BDNF Knockdown in the VTA Blocks Social Stress-Induced Deficits in Social Behavior and Nucleus Accumbens ΔFosB Expression

A thesis submitted to the University of Arizona College of Medicine – Phoenix
in partial fulfillment of the requirements for the degree of Doctor of Medicine

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Class of 2016

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Abstract

Social defeat stress, a salient stressor that translates readily from animal studies to humans, alters social approach behavior and induces brain-derived neurotrophic factor (BDNF) in the ventral tegmental area (VTA), as well as the stable transcription factor, ΔFosB, in the nucleus accumbens (NAc) of rats. However, it is unknown whether VTA BDNF is required for these effects of stress. Rats underwent stereotaxic surgery to receive bilateral intra-VTA infusions of adeno-associated virus inducing green fluorescent protein (AAV-GFP) or GFP and short hairpin RNA directed against BDNF (shRNA-BDNF). Following recovery, rats were subjected to control handling or social defeat stress, consisting of a brief confrontation between an aggressive resident and an experimental intruder rat every third day for 10 days. Social interaction was assessed in a social approach assay two weeks later. Following perfusion, brains were removed and processed for immunohistochemical analysis of ΔFosB expression. VTA BDNF knockdown attenuated the effect of social stress on weight gain, and increased social approach behavior, which is normally reduced by social stress. Furthermore, social stress increased NAc ΔFosB labeling in AAV-GFP rats, but this effect was blocked by prior shRNA-BDNF treatment. This study further implicates VTA BDNF signaling in the effects of stress on social behavior. VTA BDNF appears to be required for the long-lasting effects of social stress on ΔFosB expression in the NAc. Thus, activation of BDNF signaling in mesolimbic circuits may underlie the persistent deficits of social behavior induced by stress exposure in some individuals.

Table of Contents

List of Figures and Tables

Figures

Tables

Abbreviations

AMPH – amphetamine

AAV-GFP – adeno-associated virus encoding a green fluorescent protein

AAV-GFP-shRNA-BDNF – adeno-associated virus encoding a green fluorescent protein and short hairpin RNA targeted at BDNF

BDNF – brain-derived neurotrophic factor

ΔFosB – deltaFosB, a truncated splice variant of FosB

DMEM – Dulbecco's Modified Eagle Medium

IHC – immunohistochemistry

NAc – nucleus accumbens

PBS – phosphate buffered saline

PFA – paraformaldehyde

TrkB – tropomyosin receptor kinase B

VTA – ventral tegmental area

Introduction

Social stress is a salient stressor in rats: one that plays a significant role in the evolution of behavior and readily translates to humans[1]. Exposure to such stress can be maladaptive, producing anxiety-like or depressive-like behavioral changes[1,2,3,4,5,6].

A key component thought to be involved in the response to stress is the brain's reward system, or the mesolimbic dopaminergic system. This pathway acts as the rheostat of the reward system by measuring the amount of dopamine released in response to a stimulus[7,8]. Anatomically, this neurological circuit contains nerve cells that originate in the ventral tegmental area (VTA) and send axonal projections to the nucleus accumbens (NAc)[8]. It is thought that, evolutionarily, this pathway helps reinforce pro-survival behaviors, such as eating and sex. Studies have shown that this system undergoes a variety of structural changes in response to social stress[9,10,11].

Modulation of neuroplasticity

Brain-derived neurotrophic factor (BDNF) is believed to influence such neuroadaption due to its role in regulating synaptic plasticity. Found in high concentrations in the VTA, BDNF binds to TrkB receptors, which leads to receptor dimerization and autophosphorylation that activates intracellular signaling cascades. Approximately 50% of VTA neurons co-express both tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, and BDNF. BDNF is thought to play a major role in sensitization by enhancing survival of dopamine neurons[12], thereby promoting dopamine release and metabolism in the NAc[13]. BDNF has been found to be elevated in the VTA in response to intermittent social defeat stress exposure, where an experimental animal is subjected to aggressive confrontation with a counterpart[12,14,15]. Furthermore, knockdown of TrkB receptors prevents stress-induced cross-sensitization to amphetamine[17].

BDNF is thought to regulate gene transcription through activation of transcription factors, which are proteins that bind to promoter regions of target genes and regulate their expression. Generally, the Fos family proteins are induced quickly and transiently most prominently in the NAc in response to stress as well as drugs of abuse[17, 18, 19]. Normally highly

unstable, ΔFosB, a member of the Fos family, is unique in that it has an extraordinarily long half-life and can persist in neurons for several weeks, leading to accumulation in the NAc with repeated stress exposure, making ΔFosB a useful marker of neuroplasticity in investigative studies[20, 21, 22].

