channels for double labeling. At least three adjacent sections were analyzed for each brain region per animal, and labeling density was calculated by dividing the estimated total number of labeled profiles by the total area analyzed.

Statistics

Behavioral result of cocaine self-administration and immunohistochemical data were analyzed by two-way ANOVA (between subjects factors: viral vector [AAV-BDNF vs. AAV-GFP] and behavioral treatment [handling vs. stress]). T-test was used for planned comparisons between AAV-BDNF-stressed and all other groups when significant interaction was not present, and post-hoc Tukey test was used in the presence of significant interaction. All data are reported as mean ± SEM. Results were considered significant if $p \leq 0.05$. The sample numbers varied slightly across different procedures

examining different brain regions due to the exclusion of samples with occasional artifacts during tissue collection or processing.

Results

BDNF and social defeat alone enhance acquisition of cocaine self-administration

To measure acquisition, we calculated the number of infusions an animal obtained before they successfully completed an FR1 session (15 infusions/session). We found that both the AAV-GFP-stressed(8.16 ± 2.18) and AAV-BDNF-handled (7.09 ± 1.71) groups takes fewer cocaine infusions to reach the acquisition criteria compared to GFP-handled (13.05 ± 2.24) and BDNF-stressed groups (13.6 ± 2.89), who exhibited similar acquisition rates (Figure 20.A). This was evident by a significant interaction between virus and stress ($F_{(1, 62)} = 5.37$, $p < 0.05$). AAV-BDNF-handled rats takes significantly less infusions to acquire stabilized self-administration compared to handled rats received control AAV-GFP virus ($p = 0.042$) and AAV-BDNF-stressed rats ($p = 0.041$).

Social defeat enhances motivation for cocaine

When compared to handled controls, socially defeated animals exhibited higher rates of intake and responding on a progressive ratio (PR) schedule of cocaine reinforcement, regardless of virus condition (Figure 20.B and C). There were significant main effects for stress on active lever pressing numbers ($F_{(1, 62)} = 6.979$, $p = 0.010$) and infusion received ($F_{(1, 62)} = 7.989$, $p = 0.006$), but no main effect of virus (ranging from $F_{(1, 62)} = 0.10$ and 0.16, $p = 0.69$ and 0.75 respectively) nor interaction between the two factors (ranging from $F_{(1, 62)} = 1.36$ and 2.52, $p = 0.12$ and 0.25 respectively).

VTA BDNF titrates compulsive cocaine taking behavior during 12-h access "binge"

During the 12-h access to cocaine session, the BDNF-stress group had the highest rates of intake and responding, followed by the GFP-stressed, BDNF-handled, and GFP-handled (Figure 20.D and E). There were significant main effects of stress ($F_{(1, 63)}$ = 12.731, p = 0.001) and virus ($F_{(1, 63)}$ = 4.344, p = 0.041) for responding.

Intra-VTA infusion of AAV-BDNF or intermittent social defeat stress induced significant elevation of BDNF expression in the VTA after cocaine self-administration, shown by the significant main effects of AAV-BDNF ($F_{(1, 53)}$ = 40.176, p = 0.001) and stress ($F_{(1, 53)}$ = 15.377, p = 0.001; Figure 21. A and B) using two-way ANOVA. A positive correlation between VTA-BDNF expression levels and drug taking behavior during 12 h binge was found (Pearson Correlation: r = 0.422, p = 0.001; Figure 21.C).

The number of BDNF expressing dopaminergic neuron in the rostral VTA was also increased in rats received AAV-BDNF after cocaine self-administration ($F_{(1, 39)}$ = 20.006, p = 0.001). However, stress failed to affect the number of BDNF expressing dopaminergic neurons in the rostral VTA ($F_{(1, 39)}$ = 0.24, p = 0.627; Figure 22.A and B).

VTA BDNF overexpression induced compensatory change of BDNF in the prelimbic PFC

After chronic cocaine self-administration, rats with VTA BDNF overexpression showed a significant reduction of BDNF expression levels in the prelimbic cortex (PrL-PFC) compared to rats received control GFP virus ($F_{(1, 59)}$ = 5.525, p = 0.022). However, no effect of intermittent social defeat stress on BDNF expression was observed in PrL-PFC ($F_{(1, 59)}$ = 1.544, p = 0.217). Such VTA BDNF overexpression induced reduction of

PFC BDNF is specific to the PrL-PFC subregion, because no change of BDNF expression was found in ACG-PFC ($F_{(1, 56)} = 2.889$, $p = 0.095$; Figure 23.A and B).

Persistent enhanced VTA-BDNF increased DeltaFosB expression in both shell and core of the NAc

After chronic cocaine SA, VTA BDNF overexpression induced significantly increased DeltaFosB expression in both core ($F_{(1, 48)} = 8.366$, $p = 0.006$) and shell subregions ($F_{(1, 48)} = 5.593$, $p = 0.022$) of the NAc in rats, compared to groups received GFP virus (Figure 24.A and B). However, the main effect of stress on DeltaFosB expression was not significant in core ($F_{(1, 48)} = 0.049$, $p = 0.826$) and shell subregions ($F_{(1, 48)} = 0.146$, $p = 0.704$) of the NAc after chronic cocaine SA.

Discussion

The present study showed that higher BDNF level in the VTA can facilitate the acquisition of cocaine self-administration and potentiate compulsive drug taking behavior during 12-h binge. Our behavioral data also confirmed that repeated social defeat stress facilitates acquisition and increases motivation to self-administer cocaine. Stress induced escalation of cocaine self-administration during 12-h binge is further augmented by enhanced BDNF expression in the VTA. We discovered a positive correlation between VTA BDNF expression level and drug taking behavior during 12-h binge. VTA BDNF overexpression also induces significant elevation of DeltaFosB in the NAc core and shell subregions, and a reduction of BDNF specifically in PrL cortex after chronic cocaine self-administration.

VTA-BDNF, intermittent social defeat stress-induced escalation of cocaine self-administration

Social defeat stress is an etiological factor enhancing the vulnerability to develop compulsive drug-taking behavior. Stress can sensitize the brain reward circuit, leading to enhanced dopamine transmission onto the NAc in response to drugs of abuse (Tidey & Miczek, 1996), which ultimately leads to enhanced sensitivity to rewarding effect of drugs of abuse and behavioral sensitization to drugs of abuse. Previous papers demonstrated that intermittent social defeat stress facilitates acquisition of cocaine self-administration (Haney, Maccari, Le Moal, Simon, & Piazza, 1995), increases the motivation for cocaine, and enhances the compulsive drug-taking behavior during binge with unlimited access to drug (H. E. Covington, 3rd & Miczek, 2001).

The gradual increase of BDNF in the VTA is one of the hallmark neurochemical changes after social defeat stress (Fanous et al., 2010), and the elevation of VTA BDNF potentiates psychomotor sensitization to amphetamine (J. Wang et al., 2013). Taken together, these data suggest that enhanced BDNF signaling from the VTA after stress could engender higher vulnerability to the sensitizing effect of psychostimulants. Drug self-administration has great face validity as a model to study neuroadaptation important for compulsive drug-taking behavior and the reinforcing efficacy of drugs of abuse. Therefore, to examine whether VTA BDNF expression also engenders higher vulnerability to the stress-induced rewarding effect of psychostimulants and incentive sensitization, we overexpressed BDNF in the VTA using AAV-mediated gene transfer, and measured whether such overexpression could enhance drug-taking behavior during acquisition, PR and 12-h binge sessions in cocaine self-administration.

We found that handled rats with VTA BDNF overexpression initiated cocaine self-administration a faster rate than GFP controls in term of reaching our acquisition criterion. It is a well-known phenomenon that some people experience compulsive craving for cocaine after only a few times of exposure, and such vulnerability can be influenced by stress (Tidey & Miczek, 1997). Our data suggest that the enhanced VTA BDNF signaling could be another intrinsic risk factor conferring higher vulnerability to develop compulsive drug taking behavior. Such facilitation on acquisition could be caused by augmented reinforcing effect of cocaine, possibly through BDNF's potentiating effect on the functioning of dopamine neurons located in the VTA (Altar et al., 1992; Horger et al., 1999; Martin-Iverson, Todd, & Altar, 1994).

Interestingly, rats with both stress exposure and VTA BDNF overexpression showed blunted acquisition of cocaine self-administration, compared to rats who received either stress or VTA BDNF overexpression alone. One possible explanation for the facilitation of these two factors by themselves but not together is that both stress and VTA BDNF overexpression can enhance arousal, and such arousal can improve or impair the performance of learning tasks, depending on the magnitude (Yerkes & Dodson, 1908). Arousal improving performance could reduce infusions required to learn the association between lever pressing and drug infusion. However, VTA BDNF overexpression may confer higher susceptibility to the intermittent social defeat stress, which could produce too high of an arousal state that may hamper the performance of an operant learning task (i.e., lever pressing). The blunted acquisition found in AAV-BDNF-stressed rats was likely not due to reducing the reinforcing effect of cocaine, because these rats exhibited no deficit during maintenance once cocaine self-administration was acquired, and

consumed the highest amount of cocaine during the 12-h binge session. Therefore excessive arousal may underlie the blunted acquisition of cocaine self-administration in AAV-BDNF-stressed rats. Further characterization of glucocorticoid levels could shed light into the potential impairment of performance and learning induced by stress together with VTA BDNF overexpression (Lupien, Maheu, Tu, Fiocco, & Schramek, 2007).

The break points during PR sessions in social defeat animals was significantly higher than handled rats, reflected higher motivational salience assigned to cocaine, which is consistent with previous work (H. Covington, III & Miczek, 2005). Of note, VTA BDNF overexpression has a potentiating effect on locomotor sensitization (J. Wang et al., 2013). However, VTA BDNF overexpression alone had no effect on the motivational salience attributed to cocaine. It also appears that stress-induced excessive motivational salience attributed to cocaine was slightly attenuated by VTA BDNF overexpression. Taken together, our data imply a dissociation between the locomotor and incentive sensitization, and that VTA BDNF may have distinct effects on locomotor sensitization and incentive sensitization.

We found that stress induces potent escalation of drug self-administration during the binge session, which is consistent with previous findings. Also, elevated VTA BDNF expression prior to social defeat stress induced further escalation of cocaine self-administration during the 12-h binge session, suggesting that enhanced VTA BDNF signaling may facilitate the stress induced neuroplasticity in the brain reward circuitry which enhances the reinforcing effect of cocaine, ultimately leading to further escalation of drug taking.

Neurochemical changes in mesocorticolimbic circuitry underlying escalation of cocaine self-administration

We have recently showed that AAV-mediated BDNF overexpression in the VTA increases DeltaFosB expression in both the core and shell subregions of the NAc (Wang et al., 2013). DeltaFosB is a truncated variant of the FosB transcription factor. DeltaFosB accumulation in the NAc is found after chronic exposure to virtually all kinds of drugs of abuse and stress (Nestler, 2008; Perrotti et al., 2004). Increased DeltaFosB in the NAc leads to behavioral sensitization to cocaine (Kelz et al., 1999), and increases in cocaine reward (Muschamp, Nemeth, Robison, Nestler, & Carlezon Jr, 2012). Although repeated social defeat stress-induced DeltaFosB in the NAc can be found at least 10 days after the cessation of defeat (Nikulina et al., 2012), we did not find a significant effect of stress on DeltaFosB in the NAc after chronic cocaine self-administration in our study. Chronic cocaine administration can produce epigenetic changes that increase the transcriptional potential of FosB in the NAc, leading to a greater extent of cocaine-induced of DeltaFosB expression (Damez-Werno et al., 2012). Therefore, the stress-induced DeltaFosB elevation in the NAc could be occluded by the elevation of DeltaFosB during chronic cocaine self-administration. Also, increased DeltaFosB expression in the NAc suggests persistent functional activation of this region in the presence of enhanced VTA BDNF signaling.

Human imaging studies have discovered that cocaine addicts exhibit deficits in prefrontal cortical function and consequential loss of inhibitory control over drug-taking behavior (Goldstein & Volkow, 2002). The prelimbic (PrL) cortex sends robust projections to the NAc core, and BDNF from PrL cortex is anterogradely transported to

terminals in the NAc (Altar et al., 1997), providing regulation onto the mesolimbic reward circuit (Vertes, 2004). Enhanced BDNF signaling in the PrL cortex leads to decreased cocaine-seeking in rats (Berglind et al., 2007), suggesting that enhanced BDNF signaling in this area acts to inhibit drug seeking. In contrast, a marked reduction of neural activity in PrL cortex was found after extended cocaine self-administration, and such this reduction was greater in rats exhibiting compulsive drug seeking (B. T. Chen et al., 2013). We observed a reduction of BDNF specifically in the PrL cortex of rats receiving VTA BDNF overexpression, and such a reduction of BDNF in PrL cortex may contribute to the escalation of cocaine self-administration and increased susceptibility to stress-induced vulnerability to drug. However, the mechanism underlying VTA BDNF overexpression-induced reduction of PrL BDNF in rats exposed to chronic cocaine self-administration is unknown and warrants investigation.

Conclusion

In the present study, we found that VTA BDNF overexpression reduces the number of reinforcers administered before reaching acquisition criteria, suggesting that individuals with higher VTA BDNF may have greater sensitivity to the reinforcing effects of psychostimulants, such as cocaine, and be more vulnerable to transitioning to compulsive drug use. We also discovered a graded escalation of compulsive cocaine use during the 12-h binge which positively correlates with the BDNF expression in the VTA across treatment groups, suggested a gene-behavioral response relationship. Also, VTA BDNF overexpression induces significantly elevated DeltaFosB expression in both the core and shell regions of the NAc after chronic cocaine self-administration, an enduring

transcription factor known to induce higher sensitivity to the rewarding effect of psychostimulants (Grueter et al., 2013; Kelz et al., 1999).

Our result suggests that enhanced BDNF in the VTA may represent an intrinsic mechanism conferring individuals more susceptible to the stress-induced vulnerability to drugs of abuse. Therefore, increases of BDNF in the VTA may be a risk a factor inducing higher vulnerability to develop psychostimulant dependence, especially under stressful conditions.

Chapter 5

GENERAL CONCLUSION AND FUTURE DIRECTIONS

Summary of Key Findings

The objective of the current study is to investigate the role of mesolimbic BDNF signaling as a potential risk factor underlying stress induced vulnerability to psychostimulants. Therefore, we used the social defeat stress model, which has face validity in replicating interpersonal conflict and peer pressure in humans, and examined whether AAV-mediated intra-VTA BDNF overexpression would potentiate social defeat stress induced cross-sensitization to psychostimulants and escalation of compulsive drug taking. We also studied the neurochemical changes in the major nodes of the mesocorticolimbic circuit induced by social defeat stress in the presence of enhanced VTA BDNF.

In chapter 2, the effects of VTA BDNF on single social defeat-induced cross-sensitization and on a non-sensitizing dosing regimen of AMPH-induced locomotor response were investigated. We found that enhanced VTA BDNF prolongs the time-course of single social defeat stress induced cross-sensitization and potentiates the

locomotor response to a non-sensitizing dosing regimen of AMPH. In particular, we found that VTA BDNF overexpression alone leads to increased DeltaFosB accumulation in the projection areas of the VTA: the PFC and NAc. Also persistent elevation of BDNF in dopaminergic neurons across the entire rostral VTA was observed in rats receiving both VTA BDNF overexpression and single social defeat stress. Of note, the highest magnitude of elevation of DeltaFosB was found in the NAc shell subregion of rats exposed to social defeat stress which received intra-VTA BDNF overexpression. Therefore, we hypothesized that BDNF signaling in the NAc shell is critical for induction of the neuroplasticity underlying social defeat stress induced cross-sensitization. In chapter 3, we further examined this hypothesis.

