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Molecular Adaptations Underlying Susceptibility and Resistance to Social Defeat in Brain Reward Regions

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SUMMARY

While stressful life events are an important cause of psychopathology, most individuals exposed to adversity maintain normal psychological functioning. The molecular mechanisms underlying such resilience are poorly understood. Here, we demonstrate that an inbred population of mice subjected to social defeat can be separated into susceptible and unsusceptible subpopulations that differ along several behavioral and physiological domains. By a combination of molecular and electrophysiological techniques, we identify signature adaptations within the mesolimbic dopamine circuit that are uniquely associated with vulnerability or insusceptibility. We show that molecular recapitulations of three prototypical adaptations associated with the unsusceptible phenotype are each sufficient to promote resistant behavior. Our results validate a multidisciplinary approach to examine the neurobiological mechanisms of variations in stress resistance, and illustrate the importance of plasticity within the brain's reward circuits in actively maintaining an emotional homeostasis.

INTRODUCTION

An individual's emotional response to severe, acute stress (e.g., trauma, terrorist acts) or to more prolonged chronic stress (e.g., divorce, war-time torture) is determined by genetic and environmental elements that interact in complex and poorly understood ways (Charney and Manji, 2004; Nestler et al., 2002). While a vast literature describes the effects of several kinds of acute and chronic stress on an individual's physiology and behavior, much less is known about the biological basis of individual differences in stress responses (Yehuda et al., 2006). A majority of humans exposed to stressful events do not show signs of psychopathology such as posttraumatic stress disorder (PTSD) or depression (Charney, 2004; Kessler et al., 1995; Yehuda, 2004). These "resilient" individuals (Hoge et al., 2007) display traits such as cognitive flexibility (Yehuda et al., 2006) and optimism (Charney, 2004). However, the neural substrates and molecular mechanisms that mediate resistance to the deleterious effects of stress remain unknown.

Insight into the biology of variations in susceptibility can be gained by understanding models of individual differences in response to stress (Rutter, 2006). One such rodent model is social defeat, which has the ethological relevance of examining social subordination (Malatynska and Knapp, 2005), as well as face validity in its ability to model the symptomatology of stress-related disorders like PTSD and depression (Avgustinovich et al., 2005; Martinez et al., 1998). The development of social defeat in mice has also enabled the examination of the effects of specific genetic manipulations (McLaughlin et al., 2006). By employing a novel measure of social interaction, we recently showed that socially defeated mice demonstrate a long-lasting social avoidance that is reversed by chronic (but not acute) treatment with antidepressants (Berton et al., 2006; Tsankova et al., 2006). Social avoidance induced by chronic social defeat was dependent on brain-derived neurotrophic factor (BDNF) signaling in the mesolimbic dopamine circuit, which is composed of dopamine neurons in the ventral tegmental area (VTA) and their forebrain projection regions, in particular the

nucleus accumbens (NAc). This VTA-NAc circuit plays a critical integrative role in reward- and emotion-related behaviors (Nestler and Carlezon, 2006).

Here, we take advantage of a large variance in behavioral outcomes after social defeat in inbred c57bl/6 mice to study the molecular basis of susceptibility and resistance to emotional stress. We show that resistance to social defeat is latent, long lasting, extends across several behavioral and physiological domains, and is mediated by specific molecular neuroadaptations within the brain's mesolimbic dopamine reward circuit. We propose that our findings may model resilience, operationally defined as "the process of adapting well in the face of adversity" (Charney, 2004).

RESULTS

Segregation of Defeated Mice into Susceptible and Unsusceptible Populations

An episode of social defeat is accomplished by forcing a mouse to intrude into the space territorialized by a larger mouse of a more aggressive genetic strain, leading to an agonistic encounter that ultimately results in intruder subordination. We have previously shown that c57bl/6 mice subjected to chronic social defeat (10 such defeats over 10 days) display a long-lasting reduction in social interaction (Berton et al., 2006; Tsankova et al., 2006), which is measured by comparing the time a mouse spends in an interaction zone with a social target to the time in that zone in the absence of a social target. By analyzing a large number of chronically defeated mice, we found a wide distribution of responses: when examined 24 hr after the last defeat ("day 11"), 40%–50% of defeated mice displayed interaction scores similar to nondefeated controls. Because the vast majority of control mice spend more time interacting with a social target than an empty target enclosure, an interaction ratio of 100 (equal times in the presence versus absence of a social target) was set as a cutoff: mice with scores <100 were labeled "Susceptible" and those with scores $\geq$100 were labeled "Unsusceptible" (Figure 1A). This latter group displayed median and variance values similar to controls (see Table S1 in the Supplemental Data available with this article online).

Several further analyses support the validity of distinct Susceptible and Unsusceptible subpopulations. A frequency distribution histogram of absolute time spent interacting with a social target (Figure 1B) and a two-dimensional scatterplot comparing interaction times to the time spent in the corner zones of the arena (Figure S1B) also revealed a segregation of Susceptible mice from Unsusceptible and control mice. Figure 1C shows day 11 data from a representative experiment illustrating how only Susceptible mice actively avoid the target by spending more time in the corner zones. An awake behaving social target is necessary for social avoidance, as we observed a lack of avoidance to an anesthetized target (Figure 1D). Differences in susceptibility cannot be explained by locomotor behavior (equivalent in both subgroups; Figures S1C and S1D) or variations in aggression during defeat (both groups sustained the same degree of minor injuries). This avoidance phenotype was found to be long-lasting: when Susceptible and Unsusceptible mice were retested 4 weeks later, we observed a significant correlation between day 11 and day 39 interaction ratios ($r = +0.61$, $p < 0.0001$, $n = 44$).

Age, genotypic, or vendor differences cannot explain this type of variance, because only 9-week-old c57bl/6 mice from a single vendor were used. Control, Susceptible, and Unsusceptible mice displayed similar predefeat interaction ratios and body weights (Figures S1E and S1F). Because "risk-seeking" individuals are prone to stressful life events (Charney and Manji, 2004), we examined a cohort of mice on the open-field exploration test before social defeat. Once again, we found no significant differences between control, Susceptible, and Unsusceptible mice on a variety of open-field measures, including center duration and total distance traveled (Figures S1G and S1H). Thus, unsusceptibility appears to be a "latent" trait.

Susceptible and Unsusceptible Mice Display Distinct Syndromes

To examine whether resistance to defeat-induced social avoidance generalizes to other behavioral measures, we performed an extensive phenotypic characterization of Susceptible and Unsusceptible mice (summarized in Table 1 and Table S2). On day 11, only Susceptible mice displayed a significant decrease in body weight (Figure S2B) and sucrose preference ($F_{2,33} = 5.70$, $p < 0.01$; Figure 1E), both consistent with increased depression-like behavior. In contrast, both Susceptible and Unsusceptible mice showed an increase in anxiety-like behavior, spending significantly less time in the open arms of the elevated plus maze ($F_{2,76} = 5.23$, $p < 0.01$; Figure 1F). Similarly, both subgroups of mice demonstrated a sensitized corticosterone (CORT) response to a 6 min swim stress ($F_{2,35} = 12.34$, $p < 0.0001$; Figure 1G). To evaluate autonomic arousal and circadian function, we implanted a subset of mice with subcutaneous temperature transponders (Liu et al., 2003). Both Susceptible and Unsusceptible mice showed an anticipatory form of autonomic arousal during the course of social defeat: a significant elevation of body temperature in the 30 min prior to the onset of an expected defeat episode (Figure S2C). In contrast, only Susceptible mice demonstrated a significant reduction in the circadian amplitude of temperature fluctuations ($F_{2,79} = 3.21$, $p < 0.05$; Figure 1H) and a significantly elevated hyperthermic response to the social avoidance test ($F_{2,86} = 5.30$, $p < 0.01$; Figure 1I). Interestingly, only Susceptible mice displayed significant conditioned place preference to a low dose of cocaine (Figure S2D), demonstrating sensitization to psychostimulant reward. Collectively, these data show that the development of social avoidance in Susceptible mice is associated with a syndrome of hedonic changes, weight loss, and circadian abnormalities. In contrast, increases in anxiety and corticosterone reactivity are seen in both subgroups of mice.

