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Role of the Nucleus Accumbens and Mesolimbic Dopamine System in Modulating the Memory of Social Defeat in Male Syrian Hamsters (Mesocricetus auratus)

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ROLE OF THE NUCLEUS ACCUMBENS AND MESOLIMBIC DOPAMINE SYSTEM IN
MODULATING THE MEMORY OF SOCIAL DEFEAT IN MALE SYRIAN HAMSTERS

(Mesocricetus auratus)

by

CLOE LUCKETT GRAY

Under the Direction of Dr. Kim Huhman

ABSTRACT
Psychological stressors such as social stress and bullying are prevalent in today’s society. Disorders such as PTSD, depression and social anxiety disorder can be either caused or exacerbated by social stress and treatment options are not always effective in providing relief for these disorders. Our laboratory studies a form of social stress termed conditioned defeat, whereby a defeated Syrian hamster no longer displays species-typical territorial aggression but instead is submissive and defensive toward an intruder in its own cage. We hypothesized that the nucleus accumbens is a necessary component of the circuit mediating the acquisition and expression of conditioned defeat and that dopamine is necessary within the nucleus accumbens for inducing memory processes as well as expression of behavioral responses to stressful
situations. We also hypothesized that defeat activates dopaminergic and/or nondopaminergic neurons in the ventral tegmental area (VTA) and that dopamine released by neurons projecting from the VTA to the nucleus accumbens and basolateral amygdala (BLA) increases neuronal activation of these structures during defeat. We found that dopamine, but not GABA, modulates memory of social defeat within the nucleus accumbens. However, GABA does affect the expression of behavioral responses to social defeat. Defeat also increased Fos activation of non-dopaminergic neurons, but it did not increase activation of dopaminergic neurons. Baclofen infusion into the VTA prior to defeat, which was hypothesized to specifically inhibit dopaminergic neurons, did not affect Fos activation within the nucleus accumbens and the basolateral amygdala. These experiments determined that dopamine does modulate memory of social defeat within the nucleus accumbens, but it is currently unclear what the source of this dopamine is. Future experiments are planned to determine this source of dopamine that could be a target of treatment for disorders that are caused or exacerbated by social stress.

INDEX WORDS: Nucleus accumbens, conditioned defeat, mesolimbic dopamine system
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CLOE LUCKETT GRAY

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctorate of Philosophy in the College of Arts and Sciences

Georgia State University

2014
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>BLA</td>
<td>Basolateral amygdala</td>
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<tr>
<td>CD</td>
<td>Conditioned defeat</td>
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<tr>
<td>CRF</td>
<td>Corticotropin-releasing factor</td>
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<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
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<tr>
<td>FSCV</td>
<td>Fast-scan cyclic voltammetry</td>
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<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal</td>
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<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
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<tr>
<td>IL</td>
<td>Infralimbic</td>
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<td>LHa</td>
<td>Lateral hypothalamus</td>
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<td>LTP</td>
<td>Long-term potentiation</td>
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<td>NAcc</td>
<td>Nucleus accumbens</td>
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<tr>
<td>NAI</td>
<td>Non-aggressive intruder</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>PAG</td>
<td>Periaqueductal gray</td>
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<tr>
<td>PTSD</td>
<td>Posttraumatic stress disorder</td>
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<tr>
<td>RA</td>
<td>Resident aggressor</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
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<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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CHAPTER 1

GENERAL INTRODUCTION

Overview

Disorders such as post-traumatic stress disorder (PTSD), social anxiety disorder, and generalized anxiety disorder are prevalent in today’s society and, in many cases, may stem from traumatic bullying and other social stress situations (Bjorkqvist 2001). A better understanding of the neurobiological and neuroanatomical mechanisms underlying behavioral responses to social stress may lead to novel treatment options for those who fail to respond to currently available treatments.

Animal models of social stress are utilized to study potential treatments and to determine the putative neural circuitry that controls the memory of and behavioral responses to social stress and defeat. Conditioned defeat, a phenomenon demonstrated by Syrian hamsters (Potegal, Huhman et al. 1993), is a robust model of social defeat. Syrian hamsters are very aggressive in nature, and they defend their home territory against intruders (Murphy 1976). In contrast, after a hamster has been defeated in the home cage of a larger, more aggressive resident, the defeated hamster subsequently becomes submissive toward an intruder in its own home cage, even when that opponent is smaller and non-threatening. This striking change is termed conditioned defeat.

Previous studies have delineated brain areas that are critical for the acquisition (i.e., learning of behavioral responses during the initial defeat experience) and/or the expression of conditioned defeat (i.e., recall of the defeat experience or production of submissive and defensive behavior during subsequent testing). The basolateral amygdala is the primary brain region that
appears to be responsible for the synaptic plasticity necessary for conditioned defeat (Markham, Taylor et al. 2010), but other areas modulate either the memory of defeat (Markham, Taylor et al. 2010, Markham, Luckett et al. 2012) or the expression (McDonald, Markham et al. 2012) of the submissive and defensive behavior after social defeat. The nucleus accumbens is a brain region that so far has gone unexamined in terms of its role in modulating the acquisition and expression of behavioral responses to acute social defeat. Its implication in the memory processes associated with fear (Schwienbacher, Fendt et al. 2004) and chronic social defeat (Berton, McClung et al. 2006), as well as its hypothesized role as the gateway between the limbic and motor systems (Mogenson, Jones et al. 1980), suggests that it could also be a critical part of the brain circuit modulating conditioned defeat.

The basolateral amygdala and the nucleus accumbens are innervated by the mesolimbic dopamine system (Oades and Halliday 1987), a neurotransmitter system that is activated by both aversive (Deutch, Tam et al. 1985) and rewarding stimuli (Gil, Nguyen et al. 2013). Dopamine is necessary for the expression of social avoidance after chronic defeat (Chaudhury, Walsh et al. 2013), and it promotes memory processes and long-term potentiation in both the nucleus accumbens and the amygdala. Given that disorders that arise from social stress, such as PTSD (Drury, Theall et al. 2009) and depression (Camardese, Di Giuda et al. 2014) evidence changes in dopamine it is necessary to understand if dopamine acts within the neural circuit mediating conditioned defeat to promote memory and behavioral responses to social stress. The overarching goal of this dissertation is to determine if the nucleus accumbens is a critical component of the neural circuit mediating conditioned defeat and if the mesolimbic dopamine system promotes the memory of and the behavioral response to social defeat via its actions in the nucleus accumbens and basolateral amygdala.
Why study social stress?

Social stress in humans

The primary stressors that humans endure are psychological (North, Nixon et al. 1999, Dake, Price et al. 2003, Malan, Hamer et al. 2012, Myers, Perrine et al. 2013). Social stress, specifically, is perhaps the most common psychological stress experienced, and it generally occurs as the result of bullying (Dake, Price et al. 2003, Arslan, Savaser et al. 2011, Meltzer, Vostanis et al. 2011) or workplace harassment (Rospenda, Richman et al. 2009). Psychological stress, including social but also the emotional stressors of war (Cesur, Sabia et al. 2013, Silver, Holman et al. 2013) and threatened violence (O'Donnell, Roberts et al. 2011), create or exacerbate maladaptive conditions such as post-traumatic stress disorder (Taylor, Gooding et al. 2011), generalized and social anxiety disorders (van Oort, Greaves-Lord et al. 2011), schizophrenia (Lim, Chong et al. 2009) and depression (Hollis, Wang et al. 2010, Taylor, Gooding et al. 2011). These disorders cost the U.S. hundreds of millions of dollars a year in missed work income, treatment and societal costs (Lepine and Briley 2011, Sabes-Figuera, McCrone et al. 2012).

Though there are treatments, such as selective serotonin-reuptake inhibitors and cognitive behavioral therapy, for stress related anxiety disorders, many sufferers are unable to find relief with these existing treatments. For example, it is estimated that up to 40% of patients with major depressive disorder are not responsive to currently available treatments (Miller and O'Callaghan 2013). It is, therefore, necessary to explore the neurobiological basis for these disorders, including the neural structures that govern the learning, memory, and behavioral responses to stressful experiences, as well as the neurotransmitters that modulate these memories. It is
possible that these systems could be targets for novel treatments. Only with continued research into the neurobiological and neuroanatomical mechanisms of the development and expression of these disorders can the scientific community advance treatments for these debilitating syndromes.

**Animal models of social stress**

Animal models of social stress are among the best tools to understand the mechanisms whereby stressful experiences affect behavior and how these occurrences can cause changes within the brain that lead to maladaptive responses. Rodents exhibit similar changes in their behavior after defeat to that observed in humans after a stressful incident. These responses include social withdrawal (Razzoli, Andreoli et al. 2011), anhedonia (Chaouloff 2013), increased startle response (Pulliam, Dawaghreh et al. 2010), sleep changes (Meerlo, de Bruin et al. 2001), and learned helplessness (or ‘giving up’) (Berton, Aguerre et al. 1998, Hollis, Wang et al. 2010). Rodent models of stress, including social stress, allow scientists to much more easily determine the neurobiological basis of these disorders, as these models appear to have face validity for changes that are observed in humans following exposure to stress.

Social stress is often cited as one of the most naturalistic stressors with which to study the basis of the memory of stressful experiences or the expression of behavioral responses to stress. Rodents, both in their naturalistic environment (Blanchard, Spencer et al. 1995, Blanchard and Blanchard 2003) and within the laboratory (Potegal, Huhman et al. 1993), experience social defeat and hierarchical stratification when they live in groups or even alone but in close proximity to each other. Physical stressors, such as footshock, immobilization, or tail pinch, are viewed as less representational of the natural environment, as animals are quite unlikely to
encounter these experiences when living outside the lab environment. Social stress is more similar to human stressors because, as stated above, the majority of the stress experienced by humans is psychological.

**Conditioned defeat**

The conditioned defeat model of social stress is a validated and robust model of social stress observed in Syrian hamsters (Potegal, Huhman et al. 1993). In this model, hamsters, which typically show high levels of territorial aggression, are placed into the cage of a larger, more aggressive hamster and are defeated. The following day, instead of demonstrating the usual aggression toward an intruder in its own cage, the defeated hamster becomes submissive and defensive even if the intruder is smaller and non-aggressive. Though hamsters can recognize and discriminate between a familiar resident aggressor that has defeated them and a novel (unknown) hamster (McCann and Huhman 2012), they will still avoid all conspecifics during subsequent testing. This indiscriminate avoidance is often cited as a behavior analogous to the generalized fear demonstrated by sufferers of PTSD (Jovanovic, Kazama et al. 2012).

Conditioned defeat differs from other rodent models of social stress in several ways that make it a unique and relevant model for the study of the neural mechanisms underlying the responses to psychological stress. This robust behavioral change is observed after only one, brief defeat encounter, whereas in other rodents, such as rats and mice, chronic social defeat is necessary to produce social avoidance (Berton, McClung et al. 2006). The severity of the stressor in terms of physical pain or potential tissue damage is also significantly less in the conditioned defeat model. Bites and severe injuries are frequent in other models of social defeat stress (Hammamieh, Chakraborty et al. 2012), whereas bites that break the skin are rare during
brief aggressive encounters in hamsters. The behavior exhibited by the resident hamster toward the defeated hamster is also highly stereotyped, comprising postures that cause the defeated hamster to express submissive behavior but do not cause injury. Finally, both male and female hamsters exhibit conditioned defeat (Huhman, Solomon et al. 2003), in stark contrast to other models of social stress, where this type of response is only observed in males (note, however, that in California mice, the opposite is true, in that only female mice exhibit conditioned defeat (Trainor, Pride et al. 2011)).

**Regions that modulate the memory of and/or behavioral responses to social defeat in Syrian hamsters**

**Amygdala**

Several lines of evidence indicate that the amygdala is the primary region within the brain that regulates fear memory and expression of behavioral responses to stress, including those involved in conditioned defeat. Temporary inactivation or lesions of the amygdala attenuate unconditioned fear (such as predator odor (Muller and Fendt 2006)), contextual (Helmstetter and Bellgowan 1994) and cued fear conditioning (Wilensky, Schafe et al. 1999), as well as fear-potentiated startle (Walker and Davis 2000). This structure receives projections from the hippocampus and sensory information from the thalamus (Amaral, Price et al. 1992, Fendt and Fanselow 1999), allowing it to integrate information about the environment. The amygdala also projects to regions that modulate the expression of fearful behavior, including the periaqueductal gray (PAG) (Davis 1992, Davis 2006).

The basolateral nucleus of the amygdala (BLA) has been studied extensively because of its role in modulating fear-potentiated startle (Davis 2006) as well as behavioral responses to
footshock stress (Helmstetter and Bellgowan 1994). The BLA also appears to play a critical role in modulating conditioned defeat. Infusion of the GABA_A receptor agonist muscimol into the BLA either before defeat training or testing significantly reduces the duration of submissive and defensive behavior exhibited by the defeated hamster toward the non-aggressive intruder (Jasnow and Huhman 2001). Subsequent studies found that NMDA receptor activation is also necessary within the BLA for the acquisition of conditioned defeat (Day, Cooper et al. 2011), that inhibition of Trk receptors activated by the neurotrophin brain-derived neurotrophic factor (BDNF) reduces acquisition of conditioned defeat (Taylor, Stanek et al. 2011), and that overexpression of the transcription factor cAMP-response element binding (CREB) protein can augment the acquisition of conditioned defeat (Jasnow, Shi et al. 2005). Finally, the BLA is the only area within the brain tested thus far where protein synthesis during and after defeat is required for the expression of defensive and submissive behavior the following day in response to the presence of the non-aggressive intruder (Markham and Huhman 2008). Together, these data indicate that the basolateral amygdala is a critical brain region in the neural circuit that mediates conditioned defeat.

**Infralimbic cortex**

The medial prefrontal cortex in humans, or the infralimbic cortex in rodents, is often described as the ‘brake’ on the limbic system. It projects to the GABAergic intercalated cells within the amygdaloid complex and produces feed-forward inhibition within this region, decreasing the overall activation of the amygdala (Quirk, Likhtik et al. 2003, Peters, Kalivas et al. 2009). Inactivation of the infralimbic cortex, therefore, should increase fearful behavior or submissive and defensive behavior after defeat, and activation of this region should decrease submissive and defensive behavior. Consistent with this idea, temporary inactivation of the
infralimbic cortex using infusion of muscimol prior to defeat increases expression of defensive and submissive behavior the next day by defeated hamsters, and activation of the infralimbic cortex after defeat by infusion of the GABA<sub>A</sub> receptor antagonist bicuculline increases the duration of submissive and defensive behavior (Markham, Luckett et al. 2012).

**Ventral hippocampus**

The hippocampus, specifically the dorsal hippocampus, has long been associated with memory processes. H.M., a patient who had surgery to remove his hippocampus due to a seizure disorder, was thereafter unable to form any new long-term memories (Schmolck, Kensinger et al. 2002). Spatial memory formation also occurs within this region; lesioning the hippocampus impairs radial arm maze (Cho and Jaffard 1995) and Morris water maze performance (Morris, Schenk et al. 1990), and activation of specific cells in this region occurs during navigation around an environment in rodents (Breese, Hampson et al. 1989, Anderson and Jeffery 2003).