Stress-Induced Behavioral Changes

There is also evidence that intermittent social defeat stress induces chronic alterations in social behavior. For example, rats that were previously exposed to social defeat stress will exhibit reduced social interactions with a novel conspecific animal[14]. In contrast, bilateral knockdown of intra-VTA BDNF alleviates the effects of stress for weeks following exposure[15]. BDNF is also thought to play an important role in weight regulation. Mice that consume palatable food have elevated levels of BDNF in the VTA; however, this finding is not present in mice consuming standard chow[23]. This suggests that the elevated levels of BDNF in the VTA are related to the rewarding rather than consummatory properties of eating[24]. In rats, intra-VTA depletion of BDNF prevented the reductions in body weight, which are typically seen following social defeat stress[16].

The effects of stress extend beyond the behavioral changes of deficits in weight regulation and social interaction. Exposure to stress also increases the vulnerability to substance dependence in the future, which translates readily to humans. For example, stressful life events increase vulnerability to drugs of abuse, particularly to stimulants[25]. Thus, understanding neural plasticity in the VTA holds great promise for the development of therapies for addicted individuals.

Overall, we hypothesize that knockdown of BDNF within the VTA will prevent both the neuroadaptive as well as the behavioral changes that occur following exposure to social stress. Specifically, we anticipate that knockdown of BDNF will attenuate ΔFosB levels within the NAc and prevent deficits in social behavior and weight regulation.

Research Materials and Methods

Subjects

Subjects were male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA and Hollister, CA, USA), weighting 200 – 225 g upon arrival. Rats were singly-housed in standard plastic cages (55 x 31 x 21 cm) under a reverse light-dark cycle (12h:12h, lights off at 9:00h) with unlimited access to food (Purina Rodent Diet, Brentwood, MO, USA) and water, and habituated for one week in their home cages before undergoing surgery. In a separate room, male Long-Evans rats (Charles River), termed "residents," were pair-housed with females, who had undergone prior tubal ligation to prevent pregnancy, and were used to inducing social defeat stress in Sprague-Dawley male rats. Residents were screened for aggressive behavior towards an intruder rat prior to social defeat procedures to ensure adequate performance during experiment. All experimental procedures were approved by Arizona State University Institutional Animal Care and Use Committee, and conducted in accord with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2003).

General Procedure

After adaptation to laboratory conditions 32 rats were randomly assigned to one of four groups (Figure 1). Ten days after surgery, experimental rats (groups 1 and 2) were exposed to intermittent social defeat stress every third day for ten days (4 defeats total) and control rats (groups 3 and 4) were handled at the same times inside the resident room but were not exposed to a resident. Eighteen days later rats underwent social avoidance testing with a previously encountered resident. Tissue was harvested fourteen days later (Figure 2).

Figure 1 – Overview of Experimental and Control Groups

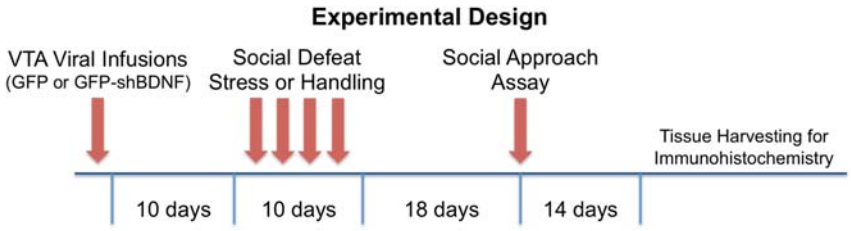

Figure 2 – Overview of Experimental Timeline

Surgery

Rats underwent stereotaxic surgery under isofluorane gas anesthesia to receive bilateral intra-VTA (AP -5.2, ML ±2.15, DV -8.7 from bregma; tilt = 10º) infusions of 0.5 µL of either AAV-GFP or AAV-GFP-shRNA-BDNF. Viruses were infused with a Hamilton syringe at a rate of 0.05 µL/min. Wounds were closed with bone wax and surgical glue. Rats were allowed 10 days for recovery from surgery prior to undergoing behavioral procedures.