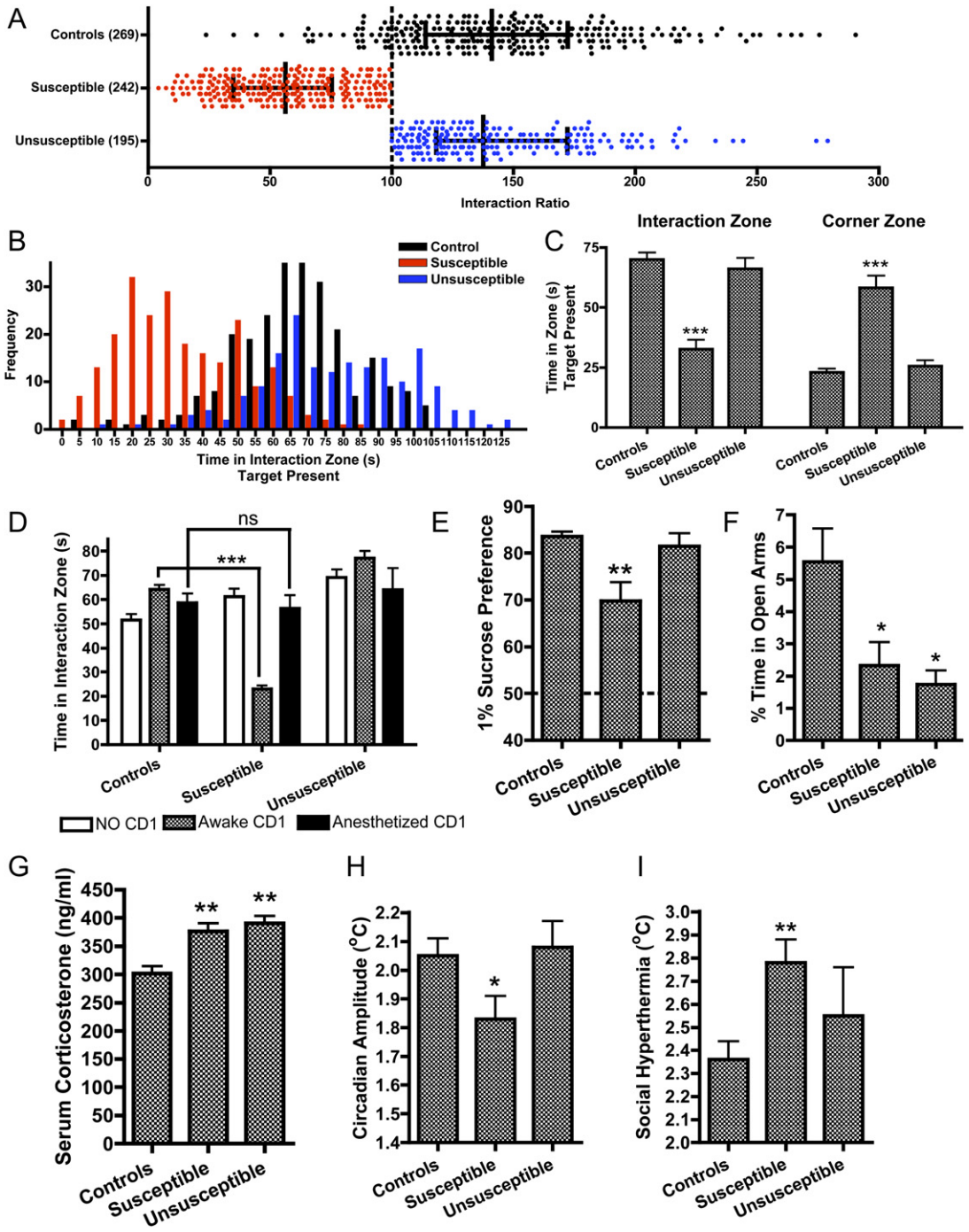


Figure 1. Identification of Susceptible and Unsusceptible Subgroups

(A) Horizontal scatterplot depicting the distribution of interaction ratios for control, Susceptible, and Unsusceptible mice over multiple social defeat experiments (error bars represent mean ± interquartile range). (B) Frequency distribution histogram for the absolute time spent in interaction zone. (C) In response to an aversive CD1 social target, only Susceptible mice avoid the interaction zone ($F_{2,54} = 33.20$, $p < 0.0001$) and prefer the corner zones ($F_{2,54} = 35.27$, $p < 0.0001$). (D) Interaction zone times for the three groups of mice in conditions of no target, an awake behaving CD1 target, and an anesthetized CD1 target (group × repeated-measure target interaction effect, $F_{4,54} = 2168.6$, $p < 0.0001$). (E) Only Susceptible mice display anhedonia as measured by a reduction in 1% sucrose preference, whereas both Susceptible and Unsusceptible mice display decreased exploration on the elevated plus maze test (F) and enhanced CORT response to a 6 min swim stress (G). Only Susceptible mice display blunted circadian rhythms (H) and significantly enhanced social hyperthermia ([I], an elevated hyperthermic response to the CD1 target). Bars represent mean + SE (standard error) with n = 10–20, * indicates significant post hoc differences with respect to nondefeated control mice, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 1. Susceptible and Unsusceptible Mice Display Distinct Syndromes

	Day 11		Day 39	
	Susceptible	Unsusceptible	Susceptible	Unsusceptible
Social avoidance	↑	↔	↑	↔
Anxiety-like behavior (time in closed arms)	↑	↑	↑	↑
Despair behavior (immobility on TST)	↔	↔	↔	↔
Despair behavior (immobility on FST)	↔	↔	N/A	N/A
Anhedonia (change in sucrose preference)	↓	↔	↔	↔
Cocaine-conditioned place preference	↑	↔	N/A	N/A
Stress-induced polydipsia (increased fluid intake)	↑	↑	↔	↔
Locomotor activity (ambulatory beam breaks)	↔	↔	↔	↔
Social hyperthermia	↑	↔	↑	↔
Circadian amplitude	↓	↔	↔	↔
Weight change	↓	↔	↔	↔
AM serum corticosterone	↔	↔	↓	↑
Swim-stress-induced corticosterone levels	↑	↑	N/A	N/A
AM serum DHEA-S	↔	↔	↔	↔
Cardiac hypertrophy (heart wt/body wt ratio)	N/A	N/A	↔	↑

This table illustrates the phenotypic differences between Susceptible and Unsusceptible mice on day 11 and also shows which of those phenotypes persist 4 weeks later (day 39). TST, tail suspension test; FST, forced swim test; DHEA-S, dehydroepiandrosterone-sulfate; Wt, weight; ↔, ↑, and ↓ (no change, significantly greater than, or less than nondefeated control group [$p < 0.05$], respectively); N/A, not available.

Increased BDNF Signaling within the NAc Mediates Susceptibility

Because chronic social defeat increases BDNF protein levels in the NAc on days 11 and 39 (Berton et al., 2006), we tested whether this response differs between Susceptible and Unsusceptible mice. Western blot analysis of NAc tissue 24 hr after avoidance testing revealed that only Susceptible mice demonstrated this BDNF increase, namely a 90% elevation in BDNF levels over controls ($F_{2,28} = 3.88$, $p < 0.05$), with no change in BDNF seen in the NAc of Unsusceptible mice (Figure 2A). We found no changes in levels of the full-length or truncated isoforms of the BDNF receptor, tropomyosin-related kinase B (TrkB.F or TrkB.T) or in levels of phospho-TrkB (Figure 2B). Consistent with an increase in NAc BDNF protein, we also observed a robust activation of signaling molecules downstream of TrkB (Chao et al., 2006). Susceptible mice displayed increased levels of phosphorylated akt thymoma viral oncogene (Akt), glycogen synthase kinase 3β (Gsk-3β), and extracellular signal regulated kinase (ERK1/2) (Figure 2C), with no significant changes in total levels of these proteins. Unsusceptible mice did not show these changes, although there was a strong trend for increased phospho-ERK levels, suggesting the possibility that ERK activation could stem partly from nonneurotrophic pathways.

We next tested the involvement of increased BDNF signaling in the NAc in the development of the Susceptible versus Unsusceptible phenotype. Bilateral intra-NAc infusions of BDNF enhanced susceptibility in response to a submaximal exposure to defeat stress (Figure 2D), without modifying locomotor activity (Figure S2E). Conversely, a blockade of increased ERK signaling in the NAc in Susceptible mice, via overexpression of a dominant-negative form of ERK2 using a herpes simplex virus (HSV-dnERK), promoted insusceptibility (Figure 2E), again with no effect on general locomotor activity (Figure S5D). These data strongly implicate BDNF induction and downstream signaling within the NAc as a mediator of defeat-induced avoidance.