Within the hippocampus, it is postulated there is a functional difference between the dorsal and ventral hippocampus, wherein the dorsal portion governs contextual and spatial memory (Ballesteros, de Oliveira Galvao et al. 2014), while the ventral portion controls the memory of fear (Bast, Zhang et al. 2001, Yoon and Otto 2007). A similar difference between the neuroanatomical functions of these hippocampal subdivisions appears to exist in Syrian hamsters, as well, in that infusion of muscimol into the ventral, but not dorsal, hippocampus impaires acquisition of conditioned defeat (Markham, Taylor et al. 2010).

**What are other potential regions that could modulate acquisition or expression of conditioned defeat?**
**Nucleus accumbens**

The nucleus accumbens (NAcc) has traditionally been associated with reward processes (Tsurugizawa, Uematsu et al. 2012), but it has recently come to the attention of researchers as an important area for modulating fear and anxiety. Temporary inactivation of the nucleus accumbens decreases anxiety-like behavior in the elevated plus maze (Lopes, da Cunha et al. 2007, da Cunha, Lopes et al. 2008), stretch-attend risk assessment behavior (da Cunha, de Nazareth et al. 2008), and fear-potentiated startle (Schwienbacher, Fendt et al. 2004). Permanent lesions impair conditioned (Bradfield and McNally 2010) and contextual (Antoniadis and McDonald 2006) fear conditioning. Therefore, it seems that not only does the nucleus accumbens govern reward behavior, it can also affect behavior associated with aversive processes, as well.

The nucleus accumbens also appears to modulate behavioral responses to social defeat. Chronic social defeat increases the immediate early gene product Fos within the nucleus accumbens (Nikulina, Covington et al. 2004), and the neurotrophic factor BDNF released within the nucleus accumbens during chronic social defeat is necessary for the expression of social avoidance in mice (Berton, McClung et al. 2006). However, no one has determined if the nucleus accumbens is necessary for the memory of an acute defeat experience or for the expression of behavioral responses to acute social defeat. *Therefore, one major goal of this project is to test the hypothesis that the nucleus accumbens modulates the acquisition and expression of conditioned defeat.*
Does dopamine modulate the activation of or protein synthesis within the neural circuit mediating conditioned defeat?

The mesolimbic dopamine system is comprised of the ventral tegmental area and its dopaminergic projections to the amygdala, nucleus accumbens and medial prefrontal cortex, among others (Oades and Halliday 1987). Dopaminergic neurons within the VTA are activated (possibly by CRF (Rodaros, Caruana et al. 2007, Wanat, Hopf et al. 2008, Walsh, Friedman et al. 2013)) during aversive situations (Brischoux, Chakraborty et al. 2009), including footshock (Deutch, Tam et al. 1985), restraint(Deutch, Lee et al. 1991), and even chronic social defeat (Anstrom, Miczek et al. 2009, Razzoli, Andreoli et al. 2011). In addition to non-human animal studies, dopamine also appears to promote fear memory processes in humans, especially within the amygdala (Takahashi, Takano et al. 2010) but also within the nucleus accumbens (Elman, Borsook et al. 2013). In addition, dopaminergic dysregulation is associated with such disorders as schizophrenia (Benes 1997, van Winkel, Stefanis et al. 2008) and post-traumatic stress disorder (Glover, Powers et al. 2003, Drury, Theall et al. 2009). Understanding whether or not dopamine augments either the memory processes or the expression of behavioral responses to social defeat will allow for better treatment in the future for these disorders, especially those triggered by stress. Drugs that reduce dopamine signaling after stress could interfere with memory processes after a trauma, preventing development of PTSD, and similar drugs could perhaps be used to ameliorate symptoms of PTSD, such as social avoidance.

Within the amygdala, dopamine has a gating action on development of long-term potentiation. Dopamine inhibits GABAergic interneurons within the BLA (Bissiere, Humeau et al. 2003, Marowsky, Yanagawa et al. 2005, Chu, Ito et al. 2012), and as dopamine is released in the amygdala concurrently with glutamate signals from sensory cortices (Li, Dabrowska et al.
2011), this allows an amplification of the signal regarding a fearful stimulus or cue to trigger memory formation. Dopamine has a similar effect within the nucleus accumbens, enhancing glutamatergic signaling to induce LTP (Schotanus and Chergui 2008). Dopamine receptor activation within the amygdala is necessary for fear conditioning, as observed using such measures as contextual conditioning (Guarraci, Frohardt et al. 2000), Pavlovian fear conditioning (Nader and LeDoux 1999, Guarraci, Frohardt et al. 2000), and fear-potentiated startle (Greba, Gifkins et al. 2001). Similar effects are seen within the nucleus accumbens; fear conditioning is reduced by dopaminergic blockade (Iordanova, Westbrook et al. 2006), and there is also evidence dopaminergic receptor activation is necessary within both the basolateral amygdala and nucleus accumbens for fear conditioning to occur (Fadok, Darvas et al. 2010).

Expression of behavioral responses to fearful situations, such as avoidance of open areas and freezing to a tone previously paired with a shock, also requires dopaminergic receptor activation of the BLA and nucleus accumbens. Within the BLA, dopamine potentiates signals from the sensory cortices (Rosenkranz and Grace 1999) and dampens weaker inputs to gate activation of nucleus accumbens medium spiny neurons (Kroner, Rosenkranz et al. 2005, Pape 2005). Consistent with the above in vitro results, there is an impairment of fear-potentiated startle (Lamont and Kokkinidis 1998, de Oliveira, Reimer et al. 2011) and contextual fear conditioning (de Souza Caetano, de Oliveira et al. 2013) by infusion of a dopamine receptor antagonist into the amygdala. Within the nucleus accumbens, dopamine acts on medium spiny neurons to enhance signaling from other regions (including the basolateral amygdala), making them more likely to fire in response to a stimulus (Horvitz 2002, Kiyatkin 2002, West, Floresco et al. 2003). This increased firing would allow for greater modulation of neural activity in regions of the fear motor output circuit, such as the PAG (Usuda, Tanaka et al. 1998, Carretie,
Albert et al. 2009), to increase expression of fearful behavior. Behavioral experiments have verified these in vitro results, in that infusion of a dopamine receptor antagonist into the nucleus accumbens decreases expression of contextual fear (Albrechet-Souza, Carvalho et al. 2013). Social stress studies have also indicated that dopaminergic signaling to the nucleus accumbens is necessary for the occurrence of social avoidance after chronic defeat; defeated mice that had optogenetic inhibition of the firing of VTA dopaminergic neurons that project specifically to the nucleus accumbens during the social avoidance test demonstrated less avoidance than did defeated mice without such inhibition (Chaudhury, Walsh et al. 2013).

What is missing?

The previous research on the role dopamine plays in affecting memory and activation of the amygdala and nucleus accumbens indicates that dopamine may act as a signal of salience (Bromberg-Martin, Matsumoto et al. 2010) to draw an animal’s attention to a stimulus, to have the animal remember it, and then to use this information later to make decisions regarding its behavior. It is unknown if nucleus accumbens and the mesolimbic dopamine system are necessary for memory formation during acute social defeat or for expression of behavioral responses to such a brief defeat. Therefore, the overarching goal of this dissertation was to determine a) if the nucleus accumbens is a necessary component of the conditioned defeat circuit and b) if dopaminergic input from the mesolimbic dopamine system to the nucleus accumbens and amygdala results in activation of these structures (via an increase in Fos protein) during conditioned defeat.

Specific Aims

Specific Aim 1
Research question: does temporary inactivation of the nucleus accumbens before defeat training or before subsequent conditioned defeat testing reduce acquisition or expression of conditioned defeat?

We hypothesize that the nucleus accumbens is a necessary component of the circuit mediating the acquisition and expression of conditioned defeat. To test this hypothesis, we infused the GABA_A receptor agonist muscimol into the nucleus accumbens to temporarily inactivate this brain region either before acquisition (defeat training) or before expression (testing) to determine if these manipulations blocked conditioned defeat. We predicted that inactivation of the nucleus accumbens would reduce the acquisition and expression of conditioned defeat as evidenced by a reduced duration of submissive and defensive behavior in drug-treated animals as compared with their vehicle-treated controls during conditioned defeat testing.

Specific Aim 2

Research question: are dopaminergic and/or nondopaminergic neurons within the VTA activated in defeated hamsters, and does inactivation of the dopaminergic neurons reduce IEG expression in regions to which it projects, specifically the nucleus accumbens and basolateral amygdala?

We hypothesize that a) defeat activates dopaminergic and/or nondopaminergic neurons within the VTA and b) dopamine released by neurons projecting from the VTA to the nucleus accumbens and BLA increases neuronal activation of these structures during defeat. To test these hypotheses, two experiments will be performed. In the first, weight-matched hamsters will be paired for 15 minutes and then sacrificed 1 hour after this encounter, when the levels of the immediate early gene product Fos are highest. Typically in this case, one animal will win the
fight and one will lose, giving us a way to measure the activation of the VTA in hamsters that have won or lost a fight. We will then use immunohistochemistry with DAB/NiDAB co-localization to visualize Fos protein within the nuclei of dopaminergic (TH+) and nondopaminergic neurons (TH-) within the VTA.

In the second experiment, the GABA\textsubscript{B} receptor agonist baclofen will be microinjected unilaterally into the VTA to preferentially inactivate dopaminergic neurons (Margolis, Toy et al. 2012), and hamsters will be defeated and then sacrificed 1 hour after defeat. We will use immunohistochemistry to visualize Fos protein within the nucleus accumbens and basolateral amygdala, both on the side of baclofen injection into the VTA and on the contralateral side. Each animal will act as its own control because the baclofen injection is confined to one side and the dopaminergic projections from the VTA are largely ipsilateral (Koob, Balcom et al. 1975). A decrease in Fos protein expression should be observed only on the ipsilateral side to the injection. In this second experiment, we are asking 3 questions: 1) does unilateral inactivation reduce Fos protein levels in the ipsilateral BLA (as we know that social defeat increases Fos protein in the BLA (Markham, Taylor et al. 2010))? 2) Does defeat cause an increase in Fos protein within the NAcc if examined 1 hour after defeat? 3) If so, does unilateral inactivation of the VTA reduce Fos protein levels in the ipsilateral NAcc?

**Specific aim 3**

*Research question: does blockade of dopaminergic receptors in the NAcc significantly reduce conditioned defeat acquisition and/or expression?*

We hypothesize that dopamine is necessary within the nucleus accumbens for inducing memory processes as well as expression of behavioral responses to stressful situations. To test this
hypothesis, we will infuse the general dopamine receptor antagonist cis(z)flupenthixol into the NAcc before defeat training or testing and will determine if this blockade of dopamine receptor activation reduces the duration of submissive, aggressive, social and nonsocial behavior that animals express toward an NAI 24 hours after defeat.

References


urbanization, and neuroendocrine function in Black Africans: the THUSA study."


CHAPTER 2

THE ROLE OF THE NUCLEUS ACCUMBENS IN THE ACQUISITION AND EXPRESSION OF CONDITIONED DEFEAT

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Abstract

When Syrian hamsters (*Mesocricetus auratus*) are defeated by a larger, more aggressive hamster, they subsequently exhibit submissive and defensive behavior, instead of their usual aggressive and social behavior, even towards a smaller, non-aggressive opponent. This change in behavior is termed conditioned defeat, and we have found that the amygdala, bed nucleus of the stria terminalis, and ventral hippocampus, among others, are crucial brain areas for either the acquisition and/or expression of this behavioral response to social stress. In the present study, we tested the hypothesis that the nucleus accumbens is also a necessary component of the circuit mediating the acquisition and expression of conditioned defeat. We found that infusion of the GABA_A agonist muscimol into the nucleus accumbens prior to defeat training failed to affect acquisition of conditioned defeat, but infusion prior to testing significantly decreased submissive behavior and significantly increased aggressive behavior directed toward the non-aggressive intruder. These data indicate that, unlike the basolateral complex of the amygdala, the nucleus accumbens is not a critical site for the plasticity underlying conditioned defeat acquisition, but it does appear to be an important component of the circuit mediating the expression of the behavioral changes that are produced in response to a previous social defeat. Of note, this is the first component of the putative “conditioned defeat neural circuit” wherein we have found that pharmacological manipulations are effective in restoring the territorial aggressive response in previously defeated hamsters.
**Introduction**

Social conflict is a ubiquitous factor in human and non-human animal relationships (Bjorkqvist 2001), and this conflict affects a wide range of organisms both physically and emotionally. Determining the neurobiology of social stress can help lead to new treatments for disorders that arise from or are exacerbated by this stress, such as post-traumatic stress disorder (Bonafons, Jehel et al. 2009), schizophrenia (Yuii, Suzuki et al. 2007), and depression (Hunter, Durkin et al.). Social stress can have many physical effects, including increased activation of the hypothalamic-pituitary-adrenal (HPA) axis (Huhman, Bunnell et al. 1990, Ebner, Wotjak et al. 2005), decreased testosterone in males (Huhman, Moore et al. 1991, Bjorkqvist 2001), and effects on body mass (Foster, Solomon et al. 2006, Tamashiro, Hegeman et al. 2006). These physical effects are often harmful in humans and are known to exacerbate heart disease, obesity, and other ailments that cost society millions of dollars in terms of health care and lost productivity every year (Agid, Shapira et al. 1999, Agid, Kohn et al. 2000).

Our lab has used a model to study the neurobiology of social stress that we term conditioned defeat. In this model, a hamster that has been defeated, even a single time, by a larger, more aggressive opponent subsequently exhibits a striking behavioral change. Instead of producing the species-typical territorial aggression toward a novel intruder, previously defeated hamsters instead exhibit submissive and defensive behavior, even when the intruder is smaller and non-aggressive (Huhman and Jasnow 2006). This is an excellent model of social stress because the defeat by the larger, aggressive hamster does not result in wounding (and therefore the stress is more psychological than physical), it is very simple to elicit, and defeated hamsters exhibit unambiguous behavior that is identified and quantified easily. By contrast, many other rodent models of social stress involve chronic exposure to an aggressor, often involving up to 2 weeks wherein the subject is defeated each day and then spends the next 24 hours within the same cage.
as the aggressor but protected by a wire mesh divider (Vialou, Robison et al., Kudryavtseva, Bakshtanovskaya et al. 1991, Berton, McClung et al. 2006, Tsankova, Berton et al. 2006, Krishnan, Han et al. 2007, Krishnan, Han et al. 2008, Covington, Maze et al. 2009). This sort of social stress experience, while also extremely useful for studying the physiological concomitants of and putative treatments for the effects of social stress, is much more severe than that studied in our laboratory. We maintain that conditioned defeat is an ethologically relevant form of fear conditioning that can be used to explore how even a single, brief exposure to social stress can lead to long-term changes in future behavior.