Virus Vector

Vectors, packaged in plasmids providing AAV2 replicase and AAV9 caspid functions, and a third plasmid encoding Adenovirus helper functions (pHelper; Stratagene, La Jolla, CA), were co-transfected into AAV-293 cells (Stratagene), molar ratio 1:1:1. Vector plasmids, wherein shRNAs were flanked between a hybrid chicken beta-actin promoter with CMV-IE enhancers, and bGH polyA, were initially provided by Ronald Klein, LSUHSC. shRNA was under control of a Pol III murine U6 promoter. The cDNA sequence was ACG-GTCACAGTCCTGGAGAAATTTGACGGTCACAGACCTGGAGAAATTCAAGAGATTTCTCCAGGACTGTGA CCGTTTTTTCTAGAAAAAACGGTCACAGTCCTGGAGAAATCTCTTGAATTTCTCCAGGTCTGTGACCGT. AAV Vectors containing shRNAs also included coding information for eGFP, controlled by a viral promoter. A similarly packaged AAV-eGFP plasmid was used as a control virus. Cells were harvested 48 hr post-transfection, and cell pellets were re-suspended in DMEM. Intracellular virus particles were released by three consecutive rounds of freeze-thaw, followed by centrifugation at 13,000 rpm for 10 min. Vector stocks were stored at 80ºC, and tittered by real-time PCR (ABI Prism 7700 Sequence Detection System; Perkin-Elmer Applied Biosystems, Foster City, CA). Titers were approximately 10^{12} DNase Resistant Particles/mL.

Intermittent social defeat stress

Experimental rats were defeated as described previously[10,12,14,16,26]. After removing the female from the resident's cage 30 min prior to social stress exposure, the experimental intruder rat was placed into the home cage of a resident male rat. For the first 5 min, the intruder rat remained under a stainless steel protective cage (25 x 15 x 15 cm) in order to expose the intruder to threats from the resident. The protective cage was then removed and the resident attacked the intruder within 30 – 120 sec. Defeat of the experimental rat was identified as the display of a submissive supine posture for at least 4 seconds, which usually occurred following 4-5 bites within a maximum of 5 mins. (Figure 3) Following display of this supine posture the intruder rat was placed under the protective cage within the resident's cage and remained there for an additional 20 min. Intruder rats were not never exposed to the same resident twice and were promptly returned to their home cage following each defeat.

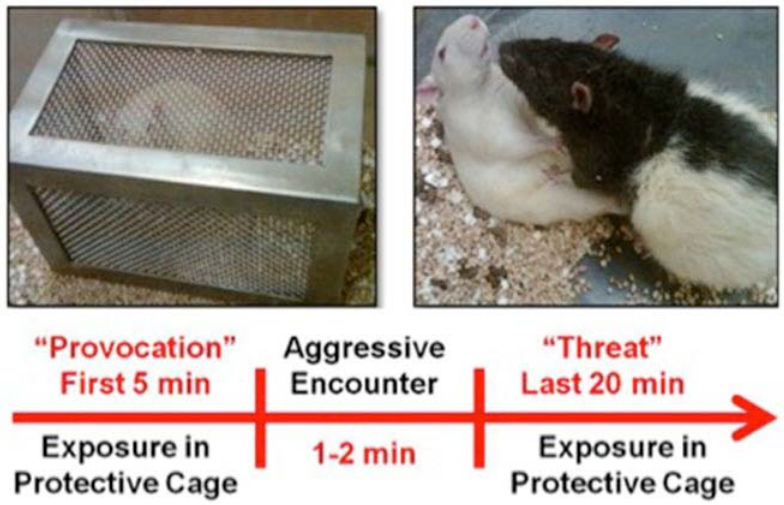

Figure 3 – Overview of Social Defeat Paradigm
Left: Rat in protective cage; Right: Attacks by the resident persisted until the animal reliably engaged in subordinate behavior; Bottom: Timeline of events.
Images/figures courtesy of C.E. Johnston.

Behavioral effects

Weight analyses for stress-induced weight changes were undertaken for only the 10-day stress or handling period. This is because stress-induced weight changes have not been found to persist past cessation of social defeat exposure[14]. Eighteen days after the last social defeat episode (5 weeks after surgery), we assessed social approach behavior in rats using a procedure adapted from Berton and colleagues[2]. Rats were exposed to a previously encountered resident rat, which was placed under a wire mesh cage (25 x 15 x 15 cm) against the short wall of a clear plastic open field (48.3 x 37.5 x 21.0 cm). Each experimental rat was introduced into the arena and its trajectory was tracked for two 5 min consecutive sessions. During the first session, the arena contained an empty wire mesh cage. Conditions were identical in the second session except that a resident rat was placed into the wire mesh cage. Videotracking data (Clever Systems, Inc. Reston, VA) reflected time spent by the experimental rat in the "cage zone" (roughly 2/3rds of the arena) and "avoidance zone" (roughly 1/3 of the arena) (Figure 4).