To explore mechanisms by which chronic social defeat increases BDNF levels in the NAc, we first measured BDNF mRNA levels in this region by qPCR. Control, Susceptible, and Unsusceptible mice displayed equivalent levels of BDNF mRNA ($p > 0.5$), suggesting that the increased NAc BDNF protein associated with social avoidance is not dependent on local transcriptional regulation. To test this prediction, we examined the behavioral effects of an established method to locally delete the *bdnf* gene from the NAc by stereotaxically infusing adeno-associated virus (AAV) that expresses Cre-recombinase into the NAc of floxed BDNF mice (Berton et al., 2006; Graham et al., 2007). When AAV-CreGFP- and AAV-GFP-infected mice were subjected to the social defeat paradigm, *Bdnf* gene knockdown within the NAc did not alleviate defeat-induced avoidance (Figure 3B). This is in striking contrast to a knockdown of *Bdnf* within the VTA, which we have recently shown to prevent defeat-induced avoidance (Berton et al., 2006). To further characterize the

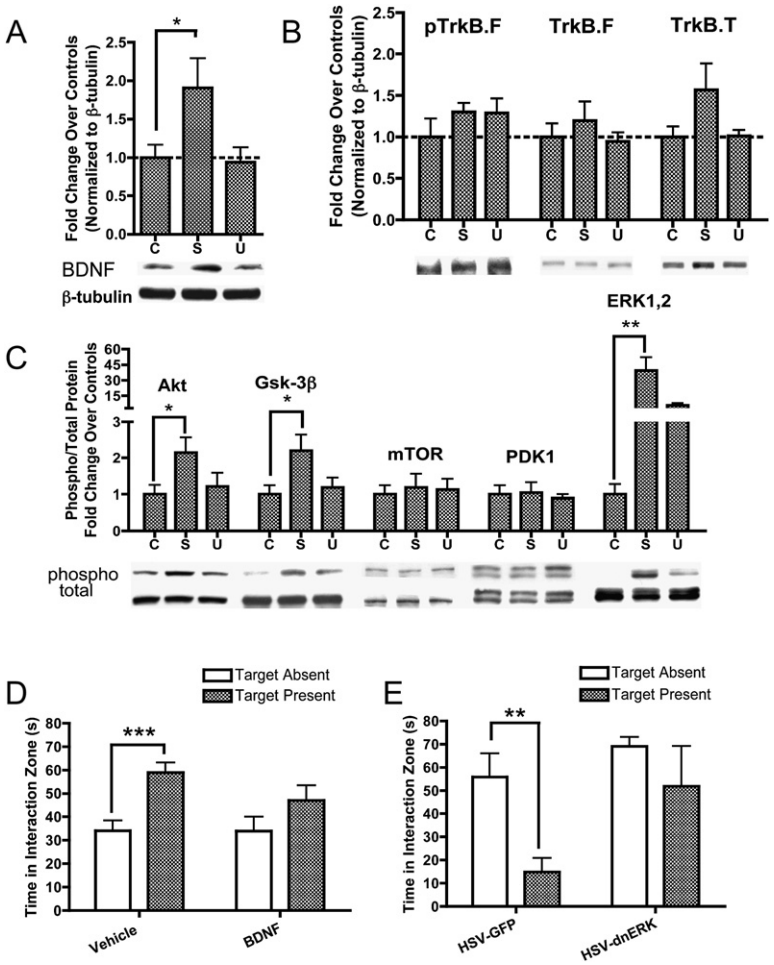


Figure 2. Increased NAc BDNF: A Molecular Signature of Susceptibility

(A) Results from immunoblotting experiments showing a 90% increase in BDNF levels in Susceptible mice compared to controls, without any changes in Unsusceptible mice and without changes in levels of phospho-TrkB.F (pTrkB.F) or total TrkB.F or TrkB.T (B). (C) Increases in NAc BDNF levels in Susceptible mice are accompanied by the significant activation of downstream signaling molecules, including Akt, Gsk-3β, and p44/42 MAPK/ERK1,2. Mammalian target of rapamycin (mTOR) and phosphoinositol dependent kinase-1 (PDK1) proteins were not significantly activated. (D) A single intra-NAc infusion of recombinant BDNF (1.5 μg/side) decreases social interaction (promotes susceptibility) following a submaximal exposure to defeat (vehicle: $t_{20} = 3.96$, $p < 0.0001$ and BDNF: $t_{20} = 1.44$, $p > 0.1$). (E) While the NAc-specific overexpression of HSV-GFP in Susceptible mice did not alleviate social avoidance ($t_8 = 3.49$, $p < 0.01$), HSV-dnERK promoted an Unsusceptible phenotype ($t_8 = 0.96$, $p > 0.3$); groups were matched for day 11 interaction times (12.2 ± 4.6 and 16.9 ± 7.2 s). Bars represent mean + SE (n = 5–11), * indicates significant post hoc comparisons to respective control groups, *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$. C, Controls; S, Susceptible; U, Unsusceptible.

relative contributions of BDNF within these structures to the behavioral sequelae of social defeat, we defeated floxed BDNF mice that had been infused with AAV-CreGFP or AAV-GFP into either the VTA or the NAc. As shown previously, a VTA-specific *Bdnf* knockdown led to an increase in the proportion of Unsusceptible mice after defeat (11% in AAV-GFP- versus 34% in AAV-CreGFP-injected mice), an effect not seen after a NAc *Bdnf* knockdown (data not shown). Likewise, *Bdnf* knockdown in VTA ameliorated the weight loss ($t_{13} = 2.19$, $p < 0.05$; Figure 3C) and sucrose preference deficit ($t_{18} = 2.46$, $p < 0.05$; Figure 3D) associated with the Susceptible phenotype. Coincident with these behavioral findings, we observed a substantial reduction in the ability of chronic social defeat to increase BDNF levels in the NAc in mice with a local VTA *Bdnf* knockdown ($t_{13} = 3.53$, $p < 0.01$; Figure 3E). These results strongly implicate the VTA as a crucial source of BDNF to the NAc during defeat.

Genome-Wide Expression Analyses Reveal the VTA as a Key Substrate for Resistance to Social Defeat

Our behavioral and molecular findings indicate that, when compared to Susceptible mice, Unsusceptible mice display a prominent lack of phenotype (i.e., changes in social avoidance, sucrose preference, and BDNF signaling are observed in Susceptible mice only). While these data shed light on features associated with vulnerability, they offer little insight into molecular mechanisms underlying Unsusceptibility. In the absence of candidate "resistance genes," we designed a microarray experiment to explore global patterns of gene expression in the NAc and VTA of control, Susceptible, and Unsusceptible mice on day 11. Our goal was to describe two main categories of genes: (1) genes regulated similarly in Susceptible and Unsusceptible groups (as a result of exposure to stress) and (2) genes regulated differentially in Susceptible and Unsusceptible mice (which may mediate differences in behavior). Our results, summarized as Venn diagrams in Figure 4A, revealed that the Unsusceptible phenotype was associated with the regulation of far more genes. While the NAc showed a substantially larger list of regulated genes, expression patterns in the VTA were particularly notable in the virtual absence of genes regulated similarly in Susceptible versus Unsusceptible mice. An expression-based dendrogram (Figure S4A) confirmed that VTA gene expression patterns more strongly correlated with our behavioral observations. Figure 4B displays