We have used the conditioned defeat model to begin to define a neural circuit that underlies acute defeat-induced behavioral plasticity (i.e., how a single social experience can dramatically “switch” an animal’s behavior). We have found that the basolateral amygdala (Jasnow and Huhman 2001) and the ventral hippocampus (Markham, Taylor et al. 2010) are necessary for both the acquisition and expression of conditioned defeat. We have also shown that the bed nucleus of the stria terminalis is necessary for the expression of conditioned defeat (Markham, Norvelle et al. 2009). To date, the only brain area that we have determined to be critical for conditioned defeat-induced neural plasticity is the basolateral amygdala, as inhibition of protein synthesis within this area before training with the resident aggressor will block expression of social stress behaviors the following day (Markham and Huhman 2008). The other brain areas mentioned above (ventral hippocampus, bed nucleus of the stria terminalis) seem to modulate this memory or its output, but they may not be crucial for storage of its memory. Another brain area that might play a role in conditioned defeat is the nucleus accumbens. It is a brain area that is most commonly associated with reward and drug addiction (Salamone, Cousins et al. 1997, Ikemoto and Panksepp 1999, Cardinal, Parkinson et al. 2002, Cardinal, Winstanley et al. 2004,
Robbins, Ersche et al. 2008), but prior research has also demonstrated that the nucleus accumbens is necessary for both the acquisition (Bradfield and McNally) and expression (Antoniadis and McDonald 2006) of conditioned fear (but see also (Josselyn, Falls et al. 2005)) and is activated during fear recall in humans (Liberzon, Taylor et al. 1999). It is also known that neurotrophic factors in the nucleus accumbens are necessary for acquisition of social avoidance in reaction to prolonged social stress (Berton, McClung et al. 2006, Krishnan, Han et al. 2007) in mice. Therefore, the purpose of the present study was to test the hypothesis that the nucleus accumbens is necessary for the acquisition and the expression of conditioned defeat in hamsters.

Methods and Procedures

2.1. Animals and housing conditions

Subjects were adult male Syrian hamsters (Mesocricetus auratus; Charles River Laboratories, Wilmington, MA) that weighed 110-135 grams and were about 9 weeks old at the time of testing. All subjects were individually housed for at least one week prior to the start of testing in a temperature (20° ± 2°C) and humidity-controlled room with ad libitum access to food and water. Animals were kept on a 14:10 light:dark cycle (lights out at 10:00 h). Resident aggressors (RA) used for defeat training were older (>6 mo), singly housed males weighing between 160 and 180 g. Younger males (~2 mo) that weighed between 100 and 110 grams were group-housed (four to five per cage) and were used as non-aggressive intruders (NAI). The cages of the experimental animals and the resident aggressors were not changed for 1 week prior to testing so that animals could scent mark their cages and establish residence. No RA or NAI was used more than twice on a single training or testing day, respectively. All procedures and
protocols were approved by the Georgia State University Institutional Care and Use Committee and conform to PHS guidelines.

2.1. Surgical procedures

Subjects were anesthetized with sodium pentobarbital (90 mg/kg, i.p.), placed into a stereotaxic frame, and bregma and lambda were leveled within the same plane. Stainless steel guide cannula (26-gauge, 4.0 mm long below the pedestal) were implanted bilaterally into the brain and aimed at the nucleus accumbens (2.4 mm rostral and ±3.2 mm lateral relative to bregma and at a 20° angle toward the midline). In order to prevent damage to the area of interest, the guide cannula was lowered to only 2.7 mm below dura. On the day of injection, a 33-gauge injection needle was used that projected 2.3 mm below the guide cannula, reaching a final depth of 5 mm below dura. Following surgery, dummy stylets were placed in the guide cannula to help maintain patency. Hamsters were allowed 7-10 days to recover from surgery prior to the start of behavioral testing. Beginning two days after surgery, the hamsters were handled each day by gently restraining them and removing and then replacing the dummy stylets in order to maintain patency and to habituate the subjects to the injection procedure.

2.3. Social defeat and behavioral testing

The conditioned defeat model has been extensively described elsewhere (Huhman, Solomon et al. 2003). Briefly, on the day of social defeat training, animals were transported to the testing suite within the vivarium and were allowed to acclimate to the environment for 1 h. All training and testing sessions were performed under dim red illumination during the first 3 h of the dark phase of the light–dark cycle (Landau 1975). Training consisted of one 15-min exposure to the RA in the aggressor’s home cage, upon which time the RA reliably attacked the experimental
hamsters within 60 sec. The following day, animals were again transported to the same testing suite, and a NAI was placed into the subject’s home cage for 5 min. An animal was considered to show conditioned defeat if it exhibited no aggressive behavior and displayed an increase in submissive and defensive behavior when the NAI was introduced into its home cage. Conversely, a reduction in conditioned defeat was operationally defined as a significant reduction in the duration of submissive and defensive behavior. In contrast, non-defeated animals typically exhibit minimal submissive behavior and show high levels of territorial aggression directed toward the NAI. All training and testing sessions were videotaped via a CCD camera mounted overhead. These videos were scored by an experimentally blind observer using the behavioral scoring program Noldus ObserverPro. A second observer scored a random subset of these videos and interrater reliability for scored behavior between the two observers was above 90%. The total duration of four classes of behavior were measured during the test session: (1) social behavior (stretch, approach, sniff, nose touching, and flank marking); (2) non-social behavior (locomotion, exploration, grooming, nesting, feeding, and sleeping); (3) submissive/defensive behavior (flight, avoidance, tail up, upright, side defense, full submissive posture, stretch attend, head flag, attempted escape from cage); and (4) aggressive behavior (upright and side offense, chase and attack, including bites).

2.4. Drug infusion

Initially, we infused a dose of 1.1 nmol muscimol into the nucleus accumbens before defeat. This dosage had been used successfully to significantly reduce conditioned defeat after microinjection in the ventral hippocampus (Markham, Taylor et al. 2010) and BNST (Markham, Norvelle et al. 2009). In the nucleus accumbens, however, this dose produced a confounding stereotypy (in the form of a marked mouthing or chewing response) that increased the aggression
of the resident aggressor toward the experimental animal forcing us to reduce the dosage to 0.55 nmol, which caused no stereotypy. Infusion of this dose into other brain areas has been shown to be effective in altering behavior. For example, 0.5 nmol of muscimol has been infused unilaterally into the dorsomedial hypothalamus to significantly decrease escape from the open arms in the elevated T-maze (Nascimento, Zangrossi et al. 2010), a measure of anxiety. In addition, a bilateral infusion of 0.5 nmol muscimol into the amygdala is also effective in reducing intake of palatable food in rats (Minano, Meneres Sancho et al. 1992). Therefore, in the present study muscimol (Sigma, 0.55 nmol in 150 nl of saline) or vehicle control (150 nl of saline) was infused bilaterally into the nucleus accumbens over a 1-min period using a 1-μl syringe and a PHD 2000 Harvard Apparatus microinfusion pump connected to a 33-gauge injection needle via polyethylene tubing. The injection volume of 150 nl was chosen to minimize spread to adjacent structures (such as the BNST, which we have also found is necessary for expression of conditioned defeat) and to maximize anatomical specificity. The needle was kept in place for an additional minute before being removed to ensure diffusion of the drug or vehicle after which the dummy stylet was replaced. A successful injection was indicated by movement of an air bubble separating drug and water down the tubing and/or patency of the needle before and after the injection. Training or testing began 5 minutes after drug or vehicle control infusion.

2.5. Site verification

At the end of each experiment, hamsters were administered an overdose of sodium pentobarbital and infused with 150 nl of India ink bilaterally into the nucleus accumbens to verify the placement of the needle. The brains were post-fixed in 10% buffered formalin for at least 3 d before being sectioned on a cryostat. Thirty-μm sections were taken and stained with cresyl violet and coverslipped with DPX. Sections were then examined by two raters who were blind to
experimental conditioned under a light microscope for placement verification. Only animals with ink injections that were 0.3 mm or less from the nucleus accumbens core or shell were included in the statistical analysis. Animals with one or both ink injections outside of the nucleus accumbens or with ink within the anterior commissure were included in a site control group (anatomical controls).

2.6. Role of the nucleus accumbens in the acquisition of conditioned defeat

The goal of this experiment was to determine whether the temporary inactivation of the nucleus accumbens using the GABA<sub>A</sub> receptor agonist muscimol would significantly reduce the acquisition of conditioned defeat. Animals (n=38) were matched by weight and randomly assigned to vehicle or drug conditions. Hamsters received either muscimol or saline injections bilaterally into the nucleus accumbens 5 minutes prior to being placed into the home cage of a resident aggressor for 15 minutes. On the following day, animals were tested drug-free in their own cage against a non-aggressive intruder for 5 minutes.

2.7. Role of the nucleus accumbens in the expression of conditioned defeat

The goal of this experiment was to determine whether the temporary inactivation of the nucleus accumbens using muscimol would significantly reduce the expression of conditioned defeat. Animals (n=42) were matched by weight and randomly assigned to vehicle or drug conditions. Hamsters were placed drug-free in the home cage of a resident aggressor for 15 minutes of defeat training. The next day, they received either muscimol or vehicle injections into the nucleus accumbens 5 minutes prior to the 5-minute test with the non-aggressive intruder (NAI).

2.8. No-defeat control experiment
The goal of this experiment was to determine whether the temporary inactivation of the nucleus accumbens using muscimol before testing would increase aggressive behavior in animals (n=22) that had not been defeated. Stereotaxic surgery to implant cannula guides was performed as described previously, and following recovery subjects were placed in the empty cage of a resident aggressor for 15 minutes as a control for the effect of exposure to a novel conspecific’s cage. On the following day, subjects were administered either 0.55 nmol muscimol in 150 nl saline or vehicle 5 minutes before a 5 min test with a NAI.

2.8 Statistical analysis

All data was analyzed using SPSS and tested for homogeneity of variance. Data that did not demonstrate homogeneity of variance were analyzed with non-parametric Mann-Whitney U statistical tests. Data that met the criteria for homogeneity of variance were analyzed using a one-way ANOVA. Statistical results given below that contain the F statistic met the criteria for homogeneity of variance, while results containing the U statistic did not meet this criteria and therefore were analyzed with the non-parametric Mann-Whitney U test. Significant differences for all analyses were set at p < 0.05.

Results

3.1: The nucleus accumbens is not necessary for the acquisition of conditioned defeat

Histology

Figure 2.1 shows the location of ink injection sites for animals in Experiment 1 and 2. Misses for Experiment 1 largely were in the bed nucleus of the stria terminalis, the caudate putamen, and the lateral ventricle. Data from animals with bilateral placements in the nucleus accumbens core
were compared with animals with placements in the accumbens shell. Ink injections that were all or mostly in the shell were counted as shell injections, whereas ink injections that were all or mostly in the core were counted as core injections. As there were no significant differences between these groups, data for the nucleus accumbens core and shell were pooled for statistical analyses.
Figure 2.1 Muscimol in nucleus accumbens histology. Histological reconstruction of injection sites of animals receiving infusions into the nucleus accumbens shell or core 5 min before defeat (Experiment 1) or testing (Experiment 2). Black dots represent the site of injection of one or more animals. Boxes represent one or more anatomical misses. Drawings adapted from (Morin and Wood 2001).

**Behavioral results**

As shown in Figure 2.2, animals with vehicle or muscimol infusions into the nucleus accumbens before defeat were not significantly different in their interactions with the non-aggressive intruder the following day, including expression of submissive/defensive behavior (p>0.05).
Figure 2.2: Muscimol in nucleus accumbens acquisition behavioral results. Experiment 1 examined the effect of muscimol infusion on acquisition of conditioned defeat. Animals received infusions of vehicle or muscimol 5 minutes before being defeated by a resident aggressor for 15 min. Bar graph shows total duration (mean ± S.E.M.) of submissive/defensive, aggressive, social, and non-social behavior exhibited by defeated hamsters during a subsequent 5-min test with a non-aggressive intruder. No significant differences were observed between any of the groups.

3.2: The nucleus accumbens is necessary for the expression of conditioned defeat

Histology

Figure 2.1 also shows the location of ink injection sites for animals in Experiment 2. Misses for Experiment 2 largely went into the anterior commissure, caudate putamen, and the bed nucleus of the stria terminalis. Animals given muscimol that also had misplaced cannulae did not differ from vehicle controls in the duration of submissive/defensive or other behavior measured, indicating that the effect of muscimol in the nucleus accumbens group was anatomically specific (see Table 2.1 for means ± SEM).
Table 2.1: Muscimol in nucleus accumbens expression behavioral data for anatomical hits and misses. Experiment 2 examined the effect of muscimol infusion into nucleus accumbens on expression of conditioned defeat. Asterisks indicate significant difference from vehicle. The lack of effect observed in unilateral and bilateral misses infused with muscimol demonstrates that the effect of muscimol in the nucleus accumbens is anatomically specific.

<table>
<thead>
<tr>
<th>Group</th>
<th>Aggression (Mean ± SEM)</th>
<th>Submission (Mean ± SEM)</th>
<th>Social (Mean ± SEM)</th>
<th>Nonsocial (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle Bilateral Hit (n =11)</td>
<td>0 ± 0</td>
<td>80.73 ± 15.16</td>
<td>39.82 ± 7.27</td>
<td>179.45 ± 12.64</td>
</tr>
<tr>
<td>Muscimol Bilateral Miss (n=4)</td>
<td>0 ± 0</td>
<td>56.5 ± 27.55</td>
<td>98.5 ± 52.02</td>
<td>104.0 ± 50.35</td>
</tr>
<tr>
<td>Muscimol Unilateral Miss(n=5)</td>
<td>0 ±0</td>
<td>78.6 ± 16.01</td>
<td>24.6 ± 11.05</td>
<td>196.8 ± 22.07</td>
</tr>
<tr>
<td>Muscimol Bilateral Hit (n=14)</td>
<td>35.57 ± 17.86*</td>
<td>36.57 ± 10.46*</td>
<td>113.64 ± 23.77</td>
<td>114.07 ± 18.77*</td>
</tr>
</tbody>
</table>
Animals with nucleus accumbens core hits were compared with animals with shell hits. There were no significant differences between these groups (see Table 2.2 for exact means and SEM), so all bilateral hits within the nucleus accumbens core and/or shell were pooled and included in analyses.

Table 2.2: Muscimol in nucleus accumbens expression core versus shell results. No significant differences in behavior were seen between drug animals with hits within the nucleus accumbens core or shell. Thus, bilateral hits in either core or shell were pooled for statistical analyses.

