VULNERABILITY AND RESILIENCE TO SOCIAL DEFEAT:

THE ROLE OF NEUROPLASTICITY WITHIN

THE MESOLIMBIC DOPAMINE CIRCUIT

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VULNERABILITY AND RESILIENCE TO SOCIAL DEFEAT: THE ROLE OF NEUROPLASTICITY WITHIN THE MESOLIMBIC DOPAMINE CIRCUIT

by

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Abstract

The pathophysiology of major depression and post-traumatic stress disorder are poorly understood. In particular, while stressful life events are an important cause of psychopathology, most individuals exposed to adversity maintain normal psychological functioning. The molecular mechanisms underlying this "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 which differ along several behavioral and physiological domains. Through a series of molecular and electrophysiological techniques, we identify signature adaptations within the mesolimbic dopamine circuit that are uniquely associated with vulnerability and, by a combination of viral-mediated gene transfer and genetic mouse models, we demonstrate how these adaptations are causally linked to a vulnerable phenotype. We also show that molecular recapitulations of adaptations associated with the unsusceptible phenotype are sufficient to promote resilient 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.

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Chapter I: Introduction

"Florida Flowers"

This photograph captures the front courtyard of the Boca Raton Resort and Club in Boca Raton, FL, and was taken during the 46th Annual Meeting of the American College of Neuropsychopharmacology (ACNP 2007). On December 12, 2007, I presented my work on the contribution of mesolimbic BDNF signaling in the development of stress vulnerability at a panel entitled "Yin-Yang of BDNF Signaling". Other speakers at this panel included Moses Chao PhD (NYU), Keri Martinowich PhD (NIMH) and Francis Lee MD PhD (Cornell). Photograph by Vaishnav Krishnan.

Major Depressive Disorder: A Unique Biomedical Challenge

Approximately 1 in 6 individuals in the United States will succumb to major depressive disorder during their lifetime (Kessler et al., 2005). Core symptoms include depressed mood, anhedonia (the reduced ability to experience pleasure from natural rewards), irritability, difficulties concentrating, as well as abnormalities in appetite and sleep (Nestler et al., 2002) ("neurovegetative symptoms"). In addition to the mortality associated with suicide (Spirito and Esposito-Smythers, 2006), depressed patients are more likely to develop coronary artery disease (Rudisch and Nemeroff, 2003) and type II diabetes mellitus (Knol et al., 2006), and depression complicates the prognosis of a host of other chronic medical conditions (Evans et al., 2005). The chronic, festering nature of depression contributes substantially to the global burden of disease and disability (Lopez and Murray, 1998).

Despite depression's widespread prevalence and impact, our understanding of its pathophysiology is rudimentary when compared with our knowledge of other common chronic, conditions, such as Type II Diabetes Mellitus, which shares depression's multifactorial etiology (Figure 1). There exist three main explanations for this discrepancy. First, observing pathological changes within the brain remains significantly more difficult than for all other organs. Currently available techniques to document aberrant function of brain circuits rely on either postmortem studies (which possess numerous limitations) or neuroimaging techniques, which rely on visualizing increases or decreases in activity using indirect markers of neuronal activation (Phelps and LeDoux, 2005). These approaches have provided important insights into candidate brain regions, however, simple increases or decreases in regional brain activity are probably insufficient to explain depression's complex array of symptoms.

Figure 1

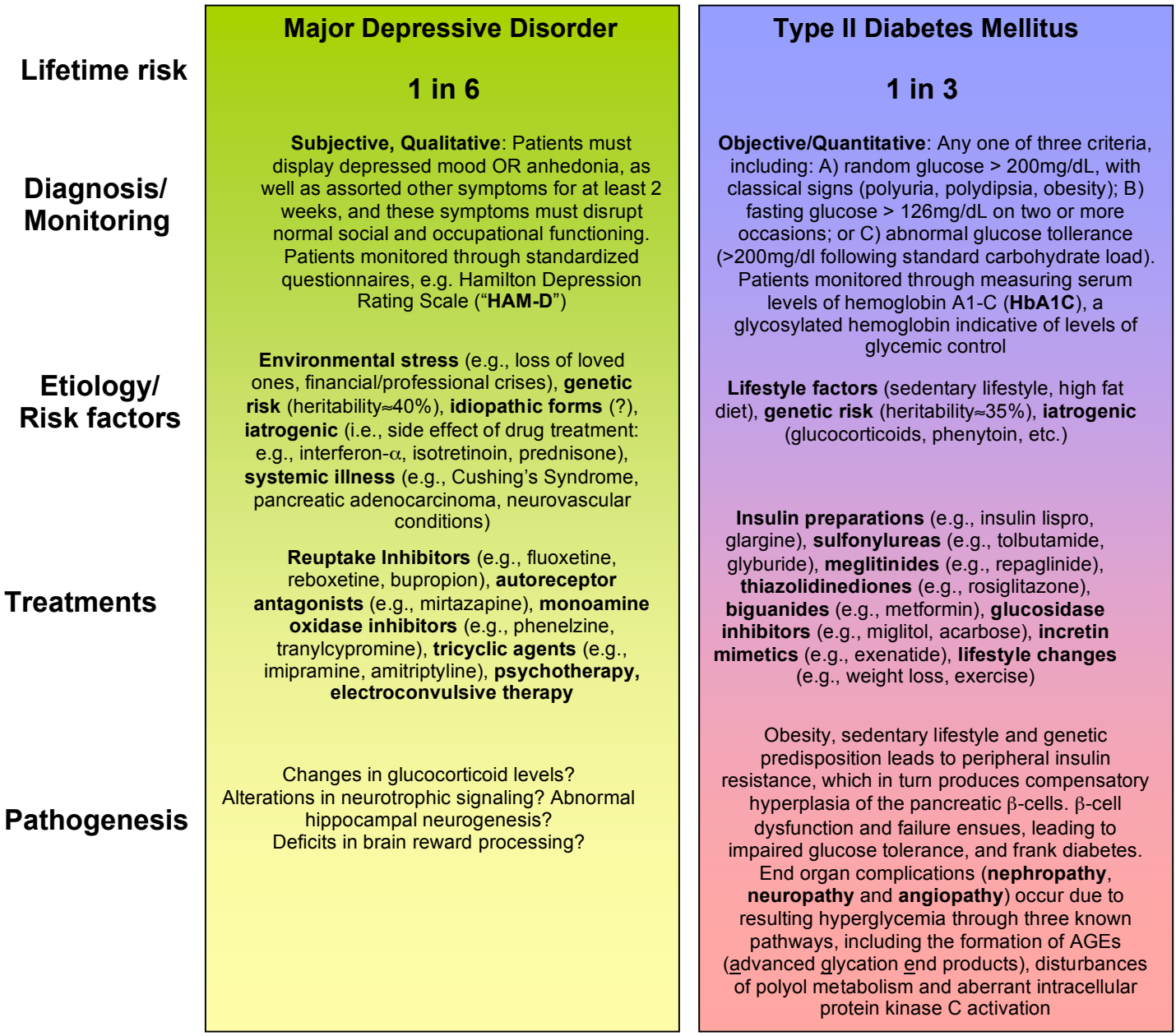


Figure 1. A "depressing" comparison. Depression and type II diabetes mellitus share several commonalities: high prevalence rates, multifactorial etiologies (including disease modifying/vulnerability genes that interact with environmental triggers). In addition, a large array of therapeutic modalities are employed to treat both these disorders. In contrast, techniques to diagnose and monitor the progression of diabetes rely on quantitatively precise measures of several serum molecules, while the diagnosis and monitoring of depression is gauged through subjective interviews and semiquantitative rating scales. In comparison to diabetes, our knowledge of depression's pathophysiology (i.e., the mechanisms by which environmental and genetic triggers produce disease symptoms) remains largely enigmatic. This is a reflection of several factors, including disease heterogeneity, enigmatic etiology, and largely symptom-based diagnosis and patient monitoring.

Secondly, the vast majority of depression occurs idiopathically, with our limited understanding of etiology coming as a list of risk factors including stressful life events, a few reproducible though weak genetic vulnerability factors (Lopez-Leon et al., 2007), endocrine abnormalities (hypothyroidism, hypercortisolism), cancers (e.g., pancreatic adenocarcinoma, breast tumors), and drug side effects [e.g., isotretinoin for acne, interferon-α for hepatitis C] (Drevets, 2001; Evans et al., 2005; Nestler et al., 2002). The official diagnosis of depression is somewhat arbitrary, and rests solely on the documentation of a certain number of psychiatric symptoms that significantly impact social and occupational functioning for a certain period of time (Nestler et al., 2002). Many of these diagnostic criteria can overlap with related conditions such as anxiety disorders, which display substantial comorbidity and symptomatic fluidity with depression (Hasler et al., 2004; Ressler and Mayberg, 2007). Therefore, two "depressed" patients may only display one symptom in common (Drevets, 2001), and if one patient experiences a manic episode, his diagnosis switches to bipolar disorder, which is presumably a distinct pathophysiological entity. Therefore, such a symptom-based approach to diagnosis poses obvious obstacles to the interpretation of genome-wide association studies, neuroimaging experiments, and postmortem investigations.

The Neural Circuitry of Depression

Several brain regions and circuits play a role in the regulation of emotion, reward, and executive function, and dysfunctional changes within these highly interconnected "limbic" regions (Figure 2) have been implicated in depression and antidepressant action (Berton and Nestler, 2006). Postmortem (Sheline, 2003) and neuroimaging (Harrison, 2002) studies of depressed patients have reported reductions in gray matter volume and glial density in the

prefrontal cortex and hippocampus, regions that are thought to mediate the cognitive aspects of depression such as feelings of worthlessness and guilt. However, these findings are by no means consistent, and are often complicated by comorbid diagnoses and medication confounds, and there has been minimal success in demonstrating any clear cause-effect relationships of these "pathological" changes. Some of these changes are apparent in one hemisphere only (Drevets, 2001; Harrison, 2002; Sheline, 2003), and the pathophysiological implications of such anatomical lateralities are unclear and have largely been neglected by preclinical studies.

In contrast to structural studies, experiments assessing brain function, such as functional magnetic resonance imaging (fMRI) or positron emission tomography (PET), demonstrate that activity within the amygdala and subgenual cingulate cortex (cg25, a subregion of prefrontal cortex) is strongly correlated with dysphoric emotions; indices of neuronal activity within these regions are elevated by transient sadness in healthy volunteers and are chronically elevated in depressed individuals, which ultimately revert back to normal levels with successful treatment (Drevets, 2001; Ressler and Mayberg, 2007). Inspired by these findings, it was demonstrated that deep brain stimulation (DBS) applied to cg25 produced a sustained remission of depressive symptoms in a small cohort of treatment-resistant patients (Mayberg et al., 2005). DBS treatment, achieved through the stereotaxic surgical placement of stimulating electrodes, inhibit stimulated areas through depolarization blockade, also provided an acute ameliorative effect on clinical ratings when applied to the nucleus accumbens [NAc] (Schlaepfer et al., 2008), a striatal subregion important for reward and implicated in depression's hedonic deficits (Nestler and Carlezon, 2006). While this highly invasive therapy remains at a preliminary stage, studies have reported altered activity by PET in neural substrates beyond those that were stimulated (Mayberg

et al., 2005; Schlaepfer et al., 2008), emphasizing the complex interconnected nature of these limbic circuits.

Figure 2

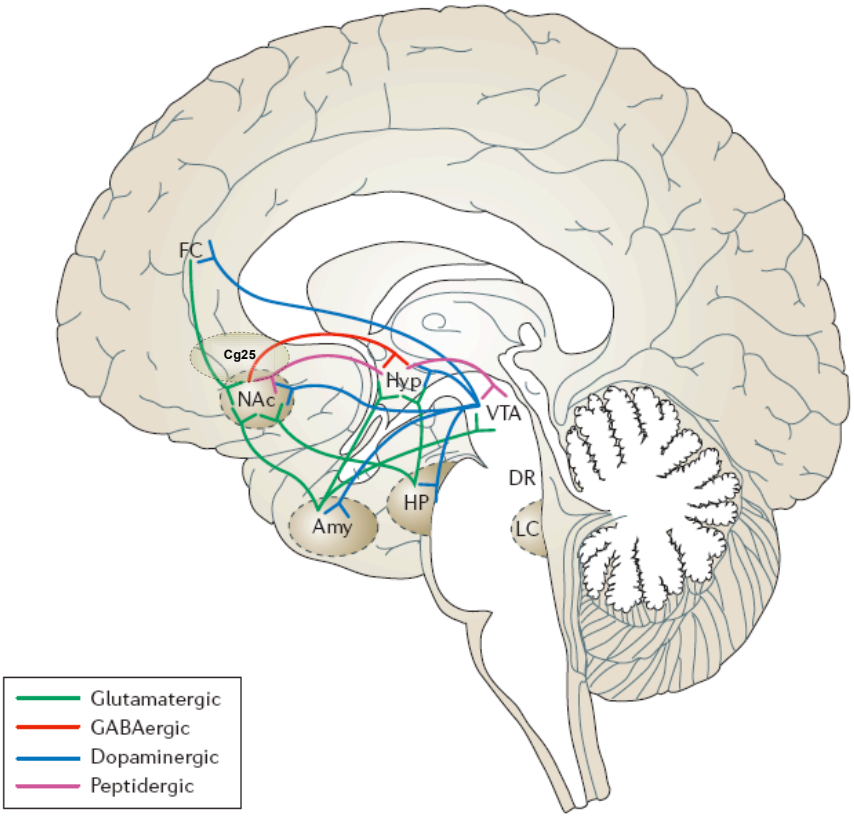


Figure 2. The neural circuitry of major depression is made up of "limbic" regions involved in reward, emotion and executive function. These nuclei are thought to function as highly interconnected parallel circuits, such that changes in affect and emotion such as "sadness" or "anxiety" cannot be uniquely ascribed to a single brain region. This property is in stark contrast to other brain regions involved in sensorimotor function, such as visual cortex or motor cortex, where specific functions are highly localized. Deep brain stimulation (DBS) of two forebrain regions, the nucleus accumbens (NAc) (Schlaepfer et al., 2008) and the subgenual cingulate cortex (cg25) (Mayberg et al., 2005), produce antidepressant effects in cases of treatment resistant depression. DBS application to these regions is thought to function through inhibiting the activity of these regions as well as altering activity patterns of nearby limbic regions. Neuroimaging studies strongly implicate the amygdala as an important limbic node for the processing of emotional salient stimuli such as sad faces (Drevets, 2001). An important theme within these circuits is that forebrain networks (consisting of mostly glutamatergic and GABAergic connections) are modulated by monoaminergic input (dopamine, serotonin and norepinepherine). Much attention has also been devoted to the role of the hippocampus, where a variety of stressors produce reductions in neurotrophin levels and neurogenesis. Several brain regions within this circuit are subject to a second level of modulation by peripherally released metabolic hormones such as ghrelin (Lutter et al., 2008b) (from stomach) and leptin (Lu et al., 2006) (from white adipose tissue), as well as more classical adrenal (glucocorticoids) and gonadal hormones (androgens, estrogens). The various nuclei of the limbic circuit, as well as the specific neurotransmitters used by each set of neurons are highly conserved within the mouse brain, justifying the utility of rodent models in examining neural systems involved in depression-related behaviors. Abbreviations: LC (locus ceruleus), VTA (ventral tegmental area), DR (dorsal raphe), Amy (amygdala), HP (hippocampus), Hyp (hypothalamus). Image reproduced from Berton and Nestler, 2006.

These forebrain networks are significantly modulated by monoamine projections from midbrain and brainstem nuclei (dopamine from the *ventral tegmental area* [VTA], serotonin from the *dorsal raphe/periaqueductal gray* [DR], and norepinephrine from the *locus ceruleus* [LC]). In addition to controlling alertness and awareness, these transmitters modulate the salience of emotional stimuli by acting through a complex array of G protein-coupled receptors. More recent studies have investigated the role of specific hypothalamic subnuclei in mediating the neurovegetative signs of depressive syndromes. Demonstrating neuropathological changes within these regions has been challenging (Drevets, 2001; Harrison, 2002). Finally, while depressive symptoms are likely mediated by dysfunction in a diffuse series of neural networks, the field has often employed a simplistic "localization of function" approach to examine limbic substrates (e.g., amygdala ≈ "*anxiety*", NAc ≈ "*reward*"). Such artificial distinctions possess limited heuristic value, and likely reflect the limitations of current technologies that require considerable enhancements in order to begin to truly appreciate systems level dysfunction.

Preclinical Models

Certain symptoms of depression such as guilt, suicidality, and feelings of worthlessness may be strictly limited to the human condition, but other key features can be successfully modeled in rodents. Depression is substantially heritable (Mill and Petronis, 2007), and while the field has identified a relatively small number of reproducibly occurring depression-related polymorphisms, these elements individually confer very small alterations in risk (Lopez-Leon et al., 2007). In the absence of bona fide depression genes that can be used to generate disease models (such as the case for Huntington's disease or Fragile X syndrome), depression models are often evaluated by three main criteria. The first is *etiological validity* (also known as *construct*

validity), which requires that a model's depression-like behaviors be caused by the same etiologies that trigger human depression. This is a challenging requirement, given the absence of definitive etiologies for human depression! Genetic predispositions interact with environmental risk factors, such as stressful life events, which play a clear causal role in the initiation of depressive episodes in some patients (Kendler et al., 1999). However, as the tendency to live in high-stress environments appears to also possess a heritable component ("risk/sensation seekers") (Mill and Petronis, 2007), many "environmentally-precipitated" depressive episodes may not simply be the consequence of stress exposure alone. Other non-genetic contributions to depression remain more poorly understood, including a role for stochastic epigenetic regulation during development, as discussed below.

In spite of these complexities, rodent models of depression employ a simple approach. In general, they gauge an animal's response to the application of inescapable stress, such as forced swimming, tail suspension, or aggressive social encounters; the selective advantages and disadvantages of these models have been extensively reviewed (Berton and Nestler, 2006; Cryan and Holmes, 2005). Within these paradigms, there is an important distinction. "Models" that employ an acute period of discomfort (e.g., six minutes of forced swimming or tail suspension) should really be thought of as "tests" of coping behavior; these paradigms are limited in their ability to recapitulate a long-lived multidimensional depression-like syndrome. Their obvious strength lies in their *pharmacological validity*, i.e., their sensitivity to acutely administered known antidepressant compounds (Berton and Nestler, 2006; Cryan and Holmes, 2005; Lucas et al., 2007) that allows for the rapid screening of novel therapeutic agents. In contrast, chronic stress models [e.g., chronic mild stress (Willner, 2005) or chronic social defeat (Krishnan et al., 2007)] are more technically challenging, but do produce several depression-related phenotypes

and display the unique sensitivity to chronic but not acute antidepressant administration, comparable to the therapeutic delay of 4-6 weeks that is required for all available antidepressant drugs to treat depression in humans (Nestler et al., 2002). To test genetic contributions, both acute and chronic stress paradigms have been applied to genetic models of depression generated through selective inbreeding (Overstreet et al., 2005), as well as to targeted knockout or transgenic mice where genes of interest have been selectively manipulated.

Finally, depression models also display *face validity* when those behavioral changes that are brought about by stress or genetic manipulation superficially resemble depressive symptoms. For instance, an animal's reduced intake of a weak solution of sucrose (sucrose preference test) following chronic stress (Krishnan et al., 2007; Strekalova et al., 2004) is thought to model anhedonia. Despite their obvious limitations, these preclinical models of depression have provided important insight into depression's pathophysiology and the mechanism of action of antidepressants.