In chapter 3, TrkB receptor knockdown in the NAc shell was found to prevent intermittent social defeat stress-induced cross-sensitization to AMPH, which confirmed our hypothesis. We also examined several important neurochemical up-regulations induced by intermittent social defeat stress which are also related to BDNF signaling in the mesolimbic circuit. We discovered that intermittent social defeat stress-induced increase of BDNF and GluA1 in the VTA were prevented by TrkB knockdown in the NAc. The level of phospho-ERK, the activated form of a downstream effector of BDNF-TrkB signaling cascade, was significantly reduced by NAc-TrkB knockdown, whereas total level of ERK remained unaffected by TrkB knockdown. Also intermittent social defeat stress induced DeltaFosB accumulation in the NAc was prevented by intra-NAc TrkB knockdown. A schematic representation of these neurochemical changes induced by TrkB knockdown in stressed animals can be seen in Figure 25. So far, we examined the role of mesolimbic BDNF on stress-induced augmentation of locomotor response to

AMPH. However, locomotor sensitization cannot recapitulate several important aspects of addictive behaviors, such as motivation and reinforcing effect. In contrast, the self-administration model, which can measure incentive sensitization, has greater face validity as a model for motivational changes underlying compulsive drug seeking in addicts. Therefore, we used a cocaine self-administration model to further study VTA BDNF's role during stress induced vulnerability to psychostimulants.

In chapter 4, the role of enhanced VTA-BDNF on stress-induced vulnerability to develop compulsive drug taking behaviors was examined using a cocaine self-administration model. With VTA BDNF overexpression and intermittent social defeat stress, we measured the susceptibility to initiate cocaine self-administration during the acquisition session, the motivational salience attributed to cocaine during PR, and the reinforcing properties of cocaine during a 12-h binge, during which unlimited access to cocaine was given. We discovered that VTA BDNF overexpression alone facilitates acquisition of cocaine self-administration. Also compulsive drug taking behavior during a binge session is positively correlated with BDNF expression level in the VTA. Interestingly, rats with VTA BDNF overexpression which are also exposed to intermittent social defeat stress showed blunted acquisition compared to their counterparts receiving either one of these two manipulations alone. Stress increases the motivation to take cocaine, observed during a PR sessions. However, VTA BDNF overexpression does not induce further increase in incentive salience toward cocaine. AAV-mediated BDNF overexpression in the VTA leads to a higher level of DeltaFosB in both shell and core subregions of the NAc and lower level of BDNF in PrL cortex than in rats receiving control AAV-GFP virus. These data suggest that the NAc is the region

responsive to enhanced BDNF signaling from the VTA, and reduction of BDNF from the PrL cortex may contribute to the compulsive drug taking behavior.

Mechanisms of BDNF Underlying Potentiation of Dopamine Transmission in the Mesolimbic Circuit

BDNF was first characterized as a nerve growth factor which can enhance the survival and activity of dopaminergic neurons (C. Hyman et al., 1991). BDNF activates the TrkB receptor–protein tyrosine kinase as the primary means of signal transduction, and mRNA for both BDNF and TrkB are expressed in dopaminergic neurons cell body regions and terminal fields (Ip et al., 1992). BDNF can enhance the activity of dopaminergic neuron and can facilitate the synthesis and the metabolism of dopamine (Martin-Iverson et al., 1994; Shen, Altar, & Chiodo, 1994). Therefore, enhanced BDNF signaling in the VTA could potentiate the activity of DA neurons and enhance dopamine transmission. In the mesolimbic DA system, BDNF synthesized in VTA DA neurons can be anterogradely transported to the projection areas, such as the NAc and other forebrain structures, such as the PFC and the amygdala (Altar et al., 1997).

Besides direct potentiation of dopamine neuron activity, BDNF also can enhance the sensitivity of DA neurons to excitatory input, therefore increases the excitability of DA neurons, ultimately leading to enhanced dopamine transmission in response to neural activation. BDNF can up-regulate expression of all four subunits of AMPAR, also can induce the assembly of GluA1-containing calcium-permeable AMPAR onto the membrane (Fortin et al., 2012). The insertion of calcium-permeable AMPAR onto the membrane will facilitate long-term potentiation, and increase membrane conductance and synaptic neurotransmission. Also BDNF signaling in the VTA can increase the sensitivity

of VTA DA neurons to excitatory signaling input, which is NMDAR-dependent (Pu et al., 2006). Therefore, BDNF can potentiate the response of VTA DA neurons to excitatory input, such as glutamate released from prefrontal sources, leading to enhanced dopamine release from VTA DA neurons.

There is also evidence suggesting that BDNF can suppress the inhibition from the GABAergic neurons in several regions of mesocorticolimbic circuit. In the mPFC, enhanced BDNF signaling induces down-regulation of the surface level of GABAA receptors in mPFC pyramidal neurons, therefore enhances activity-induced long-term potentiation (LTP) of excitatory synapses in postsynaptic mPFC neurons (H. Lu, Cheng, Lim, Khoshnevisrad, & Poo, 2010). Such effect will lead to the disinhibition of mPFC glutamatergic neurons from GABAergic inhibitory control, ultimately leading to greater excitatory output to VTA DA neurons.

BDNF, stress, and vulnerability to drugs of abuse: Implications from our studies

Elevation of BDNF in the VTA is observed in stressed animals associated with persistent cross-sensitization to psychostimulants, and overexpression of BDNF in the VTA can potentiate locomotor sensitization to psychostimulant in stressed animals. However, intra-VTA BDNF overexpression alone does not induce significant augmentation of locomotor response to psychostimulants. These data suggest that BDNF signaling from the VTA acts as a risk factor conferring higher susceptibility to the sensitizing effect induced by stress, rather than directly potentiating sensitization to psychostimulant.

In our studies, besides psychomotor sensitization (or behavioral sensitization), we also examined incentive sensitization (motivation to take drug). Our behavioral data show

that stress potentiates locomotor response to psychostimulant and induces escalation of drug taking during self-administration, which were consistent with previous studies (H. Covington, III & Miczek, 2005; Nikulina et al., 2004; Yap & Miczek, 2007). Importantly, BDNF can potentiate stress induced psychomotor sensitization, but has no effect on stress induced incentive sensitization. These data suggest that BDNF may not be involved in stress induced changes of incentive salience toward drugs of abuse.

In chapter 3 we further demonstrated that depletion of BDNF-TrkB signaling in the NAc shell prevented social defeat stress induced cross-sensitization and BDNF elevation in the VTA. This result suggests the importance of BDNF signaling in the mesolimbic circuit during stress-induced cross-sensitization to AMPH. Based on previous data, elevation of BDNF was not observed immediately after repeated social defeat stress, but became significant several weeks after social defeat stress. Our data suggest that the increase of BDNF in the VTA is not the immediate consequence of social defeat stress, and it is possible that the BDNF-TrkB signaling mediated neuroadaptation initiates and mediates the gradual increase of BDNF in the VTA, which ultimately leads to higher locomotor response to psychostimulants.

As shown from previous studies, stress sensitizes brain reward circuit and generates greater dopaminergic response to psychostimulants (Tidey & Miczek, 1996), which could be one of the molecular mechanisms underlying stress induced vulnerability to drugs of abuse. Considering the role of BDNF in the survival and activity of dopaminergic neurons (Altar et al., 1992; Shen et al., 1994; Siuciak, Boylan, Fritsche, Altar, & Lindsay, 1996), persistently enhanced VTA BDNF signaling leads to augmented dopamine transmission in the mesolimbic circuit, and therefore potentiates the effect of

stress on dopamine transmission in the mesolimbic circuit. Whether stress-induced enhancement in mesolimbic dopamine transmission is potentiated in the presence of enhanced VTA BDNF requires further investigation.

We further measured the factors in different niches of the intracellular signaling pathway which are found to be involved in sensitization to drugs of abuse, including certain receptor subunits, kinases, and transcription factors. Among these factors examined, we found that stress induced DeltaFosB accumulation in the NAc can be further augmented by VTA BDNF overexpression; simultaneously it can be prevented by the attenuation of BDNF-TrkB signaling in the NAc. These data suggest that DeltaFosB in the NAc could be regulated by the VTA BDNF signaling cascades. Considering the link between DeltaFosB and addictive behaviors, DeltaFosB could be the effector mediating the long-lasting sensitizing effect of drugs of abuse in stressed animals. However, the molecular mechanism underlying such regulation remains unknown, and requires further investigation.

Clinical Impilications of BDNF in Relapse Diagonosis, Susceptibility to Addiction and Conclusions

As discussed in chapter 1, the BDNF Val66Met polymorphism has been associated with increased vulnerability to develop substance abuse, psychiatric disorders and mood disorders in humans. 66Val allele carriers showed comparatively higher activity-induced BDNF release in the CNS than 66Met allele carriers, whose activity-induced BDNF release in the CNS is impaired. A higher frequency of BDNF 66Val homozygosity was found in population of addicts, compared with normal control subjects (S. J. Tsai, 2007). Also, AMPH induces more pronounced self-ratings of arousal and

energy in BDNF 66Val homozygotes than in people with BDNF Met/Val and Met/Met genotypes (Flanagin et al., 2006). Although the alterations in BDNF signaling caused by BDNF Val66Met polymorphism is different from viral vector-mediated region specific BDNF overexpression used in the current study, data from both fields suggested that heightened BDNF signaling contributes to the susceptibility to drug addiction.

Since BDNF can cross the blood–brain barrier in both directions (Pan, Banks, Fasold, Bluth, & Kastin, 1998), BDNF is also found in serum and plasma of human blood (Radka, Hoist, Fritsche, & Altar, 1996), and serum BDNF level is positively correlated with brain BDNF level (Karege, Schwald, & Cisse, 2002). Chronic cocaine use decreased serum BDNF, but during the abstinence the serum BDNF level increased significantly, and this level was positively correlated with craving (Corominas-Roso et al., 2012; D'Sa, Fox, Hong, Dileone, & Sinha, 2011). Therefore, serum levels of BDNF have predictive value for the loss of control measure of craving, and can be used as a biomarker for relapse. Interestingly, the gradual increase of BDNF levels in the serum during early abstinence is coincident with the elevation of BDNF in several regions of mesocorticolimbic circuit during "craving incubation" (Jeffrey W. Grimm et al., 2003).

The current studies discovered that enhanced VTA BDNF expression potentiates stress induced cross-sensitization to AMPH and escalates compulsive cocaine taking behavior, implicating that elevated VTA BDNF signaling acts as a risk factor in stressed individuals conferring higher stress induced vulnerability to drugs of abuse. Also based on the data in chapter 3, TrkB in the NAc shell could be a therapeutic target, and medication which inhibit TrkB activation in the NAc could prevent sensitized response to psychostimulant developed in stressed individuals.

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Table 1

Numbers of DeltaFosB-Positive Cells per mm^2 Present after Two AMPH Injections.

Brain region	AAV-GFP-Handled	AAV-GFP-Stressed	AAV-BDNF-Handled	AAV-BDNF-Stressed
Nucleus accumbens core	419.4 ± 50.1	479.2 ± 53.8	537.5 ± 44.3	473.8 ± 95.7
Anterior cingulate Cortex	303.3 ± 53.9	300.0 ± 24.0	446.7 ± 16.2*	359.5 ± 47.3
Prelimbic cortex	308.3 ± 50.2	347.9 ± 20.3	380.0 ± 38.9	435.7 ± 64.3
Infralimbic cortex	262.5 ± 29.9	316.7 ± 22.9	350.0 ± 29.8	385.7 ± 61.3

*significantly different from AAV-GFP-handled, t-test, $p \leq 0.05$

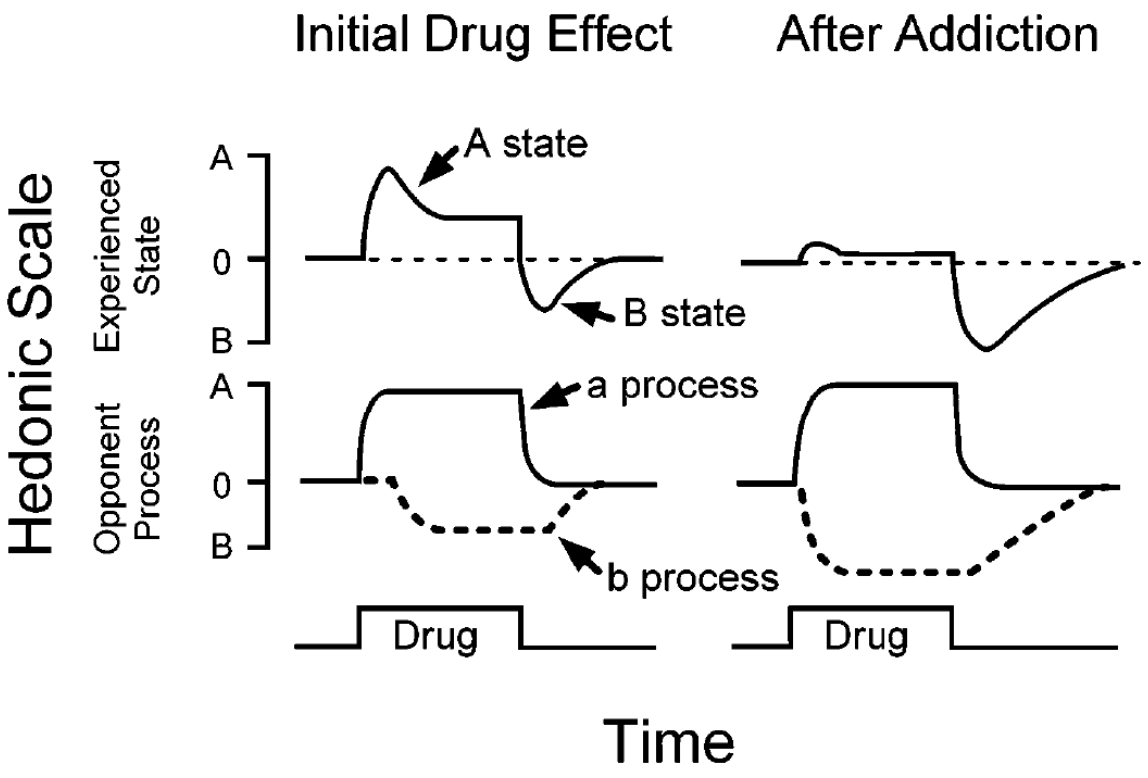

Initial Drug Effect
After Addiction
Hedonic Scale
Experienced State
A
0
B
A state
B state
Opponent Process
A
0
B
a process
b process
Drug
Drug
Time

Figure 1. Opponent process model of addiction. According to the opponent process theory the affective (hedonic or emotional) response to a drug is the underlying a-process, which in turn elicits the opponent b-process. These two underlying processes add together to cause the pleasant A-state followed by an opponent unpleasant B-state. Initially the pleasant A-state is large, followed by a small B-state. With repeated drug use and in addiction, however, the opponent b-process increases in magnitude and duration, leading to an experience dominated by the unpleasant symptoms associated with withdrawal. Adapted from Solomon and Corbit 1973.