<table>
<thead>
<tr>
<th>Group</th>
<th>Aggression (Mean ± SEM)</th>
<th>Submission (Mean ± SEM)</th>
<th>Social (Mean ± SEM)</th>
<th>Nonsocial (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle bilateral hit (n = 11)</td>
<td>0 ± 0</td>
<td>80.73 ± 15.16</td>
<td>39.82 ± 7.27</td>
<td>179.45 ± 12.64</td>
</tr>
<tr>
<td>Muscimol bilateral core (n = 3)</td>
<td>39.33 ± 39.33</td>
<td>34.67 ± 17.33</td>
<td>129.67 ± 8.67</td>
<td>97.0 ± 29.82</td>
</tr>
<tr>
<td>Muscimol one side core, one side shell (n = 3)</td>
<td>45.67 ± 44.18</td>
<td>10.67 ± 6.49</td>
<td>172.0 ± 69.96</td>
<td>71.67 ± 30.75</td>
</tr>
<tr>
<td>Muscimol bilateral shell (n = 8)</td>
<td>30.38 ± 25.53</td>
<td>47.0 ± 16.34</td>
<td>85.75 ± 31.56</td>
<td>136.38 ± 27.67</td>
</tr>
</tbody>
</table>
Behavioral results

As shown in Figure 2.3, there were significant differences between animals that received muscimol and animals that received vehicle before testing in their behavior toward the NAI. Animals that received muscimol exhibited significantly less submission during testing than did animals receiving vehicle injections ($F(1,23)=6.114$, $p=0.021$). Animals that received muscimol also exhibited significantly more aggressive behavior ($U=38.5$, $p=0.03$) and less nonsocial behavior than did vehicle controls ($U=35.0$, $p=0.021$). There were no significant differences between vehicle and muscimol animals for social behavior ($U=43.0$, ns, $p=0.066$), though animals receiving muscimol did show a trend for increased social behavior (see Figure 2.3).
Figure 2.3: Muscimol in nucleus accumbens expression behavioral results. Total duration (mean ± S.E.M.) of submissive/defensive, aggressive, social, and non-social behavior exhibited by defeated hamsters during a 5-min test with a non-aggressive intruder in Experiment 2 (expression study). Animals received infusions of vehicle or muscimol 5 minutes before being tested with a non-aggressive intruder. *Significantly different from vehicle control (p < 0.05).

There was no evidence of stereotypy exhibited by the animals that received muscimol. The fact that nonsocial behavior decreased while aggressive behavior increased in these individuals suggests that there was not a non-specific effect of muscimol on behavior (i.e., lethargy or ataxia). The increase in aggressive and social behavior (and the decrease in submission) was pervasive across the drug group; 8 of the 15 animals infused with muscimol showed aggressive or social behavior toward the non-aggressive intruder. The other six animals infused with muscimol did show some submission, but overall the average submission expressed by these animals was significantly less than that exhibited by vehicle animals.

3.3: No-defeat control study

Histology

Of the 22 animals used for the no-defeat control study, 1 animal lost its cap. Of the remaining 21 animals, there were 14 animals with bilateral hits (6 muscimol, 8 saline), 3 animals with unilateral hits, and 4 animals with bilateral misses. The behavioral results include only those animals with ink injections that were bilateral hits into the nucleus accumbens shell or core.

Behavioral results

As shown in Figure 2.4, there were no significant differences between animals that received muscimol or saline before testing in their expression of social, non-social, submission, or aggression behavior toward the NAI.
Figure 2.4: No-defeat muscimol in nucleus accumbens expression results. Effect of muscimol infusion into the nucleus accumbens on expression of behavior in non-defeated hamsters. Total duration (mean ± S.E.M.) of submissive/defensive, aggressive, social, and non-social behavior exhibited by non-defeated hamsters during a 5-min test with a non-aggressive intruder. No significant differences were found between groups receiving vehicle or muscimol.

Discussion

The present experiments indicate that infusion of the GABA_A receptor agonist muscimol into the nucleus accumbens blocks the expression but not the acquisition of conditioned defeat. These data are the first to suggest that the nucleus accumbens is a necessary component of the neural circuit underlying the expression of conditioned defeat and thus plays an important role in behavioral responses to social stress in hamsters. These data also indicate, however, that neural
activity in the nucleus accumbens during defeat training is not necessary for the formation of defeat-induced behavioral changes in this species. Finally, these data are particularly important in that they identify for the first time a component of the neural circuit mediating conditioned defeat wherein pharmacological manipulation both reduces social avoidance and restores territorial aggression in previously defeated hamsters.

The finding that the acquisition of conditioned defeat is not blocked by temporary inactivation of the nucleus accumbens is perhaps surprising based on findings from the conditioned fear literature indicating that the nucleus accumbens (specifically the shell region) is required for acquisition of conditioned fear (Bradfield and McNally), including fear-potentiated startle (Schwienbacher, Fendt et al. 2004) (but see also (Josselyn, Falls et al. 2005)). It has also been shown that activation of neurons by neurotrophins (Berton, McClung et al. 2006) or transcription factors (Vialou, Robison et al.) is required in the nucleus accumbens to elicit social avoidance after social defeat, suggesting that activation of the nucleus accumbens during the defeat experience is necessary for subsequent defeat-induced behavioral changes to occur. There are several possible explanations for the inconsistencies in the data. First, the dose of muscimol used in the present study could have been too low to effectively inactivate the nucleus accumbens during acquisition. We were limited to the lower dose, however, because the higher dose caused stereotyped responses that in turn elicited greater aggression from the resident aggressor. Future studies could use an alternative method (such as lidocaine or an NMDA antagonist, which was effective in reducing acquisition of conditioned defeat in the BLA (Jasnow, Cooper et al. 2004)) to inhibit the nucleus accumbens in order to reexamine the possibility that this area is involved in the acquisition of CD in hamsters. The dose used in the acquisition experiment, however, was the same dose that was shown to effectively inhibit the
expression of CD, suggesting that this dose of muscimol was sufficient to inhibit the nucleus accumbens. In addition, there are also previous studies indicating that a similar dose of muscimol alters panic/anxiety behavior or feeding behavior after infusion into the hypothalamus (Nascimento, Zangrossi et al. 2010) or the amygdala (Minano, Meneres Sancho et al. 1992), respectively.

Another explanation for this inconsistency is that the nucleus accumbens may not govern acquisition of social stress-induced behavioral changes in hamsters as it does in other species. There are other instances in which differences in the importance of certain brain areas in rats and mice versus hamsters (Adams 1979, Adams 2006) have been noted, though this was observed in aggression, not in fear or social stress-induced behavior. A third reason for the conflicting acquisition results could be the nature of the stimuli leading to conditioned defeat. Previous studies have suggested that the nucleus accumbens may be necessary for learning of cued fear stimuli, while contextual fear learning occurs elsewhere. It is not clear at this point what the critical stimuli are that cause conditioned defeat as the behavior generalizes to very different testing conditions. This is certainly an interesting problem for future study. Finally, this inconsistency might be due to differences in the stressors used (i.e., physical stress versus psychological stress) or in the duration of the stressor (i.e., repeated defeat vs. acute defeat). Differences in the importance of brain areas mediating physical stress versus psychological stress responses are frequently noted in the literature (Canteras and Goto 1999, Blanchard, Li et al. 2003, Blanchard, Canteras et al. 2005, Motta, Goto et al. 2009), as are differences in the brain areas that are affected by or required for responses to repeated/chronic versus acute stressors (Choi, Furay et al. 2008).
The finding that the nucleus accumbens is required for the expression of conditioned defeat is exciting given that this is the first demonstration that manipulation of the nucleus accumbens can alter defeat-induced behavior. Whereas some studies have indicated that the nucleus accumbens is activated during social stress (Tidey and Miczek 1996) and other studies have indirectly found that neuronal activation of this brain area is required during a defeat experience for social stress-induced behavioral changes to occur (Berton, McClung et al. 2006, Krishnan, Han et al. 2007), no prior study to our knowledge has demonstrated that the nucleus accumbens is necessary for the expression of behavioral responses to social stress. Our findings are congruent with previous data, however, demonstrating that the expression of fear-potentiated startle requires a functional nucleus accumbens (Schwienbacher, Fendt et al. 2004) and that expression of conditioned fear is impaired following a nucleus accumbens lesion (Antoniadis and McDonald 2006).

The demonstration that inactivation of the nucleus accumbens during conditioned defeat testing causes an increase in aggressive behavior in Syrian hamsters is an important and novel finding that extends our previous model of the neural circuit underlying conditioned defeat. It is particularly important to note that our previous studies have demonstrated that inactivation of other brain areas in the putative conditioned defeat neural circuit, such as the amygdala (Jasnow and Huhman 2001), bed nucleus of the stria terminalis (Markham, Norvelle et al. 2009), and the ventral hippocampus (Markham, Taylor et al. 2010) before conditioned defeat testing lead to a reduction in submission, but this occurs without a concomitant increase in aggression. Thus, our putative conditioned defeat neural circuit has previously lacked a site in which the defeat-induced reduction in aggression might be mediated. The current results thus represent the first experimental manipulation that has led to the resumption of territorial aggression toward an NAI
by a previously defeated animal. This finding is consistent with data indicating that inactivation of the nucleus accumbens (Albert and Chew 1980, Albert, Walsh et al. 1983, Lee, Yamamoto et al. 1983) can stimulate aggression in rats and that increased numbers of androgen receptors within the nucleus accumbens are expressed following winning an aggressive bout (Fuxjager, Forbes-Lorman et al. 2010). It is very important to note, however, that in the current study the nucleus accumbens selectively stimulated aggression in defeated hamsters and that there was no increase in aggression in non-defeated animals that received muscimol before testing.

Prior reports have speculated on how the nucleus accumbens modulates a variety of forms of conditioned fear. One conception is that the nucleus accumbens is an important interface between limbic and motor areas of the brain (Mogenson, Jones et al. 1980) and is a site within which glutamatergic innervation from the BLA (Kiyatkin 2002), which may be activated during expression of conditioned defeat, and enhanced dopaminergic activation from the ventral tegmental area (Oades and Halliday 1987) attributed to social stress (Anstrom, Miczek et al. 2009) come together and synapse on projection neurons within the nucleus accumbens (Kalivas and Nakamura 1999, Kiyatkin 2002, West, Floresco et al. 2003). This convergence of glutamatergic and dopaminergic signaling seems to gate downstream inhibition (Groenewegen, Wright et al. 1996, Horvitz 2002) of motor output areas, including those involved in aggression (Lee, Yamamoto et al. 1983). The nucleus accumbens may act to inhibit aggression following social defeat, as stated above, and when its activation is blocked by muscimol, as in this study, aggression is no longer inhibited and the experimental animal behaves aggressively toward an intruder.

In summary, the findings of this paper indicate that the nucleus accumbens is necessary for expression of conditioned defeat in Syrian hamsters, but not its acquisition. It also appears to
be a particularly important part of the neural circuit mediating conditioned defeat expression in
that the nucleus accumbens affects not just submissive and avoidant behavior but also their
opposite, given that infusion of muscimol before conditioned defeat testing significantly
increased aggression directed toward an intruder. This finding is a first for our lab, as we have
not previously found a treatment that would rapidly and reliably restore territorial aggression
after a previous social defeat.

Acknowledgements

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CHAPTER 3

DOPAMINE IN THE NUCLEUS ACCUMBENS MODULATES THE MEMORY OF SOCIAL DEFEAT IN SYRIAN HAMSTERS (Mesocricetus auratus)

Abstract

Conditioned defeat (CD) is a behavioral response that occurs in Syrian hamsters after they experience social defeat. Subsequently, defeated hamsters no longer produce territorial aggression but instead exhibit heightened levels of avoidance and submission, even when confronted with a smaller, non-aggressive intruder. Dopamine in the nucleus accumbens is hypothesized to act as a signal of salience for both rewarding and aversive situations to allow an animal to remember and respond to significant events. The purpose of the present study was to test the hypothesis that dopamine in the nucleus accumbens modulates the acquisition and expression of behavioral responses to social defeat. In Exp. 1, bilateral infusion of the non-specific D₁/D₂ receptor antagonist cis(z)flupenthixol (3.75 μg/150 nl saline) into the nucleus accumbens 5 min prior to defeat training significantly reduced submissive and defensive behavior expressed 24 hr later in response to a non-aggressive intruder. In Exp. 2, infusion of 3.75 μg cis(z)flupenthixol 5 min before conditioned defeat testing with a non-aggressive intruder significantly increased aggressive behavior in drug-infused subjects. In Exp. 3, we found that the effect of cis(z)flupenthixol on aggression was specific to defeated animals as infusion of drug into the nucleus accumbens of non-defeated animals did not significantly alter their behavior during exposure to a non-aggressive intruder. These data demonstrate dopamine in the nucleus accumbens modulates both acquisition and expression of social stress-induced behavioral changes.
Introduction

Social stress is one of the most common stressors that humans face, and such stressors as bullying and workplace harassment can cause or exacerbate post-traumatic stress disorder (Bonafons, Jehel et al. 2009), social or generalized anxiety disorder (van Oort, Greaves-Lord et al. 2011), schizophrenia (van Winkel, Stefanis et al. 2008, Lim, Chong et al. 2009) and depression (Carvalho, Pinto-Gouveia et al. 2013). Even though some sufferers are able to find appropriate treatment, others are unable to find relief from their symptoms. Understanding how social stress leads to changes in brain and behavior, including illuminating the neurobiological concomitants of the memory processes and the expression of behavioral changes (e.g., social avoidance and generalization of fear) that occur after exposure to social stress is critical for finding new treatments for these debilitating conditions.

Animal models allow scientists to develop and test new treatments to counteract the deleterious effects of exposure to social stress, an ethologically relevant stressor that animals encounter in the wild (Sapolsky 1982). Syrian hamsters, for example, display a pronounced behavioral response to social defeat, which we term conditioned defeat. Instead of producing the species-typical territorial aggression toward a novel intruder, a hamster that has been defeated demonstrates only submissive and defensive behavior toward an intruder placed in its home cage. Even a smaller, non-aggressive intruder triggers this type of generalized fear of all other hamsters. This indiscriminate avoidance is analogous to the generalized fear exhibited by sufferers of PTSD.

Research delineating the neural circuit underlying the acquisition (learning and memory of the initial defeat experience) and the expression (the demonstration of submissive and
defensive behavior after defeat in response to the presence of a non-aggressive intruder) of conditioned defeat has demonstrated that there are several brain regions that are important components of a putative neural circuit mediating this behavioral response. The basolateral amygdala (BLA) appears to be the sole region tested thus far wherein protein synthesis is necessary for acquisition of conditioned defeat (Markham, Taylor et al. 2010). This suggests that the BLA is a critical region where the memory of social defeat is ‘crystallized’ and goes on to influence ongoing agonistic behavior. Other regions, however, may also store the memory of social defeat and therefore may suggest alternative targets for the treatment of traumatic memories related to social stress.

We have previously demonstrated that GABA_A receptor activation within the nucleus accumbens decreases the expression, but not acquisition, of conditioned defeat (Luckett, Norvelle et al. 2012), suggesting that the nucleus accumbens promotes submissive behavior following defeat but is not necessary for the formation of the memory of that defeat. Recent findings indicate that there are distinct actions of GABA and dopamine within the nucleus accumbens, whereby dopamine acts on long-term potentiation processes, but GABA does not (Schotanus and Chergui 2008). This suggests that our previous conclusion that the nucleus accumbens does not modulate the acquisition of conditioned defeat may have been erroneous. Dopaminergic receptor activation in the nucleus accumbens may indeed play a role in modulating the memory of social defeat, whereas GABA receptor activation may only affect the expression of behavioral responses to social defeat.