The Role of Monoamines

The "monoamine hypothesis" of depression, which states that depression is a result of reduced central monoaminergic transmission, originated from a series of early clinical observations (Elhwuegi, 2004). Two structurally unrelated compounds developed for non-psychiatric indications, iproniazid and imipramine, displayed potent antidepressant effects and were later shown to function through enhancing central monoaminergic transmission. In contrast, reserpine, an old antihypertensive agent that depletes monoamine stores, produced depressive symptoms in a subset of patients. Today's antidepressant agents offer a better therapeutic index and lower rates of side effects for most patients than these older agents, but are still designed to

acutely increase monoaminergic transmission (Berton and Nestler, 2006), either through inhibiting neuronal reuptake (e.g., SSRIs: selective serotonin reuptake inhibitors like fluoxetine), or through inhibiting monoamine degradation (MAOIs: monoamine oxidase inhibitors like tranylcypromine). While these monoamine-based agents are potent antidepressants (Pittenger and Duman, 2008; Tidey and Miczek, 1996) and alterations in central monoaminergic function may contribute to genetic vulnerability (Ansorge et al., 2007; Lopez-Leon et al., 2007), depression is far from being *caused* by a simple deficiency of central monoamines. MAOIs and SSRIs produce immediate increases in synaptic monoamines, whereas their mood enhancing properties require many weeks of treatment. Conversely, experimental depletion of monoamines may produce a mild reduction in mood in unmedicated depressed patients, but such manipulations do not alter mood in healthy controls (Ruhe et al., 2007). Moreover, studies employing rodent stress models have demonstrated that pathological enhancements in dopaminergic and noradrenergic transmission can serve maladaptive roles in stress-related disorders by strengthening memories of aversive life events (Hu et al., 2007; Krishnan et al., 2007).

It is now believed that acute increases in synaptic monoamines induced by antidepressants produce secondary neuroplastic changes that are on a slower time scale, and involve transcriptional and translational changes that mediate molecular and cellular plasticity (Nestler et al., 2002; Pittenger and Duman, 2008). Chronically administered antidepressants produce neuroplastic changes in the levels of several molecules such as p11 [a calcium binding adaptor protein that associates with serotonin receptors (Svenningsson et al., 2006)] as well as the transcription factor CREB (cAMP response element binding protein), downstream of several stimulatory G protein-coupled receptors, in hippocampus, an effect validated in human

postmortem tissue and directly linked to antidepressant-like responses in animal models (Nestler et al., 2002). On the other hand, stress activation of CREB in NAc triggers depression-like responses, which underscores critical region-specific actions of neurotransmitters and their downstream effectors not incorporated into simplistic deficiency models (Nestler and Carlezon, 2006).

While monoamine-based antidepressants remain the first line therapy for depression, their long therapeutic delays and low (~30%) remission rates (Trivedi et al., 2006) have encouraged the search for more effective agents (Mathew et al., 2008). Among the various classes of 5HT receptors involved in antidepressant action, selective agonists of the $5HT_4$ receptor produce rapid antidepressant effects in rodents [3-4 days] (Lucas et al., 2007). Experiments on mice deficient in P-glycoprotein (P-gp), a blood-brain barrier molecule that transports numerous drugs back into the bloodstream, have shown that several antidepressant agents, including the SSRI citalopram, are substrates for P-gp. Human polymorphisms in the P-gp gene significantly alter antidepressant efficacy in depressed individuals (Uhr et al., 2008), suggesting the value of such a pharmacogenetic approach when selecting antidepressant agents.

Neurotrophins and Neurogenesis

Volumetric reductions observed for hippocampus and other forebrain regions in subsets of depressed patients have supported a popular hypothesis for depression involving decrements in neurotrophic factors – neurodevelopmentally expressed growth factors that also regulate plasticity within adult brain (Altar and DiStefano, 1998; Chao, 2003). These studies have focused largely on the role of BDNF (brain-derived neurotrophic factor), which is abundantly expressed in adult limbic structures. Support for this "BDNF hypothesis" has come from a large

preclinical literature demonstrating that several forms of stress reduce BDNF signaling in hippocampus, while chronic antidepressant treatments increase BDNF signaling (Duman and Monteggia, 2006; Nestler et al., 2002). Similar changes have been observed in postmortem hippocampus of depressed humans (Karege et al., 2005) as well as in levels of serum BDNF, the source of which remains controversial (Duman and Monteggia, 2006).

More causal evidence for BDNF's antidepressant action has come from experiments where antidepressant effects were observed upon direct infusion of BDNF into the hippocampus (Shirayama et al., 2002) or midbrain serotonergic nuclei (Siuciak et al., 1997). However, more recent findings have necessitated a revision of this hypothesis. First, a substantial number of preclinical stress and antidepressant studies have either failed to show these patterns of changes, or have shown the opposite effects (Groves, 2007; Martinowich et al., 2007). Second, mice with inducible or conditional forebrain deletions of BDNF or TrkB (tropomyosin related kinase B, the BDNF receptor) fail to convincingly display depression-like behavior (Monteggia et al., 2007; Zorner et al., 2003). Third, in other regions, for example, the VTA and NAc, BDNF exerts a potent prodepressant effect: chronic stress increases BDNF levels within the NAc (Berton et al., 2006), and the direct infusion of BDNF into the VTA-NAc increases depression-related behaviors (Eisch et al., 2003; Krishnan et al., 2007), while a selective knockout of BDNF from this circuit is antidepressant-like (Berton et al., 2006). Finally, a single nucleotide polymorphism in the BDNF gene (G196A, Val66Met), that impairs the intracellular trafficking (Chen et al., 2004) and activity-dependent release of BDNF (Chen et al., 2006; Egan et al., 2003), and decreases hippocampal volume (Chen et al., 2006; Szeszko et al., 2005), does not confer genetic vulnerability to depression (Gratacos et al., 2007; Lopez-Leon et al., 2007), and may in fact be protective against stress-related disorders (Krishnan et al., 2007; Pezawas et al., 2008) and

promote favorable antidepressant responses (Choi et al., 2006; Yoshida et al., 2006). Together, these results suggest that the current formulation of the BDNF hypothesis is too simplistic; BDNF signaling is involved in the neuroplastic responses to stress and antidepressants, but these effects are both region- and antidepressant-specifc (Duman et al., 2007; Nestler and Carlezon, 2006), further complicating the search for therapeutic agents involving BDNF-directed mechanisms (Berton and Nestler, 2006; Nestler et al., 2002).

A dramatic cellular effect of antidepressant therapies (SSRIs, exercise, electroconvulsive therapy) is the induction of adult hippocampal neurogenesis - the process by which neural progenitors of the hippocampal subgranular zone (SGZ) mitotically divide to provide new neurons which differentiate and integrate into the dentate gyrus (Nestler et al., 2002). Blockade of hippocampal neurogenesis inhibits the therapeutic-like effects of most antidepressant treatments in rodent models (Sahay and Hen, 2007). Moreover, antidepressant treatment, possibly through the actions of CREB and other transcriptional regulators (Pittenger and Duman, 2008), increases the levels of several growth factors in hippocampus which influence neurogenesis. These include BDNF [which promotes neuronal survival (Sairanen et al., 2005)], as well as VEGF (vascular endothelial growth factor) and VGF (non-acronymic), which themselves possess antidepressant and pro-neurogenic properties in rodents (Hunsberger et al., 2007; Thakker-Varia et al., 2007; Warner-Schmidt and Duman, 2007). The mechanisms by which new neurons might restore mood are largely unknown. Activity-dependent increases in neurogenesis may enhance activity propagation through hippocampal subfields (Airan et al., 2007) and allow hippocampal networks to adapt and learn new experiences (Kempermann, 2008). Indeed, this raises the possibility that neurogenesis occuring during certain forms of stress may mediate maladapative learning and promote prodepression-like sequelae (Lagace et al.,

2008). Moreover, while several types of stress reduce SGZ cell proliferation, reduced neurogenesis *per se* does not produce depression (Sahay and Hen, 2007): rodents in which hippocampal neurogenesis has been ablated [through either irradiation (Santarelli et al., 2003; Surget et al., 2008) or genetic techniques (Zhao et al., 2008)] do not exhibit anxiety- or depression-related behaviors, and do demonstrate a resistance to social defeat (Lagace et al., 2008). Moreover, indirect indices of proliferation remain unchanged in studies of postmortem hippocampus from depressed individuals (Reif et al., 2006).

Collectively, these studies highlight the weaknesses of attempts to generate a "unified theory" of depression. The neurobiological mechanisms that promote depressive symptoms in response to stress differ dramatically among different neural circuits, and may also be distinct from changes that underlie depression in the absence of extrernal stress (so-called "endogenous depression"). In addition, neuroplastic events that are required for antidepressant efficacy need not function through the reversal of stress-induced plasticity (Nestler et al., 2002), and may in fact act through separate and parallel circuits.

Neuroendocrine and Neuroimmune Interactions

The earliest reproducible abnormality associated with depression was a modest increase in serum glucocorticoid levels (Parker et al., 2003; Raison and Miller, 2003). This fueled significant interest in the role of a dysfunctional HPA (hypothalamic-pituitary-adrenal) axis in the pathophysiology of depression, a possibility that has received considerable support at least for a subset of patients. Physical or psychological stress increases serum glucorticoid levels, and some depression-like symptoms can be produced in rodents by chronic glucocorticoid administration (Gourley et al., 2007). Through the actions of glucocorticoid receptors (GRs),

excess glucorticoids can reduce SGZ proliferation rates and produce atrophic changes in hippocampal subregions (McEwen, 2007). This could contribute to hippocampal volume reductions seen in depression. Patients with Cushing's syndrome, where levels of cortisol are extremely elevated, also display depressive features and atrophic changes in hippocampus (McEwen, 2007; Nestler et al., 2002). Several metabolic abnormalities that are often associated with depression, such as insulin resistance and abdominal obesity, can at least be partially explained by elevated glucocorticoids (Brown et al., 2004; Evans et al., 2005). Depression's hypercortisolemia manifests at several levels, including impaired glucocorticoid receptor-mediated negative feedback (Brown et al., 2004), adrenal hyperresponsiveness to circulating adrenocorticotropic hormone [ACTH] (Parker et al., 2003), and hypersecretion of CRF (corticotropin-releasing factor), the hypothalamic activator of pituitary ACTH release (de Kloet et al., 2005; Nestler et al., 2002). In line with these findings, a number of novel antagonists for glucocorticoid and CRF receptors are currently being tested in clinical trials (Mathew et al., 2008).

More recent studies have revealed that hypercortisolemia is almost entirely limited to very severe depressive episodes, such as that observed in an inpatient setting (Brouwer et al., 2005) or accompanied by psychotic symptoms (e.g., hallucinations and delusions) (Nestler et al., 2002; Rush, 2007). In contrast, atypical depression, a subtype characterized by hyperphagia and hypersomnia, appears to be associated with hypocortisolemia (Brouwer et al., 2005; Gold and Chrousos, 2002), a phenomenon that is also observed in certain related conditions such as fibromyalgia, chronic fatigue syndrome, and post-traumatic stress disorder (PTSD) (Heim et al., 2000). The origins of such distinct glucocorticoid profiles may reflect the evolutionary tradeoff between the catabolic and immunosuppressant effects of glucocorticoids: while high serum

glucocorticoid levels promote the mobilization of energy resources during stressful experiences, low glucocorticoid states allow an unobstructed immune system to combat infections or physical injuries sustained during adverse encounters in the wild (Raison and Miller, 2003).

Cytokines, the humoral mediators of innate and adaptive immunity, are also known to be important modulators of mood. Cytokine receptors within the CNS are activated by both peripherally and centrally synthesized cytokines (Dantzer et al., 2008). Low doses of LPS (lipopolysaccharide) or IL-1 (interleukin-1) produce "sickness behavior" in rodents (consisting of social withdrawal, decreased exploratory and sexual behavior), brought about by the release of pro-inflammatory cytokines such as interferon-α, tumor necrosis factor-α (TNFα), IL-6, and IL-1β, which activate the HPA axis and central monoaminergic systems (Dunn et al., 2005). Roughly 30% of individuals treated with recombinant interferons develop depression as a treatment side effect (Loftis and Hauser, 2004). Clinical studies examining depression-associated increases in serum cytokine levels have been largely inconsistent (Dunn et al., 2005). This suggests that immune activation may be a signature of a small subset of depression cases, including those associated with autoimmune conditions such as multiple sclerosis or rheumatoid arthritis, where heightened system-wide inflammation, in addition to producing depressive mood changes, may increase the risk of acute coronary events (Evans et al., 2005).

Administration of cytokines such as interferon-α or IL-6 to rodents does not cause consistent depression-like features (Dunn et al., 2005). Nevertheless, recent preclinical studies indicate that blocking pro-inflammatory cytokine signaling can produce antidepressant effects. Mice with targeted deletions of IL-6 (Chourbaji et al., 2006) or TNFα receptors (Simen et al., 2006) display antidepressant-like behavioral phenotypes, and a centrally administered antagonist of the IL-1β receptor reversed the behavioral and anti-neurogenic effects of chronic stress (Koo

and Duman, 2008). Future studies of the "cytokine hypothesis" must focus on elucidating the largely unknown neural circuitry involved in the behavioral effects of cytokines, and more precisely delineate the intercellular interactions involved between brain macrophages (microglia), glia, and neurons within this circuitry.

Epigenetic Mechanisms

Among the several means by which experience can produce long-lasting changes in protein availability and function (McClung and Nestler, 2008), there has been a considerable recent interest in the role of epigenetic modifications in the pathophysiology of depression and antidepressant action. These modifications encompass covalent changes to DNA (e.g., DNA methylation), post-translational modifications of histone tails (e.g., histone acetylation and methylation), as well as non-transcriptional gene silencing mechanisms (e.g., microRNAs) (Tsankova et al., 2007). Given that these changes are often long-lasting, epigenetics has been invoked to explain several aspects of depression, including high discordance rates between monozygotic twins, the chronic relapsing nature of the illness, and peculiarly high female prevalence rates (Mill and Petronis, 2007). In essence, epigenetic changes offer a mechanism by which environmental experiences can modify gene function in the absence of DNA sequence changes, and may help explain largely inconsistent genetic association studies of depression, for example, by undermining the transcriptional impact of DNA sequence polymorphisms due to epigenetic marks on those gene promoters (Mill and Petronis, 2007). While epigenetic changes have been implicated in numerous psychiatric conditions (Tsankova et al., 2007), the field of depression research has focused on two main chromatin modifying processes. The first is DNA methylation (of cytosine residues), which appears to be important in the influence of maternal

behavior on adult emotional processing. Adult offspring of rats born to mothers with low rates of licking and grooming display enhanced anxiety and low expression of glucocorticoid receptors within the hippocampus, a process mediated by decreased methylation on the glucocorticoid receptor gene promoter (effectively repressing gene expression). This long-lasting "molecular scar" (Tsankova et al., 2007) is established within the first week of life, and is effectively reversed by cross-fostering (Szyf et al., 2007). Interestingly, this increase in methylation was also reversed by the infusion of trichostatin-A, a histone deacetylase (HDAC) inhibitor (Weaver et al., 2004).

Histone acetylation, which is associated with decondensed chromatin and transcriptional activation, appears to be a key substrate for antidepressant action (Tsankova et al., 2004). Increased histone acetylation at the BDNF promoter in hippocampus is required for the ability of chronically administered imipramine to reverse the social avoidance phenotype following social defeat (Tsankova et al., 2006). HDAC inhibitors have been demonstrated to possess antidepressant properties in several behavioral assays (Schroeder et al., 2006; Tsankova et al., 2006), and efforts are underway to develop more potent agents that are designed to target specific HDACs, such as the Class II HDAC, HDAC5 (Tsankova et al., 2007; Tsankova et al., 2006). The implications of these studies come with an important anatomical caveat: while inhibiting the actions of HDAC5 in the hippocampus may be therapeutically advantageous, mice that are globally deficient in HDAC5 appear to be more vulnerable to the deleterious aspects of social defeat (Renthal et al., 2007). Similarly, while imipramine increases HDAC5 expression in hippocampus (Tsankova et al., 2006), it significantly reduces HDAC5 expression within the NAc (Renthal et al., 2007), further emphasizing the regional specificity of stress- and antidepressant-related plasticity.

Figure 3

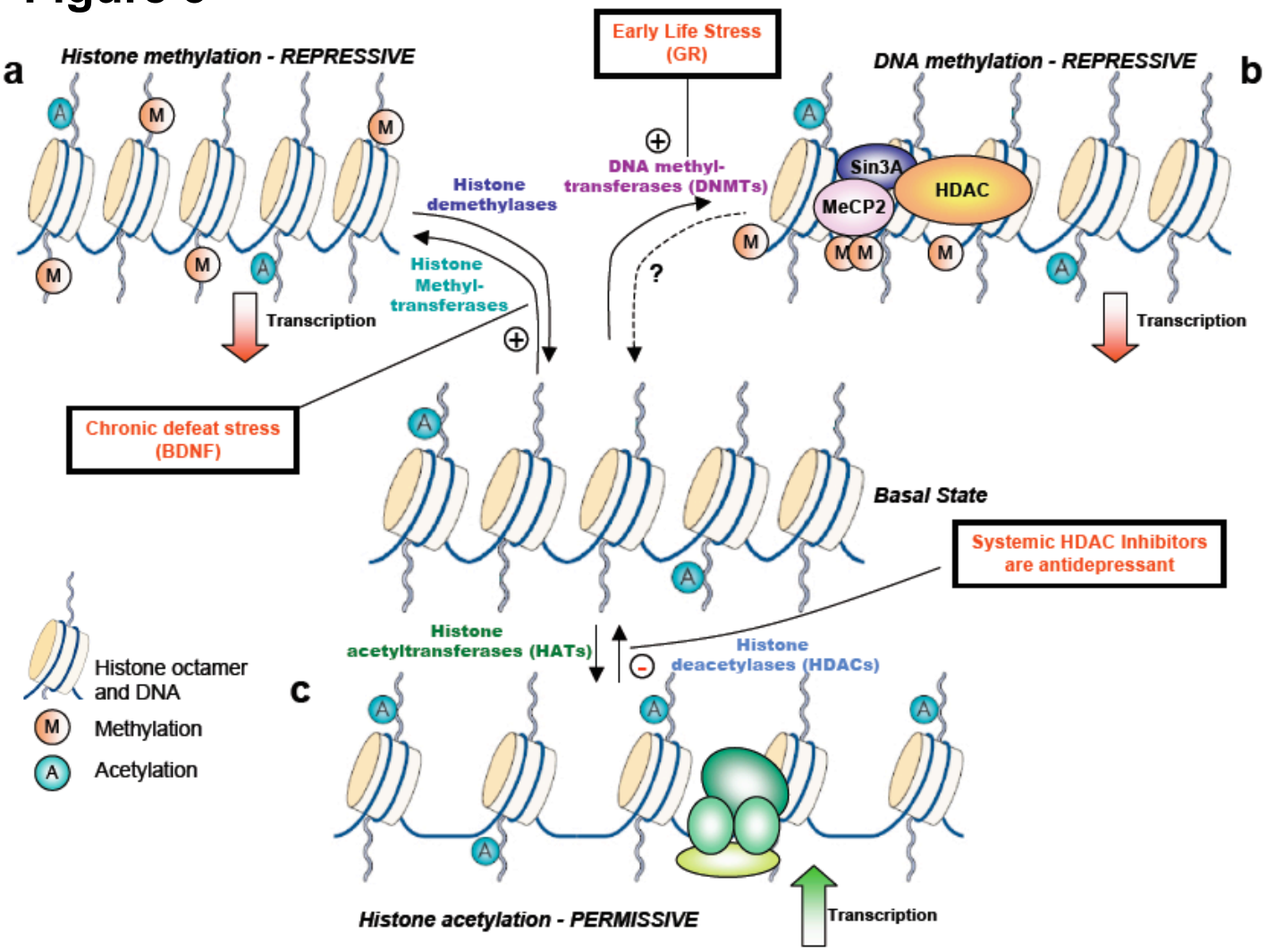


Figure 3. Epigenetic Contributions to Depression. **a,** Changes in the transcriptional potential of genes involved in neuroplastic responses can be modified through chromatin remodeling events carried out through the actions of specific enzymes. One example of a reversible change is histone methylation, which results in more condensed chromatin (heterochromatin), is important in the repression of *bdnf* expression in the hippocampus following social defeat(Tsankova et al., 2006). **b,** In contrast, repression of other genes can occur through the methylation of cytosine residues within CpG islands of promoter regions. Methylated cytosine residues attract enzymes involved in transcriptional repression such as Sin3A, MeCP2 (methyl-Cpg binding protein-2) and HDACs (histone deacetylases). DNA methylation of the glucocorticoid receptor promoter occurs in rat pups born to mothers with inherently low levels of maternal behavior (Weaver et al., 2004). While such methylation events have been shown to be reversible, the enzyme responsible for demethylating DNA has yet to be identified (Szyf et al., 2007; Tsankova et al., 2007) **c,** Addition of acetyl moieties to histone tails (histone acetylation) is catalyzed through the actions of histone acetyl transferases (HATs), and this event produces decondensed chromatin (euchromatin), allowing for unobstructed activity of transcriptional complexes. Inhibitors of HDACs (which would effectively maintain higher levels of acetylation genome-wide) possess antidepressant properties on a number of assays (Schroeder et al., 2006; Tsankova et al., 2006). Image reproduced and modified from Tsankova et al., 2007.