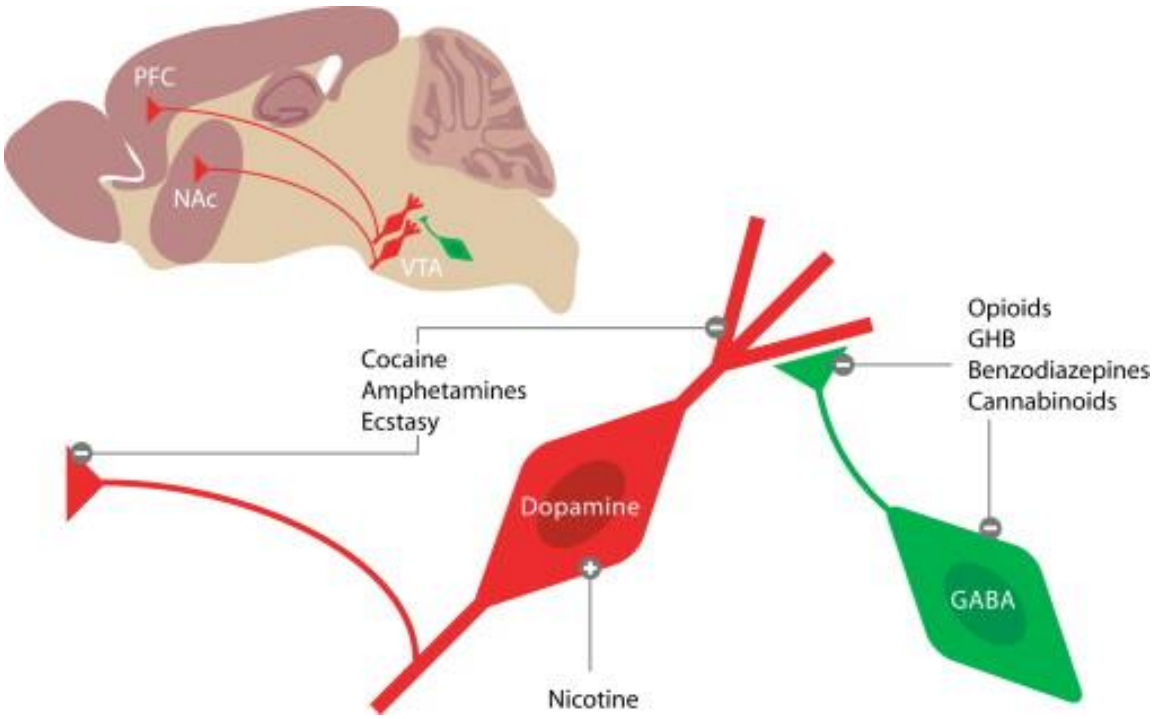

PFC
NAc
VTA
Cocaine
Amphetamines
Ecstasy
Opioids
GHB
Benzodiazepines
Cannabinoids
Dopamine
GABA
Nicotine

Figure 2. The mesocorticolimbic dopamine system as target of addictive drugs. The projection neurons in the VTA are mostly dopaminergic and under inhibitory control of local GABA neurons. Nicotine can directly depolarize DA neurons, while opioids, GHB, benzodiazepines, and cannabinoids act indirectly via pre- and postsynaptic inhibition of GABAergic interneurons (i.e., disinhibition). Cocaine, amphetamines, and ecstasy target the dopamine transporter (DAT) on axon terminals and dendrites of DA neurons. While cocaine acts as an inhibitor of the DAT, amphetamines and ecstasy promote nonvesicular release. In both cases, DA levels in the VTA, NAc, and PFC increase. Image adapted from (Luscher & Malenka, 2011).

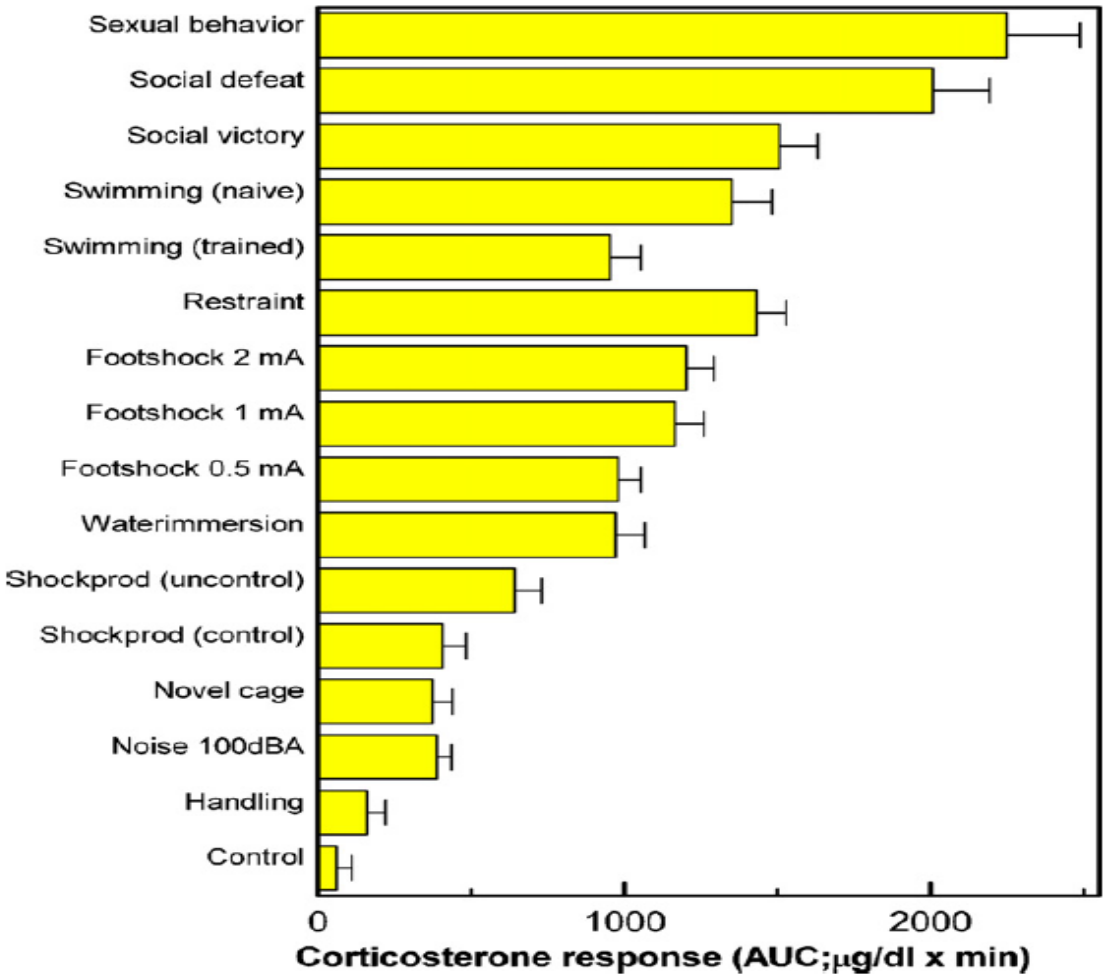

Sexual behavior
Social defeat
Social victory
Swimming (naive)
Swimming (trained)
Restraint
Footshock 2 mA
Footshock 1 mA
Footshock 0.5 mA
Waterimmersion
Shockprod (uncontrol)
Shockprod (control)
Novel cage
Noise 100dBA
Handling
Control
0
1000
2000
Corticosterone response (AUC;µg/dl x min)

Figure 3. Plasma corticosterone levels of adult male Wistar rats to different stimuli. Each test consisted of a standardized series of baseline samples, collected in home cage, and a 15min exposure to the stimulus followed by a recovery phase for the remaining hour. Blood samples were collected via a jugular vein cannula (Koolhaas, De Boer, De Rutter, Meerlo, & Sgoifo, 1997).

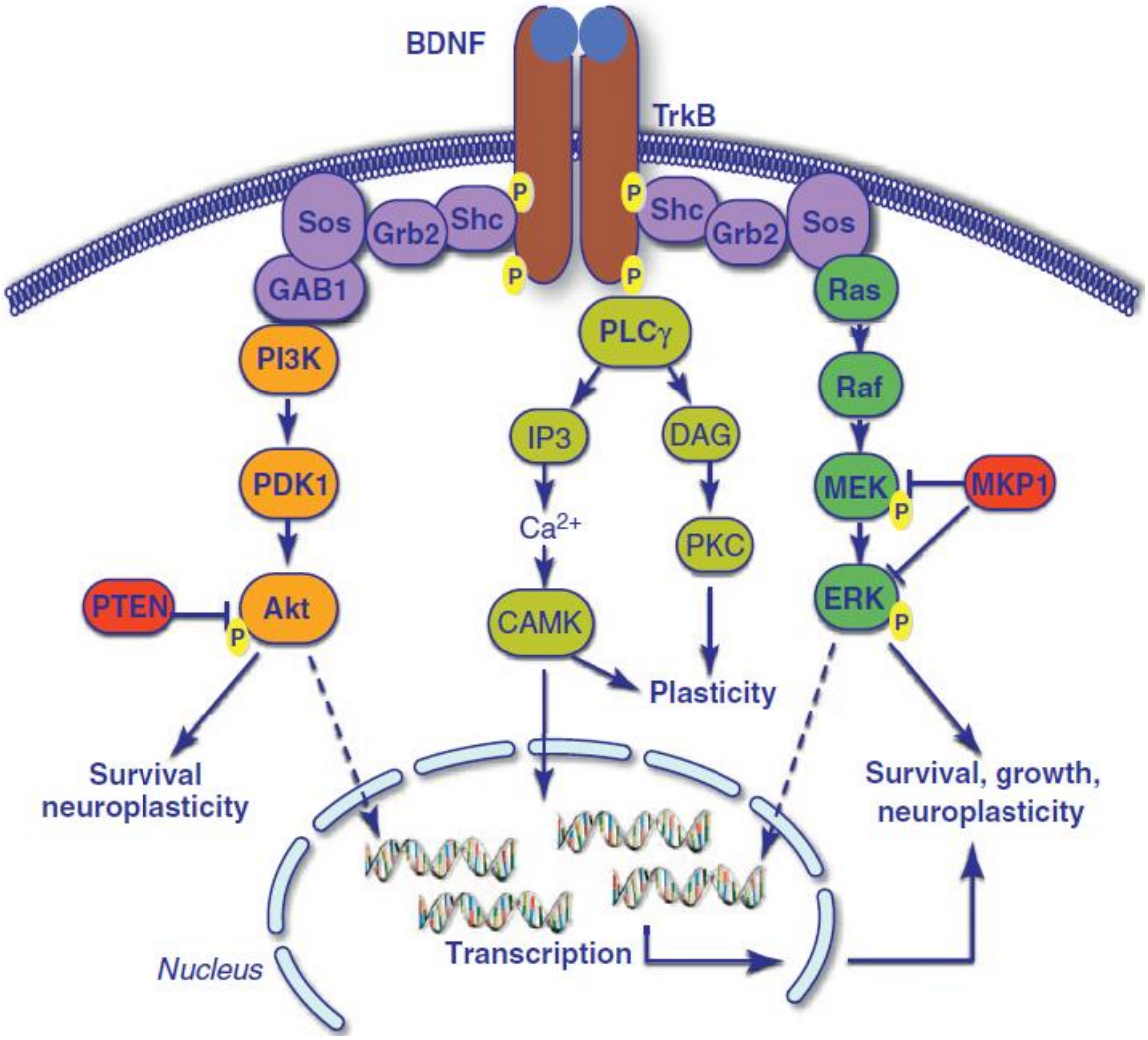

BDNF
TrkB
Sos
Grb2
Shc
GAB1
PI3K
PDK1
PTEN
Akt
P
PLCγ
IP3
DAG
Ca2+
PKC
CAMK
Plasticity
Ras
Raf
MEK
MKP1
ERK
Survival
neuroplasticity
Survival, growth,
neuroplasticity
Transcription
Nucleus

Figure 4. Brain-derived neurotrophic factor (BDNF)-Tropomyosin-related kinase B (TrkB) signaling pathways. Phosphorylation of tyrosine 515 of TrkB leads to recruitment of the Src homology 2 domain containing (Shc) adaptor protein, followed by recruitment of growth factor receptor-bound protein 2 (Grb2) and son of sevenless (SOS) and activation of the Ras–MAPK pathway (right). Shc–Grb2 can also lead to recruitment of Grb2-associated binder-1 (GAB1) and activation of the PI3K–Akt pathway (left). Phosphorylation of the TrkB tyrosine residue 816 results in recruitment of PLC gamma, which leads to the formation of inositol triphosphate (IP3) and regulation of intracellular Ca2+ and diacylglycerol (DAG),which activates CAMK and protein kinase C (PKC). Abbreviations: ERK, extracellular signal regulated kinase; MEK, MAP/ERK kinase; MKP1, MAP kinase phosphatase 1; PDK1, 3-phosphoinositide-dependent protein kinase 1; PTEN, phosphatase and tensin homolog. Image adapted from Duman and Voleti, 2012 (Duman & Voleti, 2012).