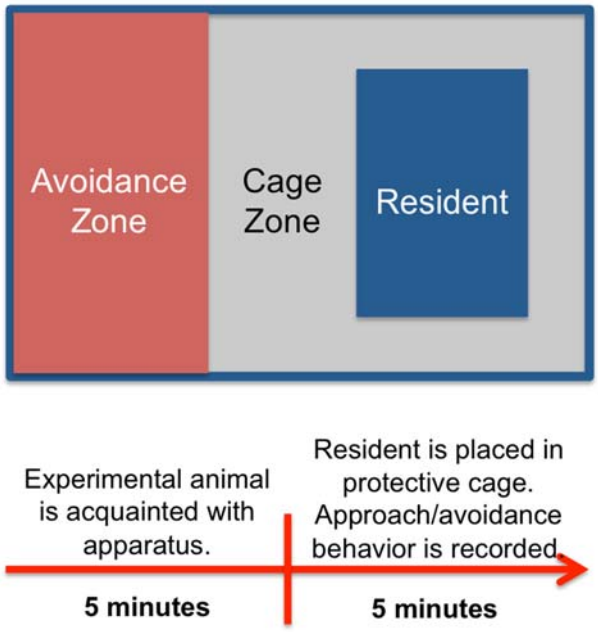

Figure 4 – Overview of Social Approach Assay
Top: Apparatus setup; Bottom: Timeline of events

Tissue harvesting

Rats were euthanized 4.5 weeks after behavioral experiments (7.5 weeks after surgery). They were anesthetized and perfused transcardially with 10 mL of 10% heparin in 0.1 M phosphate buffered saline (pH 7.4) followed by 200 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Following rapid decapitation, brains were removed and post-fixed for 1.5 h at 4º C. 20 µm coronal sections of the VTA and NAc were taken in a cryostat at 20ºC and mounted onto glass slides (Superfrost Plus; Fisher Scientific) and stored at -35ºC. Sections were visually inspected for GFP fluorescence to confirm accuracy of viral injections (Figure 5).

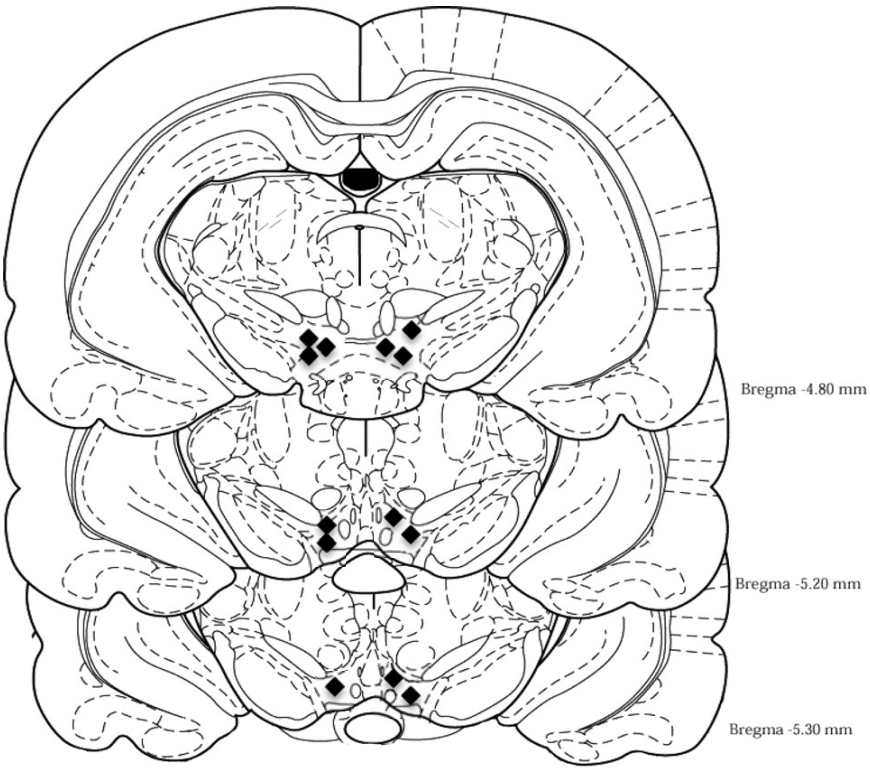

Figure 5 – Validation of Viral Infusion Location
GFP expression was measured post-surgery validating the placement of AAV into the VTA.