Our knowledge of the diversity of chromatin modifying enzymes, as well as techniques to detect and quantify chromatin modifications genome-wide are growing at an enormous pace. An

important challenge in the clinical translation of these approaches will be to improve our technological abilities to demonstrate causation, through the development of techniques to visualize these modifications *in vivo*, thereby allowing us to examine region-specific correlates of depression and antidepressant responses in humans.

Post Traumatic Stress Disorder (PTSD)

Many of the pathophysiological mechanisms described above for depression are also believed to play a key role in PTSD, which can be thought of as a stress-induced form of "learned" depression with prominent features of hyperarousal and pathological anxiety. The lifetime prevalence of PTSD within the United States is approximately one-third of major depression (Kessler et al., 2005), and it is estimated up to 20% of trauma-exposed individuals will eventually develop PTSD (Yehuda, 2004). The diagnosis of PTSD requires that an individual be exposed to a severe traumatic event and his/her stress-related symptoms persist. These include symptoms that are often branched into three categories: "re-experiencing" (e.g., nightmares, intrusive recollections), "avoidance/numbing" (e.g., emotional blunting, avoiding reminders of the event), and "hyperarousal" (e.g., exaggerated startle response, difficulty falling asleep) (Yehuda, 2002). Various elements of depression's neural circuitry also play key roles in PTSD symptoms, including the amygdala, hippocampus and the hypothalamus. Given the high degree of symptomatic overlap between depression and PTSD, as well as the largely identical treatments for these two diseases (antidepressants, anxiolytics and psychotherapy) (Yehuda, 2004), it is not surprising that rodent models involving acute or chronic stressors (e.g., social defeat, predator stress) have been applied towards both PTSD and depression, often together classified as "stress-related disorders".

In addition to the above-mentioned "emotional" symptoms, these disorders are also associated with cognitive changes. Depression is often linked to cognitive *hypofunction*, that usually manifests as difficulties with concentration and focus. Other symptoms such as ruminating negative thoughts, constant feelings of guilt, helplessness and suicidality are analogs of cognitive "dysfunction". These emotions are thought to be brought about through the "pathological" learning of maladaptive behaviors (Nestler et al., 2002). This form of "maladaptive learning" also features prominently in theories of PTSD pathogenesis, where patients are unable to "un-learn" their over-generalized and inflexible associations (Yehuda and Ledoux, 2007). Consistent with this hypothesis, some of the poorest indicators of PTSD prognosis include low levels of serum cortisol and high levels of serum catecholamines (norepinephrine and epinephrine) immediately following the traumatic event (Yehuda, 2002): this type of chemical environment (within structures such as the amygdala and hippocampus) provides a fertile environment for synapse formation and the establishment of memories. In the case of PTSD, these events serve a pathological role (Yehuda, 2004). Accordingly, treatments for PTSD have expanded to include glucocorticoids such as dexamethasone [thought to function through enhancing extinction (Cai et al., 2006)], as well as alpha- and beta-adrenergic blockers (e.g., prazosin and propranolol, respectively).

Insights into the Neurobiology of Resilience

Humans display a remarkable heterogeneity in their response to stress and adversity: while a subset of depression cases can be causally attributed to stressful life events, these events per se only moderately raise the risk of developing depression (Kendler et al., 1999). In addition, reactive dysphoric states such as PTSD only emerge in about 10-20% of trauma-exposed

individuals (Yehuda, 2004). While a large body of research describes maladaptive neurobiological changes that occur following stressful exposures (such as decreased neurogenesis and BDNF as discussed above), little attention has been devoted to the understanding the molecular mechanisms by which most individuals adapt well in the face of adversity (Charney, 2004). This phenomenon of "resilience", which has been appreciated for several decades (Curtis and Cicchetti, 2003), and a variety of psychosocial factors are known to be associated with stress-resilience, and include positive emotions and humor, cognitive restructuring abilities, spirituality and the ability to gather social support (Southwick et al., 2005). In contrast to this enormous body of clinico-psychological data, our understanding of the molecular neurobiology of resilient behavior remains at a rudimentary stage (Charney, 2004): while a number of neurotransmitter and neuropeptide systems mediate adverse outcomes following stressful episodes (Southwick et al., 2005), compensatory mechanisms by which these signaling systems are disabled remain entirely unknown. Additionally, it remains unclear whether resilient behavior is a manifestation of heightened plasticity (which would allow neuronal systems to successfully adapt following stressors) or extreme inflexibility (which would resist stress-induced neuroplastic events). This is analogous to learning paradigms that aim to dissociate heightened extinction from impaired acquisition, i.e., under conditions in which memory is impaired, did the organism learn worse, or does it simply forget at a quicker rate? Indeed, these two possibilities (high plasticity Vs. molecular inflexibility) may simultaneously occur in various regions of the brain, and identifying active resilience-related molecular processes (i.e., those that serve to restore psychological functioning to normal) may lead to the development of fundamentally novel antidepressant agents.

Recent Novel Insights

While the hypotheses described above remain active areas of research, recent novel findings have sparked interests in neurobiological systems that were previously unexplored in relation to depression. Perhaps the most dramatic example is the observation that sub-anesthetic doses of intravenously infused ketamine (a noncompetitive NMDA receptor antagonist) produce a rapid but transient antidepressant effect in treatment-resistant depression (Berman et al., 2000; Zarate et al., 2006). These striking effects suggest that depressive symptoms can be improved by altering glutamate signaling, the major excitatory neurotransmitter in brain. Ketamine's antidepressant properties have been recapitulated in animal tests of antidepressant action such as the forced swim test, where the ability of ketamine to reduce immobility requires intact AMPA glutamate receptor signaling (Maeng et al., 2008) and was associated with increased levels of hippocampal BDNF protein (Garcia et al., 2008). Given the limited evidence for glutamatergic dysfunction in depression (Maeng and Zarate, 2007), how these data contribute to our understanding of depression's pathophysiology is unclear. Nevertheless, ketamine's clinical effects have inspired new lines of preclinical research to explore the underlying neural circuitry and identify novel NMDA receptor modulators with better side effect profiles (Maeng and Zarate, 2007).

The past few years have also witnessed increased interest in examining interactions between traditional mood substrates and pathways involved in the control of feeding and metabolism. MCH (melanin-concentrating hormone) containing neurons projecting from the lateral hypothalamus (LH) to several limbic regions including NAc provide an important orexigenic (pro-appetite) signal. Global reductions in MCH signaling (Roy et al., 2007), as well

as local MCH antagonism within the NAc (Georgescu et al., 2005) produce antidepressant responses in a variety of rodent models, generating tremendous interest in the antidepressant potential of selective MCH antagonists (Berton and Nestler, 2006), which may also curb the weight gain associated with a subset of depression (Nestler and Carlezon, 2006). In contrast to the prodepressant actions of MCH, the hypothalamic neuropeptide orexin (hypocretin) may serve an antidepressant role, particularly during conditions of low caloric intake (Lutter et al., 2008a). These and other studies illustrate the general theme that an animal's metabolic status greatly impacts mood and motivation, and understanding the complex molecular interactions between peripheral metabolic signals such as ghrelin (Lutter et al., 2008b) and leptin (Lu et al., 2006), in collaboration with centrally released regulators of feeding and arousal such as MCH, orexin, NPY (Charney, 2004), and α-melanocyte-stimulating hormone (Kishi and Elmquist, 2005) may provide novel therapeutic and pathophysiological insights into mood disorders.

In conclusion, our knowledge of the pathophysiology of depression has substantially evolved from Galen's speculations during antiquity about an excess of black bile ("*melancholia*") (Nestler et al., 2002; Rush, 2007), to theories focused on "psychic pain" and "chemical imbalances", to more current hypotheses that incorporate gene-environment interactions, endocrine, immunological, and metabolic mediators, and cellular, molecular, and epigenetic forms of plasticity. However, enormous gaps in our knowledge of depression and its treatment persist. Instead of being overwhelmed by the heterogeneity of the illness, we must embrace the poly-syndromic nature of depression and employ a multidisciplinary approach to exploring the neurobiological bases for depression's many subtypes. To improve our still low remission rates (Trivedi et al., 2006), it will be imperative to look beyond monoaminergic mechanisms (Berton and Nestler, 2006), and expand our knowledge of antidepressant

pharmacogenetics. And finally, we must harness the full potential of preclinical studies, by continuing to develop improved animal models that incorporate the powerful array of molecular and anatomical tools available today, and follow a systems approach to the study of depression that acknowledges the powerful interactions between peripheral organs and the brain.

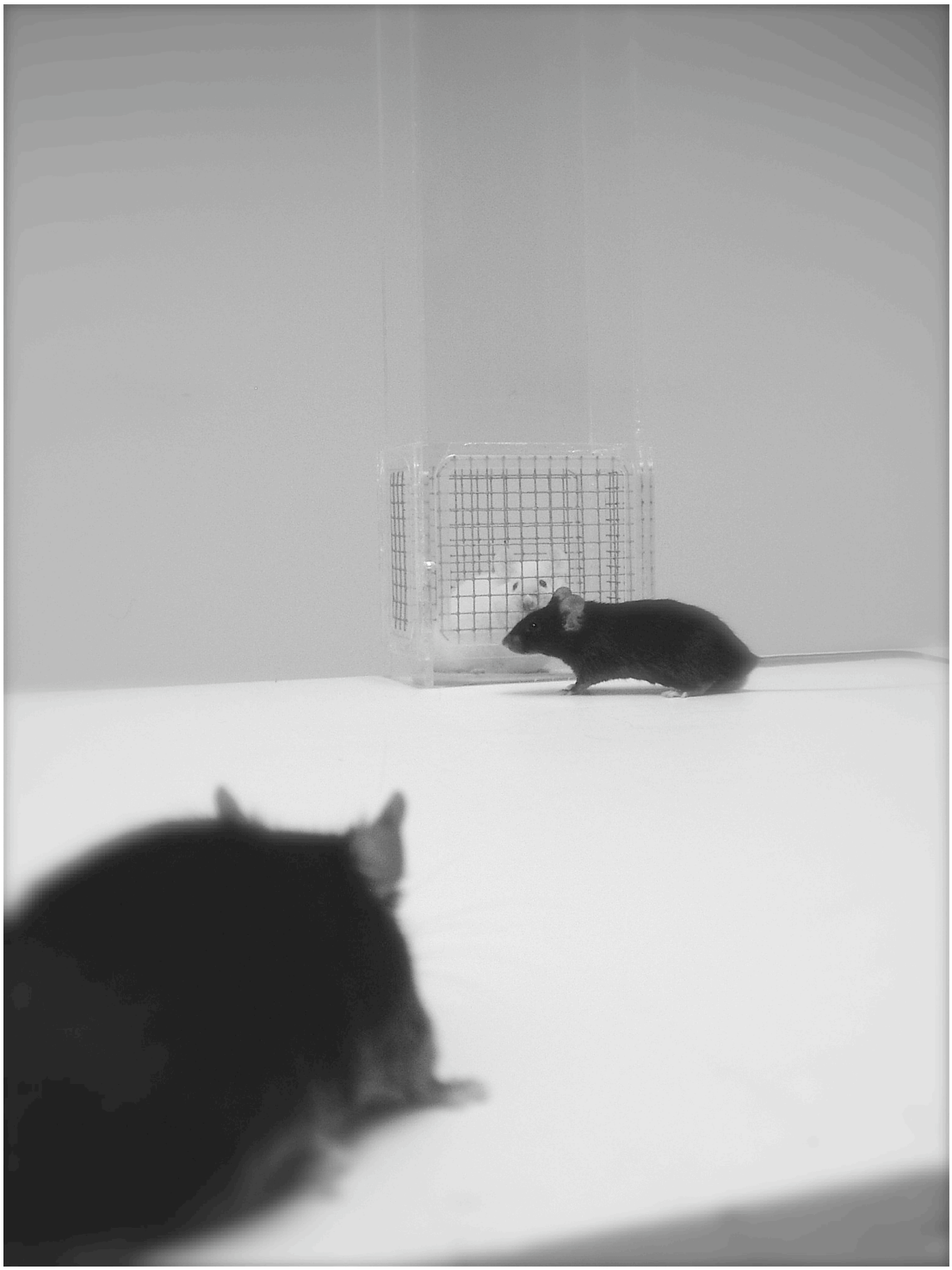
A Preface to my Work

In 1936, a Hungarian scientist named Hans Selye published a brief article in Nature titled "A Syndrome Produced by Diverse Nocuous Agents" (Selye, 1998), where he described how a variety of stressful stimuli applied to rats, such as burns, infections, injuries and intoxications, all produced a similar set of physiological responses, which he termed the "General Adaptation Syndrome". This comprised a set of systemic changes including thymolymphatic involution, gonadal atrophy, lacrimation and salivation, etc. Importantly, Selye recognized that several aspects of this syndrome could be recapitulated in non-stressed rats through a simple transfusion of serum from stressed rats. This work led to the appreciation of the role of corticosteroid signaling and the HPA axis (hypothalamo-pituitary-adrenal) on stress responses, and the significant negative impact of hypercortisolemic states on physiological and behavioral functioning (McEwen, 2007).

Over the past 70 years, as we continue to appreciate and combat the pathological consequences of stressful life events, our knowledge of *stress-induced* behavioral, physiological and molecular changes has enormously expanded. However, it is often unknown whether these neuroadaptations represent pathological "prodepressant" changes that mediate maladaptive behavior, or "pro-resilient" homeostatic mechanisms that serve to promote coping in terms of adversity. In the following chapters of my dissertation, I summarize the results of experiments

that aim to dissociate these two types of neuroplastic changes. I will first demonstrate how the social defeat model of chronic stress can be adapted for the study of individual differences, and therefore can serve as a natural model to examine behavioral and physiological changes associated with vulnerable and resilient behavior. To complement this model's established pharmacological validity (Berton et al., 2006), I will demonstrate the face validity of social defeat through a series of phenotypic characterization experiments. This model will then be employed to examine how vulnerable and resilient behavior is associated with distinct profiles of BDNF signaling within the mesolimbic dopamine reward circuit, a set of limbic substrates that are involved in encoding and transducing natural and drug-related rewards. Through a combination of behavioral, molecular and electrophysiological techniques, I will show that elevations in BDNF levels within the NAc represent a *final common pathway* for the manifestation of social avoidance, and that this molecular adaptations is mediated via increased activity-dependent BDNF release from the VTA to the NAc, which is necessary and sufficient for social avoidance. Using similar methodologies, I will reveal an important role for AKT signaling as a type of *master regulator* of susceptibility to social avoidance, by demonstrating that reductions in VTA AKT activation are sufficient to recapitulate the behavioral, electrophysiological and morphological hallmarks a susceptible phenotype. The therapeutic and scientific implications of these results are discussed.

Chapter II: The Model



"Resilience, Insusceptibility, Resistance, Hardiness and Defiance"

This photograph captures the two extreme outcomes following chronic social defeat of c57BL/6 mice. Approximately 55% of mice display social avoidance (mouse on left) to the aversive CD1 social cue, characterized by a rapid retreat to one of the corners of the social interaction arena as well as a robust hyperthermic and freezing response. Other mice (right c57BL/6 mouse) display normal/control levels of social interaction, together with a prominent lack of autonomic arousal. This response, occurring in spite of being exposed to chronic social defeat, has been described by several terms, including "resilience" or "stress-resistance".

Photograph by Vaishnav Krishnan.

An Introduction to Social Defeat

Social subordination stress, defined as stress that is experienced specifically as a result of inferior social status within a group of animals, has been utilized by investigators over a number of decades to examine ethologically relevant stress responses, i.e., in an attempt to recapitulate the naturally occurring stress of serving a subordinate role within a colony of animals. The assignment of social hierarchy within rodent communities serves the evolutionarily advantageous purpose of ensuring organization during times of stress, such that the fittest animals are ensured adequate resources (sexual mates, food, shelter, etc.). Within rodent models, a number of different variations of social stress paradigms have been developed (Martinez et al., 1998), which all rely on the territorial aggression displayed by a dominant male towards an intruder mouse. Among these paradigms, there exists an important distinction between those that quantify stress-induced behavioral changes during social stressful experience, versus those that examine behavioral adaptations following the cessation of social stress. An example of the former is the "resident-intruder" paradigm, whereby dominant males that have been previously selected for consistent and reliable aggression are allowed to territorialize their "home cage". An intruder is introduced into this cage, and the resultant physical and non-physical behavioral changes are quantified during this encounter (e.g., pin downs, subordinate posturing, tail bites, etc.).

Social defeat carried out in the Nestler laboratory falls into the latter category, whereby 9 week old c57BL6/J mice are exposed to 10 bouts of 10 minute long social defeat episodes with CD1 retired breeder mice over 10 days, and stress-induced adaptations are quantified following the termination of social stress when mice are socially isolated (Figure 4A, B). An important modification of this paradigm that we employ is the application of "sensory contact". The mouse sensory contact model, developed and validated by Kudryavtseva and colleagues (Avgustinovich

et al., 2003; Avgustinovich et al., 2005; Kudryavtseva et al., 1991), forces the subordinate mouse to experience two distinct forms of stress: the physical interaction/fighting that occurs during defeat episodes, as well as the psychological stress of being housed opposite that aggressor mouse for the remainder of the day across a perforated partition device that permits sensory experiences without actual physical contact (Figure 4C).