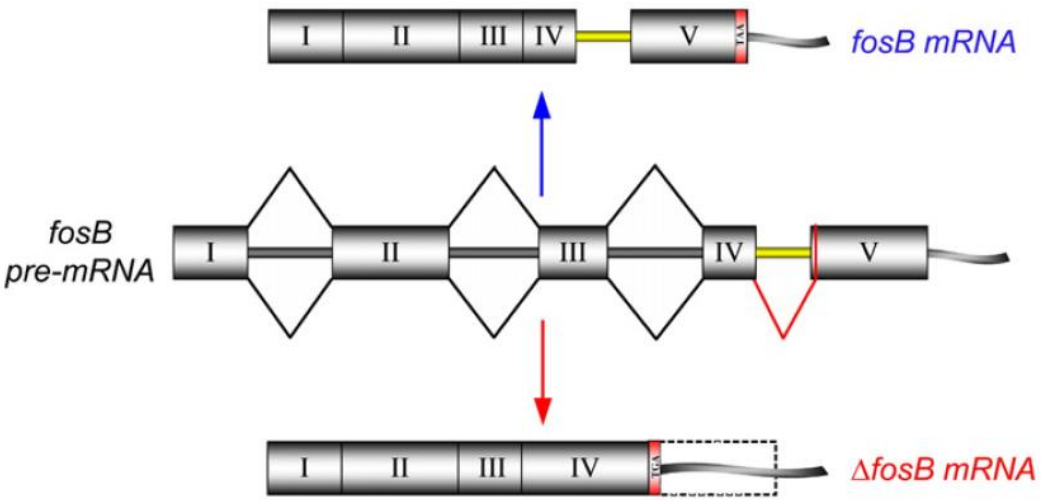

I
II
III
IV
V
fosB mRNA
fosB
pre-mRNA
I
II
III
IV
V
I
II
III
IV
ΔfosB mRNA

Figure 5. Alternative splicing of FosB RNA. DeltafosB is generated by a 140 nucleotide excision of intron in the open reading frame of full length fosB. This splice event results in a one nucleotide frameshift which creates a stop codon (TGA), therefore leads to the premature cessation of translation. Figure adapted from Alibhai et al 2007 (Alibhai et al., 2007)

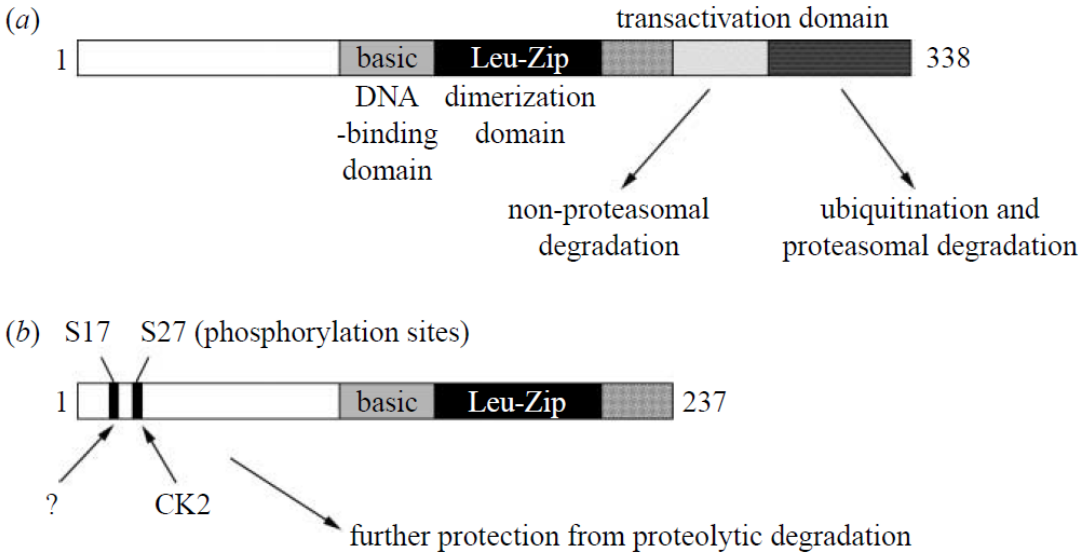

(a)
transactivation domain
1
basic
Leu-Zip
338
DNA
-binding
domain
dimerization
domain
non-proteasomal
degradation
ubiquitination and
proteasomal degradation
(b)
S17
S27 (phosphorylation sites)
1
basic
Leu-Zip
237
?
CK2
further protection from proteolytic degradation

Figure 6. Biochemical basis of DeltaFosB's unique stability. DeltaFosB lacks the C-terminal 101 amino acids sequence, which contains two degrons domains targeted by ubiquitin-mediated degradation (Carle et al, 2007). DeltaFosB can be phosphorylated at the N-terminus by casein kinase 2 or other unknown kinases, conferring extra stability (Ulery et al, 2006).Adapted from Nestler, 2008.

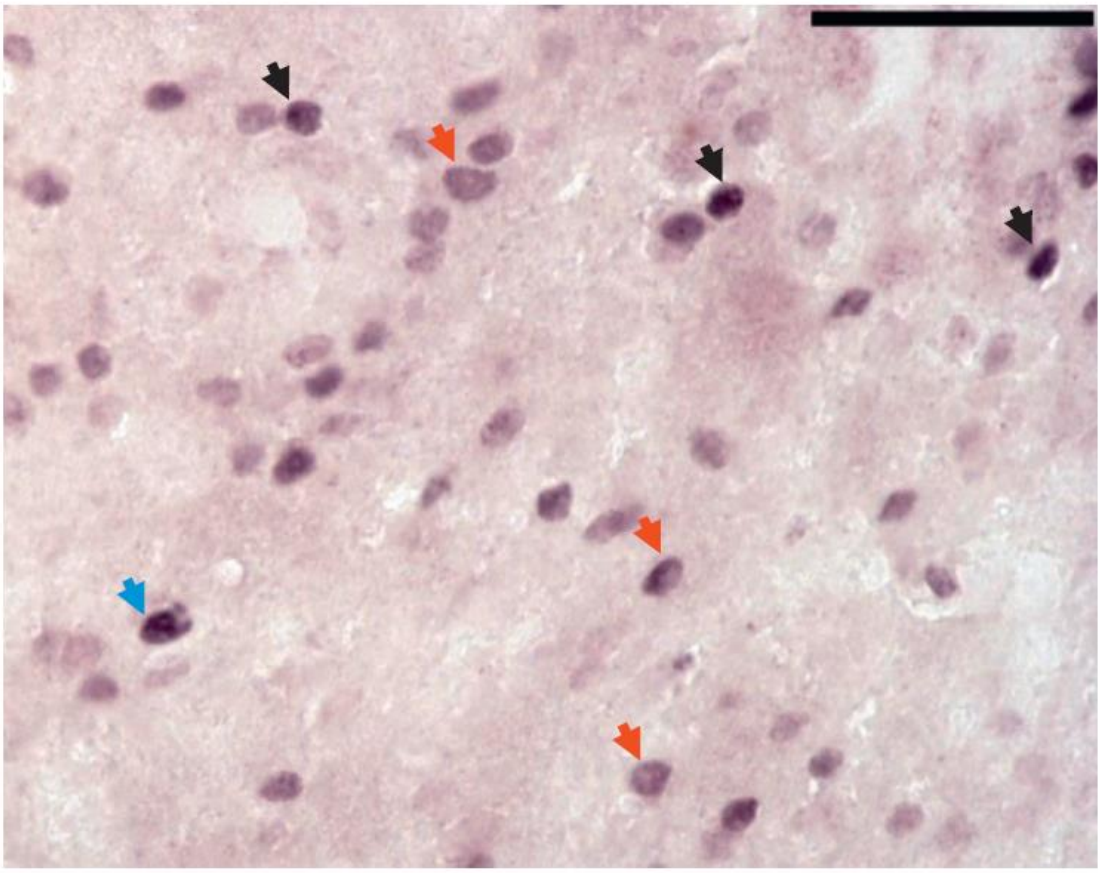
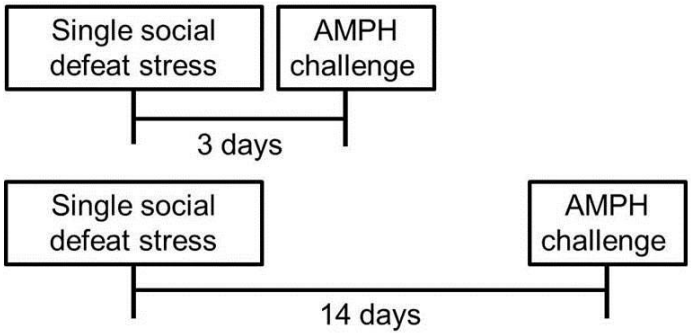
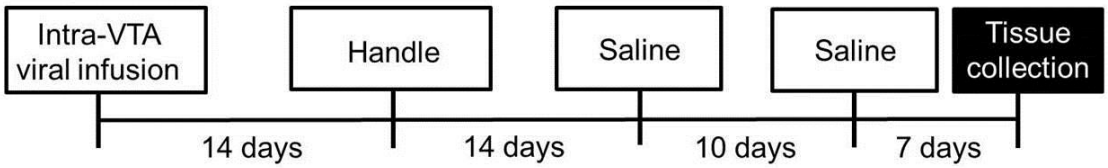


Figure 7. Representative photomicrograph of DeltaFosB and BDNF single and DeltaFosB/BDNF labeling in NAc Shell, after intermittent social defeat stress. DeltaFosB profiles are represented by a dark gray nuclear label, and BDNF cells are labeled with a diffuse, purple cytoplasmic stain with an empty nucleus; DeltaFosB/BDNF cells contain a dark nuclear DeltaFosB label surrounded by diffusely labeled purple cytoplasm. Black arrow: DeltaFosB; red arrow: BDNF; blue arrow: DeltaFosB/BDNF double-labeling. Scale bar=50 μm. Image adapted from Nikulina 2012 (Nikulina et al., 2012)

A. Experiment 1



B. Experiment 2



C. Experiment 3

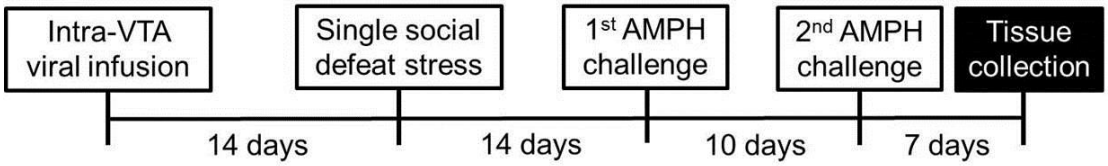


Figure 8. Schematic of experimental design. A. Experiment 1: Time-course of single social defeat stress-induced cross-sensitization to AMPH. B. Experiment 2: Effect of VTA BDNF overexpression on molecular alterations in mesocorticolimbic areas. C. Experiment 3: Effect of single social defeat stress and VTA BDNF overexpression on behavioral sensitization to AMPH and molecular alterations.

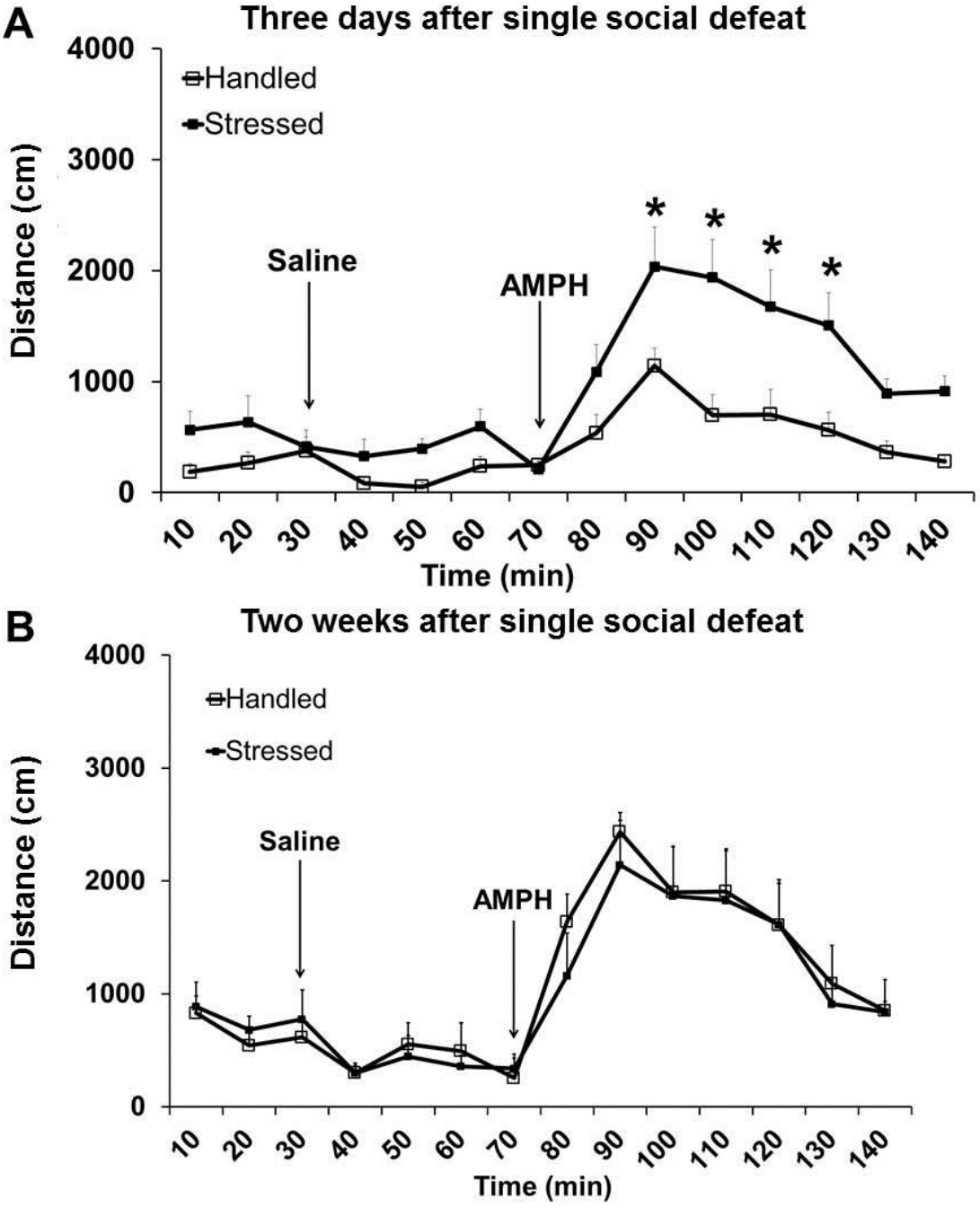

A
Three days after single social defeat
Handled
Stressed
Distance (cm)
4000
3000
2000
1000
0
Saline
AMPH
*
*
*
*
10
20
30
40
50
60
70
80
90
100
110
120
130
140
Time (min)
B
Two weeks after single social defeat
Handled
Stressed
Distance (cm)
4000
3000
2000
1000
0
Saline
AMPH
10
20
30
40
50
60
70
80
90
100
110
120
130
140
Time (min)

Figure 9. Cross-sensitization to AMPH after a single episode of social defeat stress Is observed three days, but not fourteen days after stress. Rats were exposed to a single episode of social defeat stress or handling, and then challenged with AMPH (1.0 mg/kg, i.p) 3 days or 14 days later. Distance traveled was measured before and after saline injection, and following AMPH (1.0 mg/kg, i.p.) challenge. Injection times are indicated by vertical arrows. A. Locomotor activity in response to AMPH challenge 3 days after single social defeat stress (* $p \leq 0.05$). B. Locomotor activity in response to AMPH challenge 14 days after single social defeat stress.