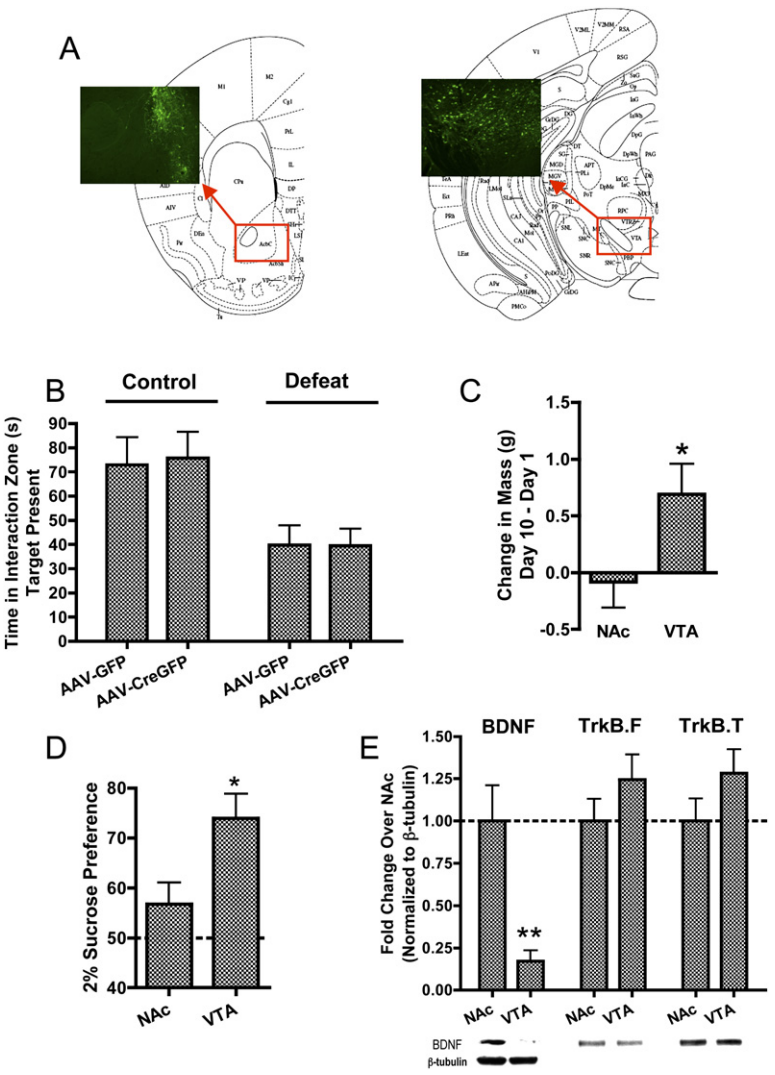


Figure 3. Effects of Region Specific BDNF Knockdowns

(A) Schematic coronal sections (Paxinos and Franklin, 2001) from NAc (left) and VTA (right), with insets showing representative high-power micrographs of viral GFP expression.

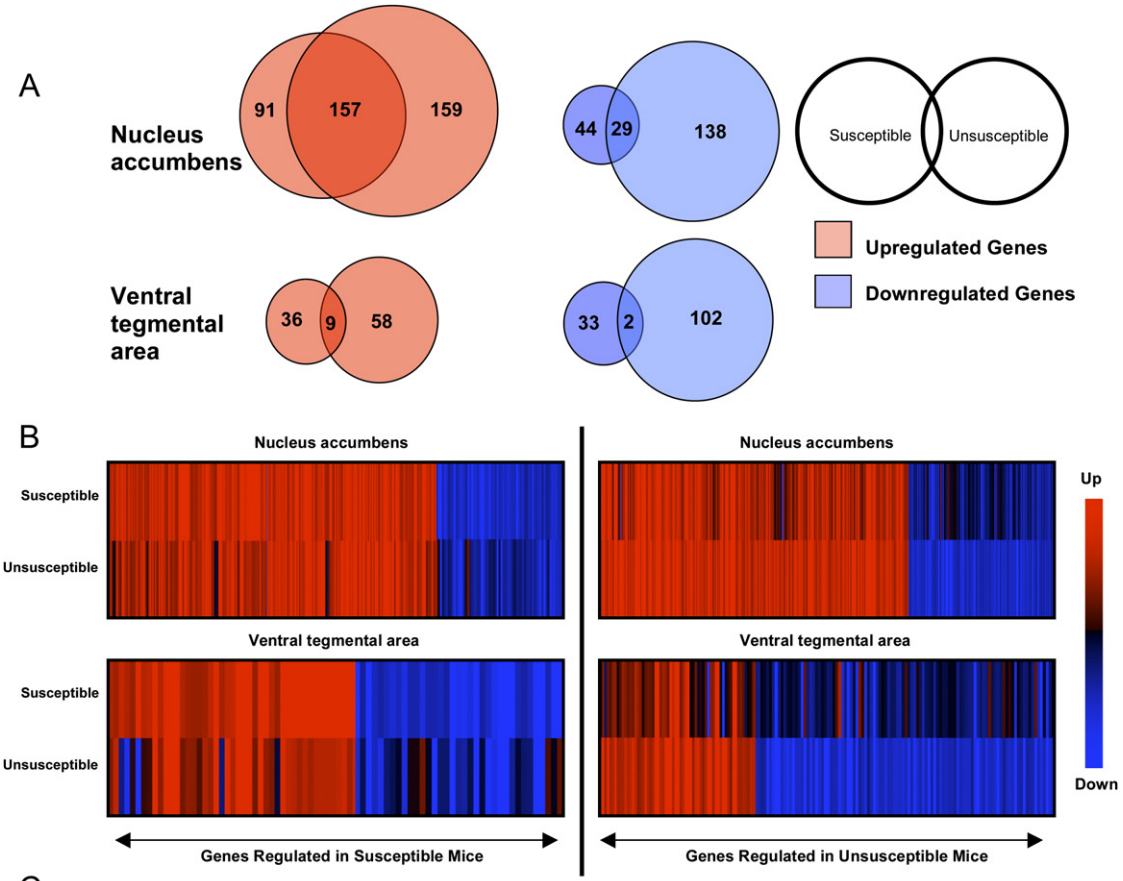
(B) Floxed BDNF mice were injected with either AAV-CreGFP or AAV-GFP into the NAc and subsequently subjected to the social defeat paradigm. In the presence of a significant effect of defeat ($F_{1,33} = 14.00$, $p < 0.001$), local knockdown of BDNF in NAc did not attenuate social avoidance ($F_{1,33} = 0.01$, $p > 0.5$).

(C–E) As compared to local BDNF knockdown within the NAc, an analogous VTA knockdown ameliorated the effects of social defeat on weight loss (C) and (D) sucrose preference. Immunoblotting NAc samples from these two groups revealed that VTA-injected mice displayed an 80% reduction in levels of BDNF protein (E). Bars represent mean + SE (n = 7–12), *p < 0.05, **p < 0.01.

a summary of regulated genes in heatmap form, which emphasizes the unique regulation of gene expression in the VTA of Unsusceptible mice. Among genes significantly upregulated in only Susceptible NAc, we found several examples whose products have previously been implicated in depressive behaviors, such as histone deacetylase-2 (*Hdac2*) and adenylyl cyclase 7 (*Adcy7*) (Hines et al., 2006; Schroeder et al., 2006). Similarly, only Susceptible VTA showed a significant upregulation in mRNA levels of galanin (*Gal*), which creates a prodepressant phenotype when infused directly into the VTA (Weiss et al., 1998), further validating our microarray results (see Supplemental Microarray Gene Lists).

Augmented Firing of VTA Dopamine Neurons Mediates Adaptations to Social Defeat

Among the genes that were significantly upregulated in the VTA of Unsusceptible mice only, we identified three voltage-gated potassium (K^+) channels (*Kcnf1*, *Kcnh3*, and *Kcnq3*). Because the induction of these proteins would be expected to reduce neuronal excitability, we hypothesized that their unique induction in Unsusceptible mice could provide a mechanism of insusceptibility, perhaps by counteracting a defeat-induced excitation of VTA dopamine neurons. To test this hypothesis, we studied the effect of social defeat on spontaneous firing rates of VTA dopamine neurons. We first obtained extracellular single-unit recordings from VTA dopamine neurons in slices obtained from control or defeated mice on day 11 (without classifying mice based on susceptibility). At this time point, chronic social defeat caused a 36% increase in the firing rate of VTA dopamine neurons ($n = 5$, $t_{78} = 2.15$, $p < 0.05$; Figure 5A). Nondopaminergic cells showed no change in firing frequency ($n = 5$, $t_{22} = 0.11$, $p > 0.5$). One defeat experience ($n = 4$, $t_{138} = 1.04$, $p > 0.3$) or a 10-week-long social isolation stress ($n = 4$, $t_{109} = 0.90$, $p > 0.3$) both failed to alter VTA firing rates, suggesting that this change is specific for chronic social defeat. Next, mice were classified as either Susceptible or Unsusceptible on day 11, and single-unit recordings were