Cell labeling

Sections were thawed and washed in 0.05 M potassium phosphate-buffered saline (KPBS). They were blocked for 1 h in 5% normal goat serum (NGS) and 0.4% Triton X-100 in KPBS and then subsequently incubated with ΔFosB (1:111 dilution, Santa Cruz, SC-48) rabbit antisera for 48 h at 4ºC. This was followed by 1 h incubation in biotin-conjugated goat anti-rabbit serum (1:40 dilution in NGS, Vectastain ABC kit; Vector Laboratories, Burlingame, CA), washing, and then 45 min incubation with an avidin-biotin-peroxidase complex, then processing with nickel-intensified DAB using substrate kit (Vector).

Image analysis

Immunohistochemical labeling was analyzed using a Zeiss Axioskop microscope and Stereo Investigator software (MBF Bioscience, Willingston, VT). A systematic random sampling approach using a stereological grid was employed to analyze at least 3 sections in the NAc shell and core. Images in selected areas in each region were digitized using a camera interfaced to the microscope using a 20x objective. Stereo Investigator software partitioned each image into 16 equal counting frames (100 x 75 µm each), half of which were randomly selected and analyzed. The number of labeled neurons was counted separately for each frame, excluding any overlapping labeled profiles on the left and bottom borders. The density of labeled profiles was averaged together for three sections of each brain region by dividing the estimated total density of labeled profiles by the numbers of analyzed areas. Labeling density was calculated by dividing the estimated total number of cells by the total area measured for conversion to the number of labeled cells per mm^2. ΔFosB-labeled profiles were identified by a dark gray-stained nucleus and a profile was considered labeled if its pixel luminance was more than 2 standard deviations different from the background luminance as calculated by Stereo Investigator software.

Statistics

Weight gain, social interaction and ΔFosB labeling were analyzed by one-way ANOVA. Weight in grams measured at the first and last (fourth) defeat. The mean difference between the two time points was compared for each group. Social interaction was measured as the percent of time spent in the interaction zone with and without a resident animal present in the cage. The mean difference in the percent of time spent in the cage zone when the resident was present or was compared for each group. Finally, the mean number of ΔFosB-labeled cells per mm^2 in the NAc core and shell for each group were compared.

Results

ΔFosB Expression in the NAc

The mean numbers of ΔFosB-labeled cells per mm^2 within the NAc core and shell for each group are indicated in Table 1.

ΔFosB-Labeled Cells

	Core Mean (SD)	P-Value	Shell Mean (SD)	P-Value
GFP + Stressed	129.0 (32.3)	<0.001	55.0 (18.7)	0.003
GFP + Handled	51.6 (10.9)		26.5 (10.6)	
BDNF shRNA + Handled	37.2 (21.0)		36.4 (19.6)	
BDNF shRNA + Stressed	26.2 (13.0)		37.0 (18.8)	

Table 1 – ΔFosB-labeled cells in the NAc core

The GFP + Stressed group had a statistically significant increased cell count in both areas when compared to other groups (p-value <0.001 and 0.003 in the core and shell, respectively).