Dopamine is a neurotransmitter of particular interest for study of the neurobiological mechanisms underlying behavioral responses to social stress because dopaminergic dysregulation has been associated with such disorders as schizophrenia (Benes 1997, van
Winkel, Stefanis et al. 2008) and post-traumatic stress disorder (Glover, Powers et al. 2003, Drury, Theall et al. 2009). The main source of dopamine to limbic regions of the brain, such as the nucleus accumbens, amygdala, and infralimbic cortex, is the ventral tegmental area (Oades and Halliday 1987). This region is activated by physical (Deutch, Tam et al. 1985) and social stress (Anstrom, Miczek et al. 2009), likely through the action of corticotropin releasing factor (Rodaros, Caruana et al. 2007, Wanat, Hopf et al. 2008). Dopamine has also been implicated in the expression of social avoidance after chronic defeat in mice (Chaudhury, Walsh et al. 2013), and social stress increases dopamine in the nucleus accumbens (Tidey and Miczek 1996) as assessed by microdialysis. However, no one has examined whether dopamine within the nucleus accumbens is necessary for the formation of a memory for a social defeat or for the expression of behavioral responses following an acute social defeat.

The general consensus in the literature is that dopamine gates excitation to allow the most salient stimuli (Floresco, Blaha et al. 2001, Horvitz 2002), both rewarding and aversive, to be remembered and to change future behavior. The evidence that dopamine can enhance both memory formation and neural activation within the nucleus accumbens indicates that dopamine might be necessary for long-term potentiation and neuronal activation of the nucleus accumbens during and after an acute social defeat experience. Thus, the purpose of this study was to test the hypothesis that dopamine in the nucleus accumbens modulates the memory of and the expression of behavioral responses to social defeat in Syrian hamsters.

Methods

2.1. Animals
Subjects were adult male Syrian hamsters (*Mesocricetus auratus*; Charles Rivers Laboratories, Wilmington, MA) that weighed 120-130 grams upon arrival and were about 9 weeks old at the time of testing. All subjects were individually housed for at least 1 week prior to the start of testing in a temperature (20 ± 2°C) and humidity-controlled room with *ad libitum* access to food and water. Subjects were weight-matched and randomly assigned to their drug groups. Animals were kept on a 14:10 light:dark cycle (lights out at 10:00 h). Resident aggressors (RA) used for defeat training were older (> 6 mo), singly housed males weighing between 160 and 180 g. Younger males (~2 mo) that weighed between 100 and 110 g were group-housed (four to five per cage) and were used as non-aggressive intruders. The cages of the subjects and the resident aggressors were not changed for 1 week prior to testing so that animals could scent mark their cages and establish residency. All procedures and protocols were approved by the Georgia State University Institutional Care and Use Committee and conform to PHS guidelines.

### 2.2. Surgical procedures

Subjects were anesthetized via exposure to 5% isoflurane mixed with oxygen, placed into a stereotaxic frame, and maintained under anesthesia via a nose cone that delivered 2-3% isoflurane. The animal was then administered 5 mg/kg ketofen and 1 ml 0.9% saline to provide pain relief and fluids, respectively. Bregma and lambda were leveled, and then stainless steel guide cannulae (26-gauge, 4.0 mm long below the pedestal) were implanted bilaterally into the brain aimed at the nucleus accumbens (2.0 mm rostral, ± 3.2 mm from bregma and at a 20° angle toward the midline). To avoid damage to the area of interest, the guide cannula was lowered 2.7 mm below dura. On the day of injection, a 33-gauge injection needle was used that projected 2.3 mm below the guide cannula, reaching a final depth of 5 mm below dura. After guide cannulae
implantation, a wound clip was placed in the skull caudal to the guide cannulae as an anchor and then dental cement was used to secure the cannulae and clip to the skull. Following surgery, dummy stylets were placed in the cannulae to help maintain patency. Hamsters were allowed 7-10 days to recover from surgery prior to the start of behavioral testing. Beginning 2 days after surgery, the hamsters were handled each day by gently restraining them and removing and then replacing the dummy stylets in order to maintain patency and to habituate the subjects to the injection procedure.

2.3 Behavioral procedures

The conditioned defeat model has been extensively described elsewhere (Huhman, Solomon et al. 2003). Briefly, on the day of social defeat training, animals were transported to the testing suite within the vivarium and were allowed to acclimate to the environment for 1 h. All training and testing sessions were performed under dim red illumination during the first 3 h of the dark phase of the light–dark cycle to reduce circadian variation in activity levels (Landau 1975). Training consisted of one, 15-min exposure to the RA in the aggressor’s home cage, upon which time the RA reliably attacked the subjects within 60 sec. The following day, animals were again transported to the testing suite, and an NAI was placed into the subject’s home cage for 5 min. All training and testing sessions were videotaped via a CCD camera mounted overhead. These videos were scored by an observer that was blind to treatment group using the behavioral scoring program Noldus ObserverPro. A second observer scored a random subset of these videos. Inter-rater reliability between the two observers was above 90%. The total duration of four classes of behavior were measured during the test session: (1) social behavior (stretch, approach, sniff, nose touching, and flank marking); (2) non-social behavior (locomotion, exploration, grooming, nesting, feeding, and sleeping); (3) submissive/defensive behavior (flight,
avoidance, tail up, upright, side defense, full submissive posture, stretch attend, head flag, attempted escape from cage); and (4) aggressive behavior (upright and side offense, chase and attack, including bites).

2.4. Drugs

Cis(z)flupenthixol (Sigma), a D1/D2 receptor antagonist, was administered bilaterally at a concentration of 0.0, 3.75 or 15 μg in 150 nl saline (the highest dose was used only to test the effect of cis(z)flupenthixol on acquisition of conditioned defeat). These dosages were taken from previous literature (Ito and Hayen 2011) indicating the effective dose of cis(z)flupenthixol in the nucleus accumbens. This volume of drug or vehicle was infused in a previous study (Luckett, Norvelle et al. 2012).

2.5. Injection procedures

Drug or vehicle was infused bilaterally into the nucleus accumbens over a 1-minute period using a 1-μl syringe and a PHD 2000 Harvard Apparatus microinfusion pump connected to a 33-gauge injection needle via polyethylene tubing. The needle was kept in place for an additional minute before removal to ensure diffusion of the drug or vehicle away from the injection site, after which the dummy stylet was replaced. A successful injection was indicated by movement of an air bubble separating drug and water down the tubing and/or patency of the needle before and after the injection. Training or testing began 5 minutes after drug or vehicle infusion.

2.6. Site verification

After testing, animals were sacrificed with an overdose of sodium pentobarbital and 150 nl of India ink was injected in each cannula to check for injection placement. Brains were post-
fixed in 10% buffered formalin for at least 3 days before sectioning on the cryostat. 30 μm sections were taken, stained with neutral red and cover slipped with DPX. An observer blind to experimental condition examined the sections with a light microscope for verification of injection sites. Animals in the drug groups were excluded if the injection was more than 300 μm outside of the border of the nucleus accumbens, but all saline animals were included so as to reduce the number of total animals in the study (see histology in results section).

2.7. Role of dopamine receptor activation in the nucleus accumbens in the acquisition of conditioned defeat

The goal of this experiment was to determine whether dopamine receptor blockade within the nucleus accumbens using the D<sub>1</sub>/D<sub>2</sub> receptor antagonist cis(z)flupenthixol reduces the acquisition of conditioned defeat. Hamsters received drug (3.75 or 15 μg) or vehicle infusion 5 min prior to being placed into the home cage of a resident aggressor for 15 minutes. On the following day, animals were tested drug-free in their own cage against a non-aggressive intruder for 5 minutes.

2.8. Role of dopamine receptor activation in the nucleus accumbens in the expression of conditioned defeat

The goal of this experiment was to determine whether dopamine receptor blockade within the nucleus accumbens during testing reduces the expression of submissive and defensive behavior toward the non-aggressive intruder. As we determined in the acquisition experiment, the effective dose of cis(z)flupenthixol in the nucleus accumbens was 3.75 μg, so only this dose or vehicle was used for the expression experiment. Hamsters were placed drug-free in the home
cage of a resident aggressor for 15 min of defeat training. Animals received the infusion into the nucleus accumbens 5 minutes prior to the 5-minute test with the NAI.

2.9. No-defeat control experiment

The goal of this experiment was to determine if the effect of 3.75 μg cis(z)flupenthixol on expression of conditioned defeat is specific only to defeated animals and/or if the drug also promotes aggression in non-defeated animals. Stereotaxic surgery to implant cannula guides was performed as described previously, and, following recovery, subjects were placed in the empty cage of a resident aggressor for 15 minutes as a control for the effect of exposure to a novel conspecific’s cage. On the following day, subjects were administered either 3.75 μg cis(z)flupenthixol or vehicle 5 minutes prior to a 5-minute test with an NAI.

2.10. Statistics

One-way ANOVAs or t-tests were performed, as appropriate. If homogeneity of variance was violated, a Kruskal-Wallis or Mann-Whitney U non-parametric test was run. Statistical significance for all tests was set at p<0.05.

Results

3.1 Dopaminergic receptor activation is necessary in the nucleus accumbens for the acquisition of conditioned defeat

3.1.1. Histology

The behavior exhibited by animals in the saline group was not statistically different if the injection site was determined to be an anatomical hit (n = 4) or miss (n = 5), so the behavioral
data for these two groups were pooled in order to reduce the number of animals that were used (data not shown). There were 2 animals from the 3.75 μg dose group and 2 animals from the 15 μg dose group that had cannulae placements that were either bilateral or unilateral anatomical misses. Only animals that received cis(z)flupenthixol and were bilateral anatomical hits within 300 μm of the nucleus accumbens were included in statistical analysis. Behavioral data from anatomical hits and misses are shown in Table 1. Localization of injection sites are shown in Figure 3.1.
Figure 3.1. Histological reconstruction of injection sites in animals that received cis(z)flupenthixol or vehicle prior to defeat or testing. Black dots represent the site of injection of one or more animals in the nucleus accumbens. Boxes represent one or more injections that were determined to be anatomical misses. Drawings adapted from (Morin and Wood 2001).

3.1.2. Behavioral results

As shown in Figure 3.2, an infusion of 3.75 µg cis(z) into the nucleus accumbens significantly decreased the duration of submission as compared to vehicle controls (H(2)=6.284, p < 0.05). There were no other significant differences among the three drug groups for any other behavior.
Figure 3.2. Effect of cis(z)flupenthixol infusion into the nucleus accumbens on the acquisition of conditioned defeat. Animals received infusions of drug or vehicle 5 minutes prior to being defeated by a resident aggressor for 15 minutes. Bar graph shows the total duration (mean ± SEM) in sec of submissive/defensive, aggressive, social and nonsocial behavior exhibited by defeated hamsters during a 5-minute test on the following day. (*) Significantly different from vehicle control (p < 0.05).

3.2. Activation of dopamine receptors in the nucleus accumbens is necessary for the suppression of aggressive behavior after defeat.

3.2.1. Histology

There were no significant behavioral differences in the vehicle group anatomical hits (n = 5) and misses (n = 5), so the behavioral data for all 10 animals was pooled (data not shown). There was a significant difference in the 3.75 μg drug group between those animals that were bilateral nucleus accumbens hits and the anatomical misses (see Table 3.1 and Figure 3.3). All anatomical hits and misses are demonstrated in Figure 3.1.
Table 3.1. Cis(z)flupenthixol in nucleus accumbens anatomical hits and misses. This table demonstrates the anatomical specificity of infusion of cis(z)flupenthixol into the nucleus accumbens on behavior. Mean ± standard error (SEM) of all drug groups and vehicle groups as well as anatomical hits and misses are listed. (*) Significantly different from vehicle (p < 0.05); (‡) Significantly different from the anatomical hit drug group.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Submission Duration (in sec)</th>
<th>Aggression Duration</th>
<th>Social Duration</th>
<th>Nonsocial Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cis(z) in NAcc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acquisition Vehicle</td>
<td>9</td>
<td>59.8 ± 20.9</td>
<td>5.9 ± 4.0</td>
<td>48.4 ± 8.6</td>
<td>186.0 ± 16</td>
</tr>
<tr>
<td>Cis(z) in NAcc</td>
<td>8</td>
<td>7.1 ± 4.0*</td>
<td>19.1 ± 19.1</td>
<td>61.5 ± 10.7</td>
<td>212.4 ± 16.5</td>
</tr>
<tr>
<td>Acquisition 3.75 µg Hits</td>
<td>2</td>
<td>1.0 ± 1.0</td>
<td>0.5 ± 0.5</td>
<td>65.1 ± 36.7</td>
<td>233.5 ± 37.2</td>
</tr>
<tr>
<td>Cis(z) in NAcc</td>
<td>8</td>
<td>27.2 ± 22.7</td>
<td>1.2 ± 1.1</td>
<td>55.5 ± 9.8</td>
<td>216.2 ± 19.3</td>
</tr>
<tr>
<td>Acquisition 15 µg Hits</td>
<td>2</td>
<td>41.6 ± 41.6</td>
<td>44.1 ± 44.1</td>
<td>33.8 ± 19.2</td>
<td>180.6 ± 21.8</td>
</tr>
<tr>
<td>Cis(z) in NAcc</td>
<td>10</td>
<td>8 ± 3.4</td>
<td>14.5 ± 7.2</td>
<td>120.1 ± 11.3</td>
<td>157.6 ± 11.4</td>
</tr>
<tr>
<td>Expression Vehicle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cis(z) in NAcc</td>
<td>5</td>
<td>0.7 ± 0.7</td>
<td>89.6 ± 32.5*</td>
<td>59.3 ± 9.6*</td>
<td>150.4 ± 36.8</td>
</tr>
<tr>
<td>Expression 3.75 µg Hits</td>
<td>4</td>
<td>20.9 ± 15.8</td>
<td>0.2 ± 0.2 ‡</td>
<td>62.1 ± 10.4*</td>
<td>216.9 ± 16.6</td>
</tr>
<tr>
<td>Cis(z) in NAcc</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expression 3.75 µg Hits</td>
<td>2</td>
<td></td>
<td>6.4 ± 9.2</td>
<td>61.6 ± 10.4</td>
<td>216.9 ± 16.6</td>
</tr>
</tbody>
</table>
3.2.2. Behavioral results

As shown in Figure 3.3, the duration of submission expressed by the vehicle control group was too low to see any effect of the drug on submission levels. Cis(z)flupenthixol infusion significantly increased aggression ($U = 9.0, p < 0.05$) in those animals with bilateral hits into the nucleus accumbens as compared with vehicle. Cis(z)flupenthixol infusion also decreased social behavior ($F(2, 16) = 9.176, p < 0.01$) in those animals with anatomical hits and misses into the nucleus accumbens as compared to vehicle.