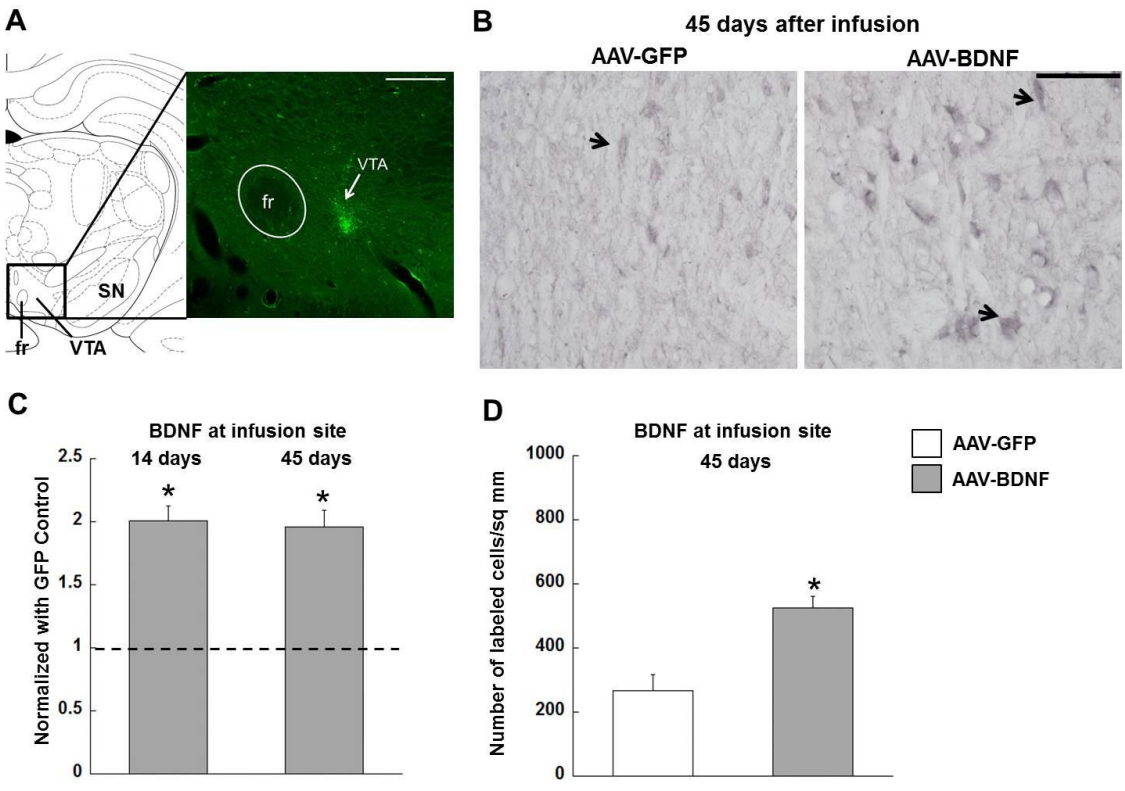

A
VTA
fr
SN
fr
VTA
B
45 days after infusion
AAV-GFP
AAV-BDNF
C
BDNF at infusion site
14 days
45 days
Normalized with GFP Control
2.5
2
1.5
1
0.5
0
*
*
D
BDNF at infusion site
45 days
AAV-GFP
AAV-BDNF
Number of labeled cells/sq mm
1000
800
600
400
200
0
*

Figure 10. AAV-BDNF infusion into the VTA produces persistent overexpression of BDNF. A. Brain atlas and representative image of AAV-GFP infusion site (arrow) in the VTA visualized by GFP fluorescence, scale bar = 200 µm; VTA: ventral tegmental area, fr: fasciculus retroflexus, SN: substantia nigra. B. Representative photomicrographs of BDNF labeling 45 days after AAV-BDNF infusion. Scale bar = 100 µm. BDNF expression near the infusion site is significantly increased after AAV-BDNF; solid arrow – BDNF- labeled neurons. C. Number of BDNF labeled cells at infusion site, normalized to AAV-GFP control, 14 and 45 days after infusion. D. Number BDNF labeled cells at infusion site 45 days after infusion, in AAV-GFP and AAV-BDNF rats. * $p \leq 0.05$ compared with control AAV-GFP-saline rats.

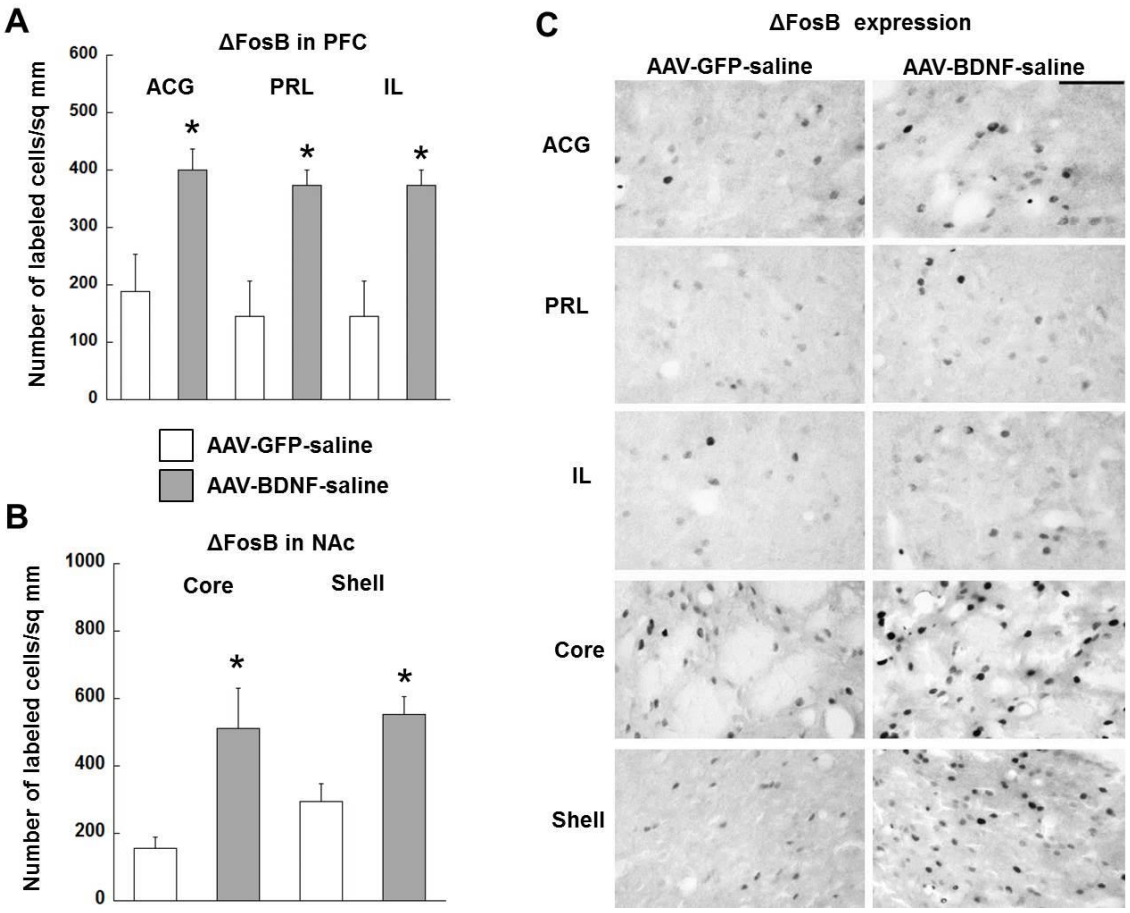

A
ΔFosB in PFC
Number of labeled cells/sq mm
600
500
400
300
200
100
0
ACG
PRL
IL
*
*
*
AAV-GFP-saline
AAV-BDNF-saline
B
ΔFosB in NAc
Number of labeled cells/sq mm
1000
800
600
400
200
0
Core
Shell
*
*
C
ΔFosB expression
AAV-GFP-saline
AAV-BDNF-saline
ACG
PRL
IL
Core
Shell

Figure 11. Persistent elevation of VTA BDNF leads to DeltaFosB accumulation in the PFC and NAc. A. DeltaFosB expression in the anterior cingulate (ACG), prelimbic (PrL), and infralimbic (IL) regions of the PFC. B. AAV-BDNF infusion significantly increased DeltaFosB expression in the NAc core and shell. * $p \leq 0.05$ compared with AAV-GFP-saline rats. C. Representative photomicrographs of DeltaFosB labeling in the PFC (ACG, PrL, IL areas) and NAc (core and shell) of AAV-GFP-saline and AAV-BDNF-saline rats; scale bar = 50 μm.

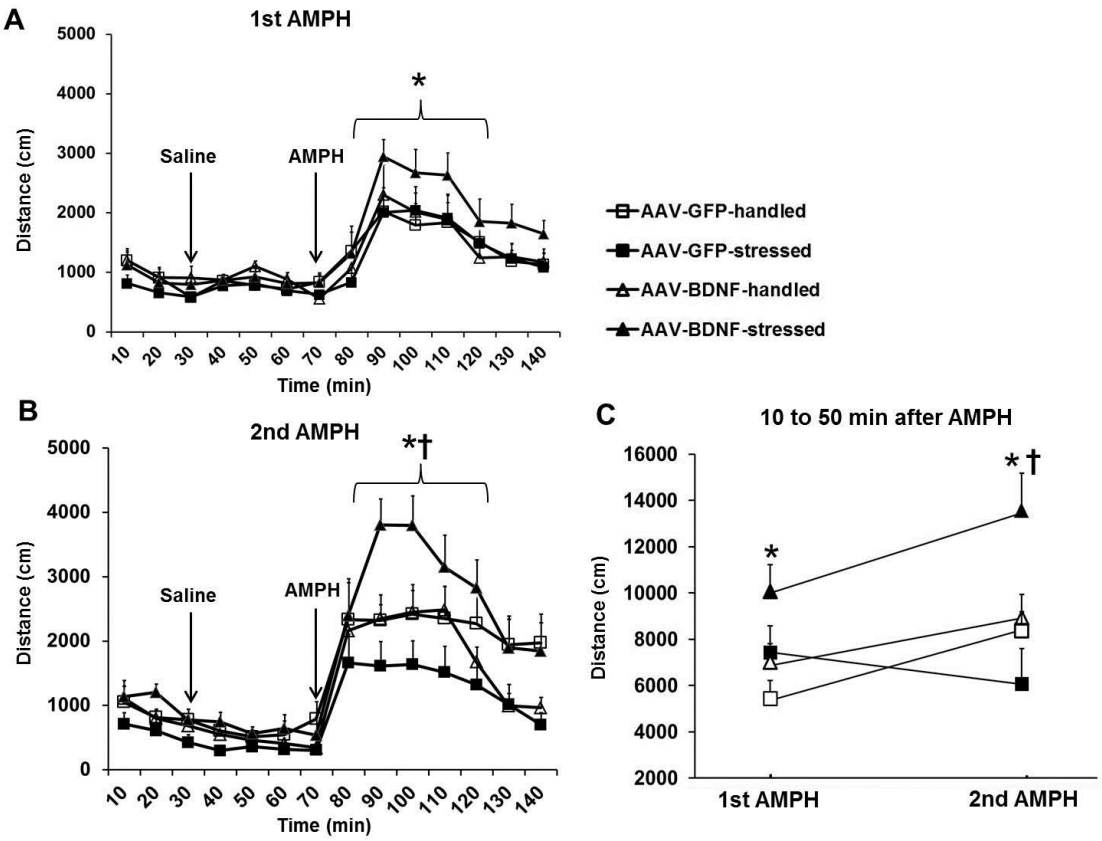

A
1st AMPH
Distance (cm)
Saline
AMPH
*
Time (min)
AAV-GFP-handled
AAV-GFP-stressed
AAV-BDNF-handled
AAV-BDNF-stressed
B
2nd AMPH
*†
C
10 to 50 min after AMPH
1st AMPH
2nd AMPH

Figure 12. AAV-mediated VTA BDNF overexpression extends the time-course of AMPH cross-Sensitization after a single social defeat stress and facilitates drug sensitization after repeated AMPH. Locomotor activity (distance in cm) over time (left) before and after saline, and following AMPH (1.0 mg/kg, i.p.) challenge (* $p \leq 0.05$ compared to all other groups, †: $p \leq 0.05$ compared to the first AMPH injection). Injection times are indicated by vertical arrows; response to AMPH (total distance traveled in cm) during the peak activity period 10 – 50 min after drug challenge. A. Locomotor activity in response to the first AMPH challenge (14 days after stress or handling). B. Locomotor activity in response to the second AMPH challenge (24 days after stress or handling). C. Interaction plot of locomotor activity during the peak activity period in response to the first and the second AMPH challenge.

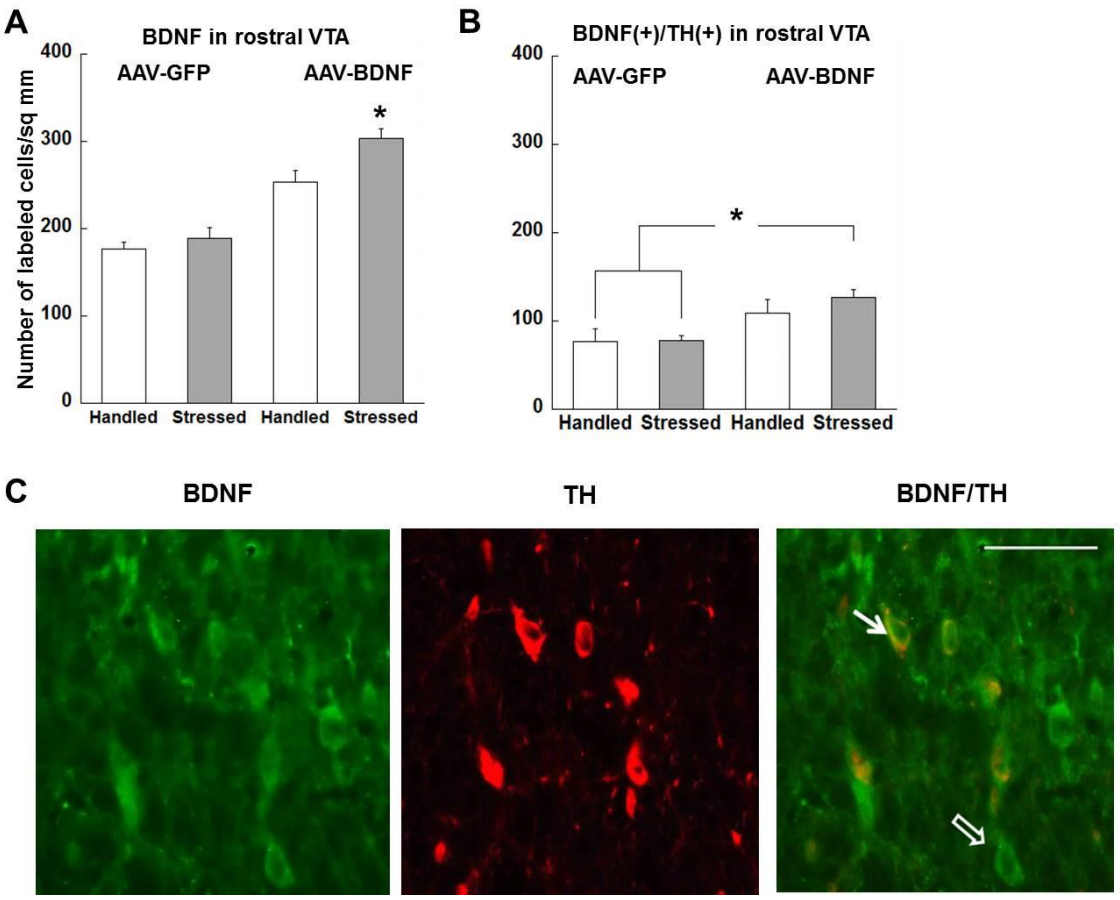

A
BDNF in rostral VTA
AAV-GFP
AAV-BDNF
Number of labeled cells/sq mm
400
300
200
100
0
*
Handled
Stressed
Handled
Stressed
B
BDNF(+)/TH(+) in rostral VTA
AAV-GFP
AAV-BDNF
400
300
200
100
0
*
Handled
Stressed
Handled
Stressed
C
BDNF
TH
BDNF/TH

Figure 13. The number of BDNF expressed neurons increases in whole rostral VTA after combined VTA BDNF overexpression, single social stress and repeated AMPH.
A. BDNF expression in whole rostral VTA, 45 days after viral infusion, with stress/handling procedure and two times AMPH challenges (*$p \leq 0.05$ compared to all other groups), labeled with fluorophore-conjugated secondary antibodies. B. BDNF/TH double labeling in VTA 45 days after viral infusion, with stress/handling procedure and two times AMPH challenges. C. Representative microphotographs of fluorescent BDNF labeling (left), TH labeling (center), and BDNF/TH double-labeling (right); scale bar = 50 µm; filled arrow: BDNF and TH double-labeled cell; open arrow: non-dopaminergic BDNF-labeled cell.