Figure 4

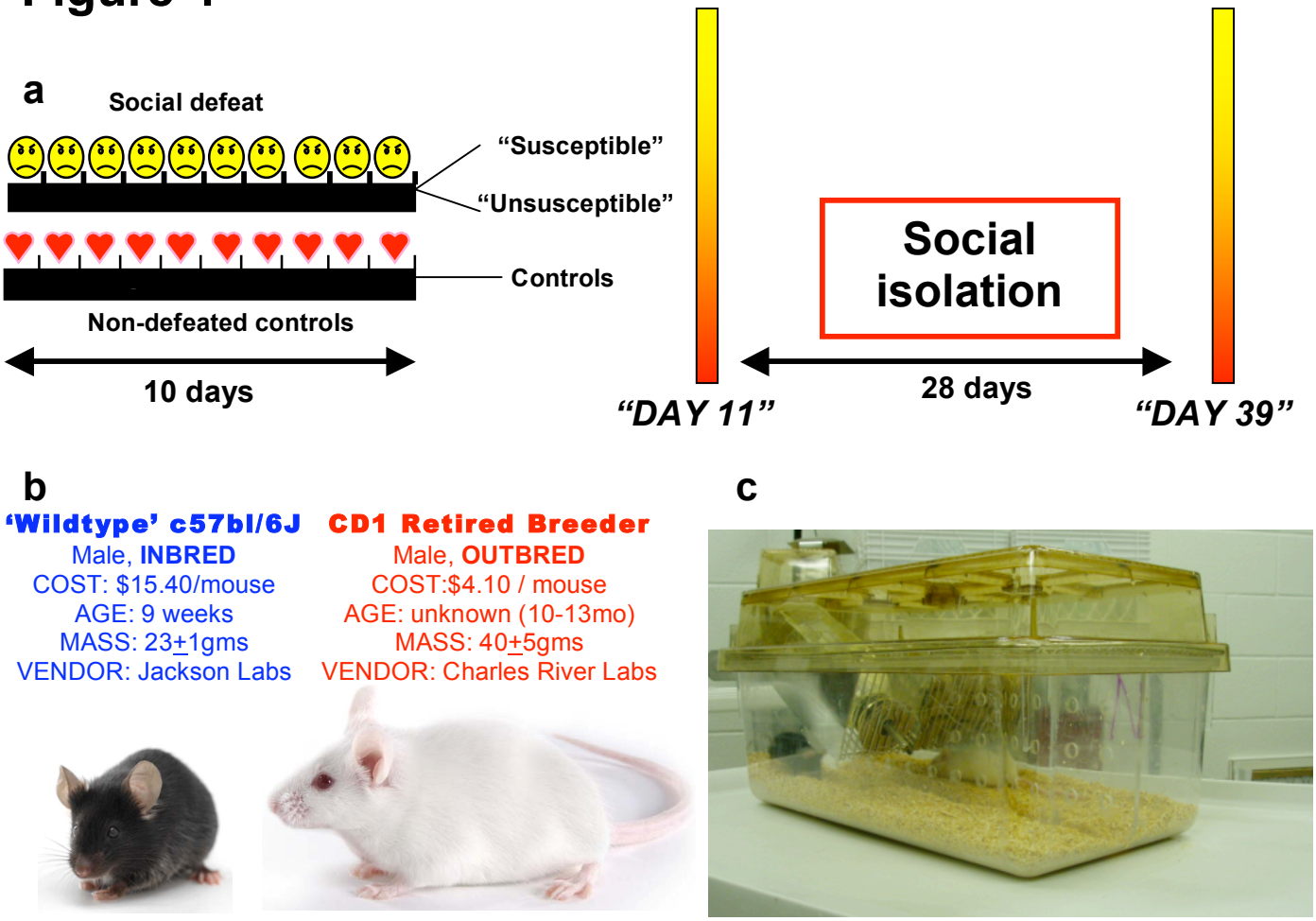


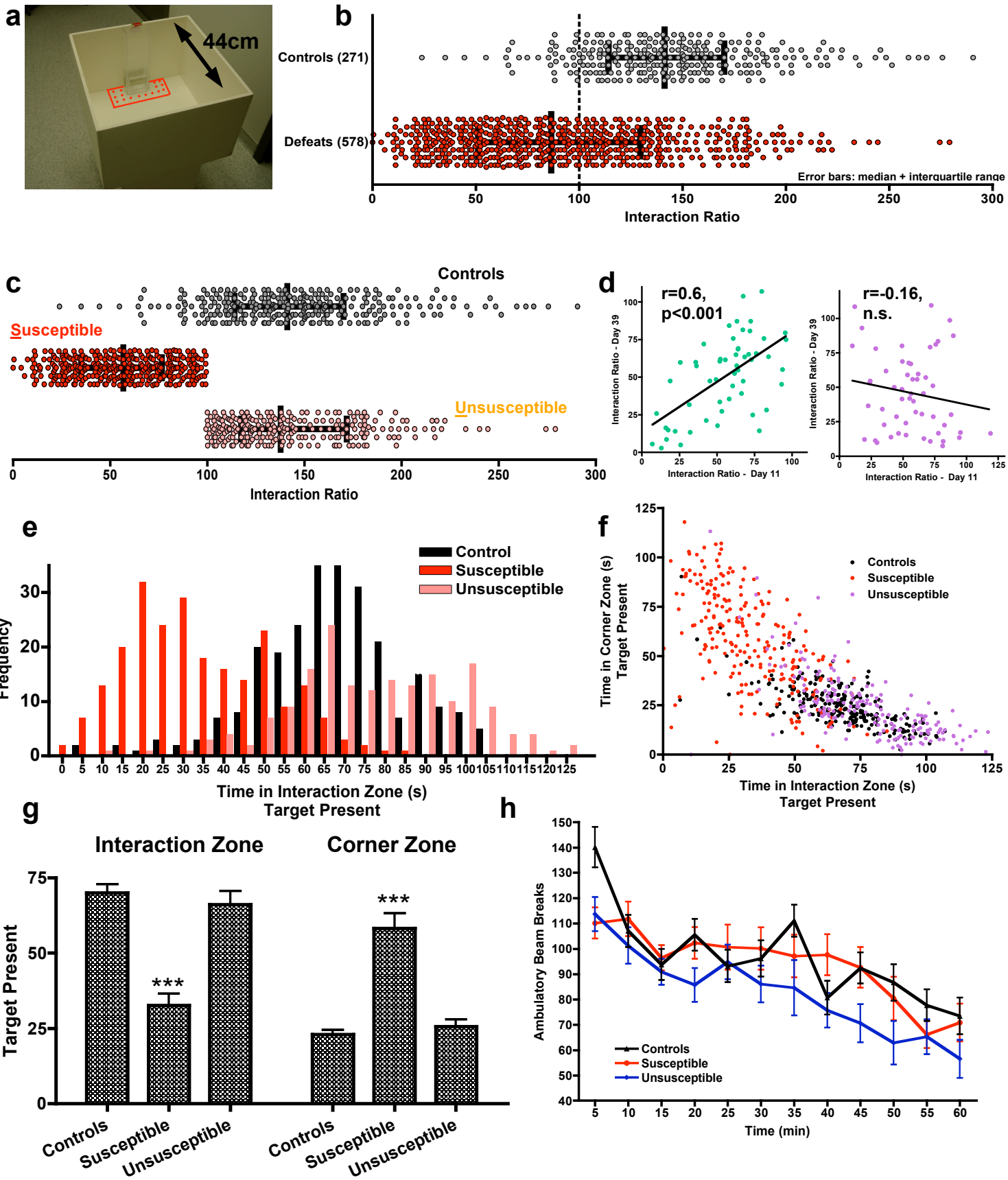
Figure 4. The social defeat model of chronic stress. **a,** Each defeated mouse is exposed to 10 daily episodes of defeat, each lasting approximately 10 minutes in duration. On Day 11, the social interaction test is employed to segregate "Susceptible" from "Unsusceptible" mice. A variety of behavioral, molecular and electrophysiological assessments can be carried out 24 hours following this interaction testing ("Day 11"), or following a prolonged social isolation period ("Day 39"). Chronic antidepressant treatments (once daily intraperitoneal injections) are performed during this interim period (Day 11 – Day 39). **b,** A comparison of certain attributes of the male mice employed in this paradigm (c57BL/6J Vs. CD1 retired breeders). **c,** Photograph of a typical "social defeat cage" where mice are housed on either side of a plexiglass perforated partition, which allows sensory contact without physical interaction.

A Segregation of Defeated Mice into Susceptible and Unsusceptible Populations

We have previously shown that c57BL/6 mice subjected to chronic social defeat (10 such defeats over 10 days) display a robust 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 (Figure 5A). This avoidance phenotype possesses two critical properties: first, social avoidance is reversed by the chronic but not acute administration of canonical antidepressant agents like imipramine and fluoxetine (Berton et al., 2006; Tsankova et al., 2006). Second, while Day 11 social interaction levels are generally measured using an unfamiliar CD1 retired breeder as a "social target", defeated c57BL/6 mice also display significant interaction deficits towards unfamiliar c57BL/6 mice, i.e., their avoidance appears to (over)generalize to physically distinct mice of a non-CD1 strain (Berton et al., 2006).

By analyzing a large number of chronically defeated mice, we found a wide distribution of responses: when examined 24 hours after the last defeat ("Day 11"), 40-50% of defeated mice displayed interaction scores similar to non-defeated controls (Figure 5B). Since 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 vs. absence of a social target) was set as a cutoff: mice with scores <100 were labeled "Susceptible" and those with scores ≥ 100 were labeled "Unsusceptible" (Figure 5C). This latter group displayed median and variance values similar to controls (Table 1). The social avoidance behavior of defeated mice on Day 11 correlated significantly with Day 39 interaction scores ($r=+0.61$, $p<0.0001$, $n=44$). Such a correlation was lost following chronic daily administration of 20 mg/kg imipramine (Figure 5D).

Figure 5


a
44cm
b
Controls (271)
Defeats (578)
Error bars: median + interquartile range
Interaction Ratio
c
Controls
Susceptible
Unsusceptible
Interaction Ratio
d
r=0.6,
p<0.001
Interaction Ratio - Day 39
Interaction Ratio - Day 11
r=-0.16,
n.s.
e
Control
Susceptible
Unsusceptible
Frequency
Time in Interaction Zone (s)
Target Present
f
Controls
Susceptible
Unsusceptible
Time in Corner Zone (s)
Target Present
Time in Interaction Zone (s)
Target Present
g
Interaction Zone
Corner Zone
Time in Zone (s)
Target Present
***
***
Controls
Susceptible
Unsusceptible
Controls
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Unsusceptible
h
Ambulatory Beam Breaks
Controls
Susceptible
Unsusceptible
Time (min)

Figure 5 (contd.)

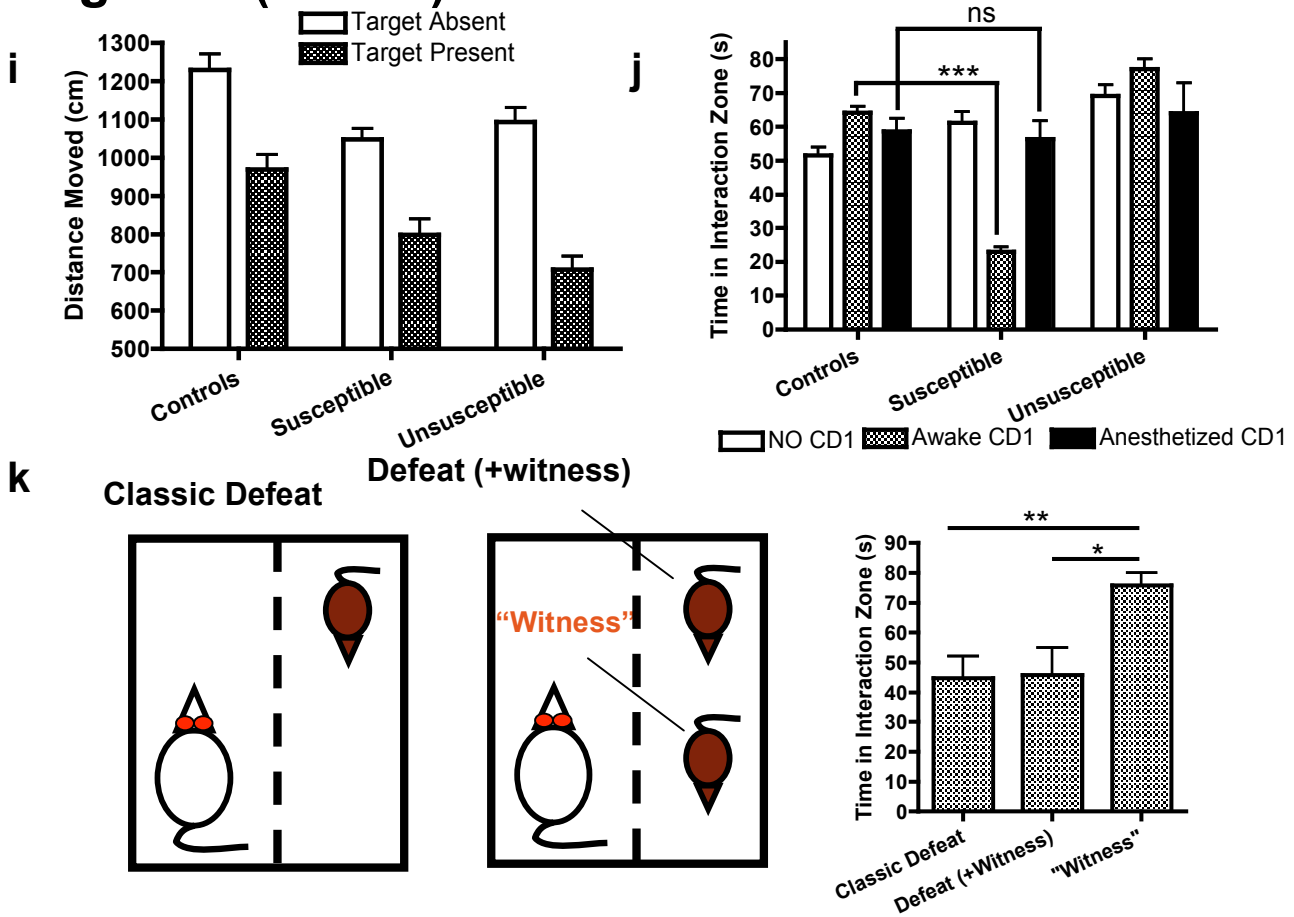


Figure 5. a, Open field box employed for social interaction testing. A rectangular "interaction zone" is demarcated around the wire mesh cage (14x8cm). The time spent in two square shaped "corner zones" (9x9cm) diagonally opposite the interaction zone is also recorded. **b,** Horizonatal scatter plot depicting IRs for a large number of socially defeated c57BL/6 mice and non-defeated controls **c,** Results of the same horizontal scatter analysis following segregation of defeated mice into two groups (Susceptible [IR<100], and Unsusceptible [IR≥100]). **d,** LEFT: Within defeated mice, Day 11 and Day 39 interaction scores are significantly correlated (i.e., their avoidance behavior remains relatively longlasting), whereas RIGHT: chronic administration of imipramine obliterates this correlation. **e,** Frequency distribution histogram for the absolute time spent in interaction zone. **f,** Two dimensional scatter plot (where every dot represents interaction [x-axis] and corner zone [y-axis] times of one mouse) demonstrating a segregation of Susceptible mice (n=242), from controls (n=269) and Unsusceptible mice (n=195). **g,** 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 ($F_{2,54} = 35.27$, $p<0.0001$). **h,** Locomotor test reveals no overall differences in ambulatory locomotor activity between control, Susceptible and Unsusceptible groups, except during the first 5 mins of the test when both stressed groups mice display decreased activity ($F_{2,54} = 5.60$, $p<0.01$). **i,** Distances moved in the interaction arena during the absence or presence of a target mouse for control, Susceptible and Unsusceptible mice illustrating that resilience to social avoidance in this paradigm cannot be explained by general locomotor effects (Defeat Factor: $F_{2,108} = 15.82$, $p<0.0001$, Target Factor: $F_{1,108} = 88.85$, $p<0.0001$, n=15-22). **j,** Interaction zone times in conditions of an absent target, an awake behaving CD1 target, and an anesthetized CD1 target (group x repeated measure target interaction effect, $F_{4,54}= 2168.6$, $p<0.0001$). **k,** Avoidance scores are similar between mice subjected to "classical" social defeat, and those that experience the social defeat paradigm together with a non-defeated "witness". This witness mouse is paired and rotated together with a defeated mouse. Groups displayed significantly different interaction times during the presence of the aversive CD1 cue ($F_{2, 29} = 5.98$, $p<0.01$, n=10), and "witness" displayed significantly greater interaction scores.

Table 1

	Susceptible	Unsusceptible	Control
25% Percentile	35.0	118.3	113.9
Median	56.5	137.6	141.2
75% Percentile	75.5	172.3	172.3
Mean	56.08	149.0	147.7
Standard Deviation	24.94	47.01	50.07
Standard Error	1.60	3.38	3.05
Lower 95% CI of Mean	52.92	142.40	141.70
Upper 95% CI of Mean	59.24	155.70	153.70

Table 1. Population characteristics of the interaction ratios of control, Susceptible and Unsusceptible mice measured across multiple identically designed social defeat experiments carried out by V.K [corresponding to Figure 5C]. These data illustrates a strong degree of population overlap between control and Unsusceptible mice: these groups were similar in measures of central tendency (mean, median) and also measures of variance (standard deviation, standard error, percentile ranks). Abbreviations: CI (confidence interval).

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 5E), and a two-dimensional scatter plot comparing interaction times to the time spent in the corner zones of the arena (Figure 5F), also revealed a segregation of Susceptible mice from Unsusceptible and control mice. Figure 5G shows Day 11 data from a representative experiment illustrating how only Susceptible mice actively avoid the interaction zone by spending more time in "corner zones" (two 9x9cm square corners diametrically opposite to the wire mesh cage). Differences in susceptibility cannot be explained by locomotor behavior (equivalent in both subgroups, Figures 5H,I) or variations in aggression during defeat (both groups sustained the same degree of minor injuries, which were subjectively quantified by a blinded experienced rater). An awake behaving social target is necessary for social avoidance, as we observed a total lack of avoidance to an anesthetized target (Figure 5J). Additionally, the development of social avoidance appears to require both the psychological and physical components of social defeat: c57BL/6 mice that reside across the CD1 retired breeder, and which

are forced to "witness" social defeat across the perforated partition do not develop social avoidance (Figure 5K).

Table 2

Pre-Defeat Measures	Pearson 'r' Coefficient	p value
Duration in Periphery(s) , Open Field	-0.02	n.s.
Latency to Center (s), Open Field	+0.11	n.s.
Total Distance Moved (cm), Open Field	-0.16	n.s.
Center/Total Distance Moved, Open Field	+0.00	n.s.
Interaction Ratio	-0.13	n.s.
Time in Interaction Zone, Target Present	-0.09	n.s.
Initial Weight (Day 1)	+0.09	n.s.
Immobility Duration (FST)	-0.08	n.s
Social Dominance	Not Correlated	N/A

Table 2. This table summarizes the results of experiments designed to explore "predefeat" characteristics that may correlate with "post-defeat" interaction ratios. For all experiments, n=30-40, and both controls and defeated mice are included in the correlation. "Pre-defeat" social dominance relationships within dyads of mice was assessed using a food restriction task, described in greater detail in Materials and methods.

The considerable variance observed within defeated c57BL/6 mice cannot be explained by differences in age, sex, genotype, or vendors, since only 9 week-old, c57BL/6J male mice from a single vendor (Jackson Labs) were used. In an effort to predict which c7BL6 mice would display this form of behavioral resilience, we obtained a series of "pre-defeat" measures prior to the start of social defeat, and explored potential correlations between these measures and "post-defeat" interaction ratios. As shown in Table 2, Day 11 interaction ratios were not significantly correlated with "Day 0" measures of body weight, open field exploration, social interaction, forced swim test immobility or putative measures of social dominance. Therefore, this form of insusceptibility to social defeat appears to act as a *latent* trait such that it is only *exposed* upon the application of social stress, a property that very accurately resembles human resilience. Importantly, susceptibility in this paradigm is not simply the consequence of a pre-existing

subordinate status, possessing lower body weight, lower motivation to socially interact or enhanced anxiety-related behavior.

Susceptible and Unsusceptible Mice Display Distinct Syndromes

Day 11

To examine whether resistance to defeat-induced social avoidance would generalize to other behavioral measures, we performed an extensive phenotypic characterization of Susceptible and Unsusceptible mice. These data are summarized in Table 3, and only important results are noted here. On Day 11, only Susceptible mice displayed a significant decrease in body weight (Figure 6A) and sucrose preference ($F_{2,33}$=5.70, p<0.01, Figure 6B), both consistent with increased depression-like behavior. Interestingly, as the sucrose preference test allows for a measure of total fluid intake during testing, we observed that both defeated subgroups displayed a stress-induced polydipsia phenotype (Figure 6C), which has also been observed in the chronic mild stress model of depression (Strekalova et al., 2006). This phenotype was found to be independent of changes in mRNA levels of arginine vasopressin (AVP, also known as antidiuretic hormone or ADH) in hypothalamic extracts (Figure 6C inset), suggesting potential extrahypothalamic mechanisms for physiological change. 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 6D). By employing commercially available high sensitivity EIAs (enzyme immunoassays) for corticosterone (CORT), we observed that baseline levels of serum CORT were similar across three groups (Figure 6E). In contrast, CORT levels were significantly lower 24 hours after a single defeat (Figure 6E inset), suggesting the presence of a heightened negative feedback response that may desensitize over time. Both

defeated subgroups demonstrated a sensitized CORT response to a 6 minute swim stress ($F_{2,35}$=12.34, p<0.0001, Figure 6E). Serum levels of dehydroepiandrosterone sulfate (DHEA-S), an adrenally derived androgen implicated in resilient behavior in humans (Charney, 2004), remained unaltered across groups (Table 3).

Table 3

	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	⇔	⇑

Table 3. Susceptible and Unsusceptible Mice Display Distinct Syndromes. This table illustrates the phenotypic differences between Susceptible and Unsusceptible mice on Day 11, and also shows which of those phenotypes persist four weeks later (Day 39). Abbreviations: TST (Tail Suspension Test), FST (Forced Swim Test), DHEA-S (dehydroepiandrosterone-sulfate), Wt (weight), ⇔,⇑ and ⇓ (no change, significantly greater than or less than non-defeated control group [p<0.05] respectively), N/A (not available).