Figure 3.3. Effect of cis(z)flupenthixol infusion into the nucleus accumbens on the expression of conditioned defeat. Animals received infusions of vehicle or cis(z)flupenthixol 5 min prior to being tested with a non-aggressive intruder. (*) Significantly different from vehicle control ($p < 0.05$); (‡) Significantly different from anatomical hit drug group.
3.3. No-defeat control study

3.3.1. Histology

There were no significant differences between saline animal anatomical hits \((n = 5)\) and misses \((n = 3)\) and so their data was pooled (data not shown). There were 6 drug animals with anatomical hits.

3.3.2. Behavioral results

As shown in Figure 3.4, there were no significant behavioral differences between those animals that received a microinjection of vehicle and those that received a 3.75 \(\mu\)g dose of cis(z)flupenthixol in the NAcc.
Figure 3.4. Effect of cis(z)flupenthixol infusion into the nucleus accumbens on expression of behavior in non-defeated hamsters. This figure demonstrates the total duration (mean ± SEM) in sec of submissive/defensive, aggressive, social and nonsocial behavior exhibited by non-defeated hamsters during a 5-minute test with a non-aggressive intruder. No significant differences were found between groups.

Discussion

These experiments demonstrate that infusion of the non-specific D1/D2 dopamine receptor antagonist cis(z)flupenthixol into the nucleus accumbens reduces the acquisition of conditioned defeat in Syrian hamsters. It is unclear if dopamine receptor activation is necessary for the full expression of conditioned defeat, as animals in the vehicle control group produced too little submission to observe an effect of the drug on submissive behavior after defeat. However, there was a significant increase in the duration of aggression and a significant decrease in the duration of social behavior in defeated hamsters that received drug. Together, these
findings indicate that dopamine receptor activation in the nucleus accumbens modulates both the acquisition and expression of behavioral responses to social defeat.

Dopamine in the nucleus accumbens is usually thought of as a neurochemical signal that modulates reward-related processes (McFarland and Kalivas 2001, Ito and Hayen 2011, Bahi and Dreyer 2012, Young, Dreumont et al. 2014), so the finding that dopaminergic receptor blockade during defeat training decreases subsequent submission is one of the first to demonstrate a role for dopamine in the formation of an aversive social memory. Previous research has demonstrated that dopaminergic receptor blockade within the nucleus accumbens reduces the memory of conditioned taste aversion (Fenu, Bassareo et al. 2001). This is the first study, however, to link dopaminergic receptor blockade during defeat to a blockade of behavioral changes after social defeat. Other social stress studies have only examined dopamine levels in the brain during defeat using microdialysis (Tidey and Miczek 1996) or fast-scan cyclic voltammetry (Anstrom, Miczek et al. 2009) without examining their part in memory modulation.

The current data provide additional clues to the mechanism whereby the nucleus accumbens modulates the acquisition of conditioned defeat. We had previously concluded that the nucleus accumbens did not play a role in the acquisition of conditioned defeat because infusion of a GABA_A receptor agonist, which is generally thought to temporarily inactivate the injected site, did not alter conditioned defeat. The current study, however, demonstrates that dopamine receptor activation within the nucleus accumbens is necessary for the learning and/or memory of social defeat. These conflicting findings can perhaps be explained by a recent study by Schotanus and Chergui, 2008, who found that dopamine regulates LTP in neurons within the NAcc while GABA does not.
We cannot at this time determine if dopamine receptor activation in the NAcc is necessary for the expression of submissive and defensive behavior after defeat because of the low submission observed in this study. We did find, however, that dopamine receptor blockade within the nucleus accumbens increases aggression and decreases social behavior after defeat. Overall, the demonstration that manipulations of neurochemical signaling within the nucleus accumbens can enhance aggressive behavior following defeat is very intriguing. In many other components of the neural circuit mediating conditioned defeat, we have shown that manipulations are effective in altering submission, but not aggression. Thus, we have been able to identify where memories of social defeat that promote submission might be formed but not where the memories that inhibit aggression are localized. This study indicates that the latter memory formation may depend, at least in part, on the nucleus accumbens. We have previously shown that temporary inactivation (using the GABA receptor agonist muscimol) of the lateral septum increases the expression of aggression following defeat, but it did not alter the acquisition of conditioned defeat (McDonald, Markham et al. 2012). In light of our present data, future studies should re-examine the role of the lateral septum in the modulation of the memory of defeat and subsequent aggression using drug manipulations that target specific neurochemical signals other than GABA.

In summary, the present findings indicate that dopaminergic receptor activation in the nucleus accumbens promotes the acquisition of behavioral changes following social defeat in Syrian hamsters. It is also evident that dopamine in the nucleus accumbens modulates aggressive and social behavior during expression of conditioned defeat. We should note that our results provide an interesting addendum to a previous study demonstrating that GABA\textsubscript{A} receptor activation decreases expression, but not acquisition, of conditioned defeat. It seems that
dopamine in the NAcc can, indeed, alter the acquisition of the memory of social defeat in Syrian hamsters, whereas neuronal inhibition via GABA_A receptor activation only decreases expression of behavioral responses to social defeat. Finally, the finding that dopamine within the nucleus accumbens modulates the learning and memory of behavioral responses to social defeat suggests that treatment with dopamine receptor antagonists might be useful for sufferers of traumatic memories associated with social stress and post-traumatic stress disorder (Benes 1997, Drury, Theall et al. 2009, Corrigan, Fisher et al. 2011, Camardese, Di Giuda et al. 2014).
References


CHAPTER 4

ACTIVATION OF THE VTA BY DEFEAT AND EFFECT OF BACLOFEN INFUSION ON FOS ACTIVATION OF THE NUCLEUS ACCUMBENS AND BASOLATERAL AMYGDALA

Abstract

We have previously determined that dopamine in the nucleus accumbens modulates the acquisition of behavioral responses to social defeat. We hypothesized that defeat increases activation of dopaminergic neurons in the ventral tegmental area (VTA) and that dopaminergic neurons activated by defeat would increase neuronal activation in efferent regions to the VTA, including the nucleus accumbens and basolateral amygdala. In our first experiment, we used immunohistochemistry with DAB/NiDAB co-localization to visualize Fos protein within the nuclei of dopaminergic (TH+) and nondopaminergic (TH-) neurons in the VTA of defeated and non-defeated animals. In our second experiment, we infused baclofen (which is hypothesized to preferentially inactivate dopaminergic neurons) into the VTA and examined the effect of this manipulation on Fos activation of the nucleus accumbens and the basolateral amygdala. We found that non-dopaminergic neurons are activated by defeat, but dopaminergic neurons are not. We also found no effect of baclofen infusion on Fos activation in two efferent regions to the VTA, the nucleus accumbens and the basolateral amygdala. This leads us to conclude that either the VTA is not the source of dopamine needed within the nucleus accumbens for acquisition of social defeat or that Fos is not the best measure of activation within the VTA.
Introduction

Dopamine receptor activation modulates the memory of acute social defeat within the nucleus accumbens. The source of this dopamine is currently unknown, but the primary source of dopamine for limbic areas is the ventral tegmental area (VTA), a region that is linked to the modulation of both rewarding (Lobo, Zaman et al. 2013) and aversive processes (Tsai, Zhang et al. 2009). The dopaminergic projection to the basolateral amygdala and nucleus accumbens (Oades and Halliday 1987), among others, is termed the mesolimbic dopamine system to distinguish it from the nigrostriatal dopamine system that originates in the substantia nigra. The ventral tegmental area is activated by stress, including footshock (Deutch, Tam et al. 1985), restraint stress (Valenti, Gill et al. 2012) and chronic social defeat (Cao, Covington et al. 2010). It is thought that corticotropin-releasing factor, a critical neurochemical signal within the hypothalamic-pituitary-adrenal stress axis (Jezova, Ochedalski et al. 1999), activates the ventral tegmental area (Wanat, Hopf et al. 2008, Boyson, Miguel et al. 2011, Walsh, Friedman et al. 2013), causing a release of dopamine into limbic brain areas. Dopamine is hypothesized to act as a signal of salience (Faure, Reynolds et al. 2008) to enhance memory processes within the nucleus accumbens (Thomas and Malenka 2003, Schotanus and Chergui 2008, Wolf 2010) and the amygdala (Guarraci, Frohardt et al. 1999, Bissiere, Humeau et al. 2003) and to promote neuronal excitation (Kalivas and Nakamura 1999, Rosenkranz and Grace 1999).

We know that mesolimbic dopamine neurons evidence increased firing after chronic social defeat (Cao, Covington et al. 2010) and that dopamine from this source is necessary for the expression of social avoidance after this repeated stressor (Chaudhury, Walsh et al. 2013).
Thus, we hypothesized that an acute defeat also activates dopaminergic and/or non-dopaminergic neurons within the VTA. We tested this hypothesis in Experiment 1 by pairing weight-matched hamsters and examining the activation of dopaminergic (tyrosine hydroxylase-positive) and non-dopaminergic (tyrosine hydroxylase-negative) neurons within the VTA of winners, losers, and controls using the Fos, a protein product of the immediate early gene c-fos, as a proxy for neuronal activation.

A recent study demonstrated that baclofen, a GABA<sub>B</sub> receptor agonist, preferentially inactivates dopaminergic neurons within the ventral tegmental area (Margolis, Toy et al. 2012). Baclofen administration either into the VTA in mice (Brebner, Phelan et al. 2000, Yang, Wang et al. 2011) or peripherally in humans (Young, Franklin et al. 2014) reduces reward and reinforcement. We hypothesized that selectively inactivating dopaminergic neurons with baclofen in the VTA of hamsters during social defeat would reduce neuronal activation within regions to which the VTA projects, therefore reducing Fos protein expression. Thus, in Experiment 2, we infused baclofen into the VTA either unilaterally or bilaterally and examined Fos activation within the nucleus accumbens and the basolateral amygdala after social defeat.

Methods

2.1 Animal housing and procedures

Subjects were adult male Syrian hamsters (Mesocricetus auratus; Charles Rivers Laboratories, Wilmongton, MA) that weighed 120-130 grams upon arrival and were about 9 weeks old at the time of testing. All subjects were individually housed for at least 1 week prior to the start of testing in a temperature (20 ± 2°C) and humidity-controlled room with ad libitum access to food and water. All animals were kept on a 14:10 light:dark cycle (lights out at 10:00
h) and all testing occurred within the first three hours of the dark phase of the daily light:dark cycle. In Experiment 1, subjects were weight-matched and randomly assigned to resident, intruder or control (both home cage and novel cage) groups. In Experiment 2, resident aggressors were used to defeat subjects in Experiment 2 (see procedure below). Resident aggressors used for defeat training were older, (> 6 mo), singly housed males weighing between 160-180 grams. All procedures and protocols were approved by the Georgia State University Institutional Animal Care and Use Committee and conform to PHS guidelines.

2.2 Surgical procedures

At the beginning of Experiment 2, subjects were anesthetized via exposure to 5% isoflurane mixed with oxygen, placed into a stereotaxic frame, and maintained under anesthesia via a nose cone that delivered 2-3% isoflurane. Animals were injected with 5 mg/kg ketofen and 1 ml 0.9% saline to provide pain relief and maintain hydration, respectively. Bregma and lambda were leveled and unilateral or bilateral guide cannulae (26-gauge, 4.0 mm long below the pedestal) were implanted into the brain aimed at the VTA. In animals implanted with a unilateral guide cannula (n = 21), the side (left or right) of implantation was counterbalanced. The stereotaxic coordinates for implantation were 2.6 mm posterior to bregma, ±1.51 mm lateral to the midline, and 2.8 mm ventral to bregma, at a 10° angle to avoid piercing the superior sagittal sinus. A second group of animals (n = 8) were stereotaxically implanted bilaterally using the same coordinates. After guide cannulae implantation, a wound clip was placed in the skull as an anchor and then dental cement was used to secure the cannulae to the skull. Following surgery, dummy stylets were placed in the guide cannulae to help maintain patency. Animals were allowed to recover for at least 2 days before commencement of handling and 7-10 days
prior to the start of behavioral testing. All animals were handled daily for 5 days by removing the dummy stylets and replacing them to habituate the animals to injection procedure.

2.3 Behavioral manipulations and drug injections

**Experiment 1**

Animals were handled by being removed from their cage, gently held and touched, and then placed back into the cage to habituate them to handling by the experimenter. On the day of behavioral experiments, subjects were transported to the testing suite 1 hour prior to the beginning of the dark period to acclimate to the testing room. Non-defeated hamsters were either left in their home cage undisturbed until perfusion at the appropriate time (home-cage controls) or placed into the vacant cage of a resident aggressor (novel cage control). The novel-cage control group was therefore exposed to the novelty of the empty cage and the smells of a dominant animal without any physical interaction with the resident. Animals in the intruder group were placed in the home cage of a weight-matched resident, and the animals were allowed to interact for 15 minutes. These interactions were filmed and scored later for the duration of submission and aggression displayed. Animals that produced aggression were determined to be dominant, and their partners, which produced submissive and defensive behavior, were determined to be subordinate.

**Experiment 2**

Animals were transported to the behavioral suite 1 hour prior to the dark period. Cannulated animals were injected with baclofen or saline 10 minutes prior to being placed into the cage of a resident hamster (defeated animals) or back into their own cage (non-defeated animals).
2.4. Drug infusions and site verification-VTA inactivation with baclofen

Hamsters were administered unilateral infusions of 0.0, 31.25 (low dose) or 62.5 ng (high dose) baclofen (Sigma) or bilateral infusions of 15.62 (low dose) or 31.25 ng (high dose) baclofen in 125 nl sterile saline over a 1-minute period with Harvard Apparatus pumps and 1-ul Hamilton syringes connected to 33-gauge, 4.2 mm needles via polyethylene tubing. The injection needle was left in place for another minute following the injection to ensure diffusion of the injectate. A successful injection was determined if an air bubble separating water from the drug moved down the tube and/or if the needle was patent both before and after the injection. After injection, animals were allowed to rest in their cages for 10 minutes prior to defeat.

2.5. Perfusion

One hour after the defeat or control manipulation in Experiment 1, hamsters were injected with sodium pentobarbital and, when the paw-pincher reflex no longer occurred, were perfused according to standard procedures with phosphate-buffered saline (PBS) and then 4% paraformaldehyde. Fixed brains were removed and post-fixed overnight in paraformaldehyde and then placed in a 30% sucrose solution until they sank. Brains were notched to allow determination of left versus right sides of the brain.

2.6. Immunohistochemistry

Brains were sectioned in a cryostat at 35 μm, and the sections were placed in cryoprotectant in a 1:4 series to ensure an adequate number of sections per animal (3-4 sections per brain area) before storage at -20°C. After washing off cryoprotectant, sections were incubated overnight at room temperature in rabbit anti-fos antibody (Santa Cruz, 1:10,000). The next day, sections were washed and incubated for 1 h at room temperature in biotinylated goat
anti-rabbit antibody (Vector, 1:600), followed by washes, incubation in ABC solution and visualization of Fos protein using Ni-DAB. For Experiment 1, an additional procedure visualized tyrosine hydroxylase within the VTA with DAB. After staining for Fos, sections were incubated overnight at room temperature with sheep anti-tyrosine hydroxylase (Santa Cruz, 1:40,000), followed the next day by a 1h incubation in biotinylated donkey anti-sheep antibody (Jackson, 1:600), followed by washes, incubation in ABC solution and then visualization of TH using DAB. The sections were mounted using a gelatin solution onto Superfrost Plus slides, allowed to dry overnight and then dehydrated and coverslipped with DPX.