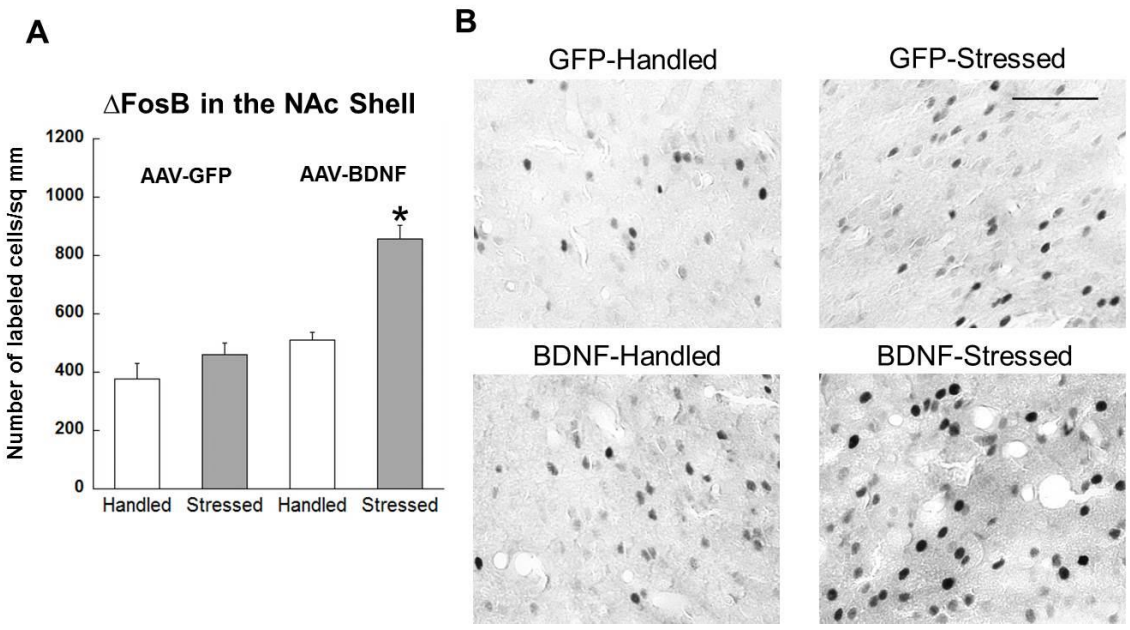

A
ΔFosB in the NAc Shell
Number of labeled cells/sq mm
1200
1000
800
600
400
200
0
AAV-GFP
AAV-BDNF
*
Handled
Stressed
Handled
Stressed
B
GFP-Handled
GFP-Stressed
BDNF-Handled
BDNF-Stressed

Figure 14. DeltaFosB expression in the NAc shell increases only in response to combined VTA BDNF overexpression, single social stress and repeated AMPH. A. Number of DeltaFosB-labeled cells in the NAc shell across groups (*$p \leq 0.05$ compared to all other groups). B. Representative photomicrographs of DeltaFosB labeling in the NAc shell of each group; scale bar = 50 μm.

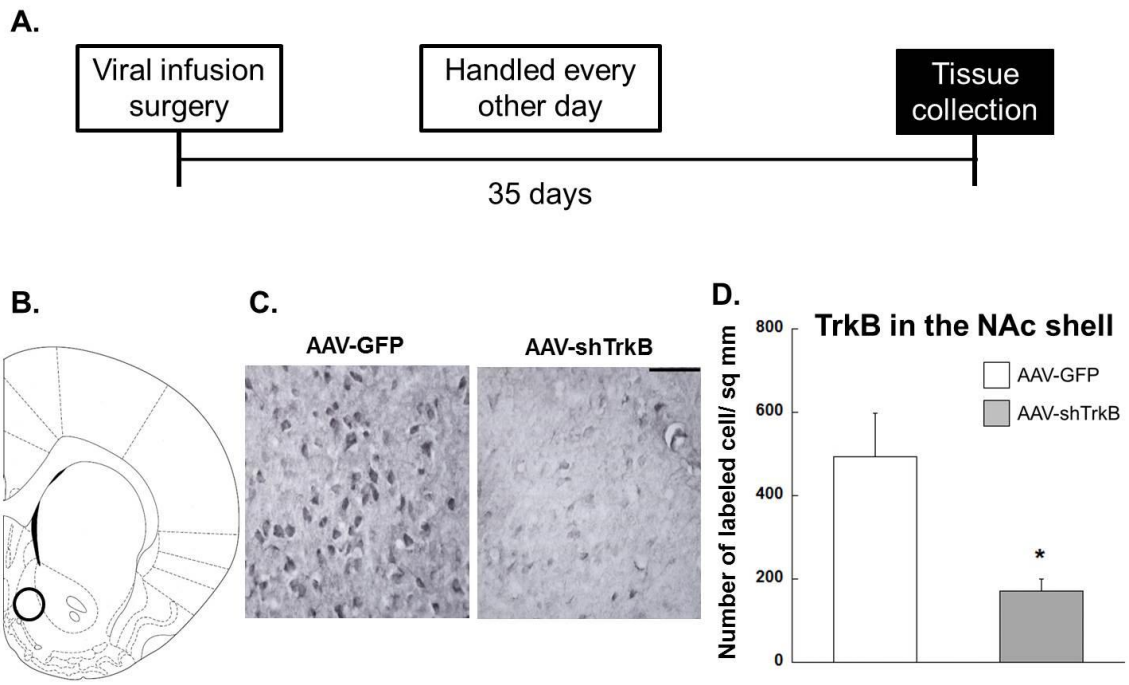

A.
Viral infusion surgery
Handled every other day
Tissue collection
35 days
B.
C.
AAV-GFP
AAV-shTrkB
D.
TrkB in the NAc shell
AAV-GFP
AAV-shTrkB
Number of labeled cell/ sq mm
800
600
400
200
0
*

Figure 15. Validation of AAV-shTrkB-mediated TrkB knockdown in the NAc Shell. A. Time-course of viral efficacy validation: 35 days after AAV-GFP/shTrkB viral infusion, tissue was collected for validation of TrkB knockdown in the NAc shell. B. Brain atlas of the NAc (left), solid circle: infusion site. C. Representative of TrkB labeling in the NAc shell of rats received AAV-GFP (left panel) and AAV-shTrkB (right panel) (scale bar = 50 μm). D. Number of TrkB-labeled cells in the NAc shell of rats received either AAV-GFP or AAV-shTrkB (* $p \leq 0.05$).

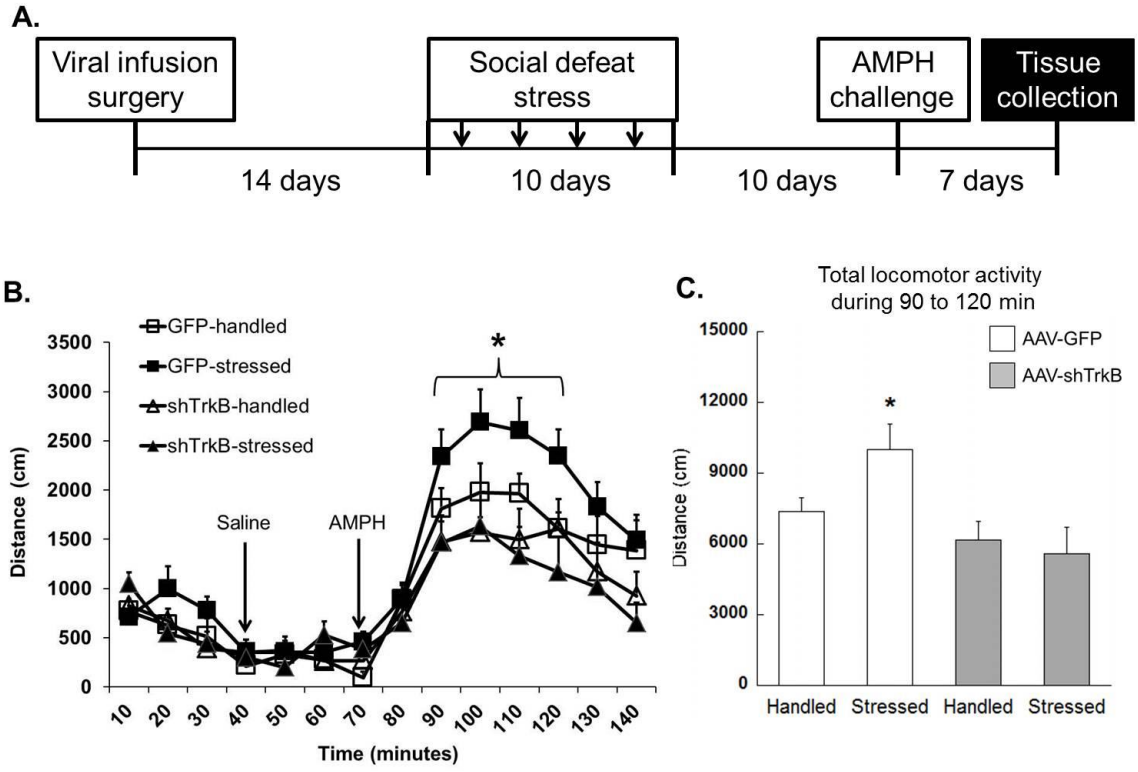

A.
Viral infusion surgery
Social defeat stress
AMPH challenge
Tissue collection
14 days
10 days
10 days
7 days
B.
GFP-handled
GFP-stressed
shTrkB-handled
shTrkB-stressed
Saline
AMPH
*
Distance (cm)
Time (minutes)
C.
Total locomotor activity during 90 to 120 min
AAV-GFP
AAV-shTrkB
Distance (cm)
Handled
Stressed
Handled
Stressed

Figure 16. TrkB knockdown prevented repeated social defeat stress-induced cross-sensitization to AMPH. A. Time-course of AAV-GFP/shTrkB viral infusion and repeated social defeat stress-induced cross-sensitization to AMPH. B. Locomotor activity (distance in cm) over time before and after saline, and following AMPH challenge (n = 7 per group). Injections indicated by vertical arrows; C. Locomotor response to AMPH (total distance traveled in cm) from 10 to 50 min after drug challenge (* $p \leq 0.05$ compared to all other groups).

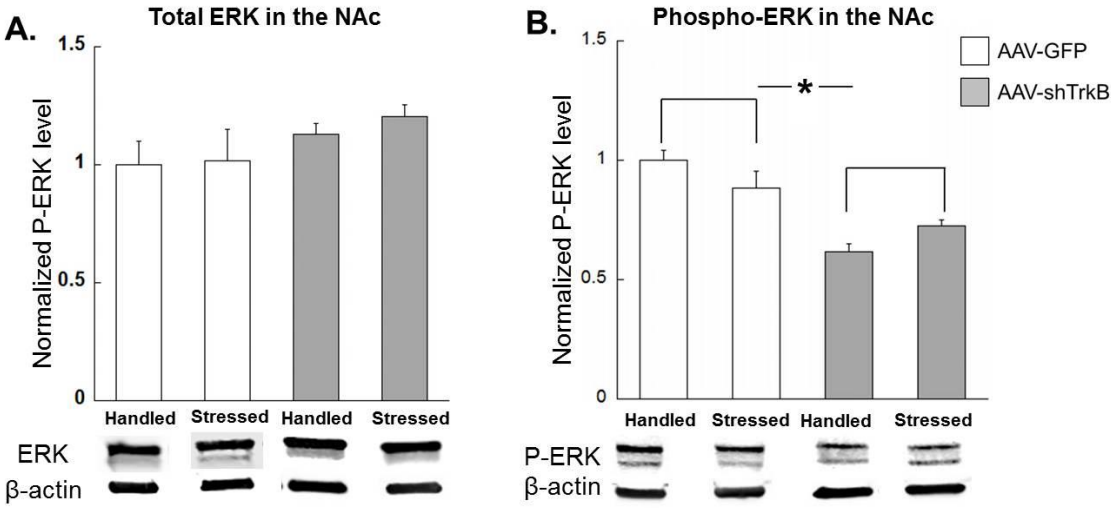

A.
Total ERK in the NAc
Normalized P-ERK level
1.5
1
0.5
0
Handled
Stressed
Handled
Stressed
ERK
β-actin
B.
Phospho-ERK in the NAc
AAV-GFP
AAV-shTrkB
*
Normalized P-ERK level
1.5
1
0.5
0
Handled
Stressed
Handled
Stressed
P-ERK
β-actin

Figure 17. Total ERK1/2 and phospho-ERK1/2 levels in the NAc. A. No difference in total ERK1/2 expression levels in the NAc was found across all groups. B. TrkB knockdown in the NAc shell caused significant reduction of levels of phospho-ERK1/2 in the NAc (n = 7 per group, * $p \leq 0.05$ compared to all other groups). Representative bands of blots were shown below the graph.