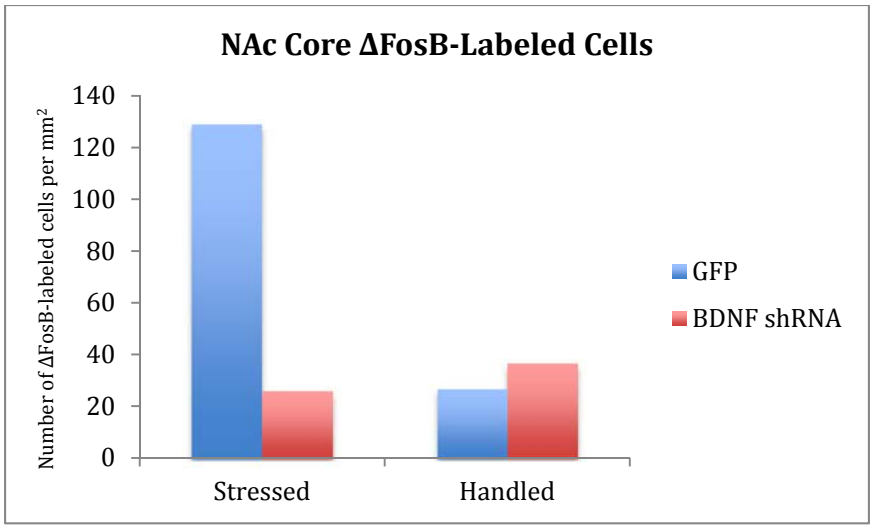

Figure 6 – Knockdown of VTA BDNF prevents social stress-induced increases in NAC core ΔFosB. After undergoing exposure to social stress or handling, GFP-Stressed rats (n = 8) exhibited significantly (** - $p < 0.001$) less ΔFosB expression than did GFP-Handled (n = 8), BDNF-shRNA Stressed (n = 8), or BDNF-shRNA Handled (n = 8) rats. The mean number of ΔFosB-labeled cells per mm^2 was identified by pixel lumination using Stereo Investigator software.

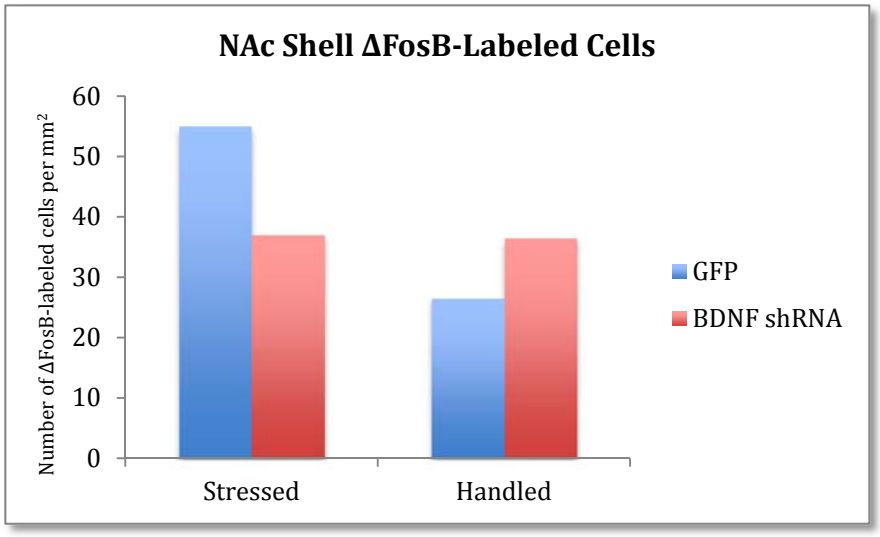

Figure 7 – Knockdown of VTA BDNF prevents social stress-induced increases in NAC shell ΔFosB. After undergoing exposure to social stress or handling, GFP + Stressed rats (n = 8) exhibited significantly (** - p = 0.003) less ΔFosB expression than did GFP + Handled (n = 8), BDNF shRNA + Stressed (n = 8), or BDNF shRNA + Handled (n = 8) rats. The mean number of ΔFosB-labeled cells per mm^2 was identified by pixel lumination using Stereo Investigator software.

Rat Brain Anatomy

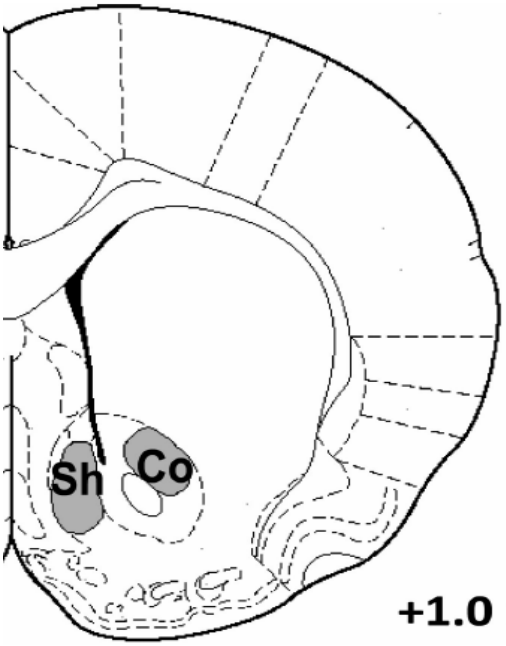

Figure 8 – Normal rat brain anatomy at +1.0 from bregma
NAc Sh = shell; Co = core

Weight Regulation

The mean grams gained between the first and fourth defeats or handling sessions is described in Table 2.