2.7. Cell counting: Experiment 1

Double and single-labeled neurons within the VTA were counted by an observer blind to experimental condition for each group from 3-4 sections throughout the anterior-posterior extent of the VTA on a Nikon Eclipse E800 Microscope with a QImaging digital camera attached. Counting domains were generated by projecting the microscopic field (10x) of neutral red-stained VTA sections onto a computer screen and delineating the ventral tegmental area and then using this standard area for the immunostained tissue. Cells counted included: brown-stained neurons with a purple-stained nucleus (Fos+/TH+), brown-stained neurons (TH+) and cells with only a purple-stained nucleus (Fos+) using neutral red stained and anatomically matched alternate sections from the same animal to determine the representative area of the VTA (see Figure 3.1). Fos counts took place at 10x; examination of Fos/TH double-labeling occurred at 20x.
2.8. Quantification of Fos in nucleus accumbens and basolateral amygdala after VTA inactivation (Experiment 2)

Pictures were taken at 10x of both sides of the brain, and anatomical markers were used to determine the location of the basolateral amygdala and the nucleus accumbens. The area was outlined on the screen and saved. All images were transferred to a computer and Fos nuclei were counted using ImageJ software (Tulogdi, Soros et al. 2012). A macro was written that removed the background equally for basolateral amygdala and nucleus accumbens sections, converted all images to black and white, and counted all particles within a set threshold with a size of 15-70 pixels and 0.2-1.0 circularity. The area of the counting domain for each side was recorded as well as the number of particles counted, and for each image, an observer monitored the analysis to ensure that no tissue damage or debris in the counting domain interfered with counting the number of Fos particles. The cell count was divided by the area in pixels and then multiplied by 100,000 to obtain a number of particles counted.

2.9. Statistics

For Experiment 1, differences between defeated and non-defeated controls for Fos+, Fos+/TH+, and TH+ cell counts were determined via a t-test were determined for Experiment 1. Cell counts were divided by the number of sections counted to correct for the fact that unequal numbers of sections were quantified for some animals. For Experiment 2, for animals receiving unilateral baclofen microinjections we calculated a difference score of the number of Fos cells counted on the side contralateral to the VTA injection minus the number of Fos cells counted on the ipsilateral. We then compared difference scores across behavioral and drug conditions using a one-way ANOVA. We also calculated the sum total of Fos cells counted bilaterally for each
area and compared behavioral and drug conditions using a one-way ANOVA. If data violated the homogeneity of variance test, a non-parametric Kruskal-Wallis test or Mann-Whitney U was run. Correlations were also done between duration of submissive behavior exhibited during defeats and the Fos+/TH+ cell counts to determine if the number of double-labeled cells was dependent on duration of submissive behavior exhibited or the duration of aggression directed by the resident hamsters toward the intruders. Statistical significance was set at p < 0.05.

Results

Experiment 1

The activation of dopaminergic neurons within the VTA following social defeat was determined by counting the number of Fos+ cells (those with a blue-black nucleus) that were surrounded by a brown-stained neuronal cell body, indicating the presence of tyrosine hydroxylase. The activation of non-dopaminergic neurons was determined by counting Fos+ cells that were not surrounded by a brown-stained neuronal cell body. Non-activated dopaminergic cells were those without a Fos+ nucleus but with a brown-stained neuronal cell body (see Figure 4.1). Novel-cage (n = 5) and home-cage (n = 4) control animals exhibited similar numbers of double-labeled cells (data not shown) and so their data were pooled for further analysis. As demonstrated in Figure 4.2, when corrected for the number of sections counted, there was no significant difference between losers (subordinate animals) of a fight and novel-cage/home-cage control animals for the number of double-labeled cells counted.
Figure 4.1: Counting domain for VTA. This photomicrograph demonstrates the counting domain created for the NiDAB/DAB stained sections (right) by delineating the area of the VTA using corresponding neutral red-stained sections (left).

Figure 4.2: Fos+/TH+ cells in the VTA corrected for number of sections counted. The number of double-labeled Fos+/TH+ cells per slide was compared between subordinate, dominant, and control animals. There were no differences between animals that lost or won the fight, nor were there differences between those animals and the novel-cage or home-cage control animals.
The number of Fos+ cells that were not stained for TH, meaning non-dopaminergic cells activated by winning or losing a fight, was significantly increased ($U=15.0$, $p = 0.043$) in animals that lost the fight versus those that were left undisturbed in their own cage/exposed to a novel cage (see Figure 4.3). The number of TH+ cells not activated by defeat was also not significantly different between the two groups as demonstrated in Figure 4.4.

Figure 4.3: Fos+ cells in the VTA corrected for number of sections counted. The number of Fos+ cells counted was not significantly different between groups.
The number of TH+ cells counted in the VTA was not significantly different between the groups. To determine the percentage of VTA TH+ neurons activated by defeat, we calculated the percentage of TH+ neurons evidencing Fos activation and compared across groups. There were no significant differences between subordinate and control animals in the percentage of TH+ cells that were also Fos+ (see Figure 4.5).
Figure 4.5: Percent of Fos+ cells that are also TH+ corrected for number of sections counted. The percentage of Fos+/TH+ cells vs. the total number of TH+ cells was calculated and compared between groups. There were no significant differences among the groups.

We also determined if the number of Fos+/TH+ neurons in the VTA was dependent on the amount of submissive behavior expressed by the subordinate animal or the amount of aggression expressed by the dominant animal. For this analysis, the number of Fos+/TH+ cells counted for subordinates was correlated with the duration of submission they expressed (Figure 4.6) and the duration of aggression expressed by the dominant animal (Figure 4.7). The correlation between the number of Fos+/TH+ cells counted and the duration of behavior was not significant for either analysis.
Figure 4.6: Correlation of Fos+/TH+ cells counted in the VTA of subordinate animals with submissive behavior expressed.
Figure 4.7: Correlation of Fos+/TH+ cells counted in the VTA of subordinate animals with duration of aggression produced toward them by their dominant opponent.

**Experiment 2**

We hypothesized that animals with unilateral baclofen infusion into the VTA should have reduced Fos protein on the side of the nucleus accumbens or basolateral amygdala ipsilateral to injection as compared to the contralateral side. We performed bilateral cell counts in the nucleus accumbens and basolateral amygdala (see Figure 4.8 for counting domains). We first determined if there was an effect on ipsilateral vs. contralateral Fos protein expression in the nucleus accumbens and basolateral amygdala of animals that received a unilateral baclofen infusion (see Figure 4.9 for a histological reconstruction of baclofen or saline injections). A difference score for the Fos count for the contralateral minus the ipsilateral side was calculated and compared among drug and behavioral groups. If our hypothesis was supported, then those animals infused with unilateral baclofen should evidence a significantly higher difference score in the nucleus accumbens and basolateral amygdala than those animals infused with saline. There was no difference, however, among any of the drug or behavioral groups (see Figures 4.10-4.12) for Fos protein in the nucleus accumbens and basolateral amygdala. We did, however, observe motor effects of unilateral baclofen infusion at the highest dose in 1 animal, whereby animals were lethargic. Subsequently, the doses were reduced to avoid non-specific motor effects.
Figure 4.8: Counting domains within the basolateral amygdala (left) and nucleus accumbens (right). Though only one side of each area is displayed, counting was performed bilaterally for both the basolateral amygdala and the nucleus accumbens. The size and location of the counting domains (illustrated in Figure 9) was similar to those of other published reports ((Valencia-Torres, Olarte-Sanchez et al. 2012, Morrison, Bader et al. 2013).
Figure 4.9: Histology of hits/misses for VTA inactivation with baclofen experiment. One or more injection sites (anatomical hits) are indicated by filled circles, while anatomical misses are indicated by squares. As anatomical misses for non-defeated animals (n = 2) did not evidence differences in Fos counts from those that were anatomical hits (n = 2), the Fos data for these non-defeated control animals were pooled.

Figure 4.10: Difference between the contralateral and ipsilateral side to infusion of the number of Fos+ cells (mean ± SEM) in the nucleus accumbens core. There were no significant differences among the drug and/or behavioral groups in the number of Fos+ cells on the contralateral – ipsilateral side.
Figure 4.11: Difference between the contralateral and ipsilateral side to infusion of the number of Fos+ cells (mean ± SEM) in the nucleus accumbens shell. There were no significant differences among the drug and/or behavioral groups.
Figure 4.12: Difference between the contralateral and ipsilateral side to infusion of the number of Fos+ cells (mean ± SEM) in the basolateral amygdala. There were no significant differences among the drug and/or behavioral groups.

We hypothesized that dopaminergic neurons from the VTA project to and activate the nucleus accumbens and basolateral amygdala. We therefore calculated the sum total of Fos protein activation within the nucleus accumbens and basolateral amygdala in animals with bilateral infusions of baclofen into the VTA and compared them with defeated animals that received saline unilaterally, defeated animals that had no surgery, and non-defeated animals injected with baclofen. If our hypothesis was supported, then those animals that received a bilateral infusion of baclofen should exhibit significant decreases in Fos in the nucleus accumbens and basolateral amygdala. Infusion of the GABA\textsubscript{B} receptor agonist baclofen
bilaterally failed to reduce Fos activation in either the nucleus accumbens core (see Figure 4.13) or shell (see Figure 4.14) or the basolateral amygdala (see Figure 4.15). Subjects that were defeated and were either home cage controls or received an infusion of saline into the VTA did not evidence higher levels of Fos in these regions than did non-defeated animals that received an infusion of baclofen (see Figures 4.13-4.15).

Figure 4.13-Sum of Fos counts (mean ± SEM) in the nucleus accumbens core for defeated animals with unilateral infusion of saline, bilateral infusion of baclofen, and non-defeated animals that received a unilateral infusion of baclofen.
Discussion

In Experiment 1, we hypothesized that an acute defeat would activate dopaminergic and/or non-dopaminergic neurons within the VTA. This was based on previous evidence that stress increases VTA neuronal activation (Wanat, Hopf et al. 2008, Valenti, Gill et al. 2012). We instead found that non-dopaminergic cells (Fos+, TH-) were activated by defeat, but dopaminergic cells (Fos+, TH+) were not. Our finding that acute defeat did not increase activation of dopaminergic neurons in the VTA is surprising given that we have demonstrated that dopamine in the nucleus accumbens is necessary for the expression of behavioral changes following social defeat (Gray, Norvelle et al. 2014). Based on our Fos results, it thus appears possible that the VTA is not the source of this dopamine. However, the VTA evidenced such low activation of dopaminergic neurons regardless of defeat (2-3% of dopaminergic neurons showed Fos activation; see Figure 4.5) that it may be that Fos is not the best method to determine
the activation of the ventral tegmental area. The substantia nigra is another region that could provide the dopamine necessary for this modulation of memory within the nucleus accumbens, but visual examination of the Fos activation of the substantia nigra did not reveal many, if any, Fos+ nuclei, let alone double-labeled Fos+/TH+(dopaminergic) neurons. It is therefore unlikely to be the source of dopamine necessary for acquisition of conditioned defeat, especially because previous studies found that it is not activated by stress (Deutch, Tam et al. 1985).

In our second experiment, we predicted that selectively inactivating dopaminergic neurons in the VTA of hamsters during social defeat using baclofen would reduce neuronal activation within regions to which the VTA projects, therefore reducing Fos protein. This was based on previous research indicating that dopamine modulates neuronal activation in the basolateral amygdala (Rosenkranz and Grace 1999) and nucleus accumbens (Horvitz 2002) and that baclofen selectively inhibits dopaminergic neurons within the VTA (Margolis, Toy et al. 2012). We anticipated that baclofen infusion into the ventral tegmental area would reduce Fos protein expression in the basolateral amygdala and nucleus accumbens ipsilateral to an infusion of baclofen as compared to the contralateral side. We also anticipated that a bilateral infusion of baclofen would bilaterally reduce Fos+ cells counted in the nucleus accumbens and/or basolateral amygdala. Surprisingly, there were no observed reductions in Fos protein following unilateral or bilateral baclofen infusion. There was also no effect of defeat on Fos activation of the nucleus accumbens or the basolateral amygdala. Non-defeated animals evidenced similar Fos protein levels in the nucleus accumbens and basolateral amygdala as defeated animals. However, because there were no drug-free, non-defeated animals, we must conclude that baclofen occluded the effect of defeat (see below).
The most likely explanation for our negative results in Experiment 2 is that baclofen did not have the intended effect on neuronal activation within the basolateral amygdala and the nucleus accumbens. Baclofen infusion is hypothesized to reduce dopamine in regions to which the ventral tegmental area projects (Westerink, Kwint et al. 1996). We did get a motor effect of baclofen infusion at the highest dose (see results), so it is an adequate assumption that dopamine release is reduced by baclofen infusion. In the basolateral amygdala, there are opposite effects of dopamine on GABAergic interneurons and excitatory pyramidal neurons. Dopamine increases activation of pyramidal neurons (Rosenkranz and Grace 2002, Kroner, Rosenkranz et al. 2005) but decreases activation of interneurons (Rosenkranz and Grace 2002, Bissiere, Humeau et al. 2003, Kroner, Rosenkranz et al. 2005). This would mean that the inhibitory effect of decreased dopamine on pyramidal neuron activation could be negated by the excitatory effect on GABAergic interneurons.

Within the nucleus accumbens, it was hypothesized that baclofen infusion into the ventral tegmental area would also reduce Fos activation. However, a recent study (Danjo, Yoshimi et al. 2014) found that optogenetic inactivation of dopaminergic neurons actually increased Fos+ cells in the nucleus accumbens, even though dopamine release was reduced by optogenetic inactivation. This effect on Fos could explain why we did not get a reduction in Fos in the nucleus accumbens after baclofen infusion into the VTA, as the methods used (inactivation of dopaminergic neurons using optogenetics or baclofen) are hypothesized to be similar in their effects on dopamine release. Therefore, instead of reducing Fos with baclofen, we may have actually increased Fos activation with our manipulation.

There was also no effect of defeat on Fos expression in Experiment 2. However, one limitation of this experiment was that there were no drug-free, non-defeated animals. Baclofen
infusion into the VTA in non-defeated animals may have increased Fos in the nucleus accumbens and amygdala and therefore occluded the effect of defeat on Fos activation. In order to conclude that the nucleus accumbens is not activated by defeat, therefore, we should compare Fos activation of drug-free defeated and non-defeated animals.
References


Gray, C. L., A. Norvelle and K. Huhman (2014). The role of dopamine within the nucleus accumbens on the memory and expression of behavioral responses to social defeat in Syrian hamsters (*Mesocricetus auratus*).