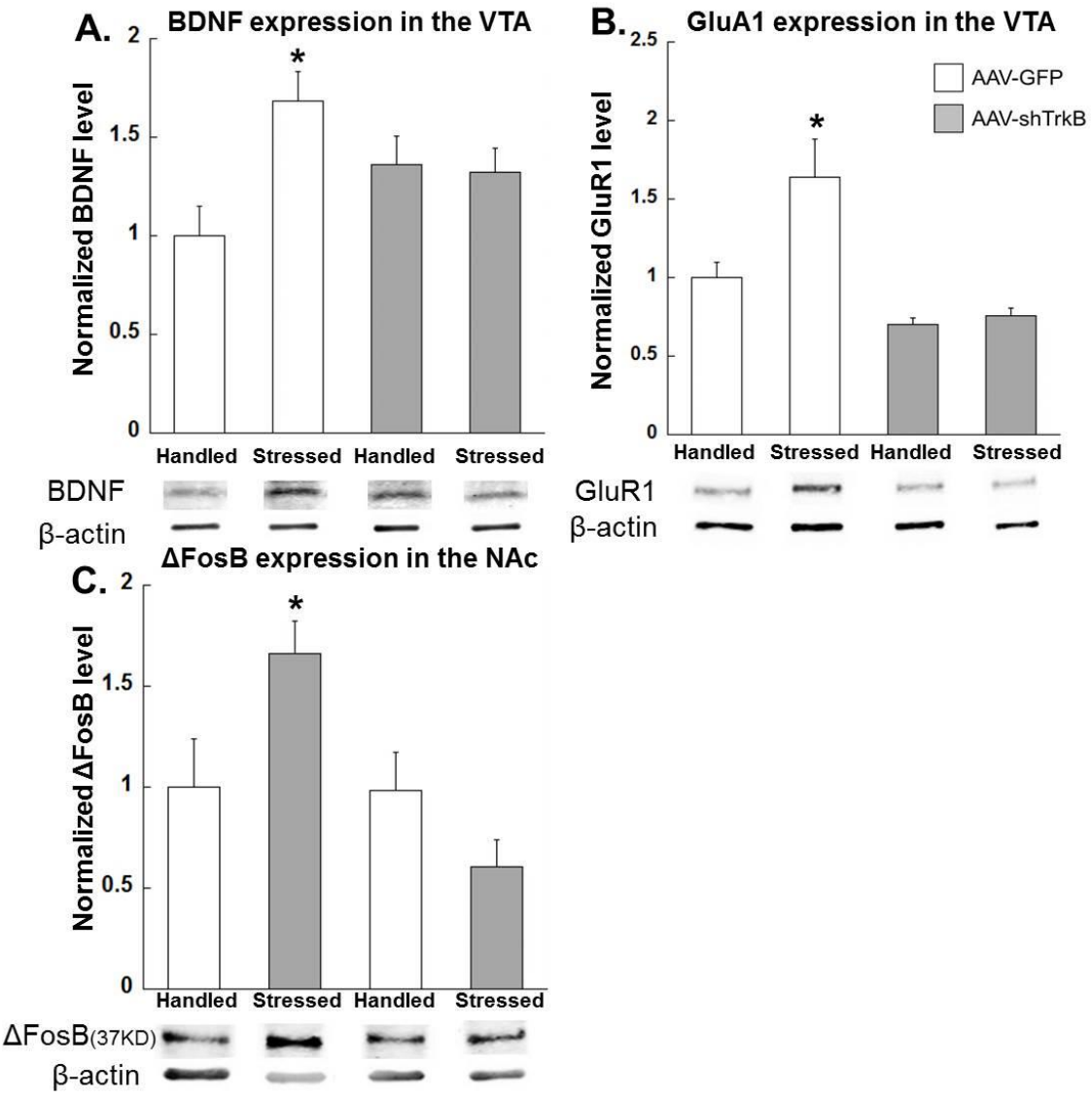
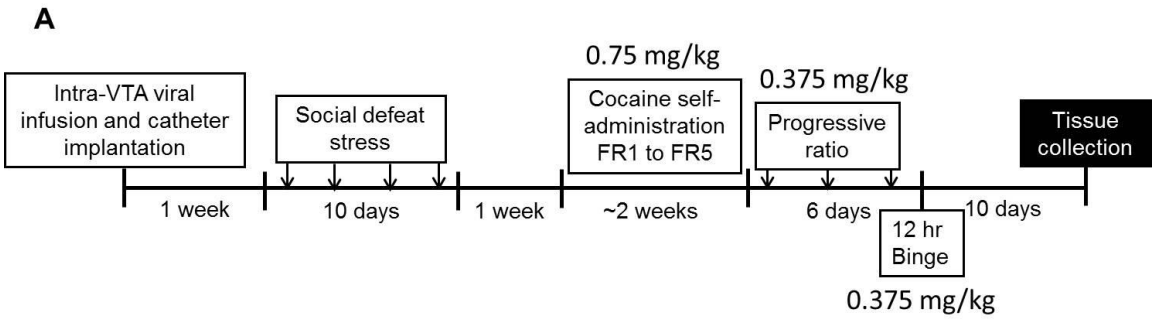

A.
BDNF expression in the VTA
Normalized BDNF level
2
1.5
1
0.5
0
*
Handled
Stressed
Handled
Stressed
BDNF
β-actin
B.
GluA1 expression in the VTA
Normalized GluR1 level
2.5
2
1.5
1
0.5
0
*
AAV-GFP
AAV-shTrkB
Handled
Stressed
Handled
Stressed
GluR1
β-actin
C.
ΔFosB expression in the NAc
Normalized ΔFosB level
2
1.5
1
0.5
0
*
Handled
Stressed
Handled
Stressed
ΔFosB(37KD)
β-actin

Figure 18. TrkB knockdown in the NAc shell prevented intermittent social defeat stress-induced neurochemical changes in the mesolimbic dopamine circuit. Social defeat stress-induced increase of the BDNF (A) and GluR1 expression in the VTA (B), and the DeltaFosB expression in the NAc (C) were prevented by TrkB knockdown in the NAc shell (n = 7 per group, * $p \leq 0.05$ compared to all other groups). Representative bands of blots were shown below the graph.


A
0.75 mg/kg
0.375 mg/kg
Intra-VTA viral infusion and catheter implantation
Social defeat stress
Cocaine self-administration FR1 to FR5
Progressive ratio
Tissue collection
1 week
10 days
1 week
~2 weeks
6 days
12 hr Binge
10 days
0.375 mg/kg

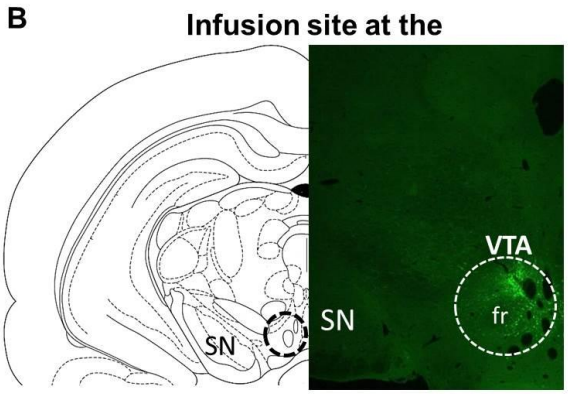

B
Infusion site at the
VTA
fr
SN
SN

Figure 19. Schematic of experimental design and the viral infusion site. A. Time-course of social defeat stress and cocaine self-administration. B. Brain atlas of the VTA, and representative image of AAV-GFP infusion site (arrow) in the VTA visualized by GFP fluorescence; VTA: ventral tegmental area, fr: fasciculus retroflexus, SN: substantia nigra.

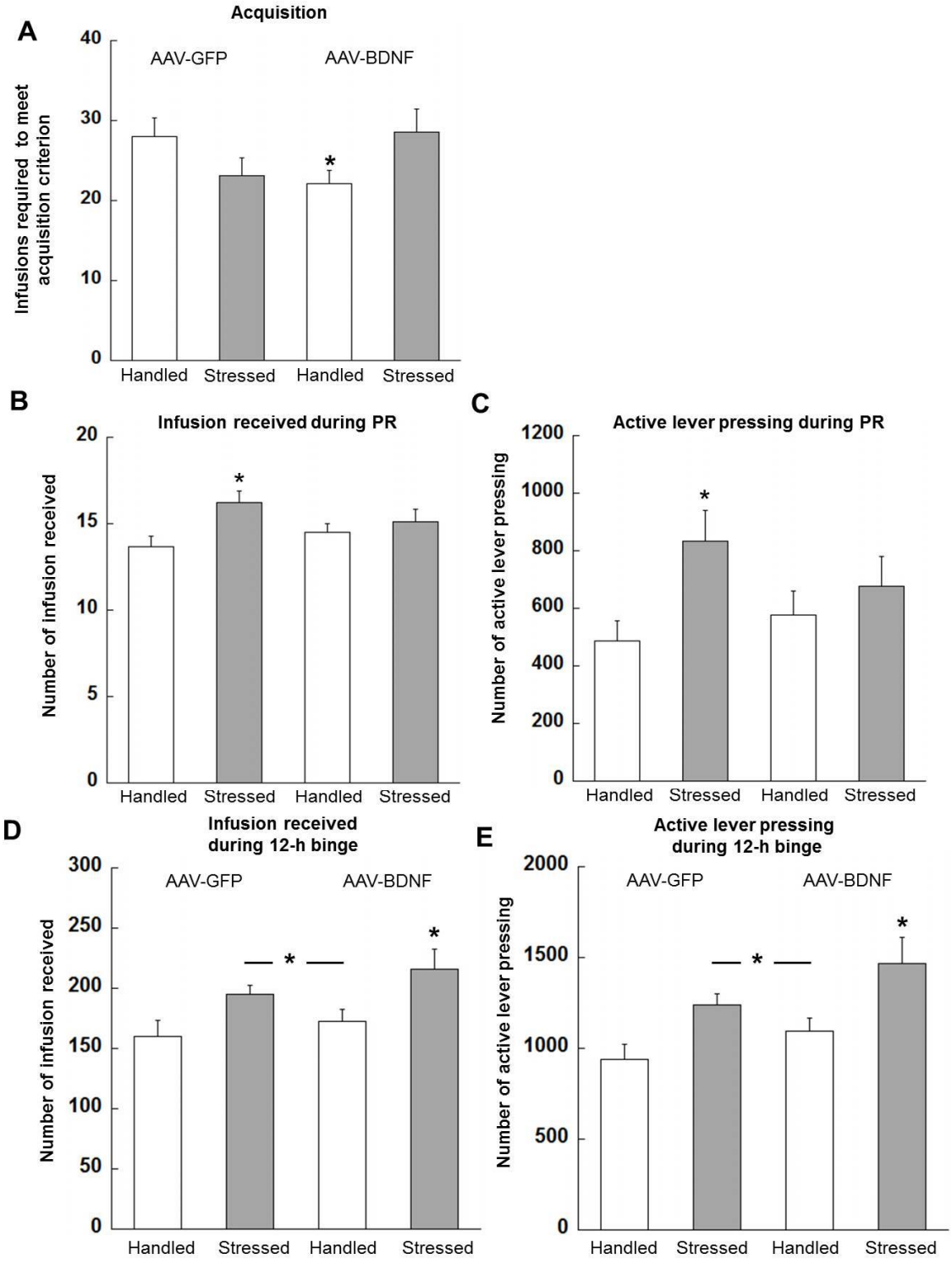

A
Acquisition
AAV-GFP
AAV-BDNF
Infusions required to meet acquisition criterion
40
30
20
10
0
*
Handled
Stressed
Handled
Stressed
B
Infusion received during PR
Number of infusion received
20
15
10
5
0
*
Handled
Stressed
Handled
Stressed
C
Active lever pressing during PR
Number of active lever pressing
1200
1000
800
600
400
200
0
*
Handled
Stressed
Handled
Stressed
D
Infusion received during 12-h binge
AAV-GFP
AAV-BDNF
Number of infusion received
300
250
200
150
100
50
0
*
*
Handled
Stressed
Handled
Stressed
E
Active lever pressing during 12-h binge
AAV-GFP
AAV-BDNF
Number of active lever pressing
2000
1500
1000
500
0
*
*
Handled
Stressed
Handled
Stressed

Figure 20. Behavioral results during acquisition, progressive ratio and twelve-hour binge sessions. A. The number of cocaine reinforcements (0.75 mg/kg/ infusion, i.v.) taken to meet our criterion of stabilized self-administration under FR1 ratio. B. The average break point for cocaine reinforcements (0.375 mg/kg/ infusion, i.v.) and C) number of active lever pressing during the progressive ratio schedule. The y-axis indicates the average infusion received (B) and number of active lever pressing (C) across three PR sessions for all groups. D. Total number of cocaine infusions (0.375 mg/kg per infusion, i.v.) and E) total number of active lever pressing during 12-h binge. Error bars indicate ±SEM. * $p \leq 0.05$ compared to AAV-GFP-handled groups; ** $p \leq 0.05$ compared to all other groups.

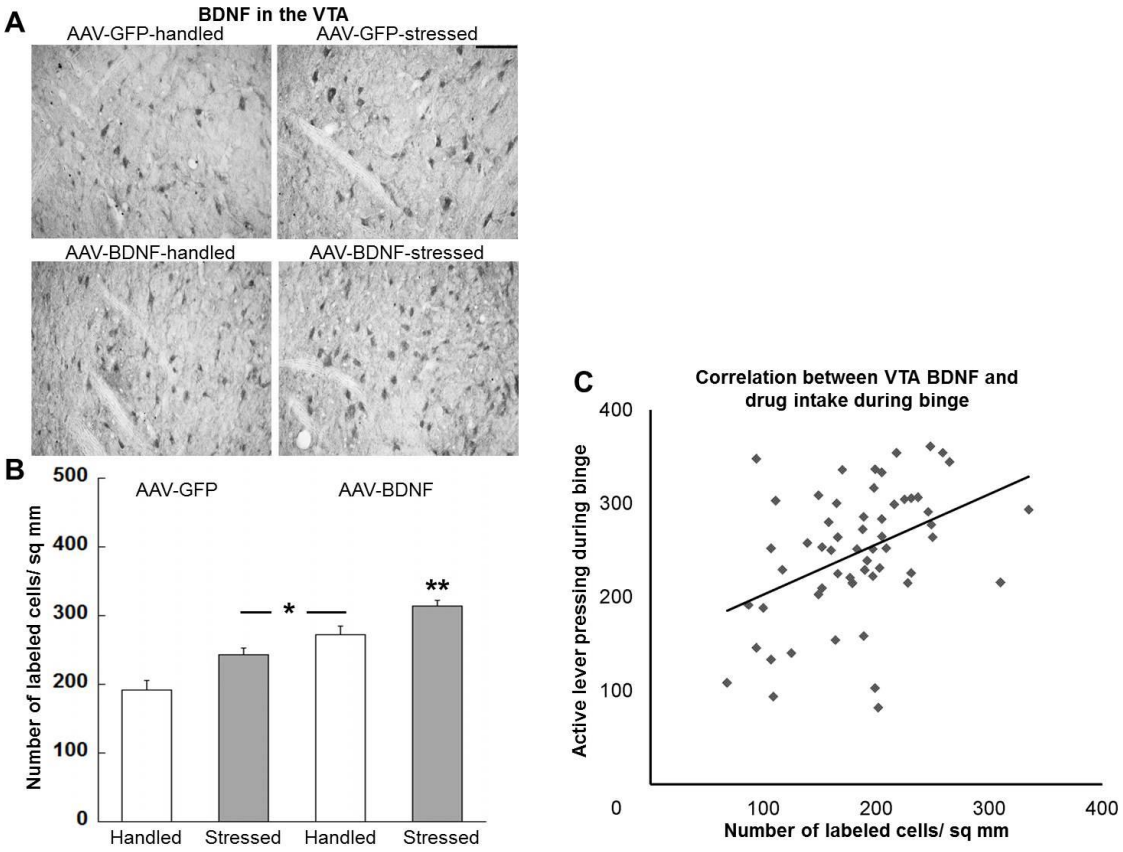
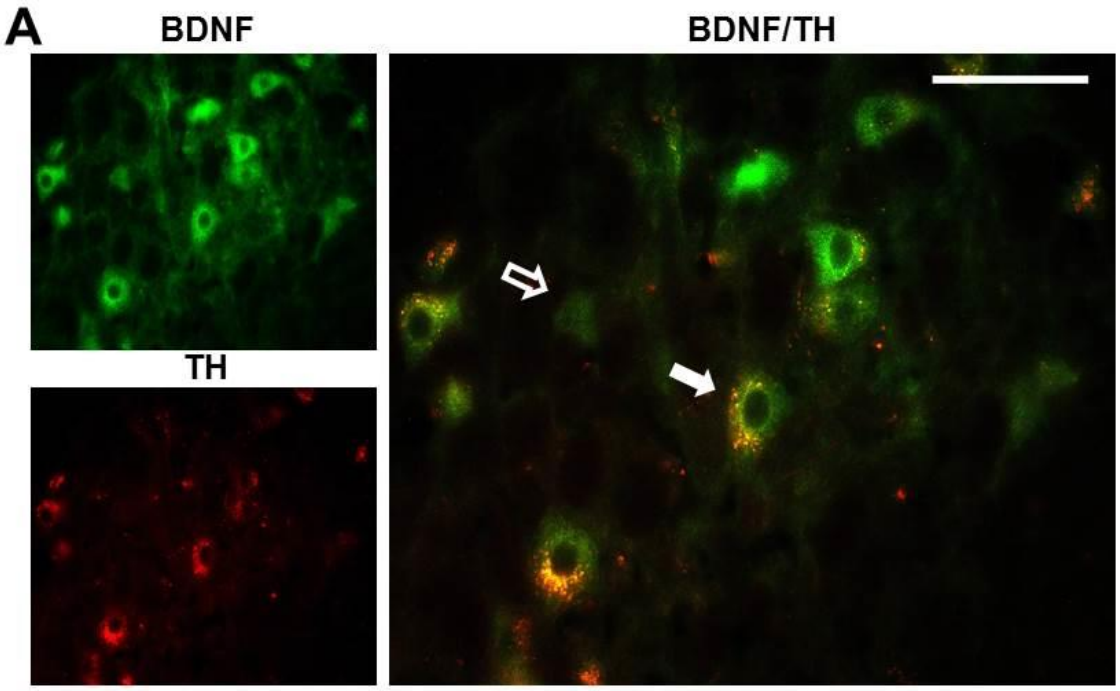

A
BDNF in the VTA
AAV-GFP-handled
AAV-GFP-stressed
AAV-BDNF-handled
AAV-BDNF-stressed
B
AAV-GFP
AAV-BDNF
Number of labeled cells/ sq mm
500
400
300
200
100
0
*
**
Handled
Stressed
Handled
Stressed
C
Correlation between VTA BDNF and drug intake during binge
Active lever pressing during binge
400
300
200
100
0
100
200
300
400
Number of labeled cells/ sq mm

Figure 21. BDNF expression in the VTA and correlation with drug taking behavior during twelve-hour binge sessions. A. Representative photomicrographs of BDNF labeling in the VTA of each group; scale bar = 100 μm. B. Number of BDNF-labeled cells in the VTA across groups. Error bars indicate ±SEM. * $p < 0.05$ compared to AAV-GFP-handled groups; ** $p < 0.05$ compared to all other groups. C. Scatter plot for the correlation between VTA BDNF expression and the number of active lever pressing during 12-h binge. The y-axis indicates the total number of active lever pressing during 12-h binge session for all rats, and the x-axis indicates the corresponding number of BDNF-labeled cells in the VTA.