To evaluate autonomic arousal and circadian function, we implanted a subset of mice with subcutaneous temperature transponders (Liu et al., 2003a). Both Susceptible and Unsusceptible mice showed an anticipatory form of autonomic arousal during the course of social defeat, and this was shown in two separate ways. On days 4-10 of the social defeat paradigm, Susceptible and Unsusceptible mice displayed a significant elevation of body temperature in the 30 min prior to the onset of an expected defeat episode ("anticipatory

hyperthermia", Figure 6F). In addition, by obtaining once daily measurements of core body temperatures at the nadir (12:00 noon) of their circadian temperature cycle (van Bogaert et al., 2006), both Susceptible and Unsusceptible mice displayed a form of "psychogenic fever" (Keeney et al., 2001) during the vast majority of social defeat days (Figure 6G), indicating the presence of chronically elevated sympathetic arousal. Following the cessation of defeat, only Susceptible mice demonstrated a significant reduction in the circadian amplitude of temperature fluctuations, ($F_{2,79}$=3.21, $p<0.05$, Figure 6H) and a significantly elevated hyperthermic response to the social avoidance test ("social hyperthermia", $F_{2,86}$=5.30, $p<0.01$, Figure 6I). In other measures, only Susceptible mice displayed significant conditioned place preference to a low dose of cocaine (Figure 6J). This phenomenon, where stressful exposures produce sensitized responses to psychostimulants and opiates ("cross-sensitization") has been observed in a variety of different paradigms (Covington and Miczek, 2005; McLaughlin et al., 2006), and further validates our model. An exploratory analysis / correlation analysis is depicted in Table 4, where Day 11 interaction ratios are correlated with other Day 11 quantitative measures. This analysis revealed that avoidance scores were most closely related to hedonic changes, such as defeat-induced deficits in sucrose preference and body weight. Importantly, social avoidance appears to be unrelated to stress-reactivity / anxiety measures (elevated plus maze, novelty-induced hypolocomotion, anticipatory hyperthermia, stress-induced polydipsia, CORT sensitization), indicating a dissociation between true "depression-related" measures (observed only in Susceptible mice) from "anxiety-related" measures (seen in both defeated subgroups) (Kalueff et al., 2006).

Figure 6

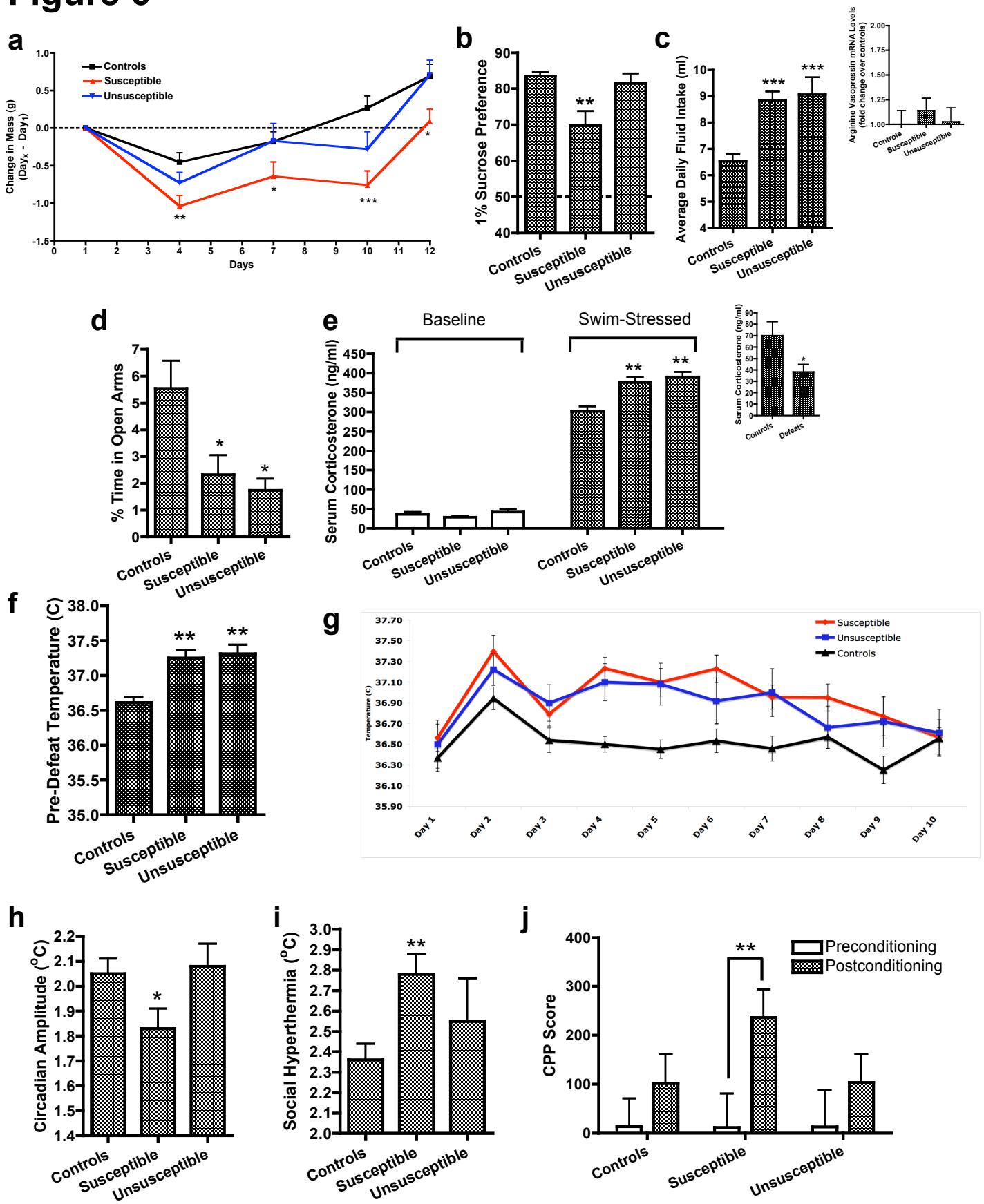

a
Controls
Susceptible
Unsusceptible
Change in Mass (g) (Day$_X$ - Day$_1$)
Days
b
1% Sucrose Preference
Controls
Susceptible
Unsusceptible
c
Average Daily Fluid Intake (ml)
Arginine Vasopressin mRNA Levels (fold change over controls)
d
% Time in Open Arms
e
Baseline
Swim-Stressed
Serum Corticosterone (ng/ml)
Serum Corticosterone (ng/ml)
Controls
Defeats
f
Pre-Defeat Temperature (C)
g
Susceptible
Unsusceptible
Controls
Temperature (C)
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
h
Circadian Amplitude (^{o}C)
i
Social Hyperthermia (^{o}C)
j
CPP Score
Preconditioning
Postconditioning

Figure 6. Social Defeat Produces a Wide Array of Behavioral Changes on Day 11. a, During the social defeat paradigm, Susceptible mice displayed a more prominent weight loss (group x repeated measure "Day" interaction effect, $F_{8,436} = 3.61$, $p<0.0001$, n=25-51). Unsusceptible mice were not significantly different from controls. b, Only Susceptible mice display anhedonia as measured by a reduction in 1% sucrose preference. c, On Day 11, both Susceptible and Unsusceptible groups display increased fluid intake ($F_{2,33} = 12.80$, $p<0.0001$, n=8-12). This "stress-induced polydipsia" (Strekalova et al., 2006) was not accompanied by changes in hypothalamic mRNA levels of arginine vasopressin (AVP mRNA, n=5-8) [inset]. d, Both Susceptible and Unsusceptible mice display decreased exploration on the elevated plus maze test. e, Whereas baseline (AM) levels of serum corticosterone were equivalent between controls, Susceptible and Unsusceptible mice were similar (n=11-15, $F_{2,55} = 1.13$, $p>0.3$), a six-minute forced swim stressor produced a sensitized CORT response in only Susceptible and Unsusceptible mice. f, Anticipatory hyperthermia (an elevation in temperature observed approximately 30 mins prior to a daily defeat episode) was observed in both Susceptible and Unsusceptible mice ($F_{2,82} = 14.76$, $p<0.0001$). g, Both Susceptible and Unsusceptible mice displayed chronically elevated body temperatures during the course of the social defeat paradigm (through daily measurements at 12:00 noon). h, Only Susceptible mice display blunted circadian rhythms and i, significantly enhanced social hyperthermia (an elevated hyperthermic response to the CD1 target). j,) Averaged Preconditioning and Postconditioning CPP (conditioned place preference) scores (time spent in drug-paired side minus saline-paired side) for control, Susceptible and Unsusceptible groups. 2 Way ANOVA revealed a significant effect of conditioning session: $F_{1,36} = 12.93$, $p<0.001$, with only Susceptible mice displaying a significant increase in CPP score (Fisher's LSD post-hoc).

Table 4

Day 11 Measures	Pearson 'r' Coefficient	p value
Time in Interaction Zone, Target Present	**+0.81**	**<0.0001**
Time in Corner Zone, Target Present	**-0.74**	**<0.0001**
% Time in Open Arms, EPM	+0.04	n.s.
Total Arm Entries, EPM	+0.23	n.s.
Total Ambulations, "Beam Break"	-0.07	n.s.
Ambulations (First 5 min)	+0.18	n.s.
Sucrose Preference	**+0.44**	**<0.01**
Duration of Immobility (s), TST	-0.17	n.s.
Duration of Immobility (s), FST	+0.05	n.s.
Circadian Amplitude (^{o}C)	+0.05	n.s.
Social Hyperthermia (^{o}C)	**-0.43**	**<0.0001**
Baseline Serum Corticosterone	+0.24	n.s.
Serum Corticosterone after Swim Stress	-0.17	n.s.
Serum DHEA-S	+0.22	n.s.
Injury Score (1-10)	+0.16	n.s.
Final Weight	**+0.21**	**<0.05**
Defeat Induced Weight Change	**+0.19**	**<0.05**

Table 4. This table summarizes the results of an exploratory analysis between the primary measure of resilience (Interaction Ratio) and other commonly employed laboratory measures of emotionality. All correlations were obtained from experiments performed with c57bl/6 mice (n=30-100), on Day 11. Abbreviations: EPM (elevated plus maze), TST (tail suspension test), DHEA-S (dehydroepiandrosterone sulfate), n.s. (non significant, $p>0.05$).

Day 39

While social defeat produced a wide variety of robust phenotypic changes on Day 11, many of these behavioral and physiological changes did not persist until Day 39 (i.e., following 28 days of social isolation). These results are also summarized in Table 3, which illustrates that only the social hyperthermia and elevated plus maze exploration phenotypes were maintained on Day 39 (Figure 7A, B). Importantly, sucrose preference scores were normalized at this time point (Figure 7C). Interestingly, serum CORT levels were significantly lower in Susceptible mice as compared to Unsusceptible mice, suggesting that our behavioral segregations resulted in distinct HPA axis profiles (Figure 7D). To extend our exploration of heightened sympathetic activation produced by social defeat, we examined a cohort of animals on Day 39 for cardiac hypertrophy, a type of cardiac remodeling event that is usually the result of excess catecholamine signaling. A simple measure of cardiac hypertrophy is obtained by precisely measuring the weight of a mouse's heart and normalizing this heart weight (HW) to an animal's body weight (BW) (Hill et al., 2000). Contrary to our expectations, only Unsusceptible mice displayed a mild but significant elevation in HW/BW ratios (Figure 7E), suggesting that the persistence of resilient behavior may be associated with prominent elevations in systemic catecholamine levels.

Figure 7

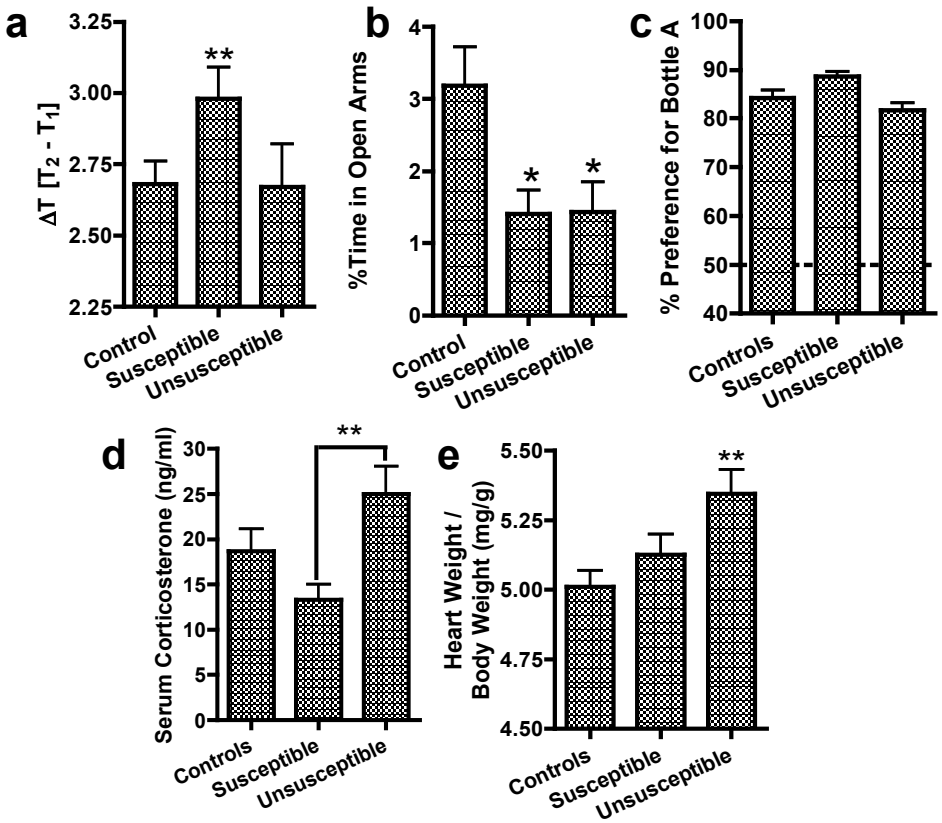
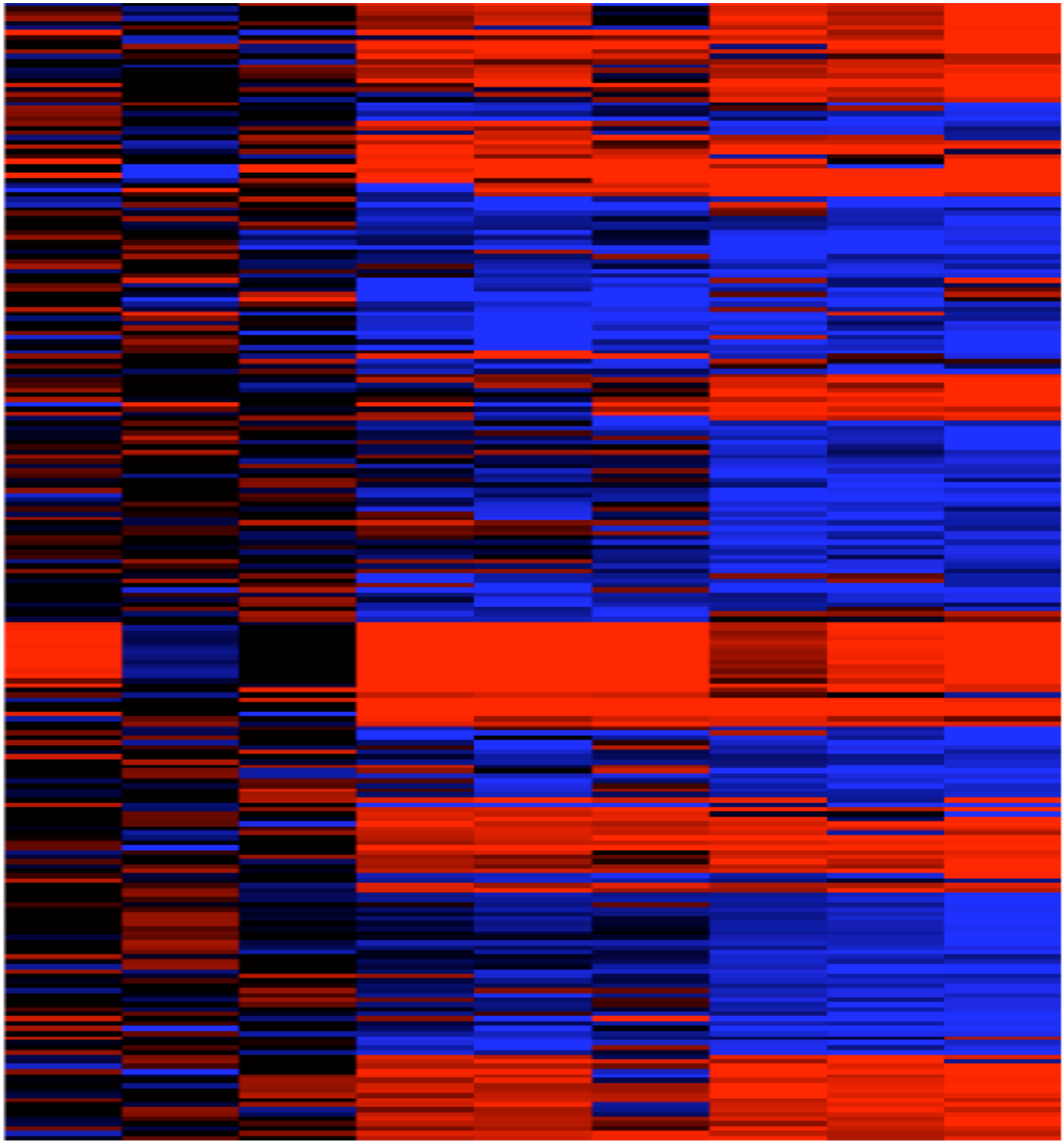


Figure 7. An Assortment of Day 39 Behavioral Phenotypes. **a,** In addition to social avoidance, social defeat produces a longlasting increase in social hyperthermia ($F_{2,\ 75}$=4.93, p<0.01) in only Susceptible mice. **b,** Similarly, on Day 39, one still observes significantly elevated levels of anxiety-like behavior on the elevated plus maze task ($F_{2,109}$ = 5.6, p<0.001). **c,** Controls, Susceptible and Unsusceptible mice display comparable sucrose preference scores ($F_{2,56}$=2.83, p>0.05), as well as daily fluid intake (data not shown). **d,** At this timepoint, daytime levels of serum corticosterone were significantly different between Susceptible and Unsusceptible mice (n=22-26, $F_{2,70}$ = 5.74, p<0.01), although neither was significantly different from controls. **e,** Unsusceptible mice displayed a significant increase in the ratio of heart weight to body weight (n=28-50, $F_{2,119}$ = 5.24, p<0.01). Mean body weights for the three groups were not significantly different (data not shown).

Collectively, these characterization data illustrate the *face validity* of this particular adaptation of the social defeat paradigm in mice (i.e., 10 x 10min long defeats between a CD1 retired breeder and a c57BL/6 mouse). While similar behavioral characterizations have been carried out by other groups that routinely perform social defeat (Avgustinovich et al., 2005; Keeney et al., 2001; Martinez et al., 1998), such a phenotypic validation is a crucial step before transitioning towards molecular parameters, particularly in light of the limited reproducibility of behavioral assays (Cryan and Holmes, 2005; Willner, 2005). In addition, we illustrate (for the first time) the application of an individual differences methodology to examining phenotypic

variation following social defeat. The separation of stress-induced anxiety from stress-induced anhedonia observed in our model has also been observed using the chronic mild stress model of depression (Strekalova et al., 2006; Strekalova et al., 2004). Finally, Susceptible and Unsusceptible mice appear most different along reward-related measures, including sucrose preference test, cocaine-conditioned place preference and weight loss. While a variety of brain regions are involved in the regulation of emotion (reviewed in the Introduction), these observations suggest that important clues about the molecular bases for vulnerable vs resilient behavior may lie within limbic reward-related neural substrates.

Chapter III: "Following BDNF"



"Red, Black and Blue"

This "heat map" diagram illustrates the results of genome-wide expression arrays performed in the **VTA** on Day 11 of the social defeat paradigm. The nine individual columns correspond to individual biological replicates of "pools" of VTA tissue (left to right: Control 1-3, Susceptible 1-3, Unsusceptible 1-3). Significantly **upregulated** and **downregulated** genes (>1.5 fold, $p<0.05$) are hierarchically clustered (grouped according to sequence homology). This experiment was performed in collaboration with Will Renthal and Quincey Laplant.