CHAPTER 5

CONCLUSIONS

Summary of findings

The overarching goal of this dissertation was to determine a) if the nucleus accumbens is a necessary component of the neural circuit mediating conditioned defeat and b) if dopaminergic input from the mesolimbic dopamine system to the nucleus accumbens and amygdala results in activation of these structures (via an increase in Fos protein) during social defeat. We found that dopamine, but not GABA, modulates memory of social defeat within the nucleus accumbens. However, GABA does affect the expression of behavioral responses to social defeat. Defeat also increased activation of non-dopaminergic neurons, but it did not activate dopaminergic neurons nor did baclofen infusion into the VTA before defeat affect Fos activation within the nucleus accumbens or basolateral amygdala.

Role of the nucleus accumbens in modulating memory of social defeat

Infusion of muscimol, a GABA_A receptor agonist, into the nucleus accumbens did not affect the memory of social defeat. This drug was used in previous studies in our lab to decrease acquisition of social defeat when infused into several components of the neural circuit mediating conditioned defeat including the basolateral amygdala (Jasnow and Huhman 2001), medial prefrontal cortex (Markham, Luckett et al. 2012), and ventral hippocampus (Markham, Taylor et al. 2010). However, given that the majority of the neurons within the nucleus accumbens are GABAergic projection neurons (approximately 90%) (Meredith and Totterdell 1999, Wolf 2010), the neuronal identity of cells within the nucleus accumbens is different from that within the basolateral amygdala. Within the basolateral amygdala, GABAergic neurons are numerous
and are, for the most part, interneurons (Rainnie, Asprodini et al. 1991). The majority of neurons in the nucleus accumbens are inhibitory projection neurons (Washburn and Moises 1992). Muscimol, therefore, could have a very different effect within the nucleus accumbens than it does in the basolateral amygdala.

A recent study determined that dopamine and glutamate are critical for memory formation in the nucleus accumbens but that GABA does not affect long-term potentiation (Schotanus and Chergui 2008). This finding puts in question our earlier conclusion that the nucleus accumbens is not involved in the acquisition of conditioned defeat during the initial social defeat (Luckett ref). Our current finding that infusion of the dopamine receptor antagonist cis(z)flupenthixol reduces the memory of social defeat supports this instead reveals that the nucleus accumbens does play a role in this process. It also extends the literature regarding the role of dopamine in fear memory, in general. No one prior to this has determined that dopamine within the nucleus accumbens modulates the learning of behavioral responses of social defeat. Our finding is also consistent with the prevailing hypothesis that dopamine acts as a signal of salience (Bromberg-Martin, Matsumoto et al. 2010). The present results are also a beginning step in determining whether or not dopamine receptor modulation could be used as a treatment for the prevention of traumatic memories after social stress or other psychological stressors.

*Role of the nucleus accumbens in the expression of behavioral responses to social defeat*

D\(_1\)/D\(_2\) receptor antagonist cis(z)flupenthixol infused prior to testing with a non-aggressive intruder increased aggression. We cannot say that dopamine modulates the expression of submissive behavior after social defeat in the present study, as submission in the animals given vehicle control injections were too low to allow us to observe a reduction. But, in combination
with our earlier finding that muscimol given before conditioned defeat testing reduces submission ((Luckett, Norvelle et al. 2012)), we can say that the nucleus accumbens is, at least in part, responsible for the inhibition of aggression after social defeat as well as the activation of submissive and defensive behavioral responses. Because the nucleus accumbens is often described as the ‘gatekeeper’ to the motor system (Groenewegen, Wright et al. 1996, Horvitz 2002), the nucleus accumbens could be the area where limbic regions converge to affect expression of aggressive behavior after defeat. Thus, it is possible that when neuronal activation is impaired with muscimol or a dopamine receptor antagonist, inhibition of downstream areas by the nucleus accumbens does not occur and aggression is expressed. This finding is notable in that this is the first brain region that we have identified that is appears to be necessary for inhibiting the species-typical aggressive response to the presence of an intruder after social defeat. Previous manipulations within the other brain areas in the neural circuit of defeat have only been effective in altering the duration of submissive and defensive behaviors following defeat and have not effected aggression. This finding also suggests, within a neural circuit mediating complex behavioral responses to social experience, that different components of the circuit may mediate different aspects of the memory of that event and may thus control different subsets of the behavioral output.

Activation of the ventral tegmental area by acute social defeat

Acute social defeat did not significantly increase Fos protein activation of dopaminergic neurons within the VTA as compared to controls, but defeat did increase the activation of non-dopaminergic neurons in the VTA. Given our results discussed above, this finding was surprising and suggested to us that dopamine from another source, such as the substantia nigra, might be modulating conditioned defeat. Visual examination of the substantia nigra, however,
did not reveal much, if any, Fos activation and given that this region was not previously implicated in activation by stress (Deutch, Tam et al. 1985), it does not appear likely that this brain region is involved. The mesolimbic dopamine system, however, has been studied in chronic social stress, and dopamine was found to be critical for social avoidance after chronic social defeat (Chaudhury, Walsh et al. 2013). Perhaps chronic, but not acute, social defeat increases activation of the mesolimbic dopamine system. It could also be that the window of Fos activation of dopaminergic neurons occurs earlier or later than when we perfused (one hour after defeat) meaning that we missed the critical time period. This was, however, the time point that was successful in the past in our laboratory to find changes in social stress-induced Fos protein activation within the basolateral amygdala (Markham, Taylor et al. 2010) and we did observe significantly higher Fos in defeated hamsters than in the non-defeated controls. Future studies are planned to determine the source of dopamine within the nucleus accumbens that modulates memory formation during acute social defeat.

**Effect of infusion of the GABA_B receptor agonist baclofen into the VTA on Fos activation in the nucleus accumbens and basolateral amygdala**

Baclofen, a GABA_B receptor agonist, had no effect on Fos activation in efferent limbic brain areas to the ventral tegmental area, most notably the nucleus accumbens. We had hypothesized that baclofen would reduce Fos activation in brain areas to which the VTA projects, as dopamine is hypothesized to increase neuronal activation of these regions. Surprisingly, we also did not observe the defeat-induced increase in Fos protein within the basolateral amygdala that we found previously, as non-defeated and defeated animals evidenced similar levels of Fos activation. This perhaps was because the non-defeated animals included in
this study were handled and were given a baclofen injection, thus there may have been an interaction between baclofen infusion and Fos activation irrespective of defeat experience.

*What do our results mean?*

We began these experiments with the hypotheses that dopamine enhances long-term potentiation and/or neuronal activation of the nucleus accumbens and the basolateral amygdala during social defeat. We also hypothesized that the source of this dopamine would be the ventral tegmental area (VTA) and that defeat would increase activation of dopaminergic neurons in the VTA (see Figure 5.1). We found that dopamine in the nucleus accumbens modulates acquisition and expression of behavioral responses to social defeat and that GABA in the nucleus accumbens modulates expression of behavioral responses to defeat. We had originally hypothesized that dopaminergic neurons within the ventral tegmental area are activated by defeat, and therefore there should be an increased activation of dopaminergic neurons in the VTA. We did not observe this increase when examining Fos activation of dopaminergic neurons during defeat, and activation of GABA B receptors within the VTA had no effect on Fos in efferent regions to the VTA, namely the nucleus accumbens and amygdala. We know that, at least in the nucleus accumbens, our hypotheses regarding the effect of dopamine were correct as far as the effect of dopamine on acquisition of social defeat. It is still unclear if our hypotheses regarding dopamine’s effect in the basolateral amygdala and the source of dopamine for the behavioral effects are supported. We still do not know the source of dopamine that is necessary in the nucleus accumbens for acquisition of defeat. Future studies should examine these questions in more detail (see future directions).
How can our results help human psychopathologies?

Disorders such as schizophrenia (van Winkel, Stefanis et al. 2008), post-traumatic stress disorder (Drury, Theall et al. 2009) and depression (Camardese, Di Giuda et al. 2014) are associated with dopaminergic dysregulation. It is evident, based on our present results, that dopamine modulates learning and memory as well as expression of social stress-induced behavioral responses. Dopamine could therefore be an alternative treatment in the future for these disorders, especially those that arise from or are exacerbated by social stress. In understanding the action of dopamine in several regions that we have found to be critical in our model, we can determine the effect that drugs that affect dopamine would have throughout the brain when used in humans. For example, it is possible that drugs that affect dopamine signaling after trauma could interfere with memory processes after stress to prevent development of PTSD or to ameliorate some of the behavioral responses to stress observed after the onset of PTSD, such as social withdrawal and generalization of fear.

Future directions

Nucleus accumbens

The experiments conducted for this dissertation illustrate that the nucleus accumbens modulates social stress-induced behavioral changes. However, the mechanisms of how the nucleus accumbens acts within the broader circuitry to modulate behavioral responses to social defeat remain unclear. We determined that the non-specific dopamine receptor antagonist cis(z)flupenthixol infused into the nucleus accumbens reduces the acquisition of social stress-induced behavioral changes. To complete our understanding of the role of dopaminergic receptor activation for conditioned defeat, we should specifically block D1 and/or D2 receptors...
during acquisition using SCH-23390 and raclopride, respectively. D1 receptor activation, in particular, is predicted to be necessary for the acquisition and/or expression of behavioral responses to social defeat stress due to the activation of adenylyl cyclase via D1 receptors (Podda, Riccardi et al. 2010), which previous studies determined is critical for long-term potentiation and therefore memory (Wong, Athos et al. 1999).

We previously found that the basolateral amygdala is the only brain area tested so far that mediates the acquisition of conditioned defeat and appears to be the ‘focal’ point for the memory of social defeat because blockade of protein synthesis within the basolateral amygdala blocks the formation of conditioned defeat. Because the present study revealed that the nucleus accumbens also modulates the acquisition of conditioned defeat, it would be informative to infuse an inhibitor of protein synthesis, such as anisomycin, prior to defeat. Protein synthesis is necessary for synaptic plasticity, so if anisomycin infused before defeat into the nucleus accumbens affects the acquisition of social defeat, this would support the hypothesis that synaptic plasticity is also taking place within the nucleus accumbens during social defeat. Either way, the finding would help answer the question of whether the plasticity underlying social stress-induced memory formation occurs within a single node of the circuit mediating this response or occurs simultaneously in more than one node.

We did not find an increase in activation by defeat of dopaminergic neurons within the VTA, the main source of dopamine to the nucleus accumbens. This could be because the fast, phasic responses of dopamine neurons in response to salient cues (Anstrom, Miczek et al. 2009, Park, Aragona et al. 2010) were not revealed by our relatively crude endpoint, Fos activation. An alternative approach to examining this question could be to measure dopamine on a subsecond timescale using fast-scan cyclic voltammetry (FSCV) immediately after a brief defeat
encounter when a barrier separates the defeated animal and its aggressor. This would allow for recording of dopamine levels during a threatening social encounter without a danger of a physical encounter damaging the recording equipment. Another option would be to record dopamine levels with FSCV during expression of conditioned defeat with a non-aggressive intruder that was caged in a mesh box. The caged intruder method was used previously in our laboratory to determine if defeated hamsters could discriminate between familiar and non-familiar aggressors (McCann and Huhman 2012). We hypothesize that a spike in dopamine in the nucleus accumbens precedes expression of submissive and defensive behavior. If this method is successful, FSCV could be used to determine levels of dopamine in other regions, including the amygdala.

**Amygdala**

We did not find evidence for an effect of baclofen infusion into the ventral tegmental area on Fos activation in the basolateral amygdala. However, due to the nondiscriminatory nature of dopamine on inhibitory interneurons as well as on pyramidal projection neurons, we do not know if this means that dopamine has no effect on neuronal activation. We hypothesized that dopamine increases synaptic plasticity in the amygdala by reducing inhibition from GABAergic interneurons and by enhancing neuronal activation in pyramidal projection neurons. To determine if dopamine does affect neuronal activation in the basolateral amygdala, we should infuse dopamine into the basolateral amygdala prior to a subthreshold defeat experience to determine if this manipulation enhances the acquisition of social defeat. This would indicate that dopamine does indeed act on regions throughout the neural circuit that modulates social defeat, and not only the nucleus accumbens.
Infralimbic/prelimbic cortex

Dopamine is hypothesized to act in an opposite manner within the infralimbic cortex (IL) than in the nucleus accumbens by disinhibiting regions downstream of the IL. The infralimbic cortex is often referred to as the ‘brake’ on the limbic system (Peters, Kalivas et al. 2009), as excitatory neurons from this region project to inhibitory GABAergic neurons in the amygdala (Quirk, Likhtik et al. 2003, Berretta, Pantazopoulos et al. 2005), and so inhibition of this region would cause increased activation of the BLA. In fact, we have shown that muscimol infusion into the infralimbic cortex enhances acquisition of conditioned defeat (Markham, Luckett et al. 2012). We predict that infusion of the dopamine receptor cis(z)flupenthixol into the infralimbic cortex should have a similar effect to that of muscimol if dopamine does, indeed, modulate neuronal activation of the infralimbic cortex during defeat.

Ventral tegmental area

It is still unclear what brain region is the source of dopamine to the nucleus accumbens during acquisition of social defeat. Our proposed use of FSCV described above would determine if there are indeed increases in dopamine during defeat or during expression of behavioral responses to social defeat. This will not tell us, however, what area is supplying the dopamine needed in the nucleus accumbens for acquisition of social defeat. An alternative method of testing our hypothesis is to stimulate the ventral tegmental area to see if that increases the acquisition of social defeat. It was previously determined that corticotropin-releasing factor (CRF) acts on dopaminergic neurons within the VTA to enhance behavioral responses to chronic social defeat (Boyson, Miguel et al. 2011). CRF is also necessary for acquisition of social defeat
(Cooper and Huhman 2010), but the exact area on which CRF acts remains unclear. If CRF increases dopamine in the nucleus accumbens by activating the VTA, then infusion of CRF before a subthreshold defeat should also increase the display of submissive and defensive behavior the following day. These additional studies could serve to further illuminate how components of the neural circuit mediating conditioned defeat act to control the acquisition and expression of this interesting behavioral response to social defeat stress.
1. CRF released by hypothalamic neurons activates dopaminergic neurons within the VTA and sensory stimulation activates the BLA.

2. Concurrent activation of the BLA via dopamine and glutamate enhances salience of stimulus:
   a. Facilitates signaling to downstream areas via increasing responsiveness of neurons to stimulus inputs.
   b. Facilitates LTP as dopamine decreases inhibition by interneurons.

3. Dopamine acts similarly within the NAcc to facilitate neuronal signaling and LTP.

4. NAcc projects to regions of the defensive behavior circuit (PAG and LHa) and therefore can facilitate behavioral responses to defeat.

5. NAcc projects to regions of the defensive behavior circuit, facilitating neuronal signaling and LTP.

6. Dopaminergic neurons within the VTA activate sensory stimuli leading to defeat or testing experience.
Figure 5.1. Summary of proposed mechanisms for how dopamine modulates the memory of and expression of behavioral responses to social defeat.

REFERENCES