C

Nucleus accumbens			Ventral Tegmental Area		
***Gene* (Definition)**	Susceptible	Unsusceptible	***Gene* (Definition)**	Susceptible	Unsusceptible
Adcy7 (Adenylyl cyclase isoform 7)	⇑	⇔	*Gal* (Galanin)	⇑	⇔
Api5 (Apoptosis inhibitor 5)	⇑	⇑	*Gdnf* (Glia derived neurotrophic factor)	⇔	⇑
App (Amyloid precursor protein)	⇔	⇑	*Kcnf1* (Voltage gated K^+ channel F1)	⇔	⇑
Cdh9 (Cadherin-9)	⇑	⇑	*Kcnh3* (Voltage gated K^+ channel H3)	⇔	⇑
Cntf (Ciliary neurotrophic factor)	⇑	⇔	*Kcnk4* (K^+ channel K4 [TRAAK])	⇔	⇑
Fbxo3 (F-box protein 3)	⇑	⇑	*Kcnq3* (Voltage gated K^+ channel Q3)	⇔	⇑
Gria4 (Ionotropic glutamate receptor-4)	⇑	⇑	*Kif1b* (Kinesin family member 1B)	⇔	⇓
Hdac2 (Histone deacetylase 2)	⇑	⇔	*Lcn2* (Lipocalin-2)	⇑	⇑
Homer3 (Homer homolog 3 [Drosophila])	⇓	⇔	*Nell2* (NEL-2 like [Chicken])	⇓	⇑
Hoxb3 (Homeo box B3)	⇑	⇔	*Pde3a* (Phosphodiesterase-3a)	⇔	⇓
Hoxc8 (Homeo box C8)	⇔	⇑	*S100a8* (S100 calcium binding protein A8)	⇑	⇑
Kcnc3 (Voltage gated Shaw K^+ channel-3)	⇓	⇓	*S100a9* (S100 calcium binding protein A9)	⇑	⇑
Lcn2 (Lipocalin-2)	⇑	⇑	*Socs3* (Suppressor of cytokine signaling-3)	⇑	⇑
Nhlh2 (Nescient helix loop helix-2)	⇓	⇓	*Titf1* (Thyroid TF-1)	⇑	⇑
Plcg2 (Phospholipase C gamma-2)	⇑	⇔	*Wnt2* (Wingless-related MMTV site 2)	⇑	⇔
Pten (Phosphatase and tensin homolog)	⇑	⇑	*Wnt7b* (Wingless-related MMTV site 7b)	⇔	⇑
Runx1t1 (Runt-related TF-1)	⇑	⇓			
S100a8 (S100 Ca^{2+} binding protein A8)	⇑	⇑			
S100a9 ((S100 Ca^{2+} binding protein A9)	⇑	⇑			
Sox18 (SRY containing box 18)	⇑	⇑			

Figure 4. Gene Expression Analysis after Social Defeat

DNA microarrays were performed on VTA and NAc of control, Susceptible, and Unsusceptible mice on day 11 after chronic social defeat. (A) Venn diagrams show the number of uniquely regulated genes in Susceptible and Unsusceptible mice (as compared to nondefeated controls), with the overlap depicting genes that were identically regulated by both conditions. Upregulated (red) and downregulated (blue) genes are shown separately (criteria for significance: $\geq$1.5-fold change compared to respective anatomical control group at $p < 0.05$). (B) Heatmaps illustrating the regulation of genes for each condition and anatomical structure, with red to blue gradient depicting an up to downregulation ($\geq$2-fold increase $\rightarrow$ $\leq$2-fold decrease). For example, the upper left panel displays significantly regulated genes in Susceptible NAc (top row) and how each of those genes is regulated in Unsusceptible NAc (bottom row). (C) Summary table showing examples of genes significantly upregulated (↑) or downregulated (↓) as compared to the nondefeated control group for each brain region. TF, transcription factor; SRY, sex-determining region-Y; TRAAK, TWICK-related amino acid-sensitive K^+ channel; NEL, neural epidermal growth factor-like; MMTV, mouse mammary tumor virus.

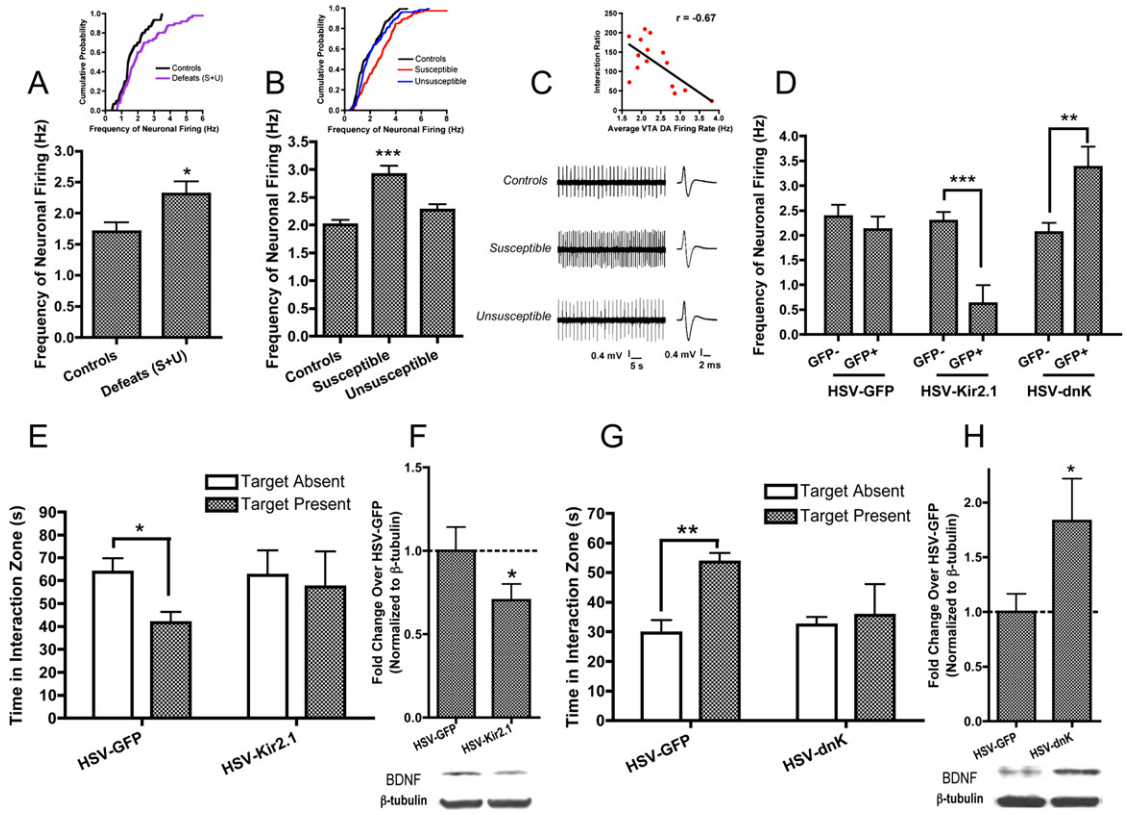


Figure 5. Increased VTA Dopamine Neuron Firing Mediates Susceptibility

(A) Social defeat results in a significant increase in VTA dopamine neuron firing rates on day 11, with the inset showing a relative cumulative distribution histogram. (B) On day 25, dopamine neurons from only Susceptible mice display significantly enhanced firing rates. (C) Sample traces and spikes; inset shows that averaged VTA firing rates for each mouse are significantly correlated with interaction ratios measured on day 11. (D) Single-unit recordings of GFP-positive or GFP-negative VTA dopamine neurons in slice culture, showing that HSV-Kir2.1 and HSV-dnK are able to significantly modulate the spontaneous activity of VTA neurons in vitro (n = 3–5 mice/group, 20–30 neurons/group). (E) While the VTA-specific overexpression of HSV-GFP in Susceptible mice did not alleviate social avoidance ($t_{11} = 2.98$, $p < 0.05$), HSV-Kir2.1 promoted resilient behavior ($t_{14} = 0.30$, $p > 0.3$), and (F) resulted in a significant reduction in NAc BDNF levels (one-tailed t test). (G) An intra-VTA infusion of HSV-dnK decreased social interaction (produced a Susceptible phenotype) following a submaximal social defeat regimen (HSV-GFP: $t_{10} = 4.52$, $p < 0.001$; and HSV-dnK: $t_{10} = 0.31$, $p > 0.5$) and (H) resulted in a significant increase in BDNF levels (one-tailed t test). Bars represent mean + SE (n = 5–11), * indicates significant post hoc comparisons to respective control groups, *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$.

performed 2 weeks later. At this time point, VTA dopamine neuron firing rates were increased in Susceptible mice (n = 5, $F_{2,381} = 14.37$, $p < 0.0001$), with no effect seen in Unsusceptible mice (Figures 5B and 5C). VTA firing rates were significantly correlated with the interaction ratio measured on day 11 ($r = -0.67$, $p < 0.01$, n = 15; Figure 5C, inset).