The Contribution of the Mesolimbic Dopamine Circuit to Depression-Related Behavior

The various limbic circuits that mediate depression's complex array of symptoms have been previously reviewed in the introduction (see: The Neural Circuitry of Depression). Among these regions, frontal cortical regions and the hippocampus have received the most attention across preclinical, postmortem and neuroimaging experiments (Nestler et al., 2002). Given that one of depression's core symptoms is *anhedonia* (a relative inability to experience naturally rewarding stimuli such as food and sexual rewards), there has been a considerable recent interest in understanding the contribution of the mesolimbic dopamine reward circuit in depression-related behavior (Dunlop and Nemeroff, 2007; Nestler and Carlezon, 2006; Ressler and Mayberg, 2007). This circuit consists of dopaminergic neurons arising from the ventral tegmental area (VTA), and their projections to a series of forebrain regions including the prefrontal cortex (PFc) and the nucleus accumbens (NAc). This dopaminergic "neuromodulatory" signal is transduced via two main classes of dopamine receptors (D_1 [Gs coupled] and D_2 [Gi coupled]) (Nestler, 2001).

In vivo electrophysiological studies have demonstrated a role for VTA dopaminergic neurons in encoding the "reward prediction error", i.e., these neurons acutely increase their firing rates in response to an unexpected reward (Le Moal and Koob, 2007; Schultz, 2002). VTA neurons are also excited in response to aversive stimuli (Grace, 2000; Schultz, 2002), although this type of activation appears to occur on a slower timescale. Such is also the case for social defeat: Miczek and colleagues employed microdialysis techniques in rats to demonstrate that the VTA-NAc dopamine projection is acutely activated during the threat of social defeat (Tidey and Miczek, 1996). Extrasynaptic dopamine levels in NAcs of subordinate intruder rats were significantly elevated during the course of an agonistic physical encounter, and this effect was

not observed in dialysates of prefrontal cortical regions, another important projection region of the VTA. In contrast to these acute effects, the long-lasting consequences of repeated defeat encounters on the tonic and phasic activity of this circuit remain sparsely understood (Grace, 2000).

Within this circuit, brain derived neurotrophic factor (BDNF) signaling is a potent regulator of molecular neuroplasticity following the exposure to stress and drugs of abuse (Bolanos and Nestler, 2004; Bolanos et al., 2003; Graham et al., 2007; Horger et al., 1999). The binding of BDNF to its receptor TrkB, causes receptor autophosphorylation and the activation of downstream signaling cascades including the Ras-Raf-MAPK cascade and the PI3K-AKT cascade (Figure 8A). While the VTA and NAc contain abundant levels of BDNF and TrkB protein, GABAergic medium spiny neurons of the NAc possess low levels of BDNF mRNA (Conner et al., 1997; Monteggia et al., 2007; Numan and Seroogy, 1999; Yan et al., 1997). BDNF immunolabelling within striatal regions is drastically reduced upon the intracerebroventricular infusion of *colchicine*, a microtubule depolymerizing agent (Altar et al., 1997). Thus, BDNF protein levels in the NAc are maintained at normal levels through anterograde axonal transport from extra-accumbal nuclei that project to the NAc, including the amygdala, cortex and most importantly, the VTA (Altar and DiStefano, 1998), and co-exocytosed during neurotransmitter release. As reviewed earlier, a direct infusion of BDNF into the VTA produces a significant increase in immobility on the rat forced swim test (Eisch et al., 2003), an effect that is mimicked by the overexpression of a dominant negative form of TrkB (truncated TrkB or TrkB.T) in nucleus accumbens. Consistent with this observation, social defeat stress applied to c57BL/6 mice produces a long-lasting elevation of NAc BDNF levels, and a

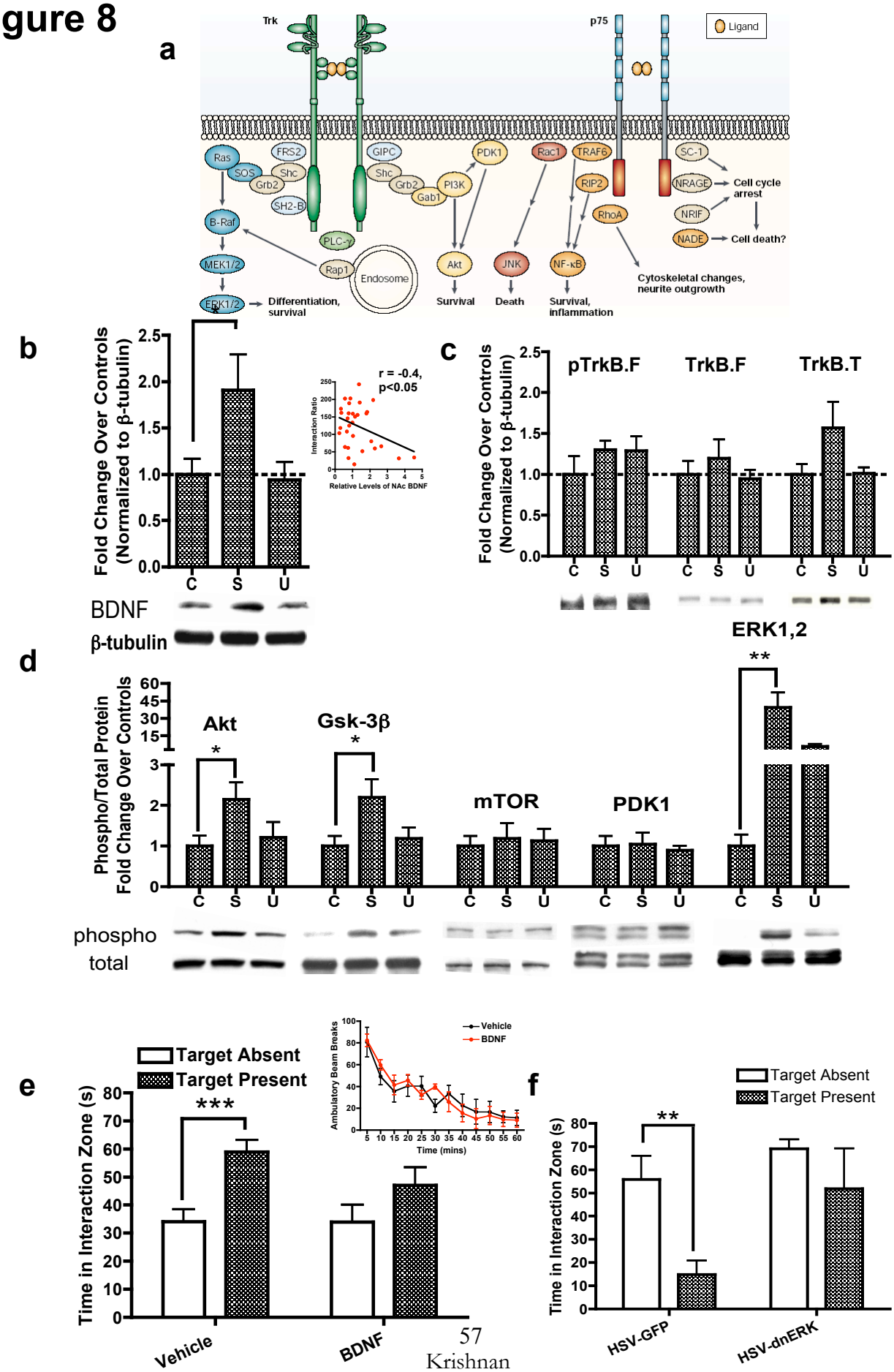
local *bdnf* knockout within the VTA produces an antidepressant effect / "pro-resilient" effect in the social defeat paradigm (Berton et al., 2006).

Increased BDNF Signaling Within the NAc Mediates Susceptibility

We tested whether defeat-induced BDNF elevations differ between Susceptible and Unsusceptible mice. Western blot analysis of NAc tissue 24 hours 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 8B), and no changes in TrkB levels or activation (Figure 8C). BDNF levels measured in this way correlated significantly with Day 11 interaction ratios (Figure 8B inset). 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 (akt thymoma viral oncogene), Gsk-3β (glycogen synthase kinase 3β), and ERK1/2 (extracellular signal regulated kinase) (Figure 8D), with no significant changes in total levels of these proteins. Unsusceptible mice did not show these changes, although there was a trend for increased phospho-ERK levels, suggesting the possibility that ERK activation could stem partly from non-neurotrophic pathways.

Figure 8. Increased NAc BDNF: A Molecular Signature of Susceptibility. **a,** Trk receptors (tropomyosin-related kinase) such as TrkB produce pro-differentiation and pro-survival signals through their activating MAPK, AKT and PLCγ signaling cascades (reproduced from Chao et al., 2003). **b,** Social defeat produces a 90% increase in BDNF levels in Susceptible mice compared to controls, without any changes in Unsusceptible mice, and **c,** without changes in levels of phospho-TrkB.F (pTrkB.F) or total TrkB.F or TrkB.T. **d,** We also observed a significant activation of downstream signaling molecules including Akt, Gsk-3β and p44/42 MAPK/ERK1,2. mTOR (Mammalian Target of Rapamycin) and PDK1 (Phosphoinositol dependent kinase-1) proteins were not significantly activated. **e,** 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, $p>0.1$). **f,** 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 &16.9±7.2sec)., C: Controls, S: Susceptible, U: Unsusceptible.

Figure 8


a
Trk
p75
Ligand
Ras
SOS
Shc
Grb2
FRS2
SH2-B
GIPC
Shc
Grb2
Gab1
PI3K
PDK1
Rac1
TRAF6
RIP2
RhoA
SC-1
NRAGE
Cell cycle arrest
NRIF
NADE
Cell death?
B-Raf
MEK1/2
ERK1/2
Differentiation, survival
PLC-γ
Rap1
Endosome
Akt
JNK
NF-κB
Survival
Death
Survival, inflammation
Cytoskeletal changes, neurite outgrowth
b
Fold Change Over Controls (Normalized to β-tubulin)
C S U
BDNF
β-tubulin
r = -0.4, p<0.05
Interaction Ratio
Relative Levels of NAc BDNF
c
Fold Change Over Controls (Normalized to β-tubulin)
pTrkB.F
TrkB.F
TrkB.T
C S U C S U C S U
d
Phospho/Total Protein Fold Change Over Controls
Akt
Gsk-3β
mTOR
PDK1
ERK1,2
*
**
C S U C S U C S U C S U C S U
phospho
total
e
Target Absent
Target Present
***
Time in Interaction Zone (s)
Vehicle
BDNF
Ambulatory Beam Breaks
Time (mins)
Vehicle
BDNF
f
Target Absent
Target Present
**
Time in Interaction Zone (s)
HSV-GFP
HSV-dnERK

We next tested the involvement of increased BDNF signaling in the NAc in the development of the Susceptible vs. Unsusceptible phenotype. Bilateral intra-NAc infusions of BDNF enhanced susceptibility in response to a submaximal exposure to defeat stress (Figure 8E), without modifying locomotor activity (Figure 8E inset). 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 8F), again with no effect on general locomotor activity ($p>0.5$). These data strongly implicate BDNF induction and downstream signaling within the NAc as a mediator of defeat-induced avoidance.

VTA: A Significant Source of Defeat-Induced BDNF Elevations in NAc

To explore mechanisms by which chronic social defeat increases BDNF levels in the NAc, we first measured BDNF mRNA levels in this region by RT-PCR. 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 (Figure 9A,B) 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 9C). 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).

Figure 9

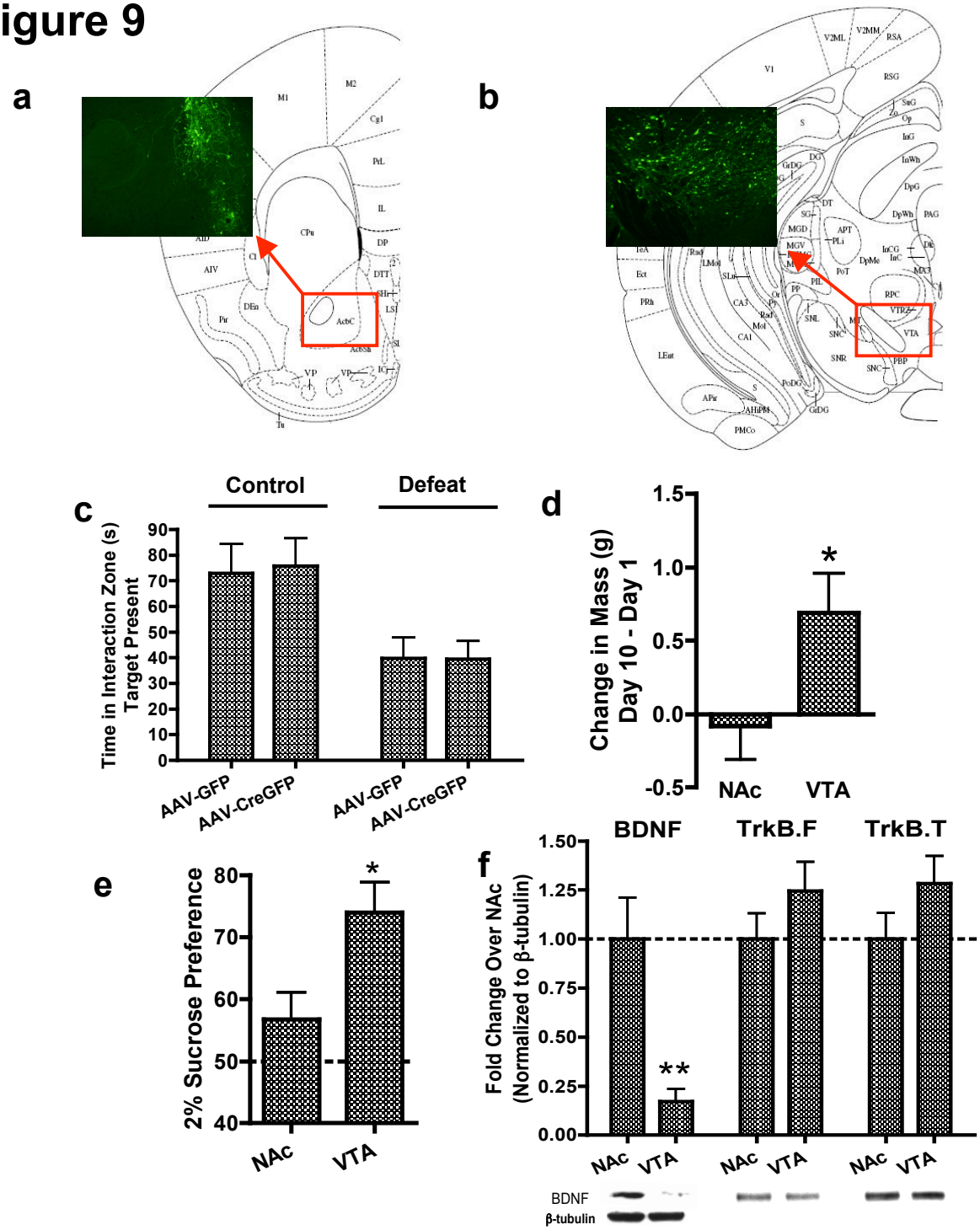


Figure 9. Effects of region specific BDNF knockdowns. **a,b** Schematic coronal sections (Paxinos and Franklin, 2001) from NAc (a) and VTA (b), with insets showing representative high power micrographs of viral GFP expression. **c,** Floxed BDNF mice were injected with either AAV-CreGFP or AAV-GFP into 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). **d,e** As compared to a local BDNF knockdown within the NAc, an analogous VTA knockdown ameliorated the effects of social defeat on weight loss (d) and sucrose preference (e). **f,** Immunoblotting NAc samples from these two groups revealed that VTA injected mice displayed an 80% reduction in levels of BDNF protein.

To further characterize the 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 vs. 34% in AAV-CreGFP injected mice), an effect not seen after a NAc *Bdnf* knockdown. Likewise, *Bdnf* knockdown in VTA ameliorated the weight loss (t_{13}=2.19, $p<0.05$, Figure 9D) and sucrose preference deficit (t_{18}=2.46, $p<0.05$, Figure 9E) 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 9F). These results strongly implicate the VTA as a crucial source of BDNF to the NAc during defeat.

To extend and confirm these findings, we examined how a VTA- and NAc-specific manipulation of the TrkB receptor would affect avoidance behavior following social defeat. Since the site of action of BDNF's prodepressant effects within this circuit appears to be the NAc, we hypothesized that a NAc-specific *TrkB* knockdown would promote resilient behavior, whereas an identical knockdown within the VTA would largely have no consequence on social defeat behavior. In the first experiment, we stereotaxically infused AAV-CreGFP or AAV-GFP alone into the NAc of floxed TrkB mice (Luikart et al., 2003), and subjected these mice to the social defeat paradigm. As shown in Figure 10A, this experiment unfortunately provided inconclusive results! We did not observe a significant defeat-induced reduction in social interaction. The observation of high social interaction scores (~100 seconds, in comparison to ~70 seconds observed in c57BL/6 mice) is also observed following social defeat of the

NPAS2KO (knockout) line of mice (Figure 10B), and is speculated to reflect the consequence of a different genetic background (129 vs c57BL/6 background of floxed BDNF mice).

Figure 10

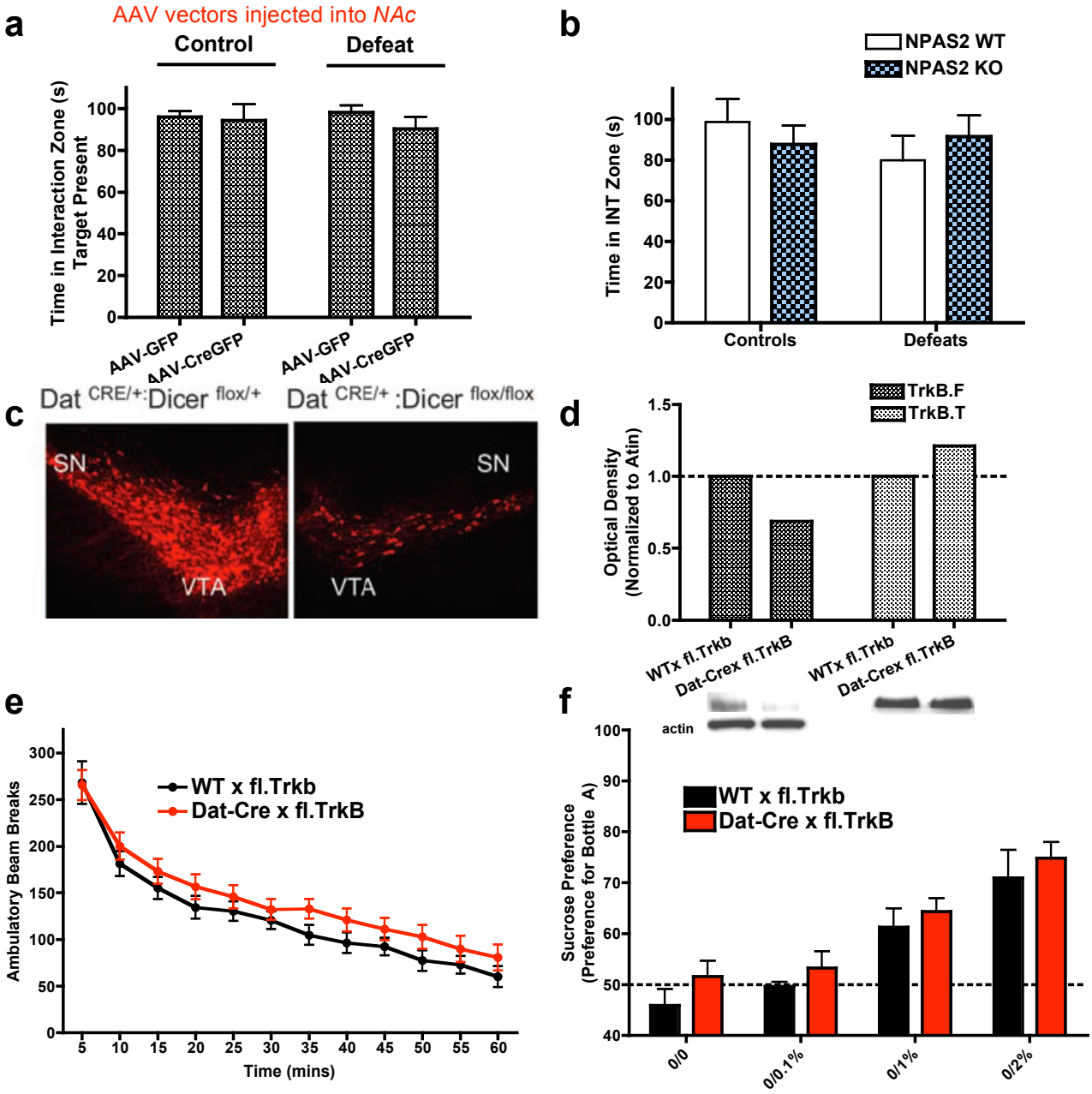


Figure 10 (contd.)