Weight Gained

Group	Weight before 1st Defeat	Weight before 4th Defeat	Difference	P-Value
	Mean (SD)	Mean (SD)	Mean (SD)	
GFP + Stressed	336.4 (17.0)	364.5 (22.4)	28.1 (9.6)	0.006
GFP + Handled	339.5 (19.6)	386.8 (26.2)	47.3 (10.5)	
BDNF shRNA + Handled	340.9 (24.4)	391.6 (17.1)	50.8 (13.1)	
BDNF shRNA + Stressed	358.4 (26.2)	405.4 (43.1)	47.0 (19.8)	

Table 2 – Mean difference weight gained between 1st and 4th defeats

All animals gained some amount of weight during the social defeat phase of the experiment; however, the GFP + defeat group gained less weight than the other three groups, which was statistically significant (p-value = 0.006).

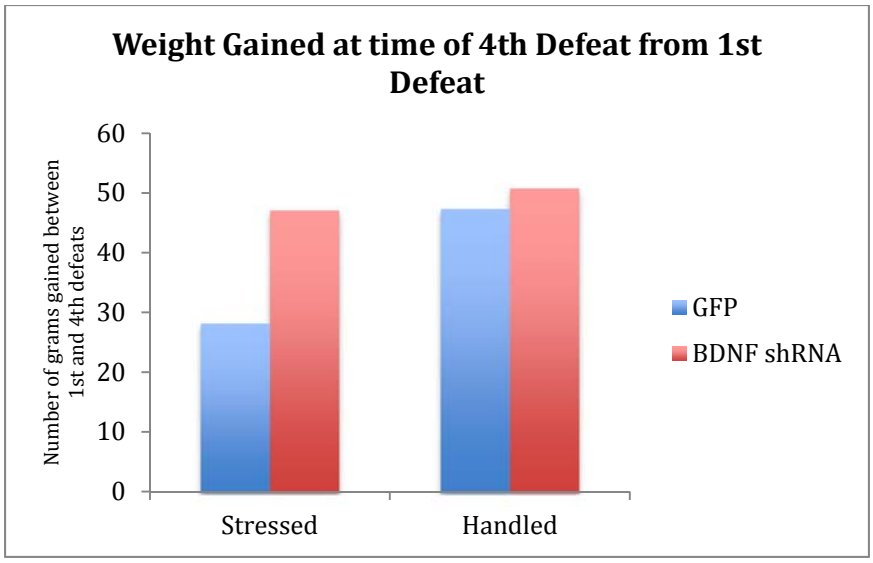

Figure 9 – Knockdown of VTA BDNF prevents social stress-induced deficit of weight gain. While undergoing social stress or handling, GFP + Stressed rats (n = 8) exhibited significantly (** - $p < 0.006$) less weight gain than did GFP + Handled (n = 8), BDNF shRNA + Stressed, or BDNF shRNA + Handled (n = 8) rats. Weight was taken at time of first and fourth defeat, which was 10 days apart.

Social Interaction

The mean percent of time spent in the interaction zone is shown below for each group in Table 3.

Percent of Time Spent in Interaction Zone

Group	Without Resident	With Resident	Percent Difference	P-value
	Mean (SD)	Mean (SD)	Mean (SD)	
GFP + Stressed	73.3% (9.9)	69.0% (18.0)	-4.3% (22.6)	0.04
GFP + Handled	55.8% (15.9)	79.8% (13.1)	+24.0% (20.8)	
BDNF shRNA + Handled	61.6% (8.5)	79.6% (7.8)	+15.0% (11.8)	
BDNF shRNA + Stressed	67.1% (17.4)	79.7% (10.1)	+12.6% (18.8)	

Table 3 – Percent of time spent in interaction zone

All but the GFP + Stressed group spent more time in the interaction zone when the resident was present. In contrast, the GFP + Stressed group spent less time in the interaction zone, which was statistically significant ($p = 0.04$).