To establish a causal link between changes in VTA excitability and social avoidance, we overexpressed an inward rectifying K^+ channel (*Kir2.1* or *Kcnj2*) in the VTA of Susceptible mice to examine whether this manipulation would promote resistance to avoidance. We chose Kir2.1 because it has been shown to reliably suppress the excitability of several types of neurons (Burrone et al., 2002; Nitabach et al., 2002; Dong et al., 2006); indeed, we found that HSV-mediated Kir2.1 overexpression robustly decreased the firing of VTA dopamine neurons (Figure 5D). We next took two groups of Susceptible mice, matched for day 11 interaction times (43.0 ± 5.3 and 43.9 ± 6.6 s), and injected one with HSV-GFP and the other with HSV-Kir2.1 into the VTA. When assayed 3 days later, HSV-Kir2.1-injected mice displayed an Unsusceptible phenotype as compared to their GFP-infected counterparts (Figure 5E), despite comparable levels of locomotor activity (Figure S5D). Also, HSV-Kir2.1-infected mice displayed significantly reduced NAc levels of BDNF ($t_{20} = 1.96$, $p < 0.05$; Figure 5F). The Unsusceptible phenotype induced by HSV-Kir2.1 was absent after transgene expression had degraded (8 days following HSV infusions; Figure S5E). Intra-VTA infusions of HSV-Kir2.1 had no effect on the behavior of Unsusceptible mice (Figure S5F). Converse effects were seen upon overexpressing a K^+ channel (*Kcnab2*) rendered dominant-negative (HSV-dnK). HSV-dnK increased the firing rate of VTA dopamine neurons (Figure 5D), promoted the development of a Susceptible phenotype on a submaximal exposure to defeat (Figure 5G) without affecting general locomotor activity

(Figure S5D), and enhanced stress-induced increases in NAc BDNF levels ($t_9 = 2.10$, $p < 0.05$; Figure 5H). Together, these findings provide direct support for our hypothesis that a defeat-induced increase in K^+ channel activity in the VTA represents a molecular mechanism for resistance to social defeat and suggest that the heightened VTA electrical activity may be a mechanism for increased BDNF release into the NAc.

Deficits in Activity-Dependent BDNF Release Promote an Unsusceptible Phenotype

Thus far, our data are consistent with a model wherein susceptibility to an avoidant phenotype is caused by upregulation of VTA neuronal activity, which results in increased BDNF signaling within the NAc. To test this hypothesis, we examined the consequence of a naturally occurring human single-nucleotide polymorphism (SNP) in the BDNF prodomain (G196A, Val66Met), which impairs activity-dependent BDNF secretion (Chen et al., 2004; Egan et al., 2003). Val/Val and Met/Met mice (Chen et al., 2006) showed comparable responses in the forced swim and sucrose preference tests (Figures S6A and S6B), suggesting that Met-BDNF does not affect baseline responses to stress or natural rewards. However, a dramatic phenotype emerged when mice were subjected to chronic social defeat: while Val/Val mice demonstrated a significant reduction in social interaction after defeat ($t_{14} = 3.9$, $p < 0.01$; Figure 6A), Met/Met mice displayed an Unsusceptible phenotype ($t_{14} = 0.2$, $p > 0.5$). Nondefeated Val/Val and Met/Met mice showed similar interaction scores ($p > 0.5$). When NAc samples from both defeated groups were analyzed for BDNF levels, Met/Met mice showed 50% lower levels of BDNF protein compared to Val/Val mice ($t_{15} = 1.78$, $p < 0.05$; Figure 6B). While this polymorphism impairs BDNF release, it did not modify VTA neuronal activity: extracellular recordings of VTA dopamine neurons from defeated Val/Val and Met/Met mice showed similar levels of firing ($n = 3$/group, $t_{72} = 0.14$, $p > 0.5$; Figure 6C). These findings further support our model of how BDNF signaling within the VTA-NAc circuit relates to vulnerability and resistance to social defeat (Figure 6E) and indicate that preventing BDNF signaling to the NAc may be a key molecular mechanism of resistance.

Depressed Humans Display Increased Levels of BDNF in NAc

To examine the clinical relevance of our findings, we obtained postmortem samples of human NAc from depressed patients and unaffected controls (Table S3A). Only samples from males were examined, and groups were matched for age, postmortem interval, RNA integrity number (RIN), and tissue pH (Table S3B). We observed a 40% increase in levels of BDNF protein in the NAc of depressed samples as compared to controls ($n = 10$, $t_{18} = 2.95$, $p < 0.01$; Figure 6D), with no changes in levels of TrkB protein (data not shown) or BDNF mRNA levels (Figure S6C). Because most of the depressed patients had been chronically treated with antidepressants (Table S3A), we tested whether this BDNF increase could reflect a drug effect. Chronic treatment (28 days) with imipramine (a standard antidepressant) had no effect on levels of BDNF or TrkB isoforms in NAc from naive mice (Figure S6D).

DISCUSSION

Upon exposure to psychological stress, why do some individuals succumb to debilitating psychiatric disease whereas others progress normally? The goal of the present study was to identify molecular mechanisms underlying vulnerability to stress-induced psychopathology, as well as molecular adaptations that promote resistance to those changes. We utilized the social defeat paradigm and segregated socially defeated c57bl/6 mice into Susceptible and Unsusceptible subgroups: resistance to defeat-induced avoidance was found to be long-lasting and latent. While Unsusceptible mice were immune to several depression-like changes (e.g., anhedonia and weight loss), they did display other signs consistent with exposure to chronic stress (e.g., elevated anxiety and CORT reactivity), which were also observed in Susceptible mice. Interestingly, on day 39, only Unsusceptible mice developed a significant increase in relative cardiac mass, suggesting that the persistence of the Unsusceptible phenotype may be associated with the potential tradeoff of prolonged β-adrenergic stimulation (Bonanno et al., 2003) and possibly its subsequent adverse consequences.

This study shows that genetically identical (inbred) mice can display phenotypic differences after exposure to chronic stress; analogous findings have been observed in the chronic mild stress model (Strekalova et al., 2004). Such examples of phenotypic variability in inbred mice have always been attributed to environmental influences that are difficult to control and measure, such as variations in prenatal and postnatal development and early dominance hierarchies (Peaston and Whitelaw, 2006; Wong et al., 2005). However, experiments performed on inbred mice raised in strictly defined environments have shown that up to 80% of random variability in quantitative traits (e.g., body weight) are unrelated to genetic and environmental influences (Gartner, 1990). This third component to natural variation is thought to maintain Gaussian distributions of biological variables independent of environmental influences and sequence constraints and is now attributed to chromatin remodeling events such as histone acetylation or methylation (Wong et al., 2005). While these modifications play an important role in an organism's stress responses (Tsankova et al., 2006), future work is needed to delineate the relative contribution of epigenetic, genetic, and environmental factors that may together explain variations in susceptibility.

A Role for Neural Reward Substrates

In our paradigm, Susceptible mice showed a deficit in natural reward (sucrose preference), coincident with an increase in drug reward (cocaine place conditioning),