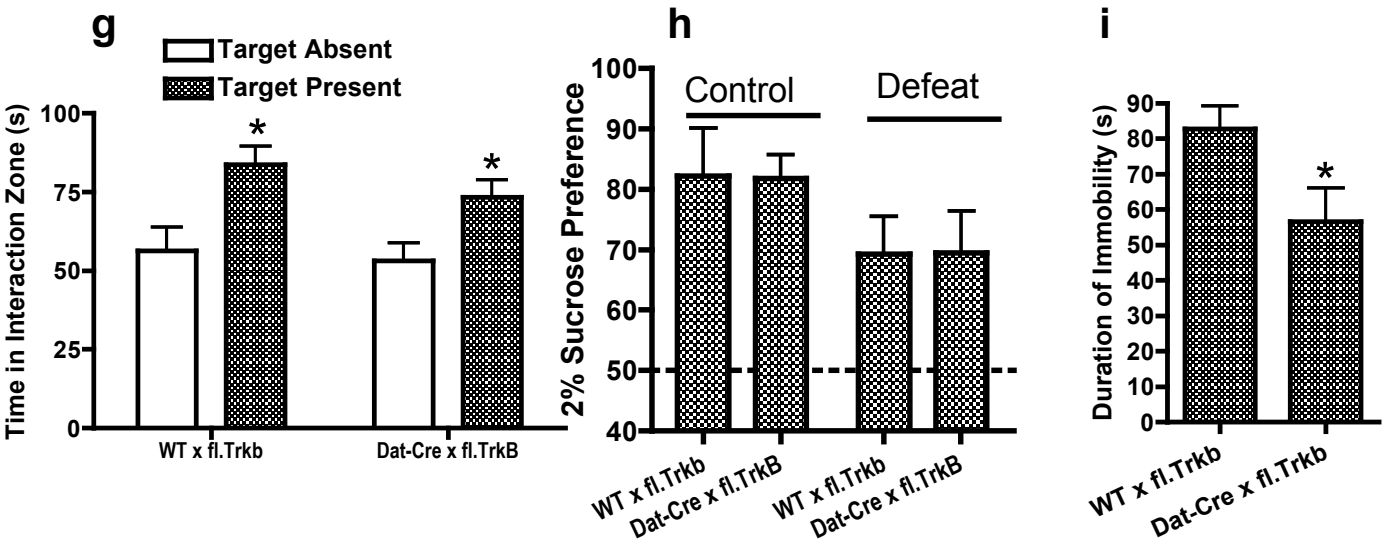


Figure 10. BDNF Signaling and Social Defeat: Effects of Local TrkB Knockdown. **a,** Floxed trkB mice were injected with either AAV-CreGFP or AAV-GFP into the nucleus accumbens and subsequently subjected to the social defeat paradigm. We observed no significant effect of social defeat ($F_{1,38}$=0.02, p>0.5), complicating the interpretation of potential viral effects. **b,** Similar effects were observed following the social defeat of the NPAS2KO line (*defeat factor,* $F_{1,42}$=0.001, p>0.8). **c,** Floxed trkB mice were crossed to the Dat-Cre line of mice to examine the effects of TrkB knockdown within the VTA. This particular DatCre line of mice has previously been employed to examine the effects of loss of *Dicer* (a gene involved in miRNA processing). Photomicrograph reproduced from Kim et al., 2005, demonstrating a loss of tyrosine hydroxylase positive neurons within midbrain dopaminergic nuclei (red staining). **d,** Total VTA homogenates were examined by immunoblotting, where we observed a ~30% reduction in levels of TrkB.F without affecting TrkB.T levels (truncated TrkB, which is mostly expressed on glia). **e,f** Local TrkB knockouts were no different on measures of locomotor activity (e) and sucrose preference (f). On the social defeat paradigm, wild type and TrkB knockouts performed comparably. While avoidance behavior was not immediately apparent (both groups displayed a paradoxical *increase* in social interaction in the presence of the social cue), we did observe the negative effects of chronic defeat on sucrose preference measures (**h**, $F_{1,23}$=3.64, p<0.05). **i**, A local knockout of TrkB produced an antidepressant effect on the forced swim test (t_{23} = 2.04, p<0.05).

To observe the effects of a VTA specific TrkB knockdown, we employed a slightly different yet analogous approach. Homozygous floxed TrkB mice were intercrossed with DAT-Cre mice (transgenic mice constitutively expressing Cre-recombinase under the control of dopamine transporter [*Dat*] promoter) (Kim et al., 2007; Zhuang et al., 2005) (Figure 10C). Such a region-specific *TrkB* knockdown produced an approximately ~30% reduction in TrkB.F protein levels in whole VTA punches (Figure 10D), without reducing levels of TrkB.T (which are mainly expressed in DAT-negative glial cells). Controls (WTxfl.trkB) and "knockouts"

(DatCrexfl.TrkB) mice displayed equivalent locomotor activity and sucrose preference (Figures 10E,F) indicating that such a selective reduction in TrkB.F protein within the VTA (and substantia nigra complex) did not grossly affect locomotor habituation and responses to natural reward. Following 10 days of social defeat, controls and knockouts performed similarly during both trials of the social interaction test (Figure 10G). Once again, perhaps due to residual 129 background effects, these mice displayed high social interaction scores. Social defeat did however produce a similar significant reduction in sucrose preference in controls and knockouts (Figure 10H), indicating that while these mice did respond to social defeat, their response may only be observable along certain behavioral indices. Interestingly, a separate naïve cohort of mice was examined on the forced swim test: here, Dat-Crexfl.TrkB mice displayed significantly lower immobility levels than their wild type counterparts (Figure 10I), consistent with prodepressant effects of BDNF infusions into the VTA (Eisch et al., 2003). These data reveal an important dissociation between the responses to acute versus chronic stressors, an effect that is also observed in later sections (see below).

Depressed Humans Display Increased BDNF Levels in NAc

To examine the clinical relevance of our findings, we obtained postmortem samples of human NAc from depressed patients and unaffected controls (Table 5). Employing a commercially available BDNF ELISA (Chen et al., 2006), 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 11A), with no changes in levels of BDNF mRNA levels (Figure 11B). We tested whether this BDNF increase could reflect an effect of chronic antidepressant treatment. Chronic

imipramine treatment (28 days) had no effect on levels of BDNF or TrkB isoforms in NAc from naïve mice (Figure 11C).

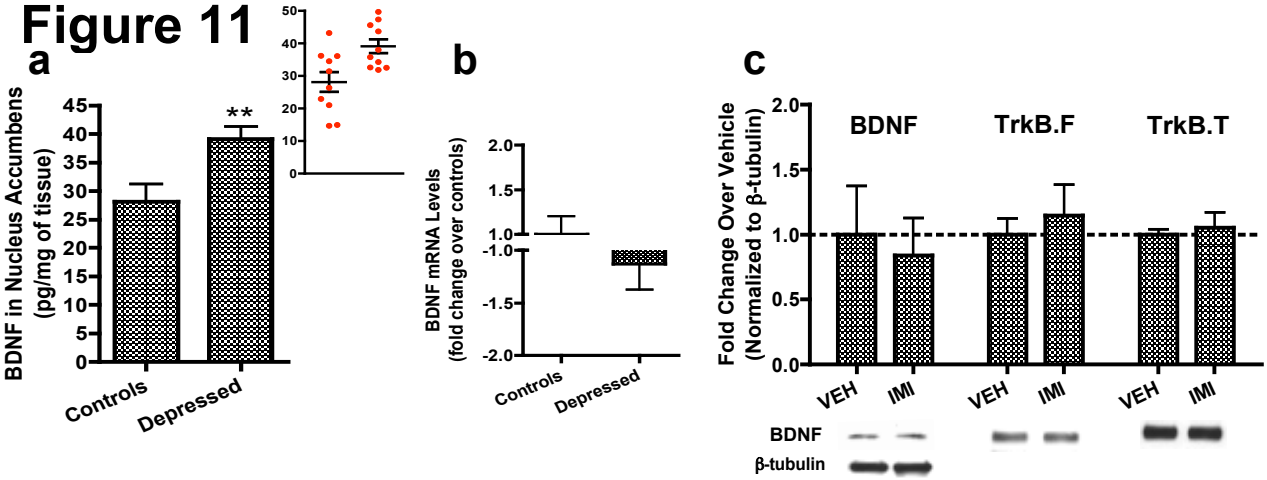


Figure 11. BDNF Protein is Elevated in Human Depression. **a,** ELISA comparing levels of total BDNF in NAc lysates from depressed humans and unaffected controls (n=10) [inset: scatter plot]. **b,** qPCR data for BDNF transcripts in RNA extracts of human depressed and control NAcs (n=9, t_{16} = 0.4016, p>0.5,), showing no significant change in levels of *bdnf* mRNA. **c,** NAc tissue punches from non-defeated c57bl/6 mice (n=6) exposed to chronic (28 days) daily intraperitoneal injections of imipramine (20 mg/kg) or vehicle (sterile saline) displayed no changes in levels of BDNF, TrkB.F or TrkB.T as measured by western blotting.

Table 5

Patient ID	Age (yrs)	Diagnosis	PMI (hrs)	RIN	pH	On ADx at TOD	Hx of ADx
2	51	NC	9	8.2	6.86	No	No
13	49	NC	12	9.5	6.61	No	No
14	49	NC	24	9.5	6.83	No	No
20	16	NC	16	8.3	6.63	No	No
39	32	NC	22	9.7	6.90	No	No
40	20	NC	21	8.2	6.81	No	No
41	60	NC	20	8.5	6.63	No	No
46	43	NC	15	6.1	6.89	No	No
47	19	NC	20	9.0	6.64	No	No
48	31	NC	16	8.1	6.92	No	No
6	15	DEP	28	8.1	6.81	Yes	Yes
8	69	DEP	22	8.8	6.73	No	No
15	90	DEP	18	4.9	6.45	Yes	Yes
18	54	DEP	6	8.9	6.84	No	Yes
34	31	DEP	14	9.0	7.13	Yes	Yes
35	24	DEP	23	9.1	6.45	Yes	Yes
36	50	DEP	23	8.8	6.89	No	No
37	51	DEP	27	7.5	6.81	No	Yes
38	19	DEP	27	9.3	6.96	No	Yes
44	33	DEP	18	8.5	6.94	No	No

Table 5. Results of Nucleus Accumbens Postmortem Study. (A) Data for each of the individual patients from which postmortem NAc samples were obtained. No significant differences were observed between depressed and control groups in measures of Age (p=0.47), PMI (p=0.25), RIN (p=0.68) and tissue pH (p=0.72). Abbreviations: PMI (postmortem interval), RIN (RNA Integrity Number), On ADx at TOD (detectable serum levels of antidepressants at time of death), Hx of ADx (History of antidepressant drug administration), NC (normal control), DEP (depressed).

Results of Genome Wide Expression Analyses

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: (A) genes regulated similarly in Susceptible and Unsusceptible groups (as a result of exposure to stress), and (B) genes regulated differentially in Susceptible and Unsusceptible mice (which may mediate differences in behavior). Our results, summarized as Venn diagrams in Figure 12A, 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 vs. Unsusceptible mice. An expression-based dendrogram (Figure 12B) confirmed that VTA gene expression patterns more strongly correlated with our behavioral observations. Figure 12C displays a summary of regulated genes in heatmap form, which emphasize the unique regulation of gene expression in the VTA of Unsusceptible mice. Among genes significantly upregulated in only Susceptible NAc, we found several examples of

molecules that have previously been implicated in depressive behaviors, such as *Hdac2* (Histone Deacetylase-2) and *Adcy7* (Adenylyl Cyclase 7) (Hines et al., 2006; Schroeder et al., 2006). Similarly, only Susceptible VTA showed a significant upregulation in mRNA levels of *Gal* (Galanin), which creates a prodepressant phenotype when infused directly into the VTA (Weiss et al., 1998), further validating our microarray results (see Supplemental Online Microarray Gene Lists in Krishnan et al., 2007).

Augmented Excitability Within VTA Dopamine Neurons: A Signature of Susceptibility

Among the genes that were significantly upregulated in the VTA of Unsusceptible mice only, we identified three voltage-gated potassium (K^+) channels (*Kcnf1, Kcnh3, Kcnq3*). Since 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 13A). Non-dopaminergic 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 performed two weeks later ("Day 25"). At this time-point, VTA dopamine neuron firing

Figure 12

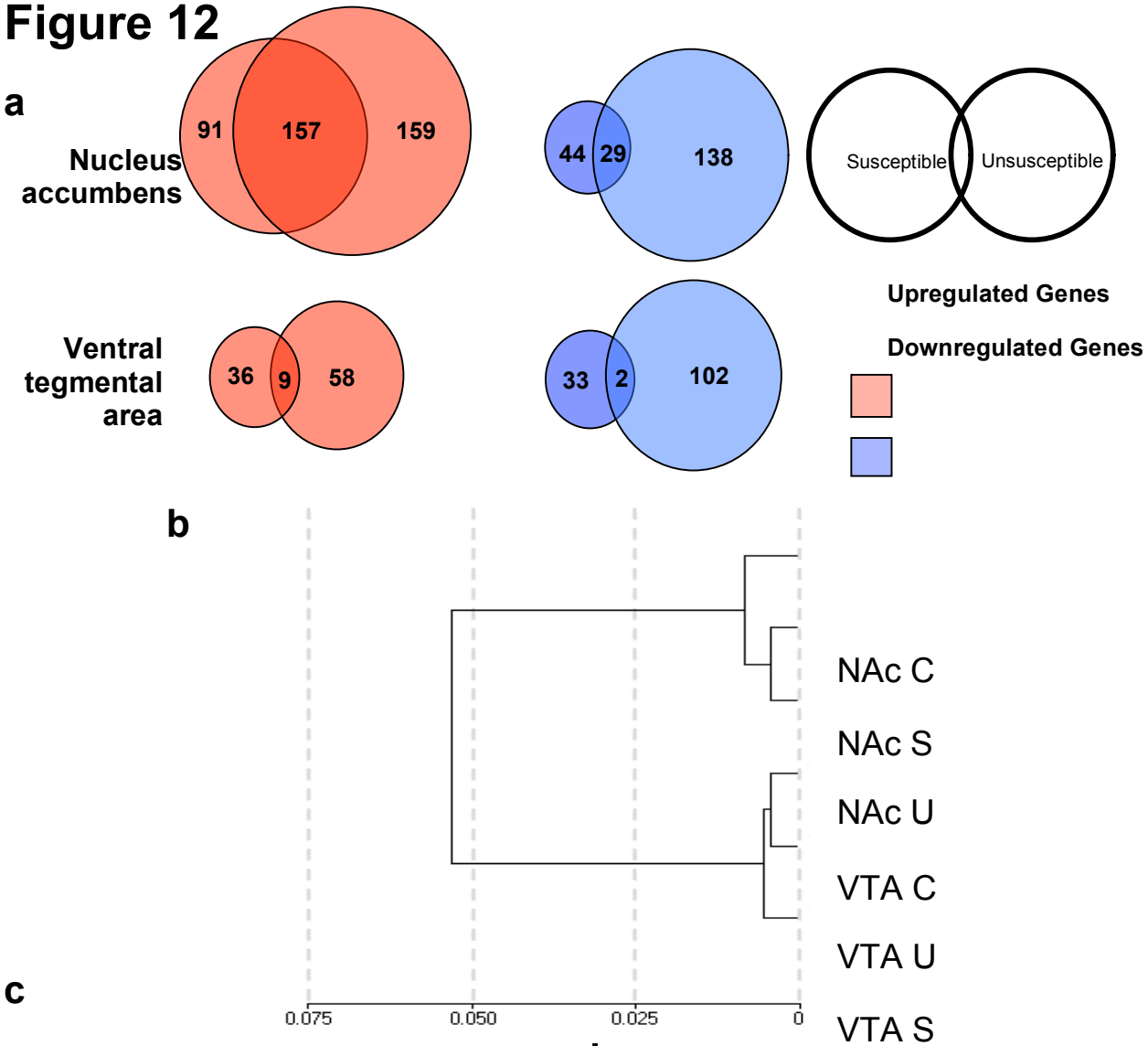

a
Nucleus accumbens
91
157
159
44
29
138
Susceptible
Unsusceptible
Upregulated Genes
Downregulated Genes
Ventral tegmental area
36
9
58
33
2
102
b
NAc C
NAc S
NAc U
VTA C
VTA U
VTA S
0.075
0.050
0.025
0
c
Nucleus accumbens
Nucleus accumbens
Susceptible
Unsusceptible
Ventral tegmental area
Susceptible
Unsusceptible
Up
Genes Regulated in Susceptible Mice
Genes Regulated in Unsusceptible Mice

Figure 12. Results of Gene Expression Microarrays Performed on Day 11. **a,** Venn diagrams show the number of uniquely regulated genes in Susceptible and Unsusceptible mice (as compared to non-defeated 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: ≥1.5 fold change compared to respective anatomical control group at $p<0.05$). **b,** A gene expression dendrogram comparing overall patterns of gene expression. In the VTA, controls and Unsusceptible mice were clustered together, whereas in the NAc, Susceptible and Unsusceptible mice were clustered together. **c,** Heatmaps illustrating the regulation of genes for each condition and anatomical structure, with red to blue gradient depicting an up to downregulation (≥2 fold increase → ≤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 Susceptible NAc (bottom row). Data obtained in collaboration with William Renthal and Quincey Laplant.

rates were increased in Susceptible mice ($n=5$, $F_{2,381}=14.37$, $p<0.0001$), with no effect seen in Unsusceptible mice (Figure 13B,C). VTA firing rates were significantly correlated with the interaction ratio measured on Day 11 ($r = -0.67$, $p<0.01$, $n=15$, Figure 13C inset).

To further explore potential mechanisms by which these neurons display a chronically elevated excitability, we measured a series of action potential parameters in current clamp mode. As shown in Table 6, VTA dopaminergic neurons from control, Susceptible and Unsusceptible mice (on Day 25) were quite comparable along a variety of different action potential parameters. Through the injection of hyperpolarizing current, we also measured the magnitude of I_H, (hyperpolarization-induced cationic current), a key conductance involved in the maintenance of VTA spontaneous pacemaker activity (Liu et al., 2003b). Overall, we did not observe a significant change in the magnitude of the Ih mediated depolarization response (data not shown). However, since VTA dopamine neurons with higher Ih currents tend to be most responsive to stress (Ungless et al., 2003), within "high Ih" neurons we observed that susceptibility was associated with a significant elevation in the Ih response (Figure 13D). While these data are strictly still at a preliminary stage, they point towards an important role for mediators of the Ih current (HCN channels or hyperpolarization activated cyclic nucleotide gated channels) in mediating this form of electrophysiological adaptation (Jackson et al., 2007).