A
BDNF
BDNF/TH
TH

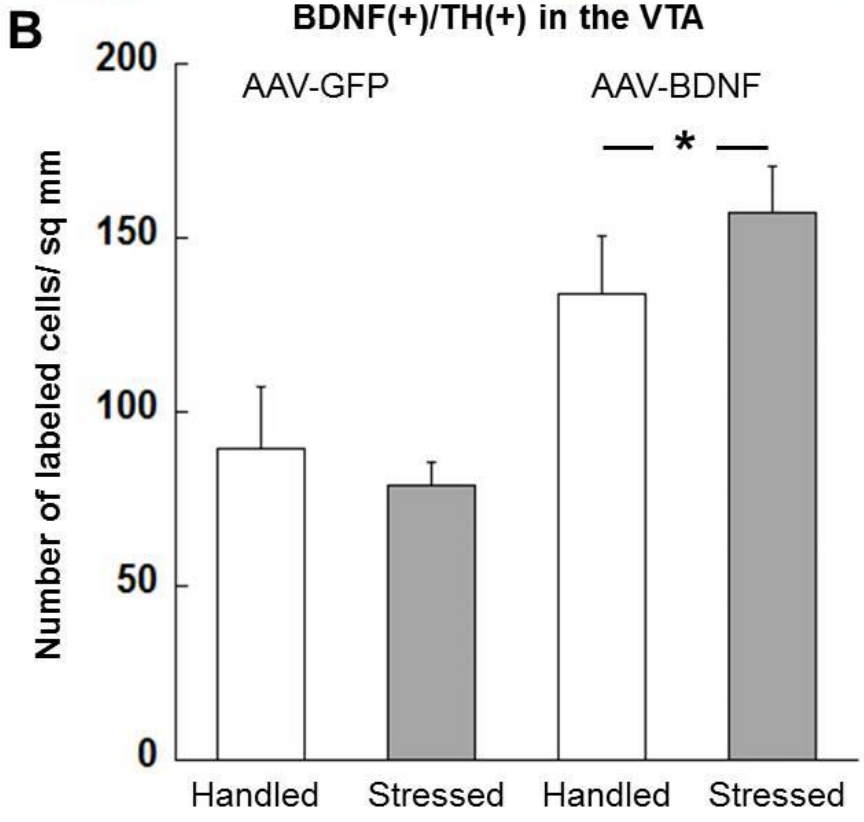

B
BDNF(+)/TH(+) in the VTA
AAV-GFP
AAV-BDNF
*
Number of labeled cells/ sq mm
200
150
100
50
0
Handled
Stressed
Handled
Stressed

Figure 22. The number of BDNF expressing dopaminergic neurons increases in the VTA after cocaine self-administration in rats with VTA BDNF overexpression. A. Representative microphotographs of fluorescent BDNF labeling (left upper), TH labeling (left bottom), and BDNF/TH double-labeling (right); scale bar = 50 μm; filled arrow: BDNF and TH double-labeled cell; open arrow: non-dopaminergic BDNF-labeled cell. B. BDNF/TH double labeling in VTA after chronic cocaine self-administration (* $p \leq 0.05$ compared to all groups received AAV-GFP control virus), labeled with fluorophore-conjugated secondary antibodies.

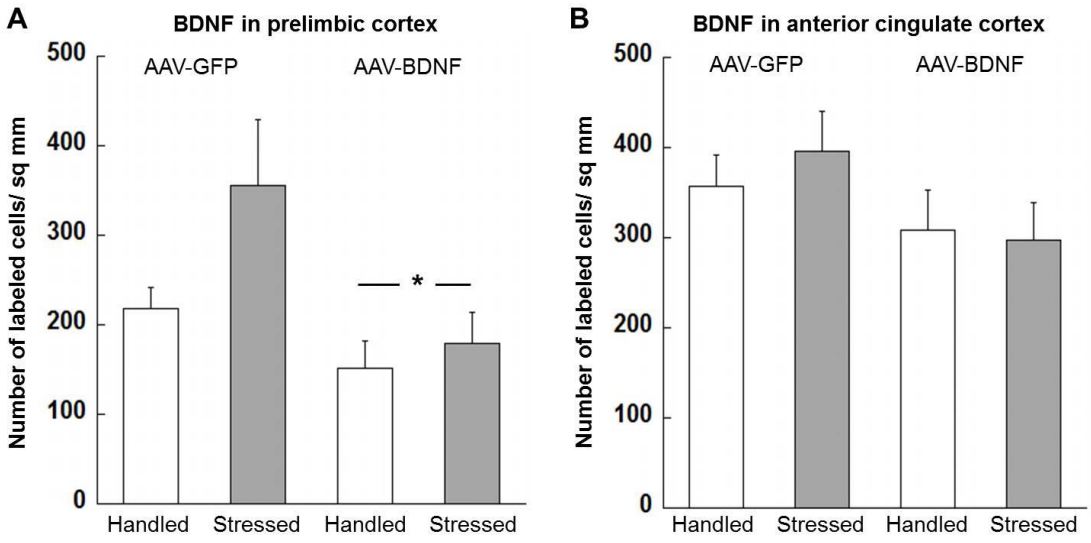

A
BDNF in prelimbic cortex
AAV-GFP
AAV-BDNF
Number of labeled cells/ sq mm
500
400
300
200
100
0
*
Handled
Stressed
Handled
Stressed
B
BDNF in anterior cingulate cortex
AAV-GFP
AAV-BDNF
Number of labeled cells/ sq mm
500
400
300
200
100
0
Handled
Stressed
Handled
Stressed

Figure 23. VTA BDNF overexpression reduces the number of BDNF expressing neurons in the prelimbic Cortex, but not in anterior cingulate cortex. A. BDNF expression in PrL cortex; B. BDNF expression in ACG cortex (*$p \leq 0.05$ compared to groups received control AAV-GFP virus).

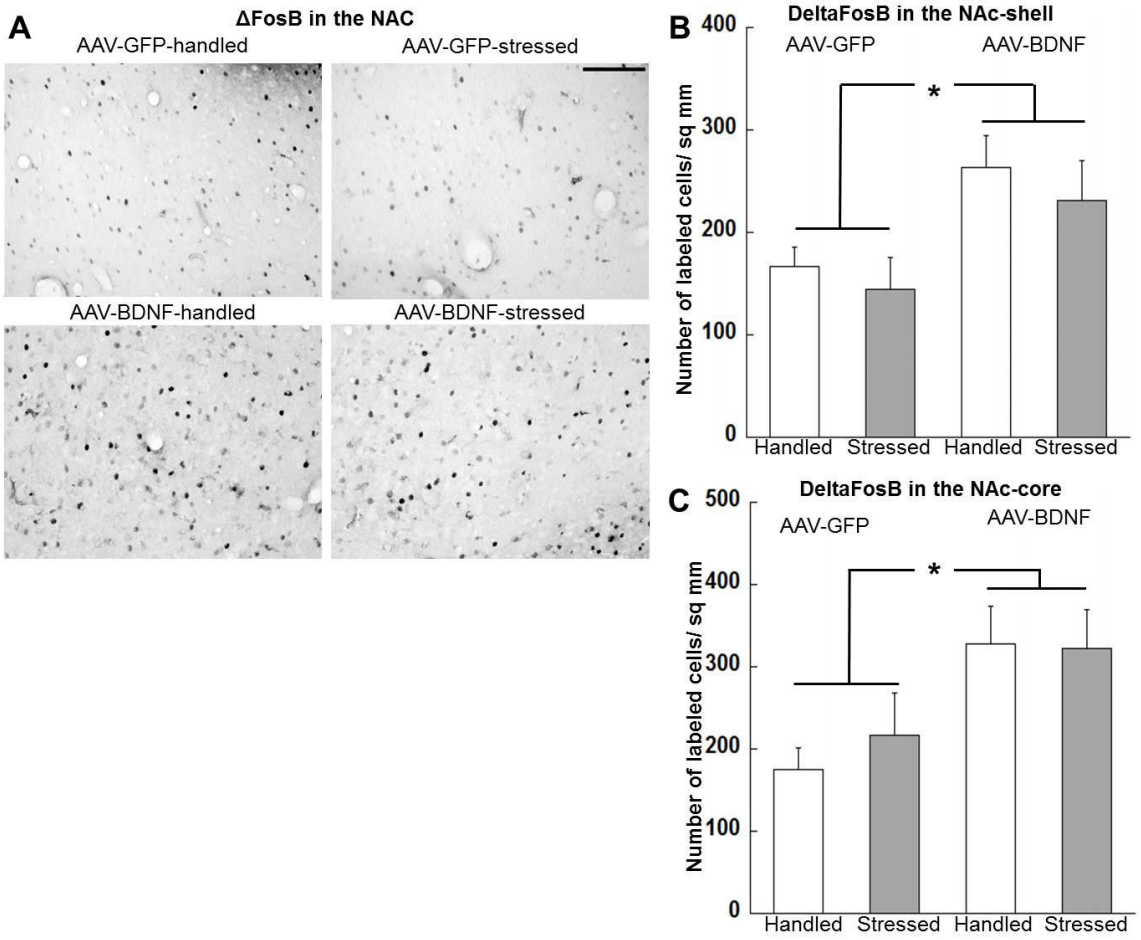

A
ΔFosB in the NAC
AAV-GFP-handled
AAV-GFP-stressed
AAV-BDNF-handled
AAV-BDNF-stressed
B
DeltaFosB in the NAc-shell
AAV-GFP
AAV-BDNF
Number of labeled cells/ sq mm
400
300
200
100
0
*
Handled
Stressed
Handled
Stressed
C
DeltaFosB in the NAc-core
AAV-GFP
AAV-BDNF
Number of labeled cells/ sq mm
500
400
300
200
100
0
*
Handled
Stressed
Handled
Stressed

Figure 24. DeltaFosB expression in the NAc increases in response to VTA BDNF overexpression after chronic cocaine self-administration. A. Representative photomicrographs of DeltaFosB labeling in the NAc of each group; scale bar = 100 µm. B. Number of DeltaFosB-labeled cells in the NAc shell and C) core across groups (*$p \leq 0.05$ compared to groups received control AAV-GFP virus).

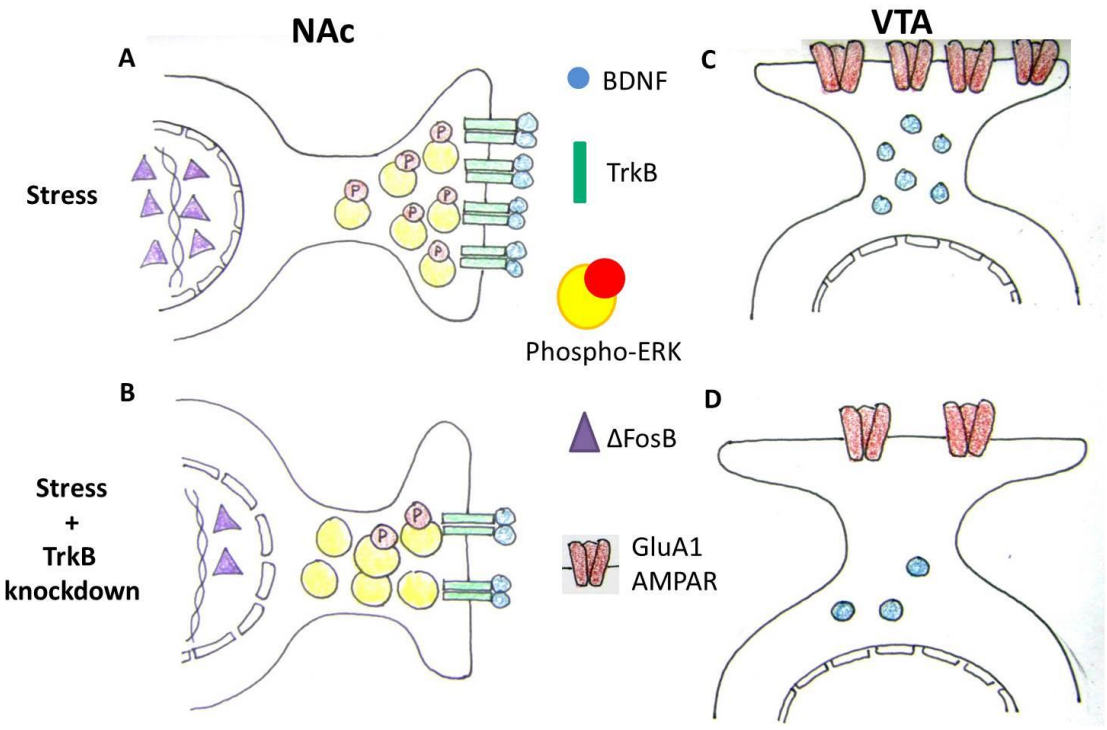

NAc
VTA
A
C
Stress
BDNF
TrkB
Phospho-ERK
B
D
Stress
+
TrkB
knockdown
ΔFosB
GluA1
AMPAR

Figure 25. Schematic of social defeat-induced neurochemical changes altered by intra-NAc TrkB knockdown. A. In the NAc, after intermittent social defeat stress, increased DeltaFosB was observed. B. TrkB in the NAc prevented stress induced increase of DeltaFosB in this area, and reduced the level phospho-ERK, but not total ERK expression level in the NAc. C. Intermittent social defeat stress increased the expression of BDNF and GluA1 in the VTA. D. TrkB knock-down in the NAc prevented stress induced elevation of BDNF and GluA1 in the VTA. Hand drawing by J.Wang.