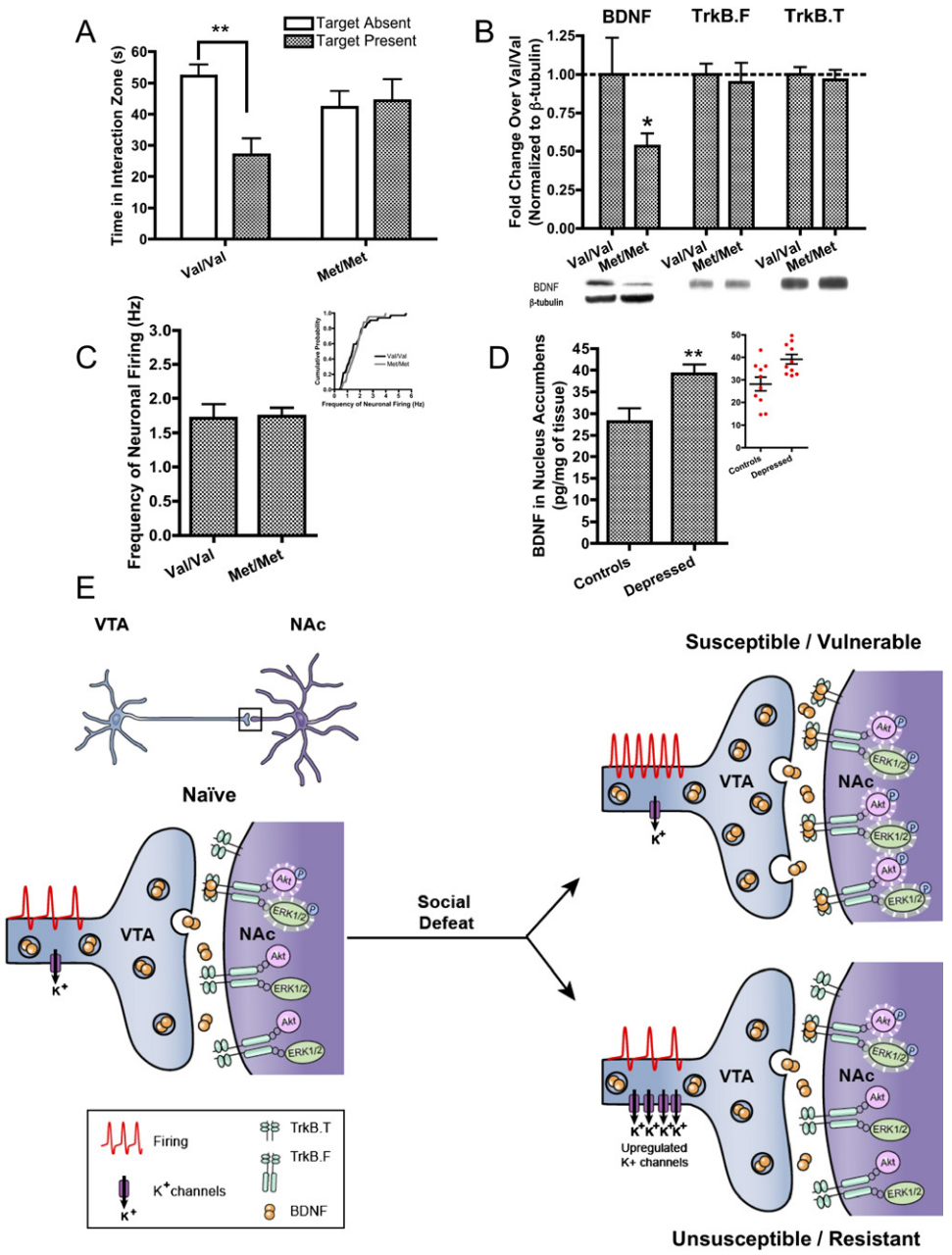


Figure 6. Human Correlates: Variant BDNF (Val66Met) and Postmortem NAc Studies

(A) Socially defeated Val/Val mice demonstrated a significant reduction in social interaction upon exposure to a CD1 target mouse, while Met/Met mice behaved comparably in both trials. (B) Day 11 western blot analysis of defeated Val/Val and Met/Met mice showing 50% lower BDNF levels in NAc of Met/Met mice (one-tailed t test). (C) VTA dopamine neurons from socially defeated Val/Val and Met/Met mice displayed comparable firing rates. (D) ELISA comparing levels of total BDNF in NAc lysates from depressed humans and unaffected controls (n = 10) (inset: scatterplot). Bars represent mean + SE (n = 7–8), * indicates significant changes compared to controls, *$p < 0.05$, **$p < 0.01$. (E) Schematic: in Susceptible mice, chronic social defeat increases the firing rate of VTA dopamine neurons, which subsequently gives rise to heightened BDNF signaling within the NAc. Unsusceptible mice display a resistance to this adverse cascade of events by upregulating various K^+ channels within the VTA.

while Unsusceptible mice showed neither of these changes. This aspect of our paradigm may serve to model the high comorbidity between mood and substance abuse disorders (Brady and Sinha, 2005). Immunoblotting experiments revealed that susceptibility to avoidance is marked by significantly increased levels of BDNF, a molecular adaptation that has also been shown to occur in rodent models of cocaine withdrawal (Grimm et al., 2003) and which promotes behavioral responses to cocaine (Graham et al., 2007; Horger et al., 1999). Postmortem NAc

samples from human depressed patients also showed increased BDNF levels, indicating that our social defeat paradigm is a useful method to understand the molecular neurobiology of human depression.

Results of gene-expression profiling studies revealed that the Unsusceptible phenotype was associated with a heightened degree of molecular plasticity; a considerably larger number of genes were regulated in this subgroup, suggesting that the expression of an Unsusceptible phenotype is an active neurobiological process that is not simply the absence of vulnerability. The selective upregulation of several voltage-gated K^+ channel subunits in the VTA of Unsusceptible mice encouraged us to explore the electrophysiological correlates of Susceptible and Unsusceptible behavior. We discovered that Susceptible mice show a long-lasting upregulation in the firing rate of VTA dopamine neurons, an effect not seen in Unsusceptible mice. With the aid of HSV-encoded K^+ channels, we demonstrated that selective and specific modulations of VTA excitability are able to significantly control susceptibility. These data support a causative role for increased VTA firing in mediating defeat-induced avoidance. Presumably, VTA firing rates are normal in Unsusceptible mice despite the upregulation of several K^+ channels, because these adaptations oppose underlying defeat-induced changes that increase the intrinsic excitability of VTA neurons. This latter mechanism requires further study, as our gene-array experiments did not reveal alterations in ion channels that could explain this phenomenon. Further work is also needed to assess whether social stressors alter the tonic-phasic pattern of VTA neuronal firing in vivo (Grace, 2000).

We also observed a strong association between VTA firing rates and NAc BDNF levels. This increase in NAc BDNF protein levels (in both Susceptible mice and depressed humans) was not accompanied by *Bdnf* mRNA changes, and a region-specific *Bdnf* gene deletion from the VTA, but not from the NAc, blocked the defeat-induced increase in NAc BDNF protein levels and led to an Unsusceptible phenotype. We propose that increases in NAc levels of BDNF protein and downstream signaling observed in Susceptible mice are due to enhanced activity-dependent BDNF release from VTA dopamine neurons, which we show are chronically activated under these conditions (Figure 6E). This model is consistent with the phenomenon of anterograde axonal BDNF transport that is reported to occur within this circuit (Altar and DiStefano, 1998), and the observation that VTA neurons are strongly activated during the threat of social subordination (Tidey and Miczek, 1996). Increased dopamine release in the NAc in the context of social defeat may promote alertness during a potentially harmful situation, and simultaneous BDNF release may promote neuroplastic changes necessary for survival. However, excessive and prolonged VTA activation and BDNF signaling to the NAc may be maladaptive, as it could produce long-lasting inflexibility and overgeneralization, causing harmless social cues to become aversive.

A Naturally Occurring Polymorphism Promotes Insusceptibility: BDNF G196A/Val66Met

The G196A SNP of the *Bdnf* gene results in the substitution of Met in place of Val in the prodomain of BDNF (Lu et al., 2005). Humans possessing this polymorphism display a selective impairment in episodic memory and abnormal hippocampal activation (Egan et al., 2003). Consistent with a role for the BDNF prodomain in intracellular trafficking and secretion, the Met-BDNF variant causes defective intracellular localization and impaired activity-dependent BDNF release (Chen et al., 2004, 2006). Mice homozygous for the Met/Met variant have recently been shown to display impaired learning and reduced hippocampal volumes (Chen et al., 2006). We show here that while Val/Val and Met/Met mice showed comparable behavior on baseline measures of emotionality, Met/Met mice displayed a striking Unsusceptible phenotype in the social defeat paradigm, which was associated with an ~50% reduction in levels of BDNF protein in the NAc. These data show that a naturally occurring impairment in activity-dependent BDNF release promotes resistance to social defeat and further strengthen our working hypothesis. Genetic-association studies report that the BDNF Met allele is associated with more favorable antidepressant responses (Choi et al., 2006; Yoshida et al., 2006), but other studies have displayed little consensus as to whether the BDNF Met allele alters rates of depression (Gratacos et al., 2007; Hong et al., 2003; Hwang et al., 2006; Strauss et al., 2005; Surtees et al., 2007). Clearly, further studies are required to examine the influence of the BDNF Val/Met polymorphism on human psychopathology in light of our findings. It is also particularly interesting that a variant of BDNF that impairs contextual learning (Chen et al., 2006) also leads to resistance to defeat-induced social avoidance. This finding may reflect an evolutionary tradeoff to ensure species survival: while a certain degree of memory impairment may adversely affect an organism's fitness, it may serve a positive function by preserving motivation for social interaction in the face of chronic stress.