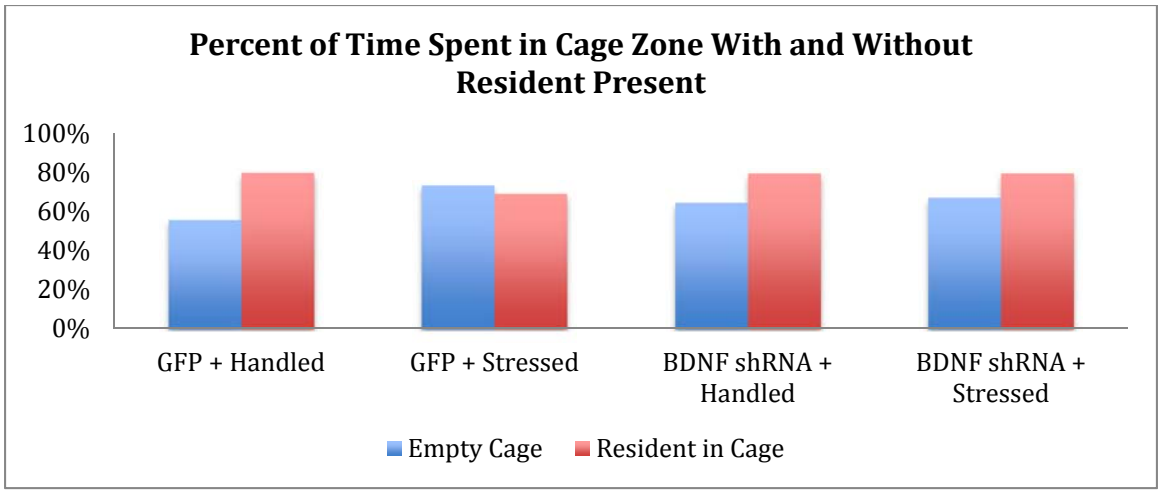

Figure 10 – Knockdown of VTA BDNF prevents social stress-induced social avoidance. GFP + Stressed rats (n = 8) spent significantly (* - $p < 0.04$) less time in the cage zone as compared to GFP + Handled (n = 8), BDNF shRNA + Stressed (n = 8), or BDNF shRNA + Handled rats (n = 8). Percent of time spent in cage zone when resident was present shark cage compared to when the resident was not present.

Discussion

The results reveal that viral knockdown of BDNF in the VTA partially attenuated weight deficits accompanying intermittent social defeat stress exposure, decreased social avoidance behavior, and reduced ΔFosB expression in the NAc.

Intermittent social defeat stress produces transient deficits in weight gain, according to our data. This effect was partially normalized by knockdown of BDNF within the VTA, suggesting that lower levels of intra-VTA BDNF may contribute to stress resilience. Fanous et al found similar results in their 2011 experiment, which was similar to ours[15]. In contrast, augmented intra-VTA BDNF has been implicated in an increased stress responsiveness in humans. For example, possessing the single nucleotide polymorphism resulting in a val^{66}met substitution in the BDNF peptide sequence leads to elevated BDNF release[27]. Men with the val^{66} allele have been found to have lower salivary cortisol response to social stress exposure[28]. Thus, a val^{66} polymorphism that results in decreased BDNF release may confer stress resilience.

Additionally, our results show that knockdown of intra-BDNF attenuated the social avoidance behavior that typically occurs with exposure to a previously encountered aggressive resident rat. These results are similar to what Fanous et al found in their 2011 study[15]. Thus, activation of BDNF signaling in mesolimbic circuits may underlie the persistent deficits of social behavior induced by stress exposure in some individuals.

Finally, our findings demonstrate that knockdown of intra-VTA BDNF led to reduced ΔFosB expression within the NAc. Prior experiments by Wang et al in 2013 found that increased levels of intra-VTA BDNF led to accumulation of ΔFosB in the NAc[16]. Together, these studies make BDNF a likely mediator of ΔFosB expression in the NAc. Thus, VTA BDNF appears to be required for the long-lasting effects of social stress on synaptic plasticity and reward-related behaviors.

Future Directions

The sample size for this experiment was small at eight animals per group. These results are similar to that of prior experiments, all of which contain similar sample sizes. Future experiments should attempt to reproduce these results with larger sample sizes. This experiment sought to elucidate the role of BDNF in the response to stress by knocking out the virus in the VTA. Additional studies may be done which explore the role of BDNF in response to stimulant administration, as this BDNF is also thought to be a key mediator of synaptic plasticity that occurs in substance dependence, specifically during sensitization.

Conclusion

VTA BDNF knockdown attenuated the effect of social stress on weight gain, and prevented social avoidance behavior that typically occurs following stress exposure. Furthermore, social stress increased NAc ΔFosB labeling in AAV-GFP rats, but this effect was blocked by prior shRNA-BDNF treatment. This study further implicates VTA BDNF signaling in the effects of stress on social behavior. VTA BDNF appears to be required for the long-lasting effects of social stress on ΔFosB expression in the NAc. Thus, activation of BDNF signaling in mesolimbic circuits may underlie the persistent deficits of social behavior induced by stress exposure in some individuals.

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