In conclusion, we propose that resistance to social defeat may serve as a model for studying the more general phenomenon of resilience in humans. The resistance described here constitutes a latent trait, which accurately recapitulates the human condition, in that resiliency is often only identifiable after exposure to stressful events (Hoge et al., 2007; Yehuda et al., 2006). Resilient individuals display a striking ability to preserve optimism in the face of adverse situations, a characteristic that may reflect reward substrates that are either especially plastic or insensitive to change (Charney, 2004; Curtis and Cicchetti, 2003). Either way, these features could be mediated by neuroplastic events within the VTA-NAc circuit. Our ability to enhance resistance to stress by reducing BDNF release from the VTA to NAc, or by blocking BDNF signaling in the NAc, provides novel insight into the development of therapeutic agents to promote human resilience in the context of severe stress. Furthermore, our studies on the BDNF

Met allele suggest that while this variant may confer unfavorable effects on hippocampal function (Chen et al., 2006; Egan et al., 2003), its effects on mesolimbic BDNF signaling may represent a compensatory biological advantage under adverse conditions.

EXPERIMENTAL PROCEDURES

Subjects and Drugs

Male 7-week-old c57bl/6 (Jackson), CD1 retired breeders (Charles River), 9- to 13-week-old floxed BDNF mice (Berton et al., 2006), and 10- to 14-week-old BDNF Met/Met and Val/Val mice (Chen et al., 2006) were used. Cocaine (5 mg/kg) and imipramine (20 mg/kg) were given ip. All experiments were performed in accordance with the UTSWMC Institutional Animal Care and Use Committee and the Institutional Review Board.

Social Defeat and Behavioral Testing

Social defeat and avoidance testing were performed according to published protocols (Berton et al., 2006; Tsankova et al., 2006). During each defeat episode, intruder mice were allowed to interact for 10 min with an aggressive CD1 mouse, during which they were attacked and displayed subordinate posturing. For the social interaction test, we measured the time spent in the interaction zone (Figure S1A) during the first (target absent) and second (target present) trials; the *interaction ratio* was calculated as 100 × (interaction time, target present)/(interaction time, target absent). IPTT-300 temperature transponders (Bio Medic Data Systems) were implanted in the dorsal interscapular region under isoflurane anesthesia. Behavioral phenotyping on days 11 and 39 were performed using standard protocols extensively described in Supplemental Experimental Procedures.

Sucrose Preference

For sucrose-preference testing, a solution of 1% or 2% sucrose or diluent alone (drinking water) was filled in 50 ml tubes with stoppers fitted with ball-point sipper tubes (Ancare). All animals were acclimatized to two-bottle choice conditions prior to testing conditions. Daily, at ~1600 hr, the fluid level was noted, and the position of the tubes were interchanged. Sucrose preference was calculated as a percentage [100 × volume of sucrose consumed (in bottle A)/total volume consumed (bottles A and B)] and was averaged over at least 3 days of testing.

Stereotaxic Surgery

Mice were anesthetized with a cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg), positioned in a small-animal stereotaxic instrument, and the skull surface was exposed. 33 gauge needles were used to bilaterally infuse 0.5 μl of virus (or BDNF) into NAc or VTA at a rate of ~0.1 μl/min (Berton et al., 2006).

Immunoblotting and Immunoassays

NAc tissue punches (core and shell) were lysed, sonicated, and centrifuged in an EMSA buffer, following which 40 μg of supernatant protein was electrophoresed on precast 4%–20% SDS gradient gels. Following transfer, PVDF membranes were washed in 1× Tris-buffered saline with 0.1% Tween-20 (TBS-T), and blocked in 5% w/v milk for 1 hr at 25°C. The membrane was then incubated in a solution of the appropriate primary antibody overnight at 4°C, peroxidase-labeled secondary antibody at 25°C for 1 hr, and bands were visualized by enhanced chemiluminescence. Serum for enzyme immunoassays (EIAs) was obtained from centrifuged trunk blood, and steroid levels were assayed with commercially available EIA kits per manufacturer's instructions. Similarly, BDNF levels from human NAc were assayed from homogenized and lysed protein extracts using previously published protocols (Chen et al., 2006).

Human Postmortem Study

Human specimens were obtained from the Dallas Brain Collection (Stan et al., 2006). After obtaining next of kin permission, tissue was collected from cases at the Dallas County Medical Examiners Office and The Transplant Service Center at UTSWMC. Blood toxicology screens were conducted in each case, and subjects with a recent or past history of drug abuse, neurological disorders, or head injury were excluded. Clinical records and collateral information from telephone interviews with a primary caregiver was obtained for each case. Two psychiatrists carried out an extensive review of the clinical records and made independent diagnoses followed by a consensus diagnosis using DSM IV criteria. To obtain specimens of human nucleus accumbens, cerebral hemispheres were cut coronally into 1–2 cm blocks. Dissected NAc was immediately placed in a mixture of dry ice and isopentane (1:1, v:v). The frozen tissue was then pulverized on dry ice and stored at −80°C. For measurements of tissue pH, a 150 mg cerebellar punch was homogenized in 5 ml of ddH_2O (pH adjusted to 7.00) and centrifuged for 3 min at 8000 × g at 4°C. The pH of this supernatant was measured in duplicate. Each sample's RNA integrity number was determined by isolating total RNA using Trizol (Invitrogen) followed by analysis with an Agilent 2100 Bioanalyzer. For protein studies, ~100 mg of NAc tissue was homogenized in 1 ml of lysis buffer (100 μg/ml PMSF, 2 μg/ml aprotinin of leupeptin, aprotinin, and pepstain in PBS) with a Polytron homogenizer (900 rpm × 12 times). Samples were then sonicated, and by using the Bradford assays, protein concentrations were found to be between 2 and 5 μg/μl.

PCR and Gene Expression Microarrays

RNA from NAc, VTA, and hypothalamus was prepared using the RNAeasy Micro Kit (QIAGEN). cDNA was obtained using a first-strand synthesis kit (Invitrogen). All PCR experiments were conducted in triplicate, and the data were analyzed by using the ΔΔCt method (Tsankova et al., 2006) and were normalized to measures of *Gapdh* mRNA. For microarrays, NAc and VTA tissue was obtained from a single experiment where 50% of stressed mice were Unsusceptible. To reduce variability and increase statistical power, we simultaneously performed three biological replicates for each group, each consisting of pools of mRNA from four mice (Peng et al., 2003). RNA quality was verified by an Agilent Bioanalyzer prior to labeling and hybridization (performed by the UTSWMC Microarray Core) onto Illumina Mouse V6-1.1 full genome arrays (Illumina). Raw expression values were subjected to a cubic spline normalization and averaged across triplicates. Genes were considered to be significantly regulated if they displayed a >1.5-fold change in expression compared to their respective anatomical control group (at $p < 0.05$).

Electrophysiology

Mice were perfused with cold artificial cerebrospinal fluid (aCSF), following which 250 μm VTA slices were placed in an aCSF-filled holding chamber. Slices were transferred into a recording chamber fitted with a constant flow rate of aCSF (2.5 ml/min). Glass microelectrodes filled with 2.0 M NaCl were used to record single-unit extracellular potentials that were monitored through a high-input impedance amplifier (Axon Instruments). Dopamine neurons were identified by their location and electrophysiological criteria: regular and spontaneous action potentials with triphasic waveforms (Ungless et al., 2004). Firing rate was recorded in the amplifier's bridge mode, and data acquisition and on-line analysis of firing rate were collected using a Digidata 1322A digitizer and pClamp 8.2 (Axon Instruments). Cell culture recordings were performed in a cell-attached configuration.

Statistics

Unless otherwise noted, we used two-tailed unpaired Student's t tests (for comparison of two groups), one-way ANOVAs followed by the Dunnett's Multiple Comparison (for three groups), and one-way repeated-measure ANOVAs (to examine significant repeated-measure

effects). Two-way ANOVAs were performed when more than one factor was examined simultaneously, followed by Fisher's Least Significant Difference post hocs.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.cell.com/cgi/content/full/131/2/391/DC1/.

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Accession Numbers

Microarray data can be accessed through NCBI's Gene Expression Omnibus Webpage at http://www.ncbi.nlm.nih.gov/geo (Accession Number: GSE8870).