Figure 13

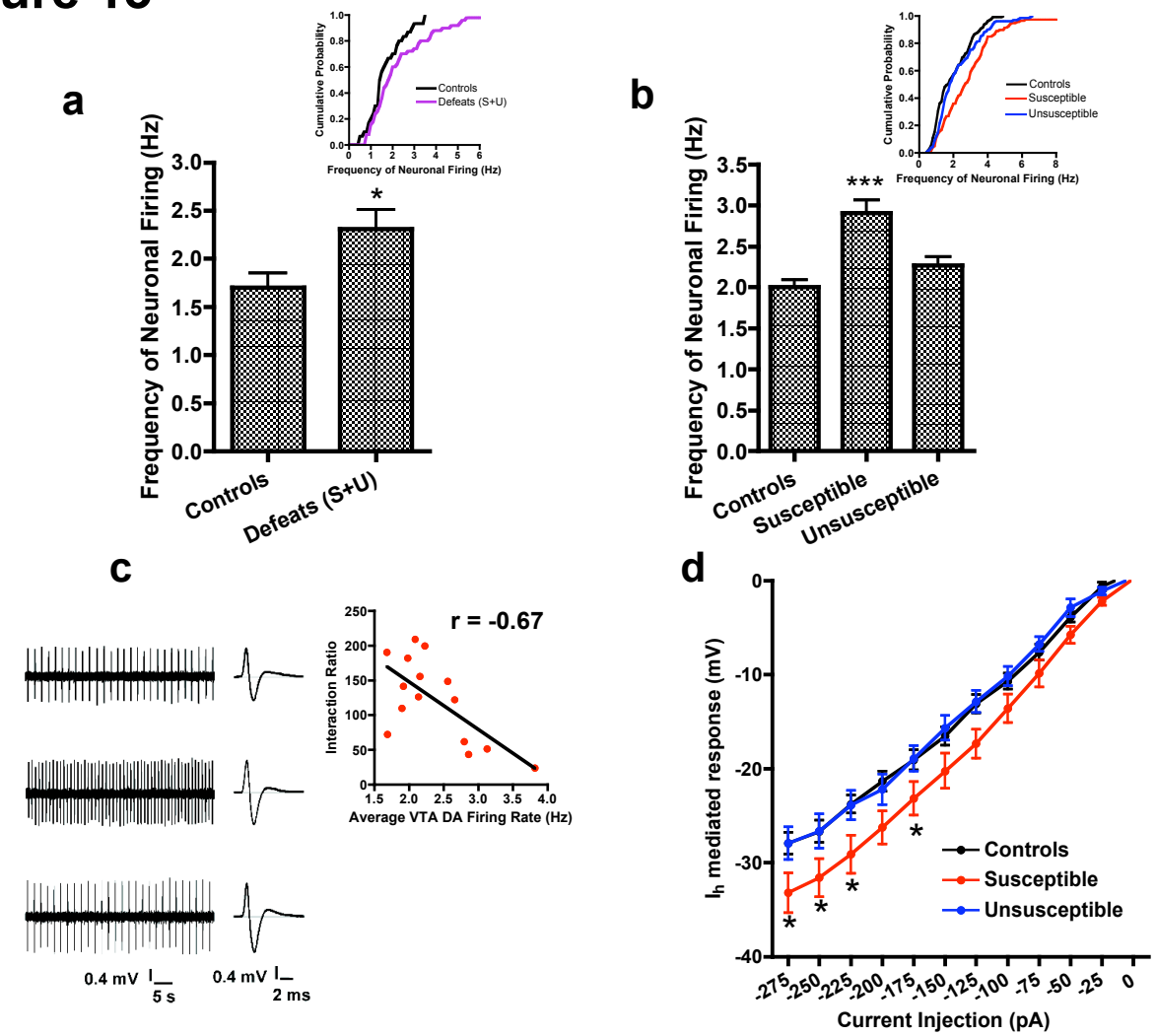


Figure 13. a, Social defeat results in a significant increase in VTA dopamine neuron firing rates on Day 11, with the inset showing a relative cumulative distribution histogram. **b,** On Day 25, dopamine neurons from only Susceptible mice display significantly enhanced firing rates. **c,** Sample traces and spikes; inset shows that averaged VTA firing rates for each mouse are significantly correlated with Interaction Ratios measured on Day 11. **d,** Patch clamped VTA dopamine neurons display a depolarizing response to a hyperpolarizing current injection (x axis), which is significantly enhanced in neurons from Susceptible mice (figure depicts only those neurons with high Ih currents). Data obtained in collaboration with Ming-Hu Han, PhD.

Table 6

Parameter	Control	Susceptible	Unsusceptible	F_{df}, p
Spike Threshold (mV)	-27.36±0.65 (22)	-26.70±0.67 (28)	-26.81±0.69 (27)	$F_{2,74}$=0.25, p=0.77
AP Peak (mV)	60.24±1.71 (21)	56.63±1.60 (28)	59.86±1.38 (27)	$F_{2,73}$=1.66, p=0.19
AP Antipeak (mV)	-29.35±0.92 (21)	-28.42±1.00 (28)	-29.93±0.84 (27)	$F_{2,73}$=0.72, p=0.49
AP Half Width (ms)	1.07±0.04 (21)	1.02±0.05 (28)	1.03±0.04 (28)	$F_{2,73}$=0.32, p=0.72

Table 6. Social defeat does not modify several action potential parameters in VTA dopamine neurons (obtained on Day 25). Control, Susceptible and Unsusceptible parameters are presented as mean ± SE (n), with the final column depicting the F and p statistic from a one way ANOVA comparing three groups. Abbreviations: mV (millivolts), ms (milliseconds), df (degrees of freedom), AP (action potential). Data obtained in collaboration with Ming-Hu Han, PhD.

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); indeed, we found that HSV-mediated Kir2.1 overexpression robustly decreased the firing of VTA dopamine neurons (Figure 14A). We next took two groups of Susceptible mice, matched for Day 11 interaction times (43.0±5.3 and 43.9±6.6 sec), and injected one with HSV-GFP and the other with HSV-Kir2.1 into the VTA.

Figure 14

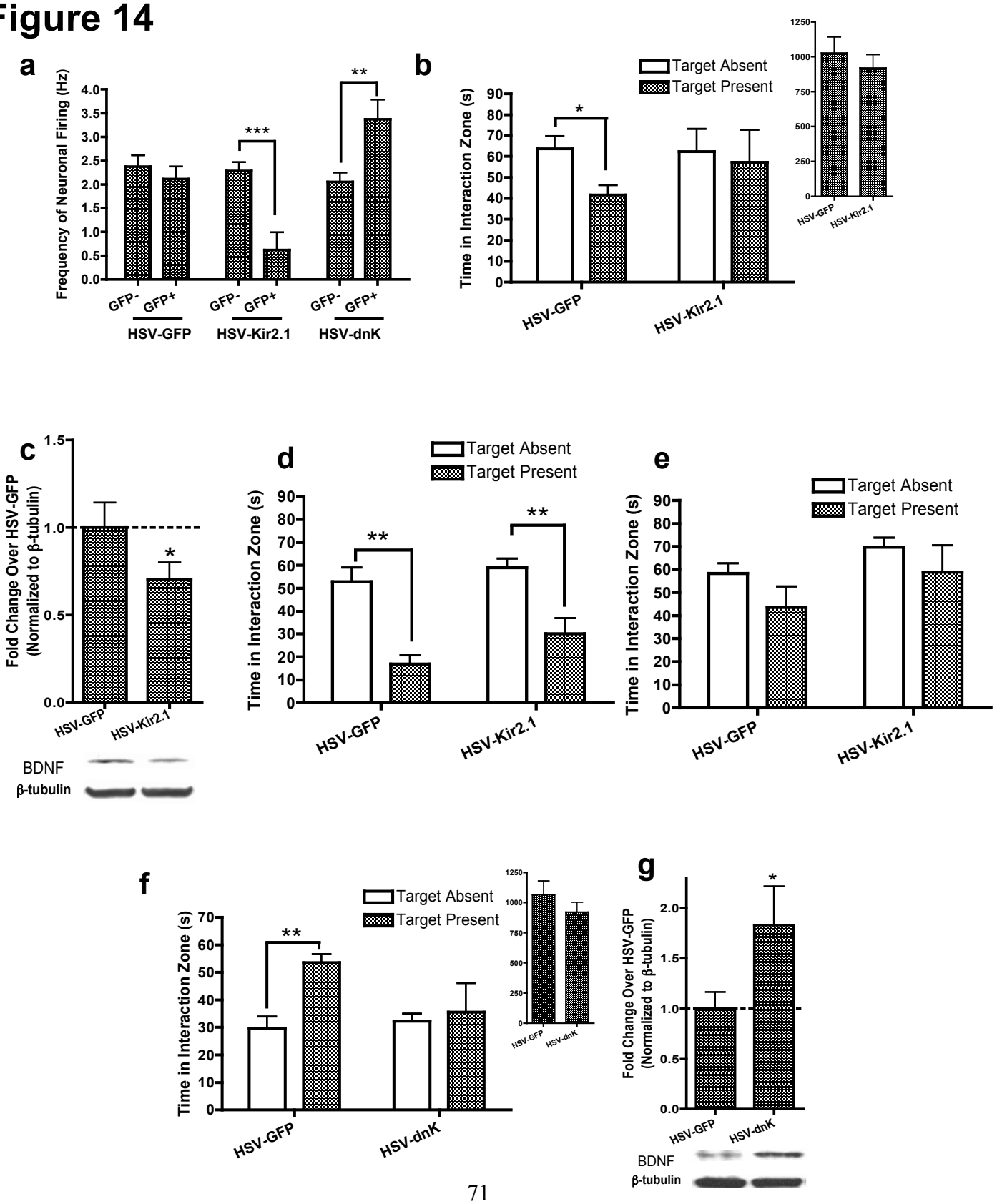

a
Frequency of Neuronal Firing (Hz)
GFP-
GFP+
HSV-GFP
HSV-Kir2.1
HSV-dnK
b
Target Absent
Target Present
Time in Interaction Zone (s)
HSV-GFP
HSV-Kir2.1
c
Fold Change Over HSV-GFP (Normalized to β-tubulin)
BDNF
β-tubulin
d
e
f
HSV-dnK
g

Figure 14. Increases in VTA Electrical Activity Mediate Social Avoidance and BDNF Elevations in the Nucleus accumbens. **a,** 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=3-5mice/group, 20-30neurons/group). **b,** 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 (**c)** resulted in a significant reduction in NAc BDNF levels (one-tailed t test). **d,** An intra-VTA infusion of HSV-Kir2.1 does not produce resilient behavior when measured 8 days after stereotaxic surgery (when viral expression has peaked and subsequently waned). Prior to surgery, both groups of Susceptible mice displayed similar interaction times (n=6, HSV-GFP:40.1±4.1sec, HSV-Kir2.1:39.6±6.4sec). **e,** A VTA specific infusion of HSV-Kir2.1 into Unsusceptible mice does not further increase social interaction scores above HSV-GFP infused control counterparts. Prior to surgery, both groups of Unsusceptible mice displayed similar interaction times (n=6, HSV-GFP:77.4±7.8sec, HSV-Kir2.1:77.2±10.2sec). **f,** 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 (**g)** resulted in a significant increase in BDNF levels (one-tailed t test). Data obtained in collaboration with Ming-Hu Han, PhD.

When assayed three days later, HSV-Kir2.1 injected mice displayed an Unsusceptible phenotype as compared to their GFP infected counterparts (Figure 14B), despite comparable levels of locomotor activity (Figure 14B inset). Also, HSV-Kir2.1 infected mice displayed significantly reduced NAc levels of BDNF (t_{20}=1.96, $p<0.05$, Figure 14C). The Unsusceptible phenotype induced by HSV-Kir2.1 was absent after transgene expression had degraded (8 days following HSV infusions, Figure 14D). Intra-VTA infusions of HSV-Kir2.1 had no effect on the behavior of Unsusceptible mice (Figure 14E). Converse effects were seen upon overexpressing a K^+ channel (*Kcnab2)* rendered dominant negative (HSV-dnK, Figure 14A). HSV-dnK increased the firing rate of VTA dopamine neurons (Figure 14A), promoted the development of a Susceptible phenotype on a submaximal exposure to defeat (Figure 14F) without affecting general locomotor activity (Figure 14F inset), and significantly enhanced NAc BDNF levels (t_9=2.10, $p<0.05$, Figure 14G). 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 (Figure 15A & B), as well as displayed similar patterns of locomotor habituation (Figure 15C). 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 15D), Met/Met mice displayed an Unsusceptible phenotype (t_{14}=0.2, $p>0.5$). Non-defeated Val/Val and Met/Met mice showed similar interaction scores ($p>0.5$, data not shown). 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 15E). 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 15F).

Figure 15

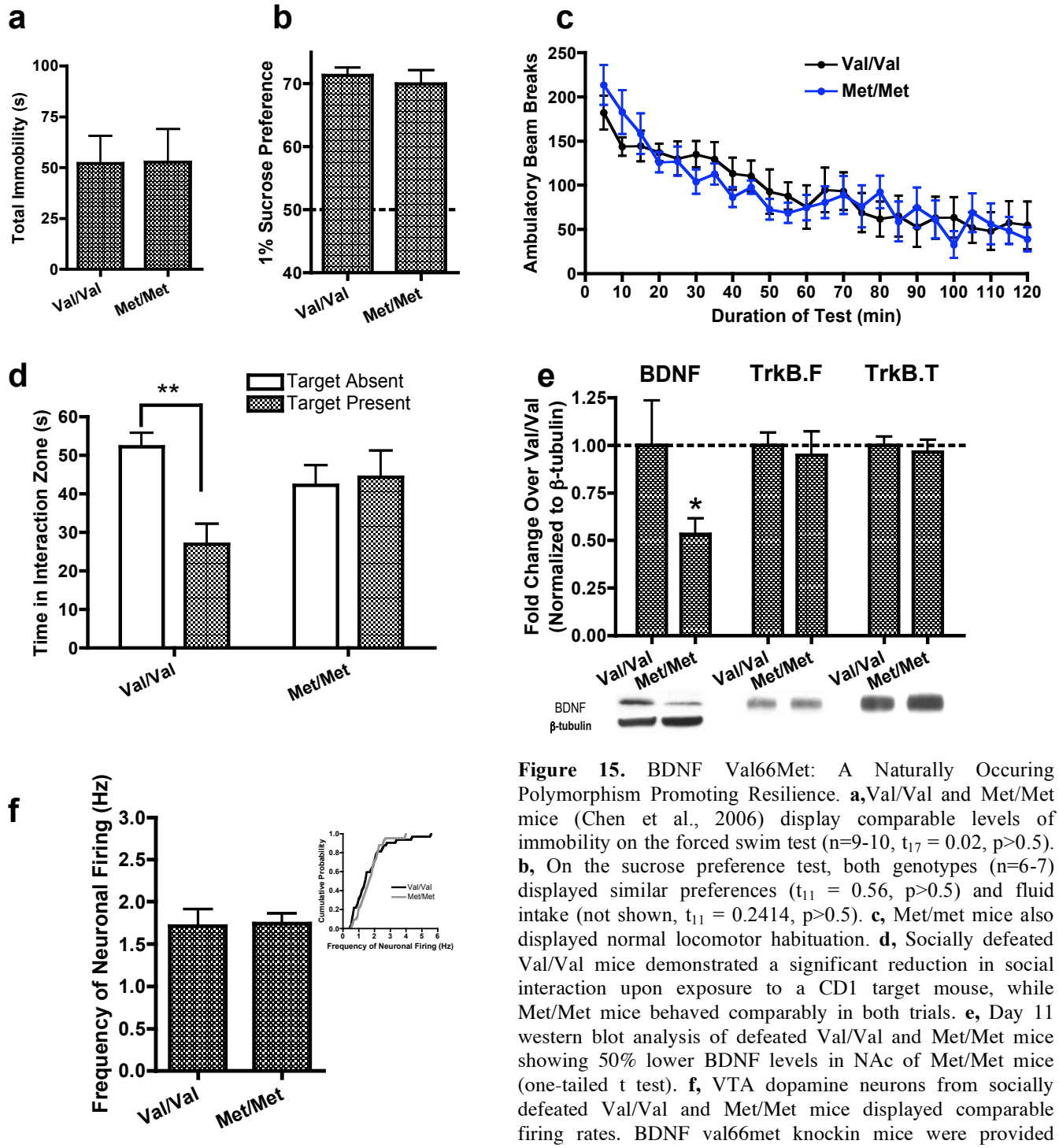


Figure 15. BDNF Val66Met: A Naturally Occuring Polymorphism Promoting Resilience. **a,**Val/Val and Met/Met mice (Chen et al., 2006) display comparable levels of immobility on the forced swim test (n=9-10, $t_{17} = 0.02$, $p>0.5$). **b,** On the sucrose preference test, both genotypes (n=6-7) displayed similar preferences ($t_{11} = 0.56$, $p>0.5$) and fluid intake (not shown, $t_{11} = 0.2414$, $p>0.5$). **c,** Met/met mice also displayed normal locomotor habituation. **d,** 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. **e,** 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). **f,** VTA dopamine neurons from socially defeated Val/Val and Met/Met mice displayed comparable firing rates. BDNF val66met knockin mice were provided Francis Lee and colleagues (Cornell).

Figure 16

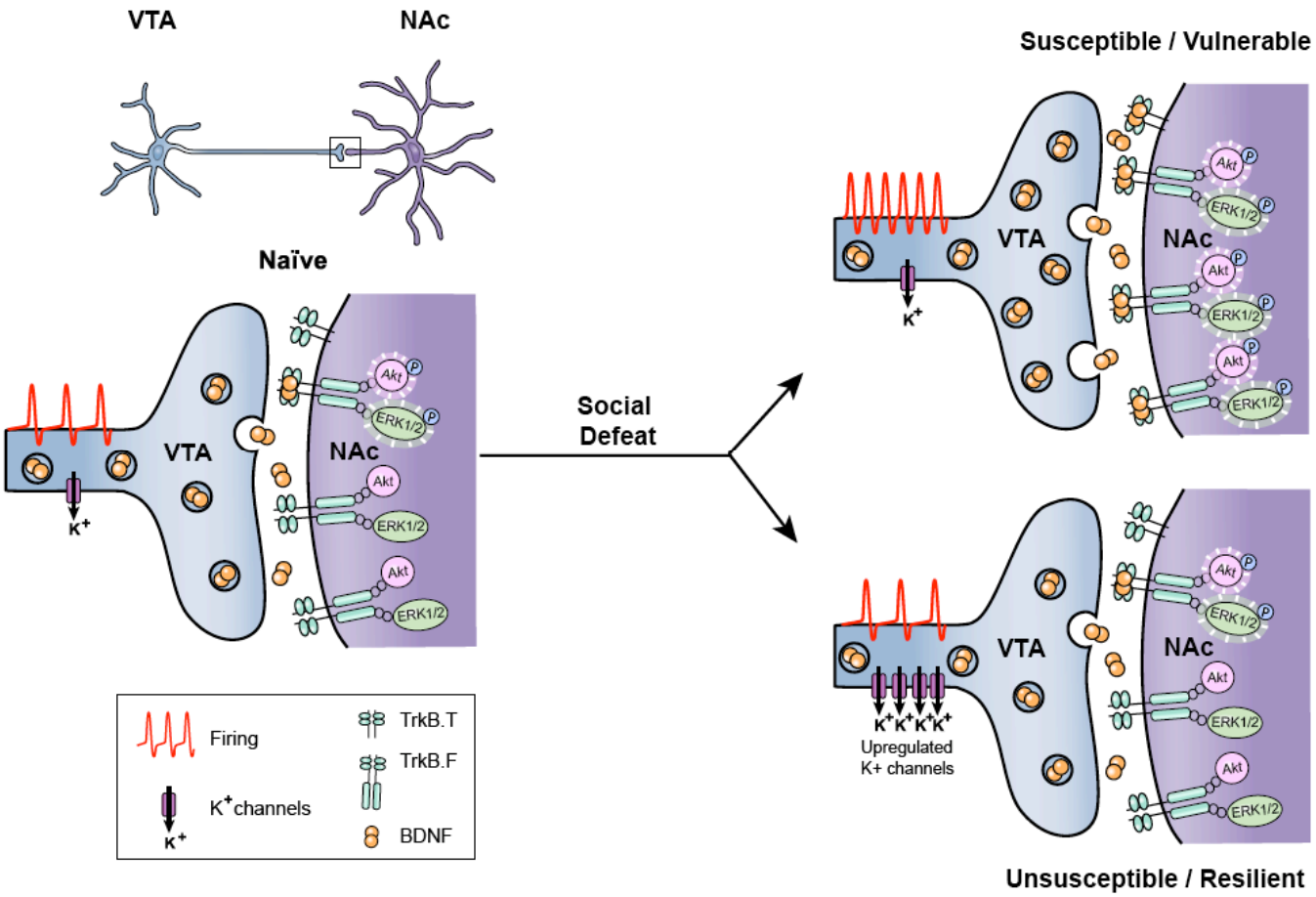
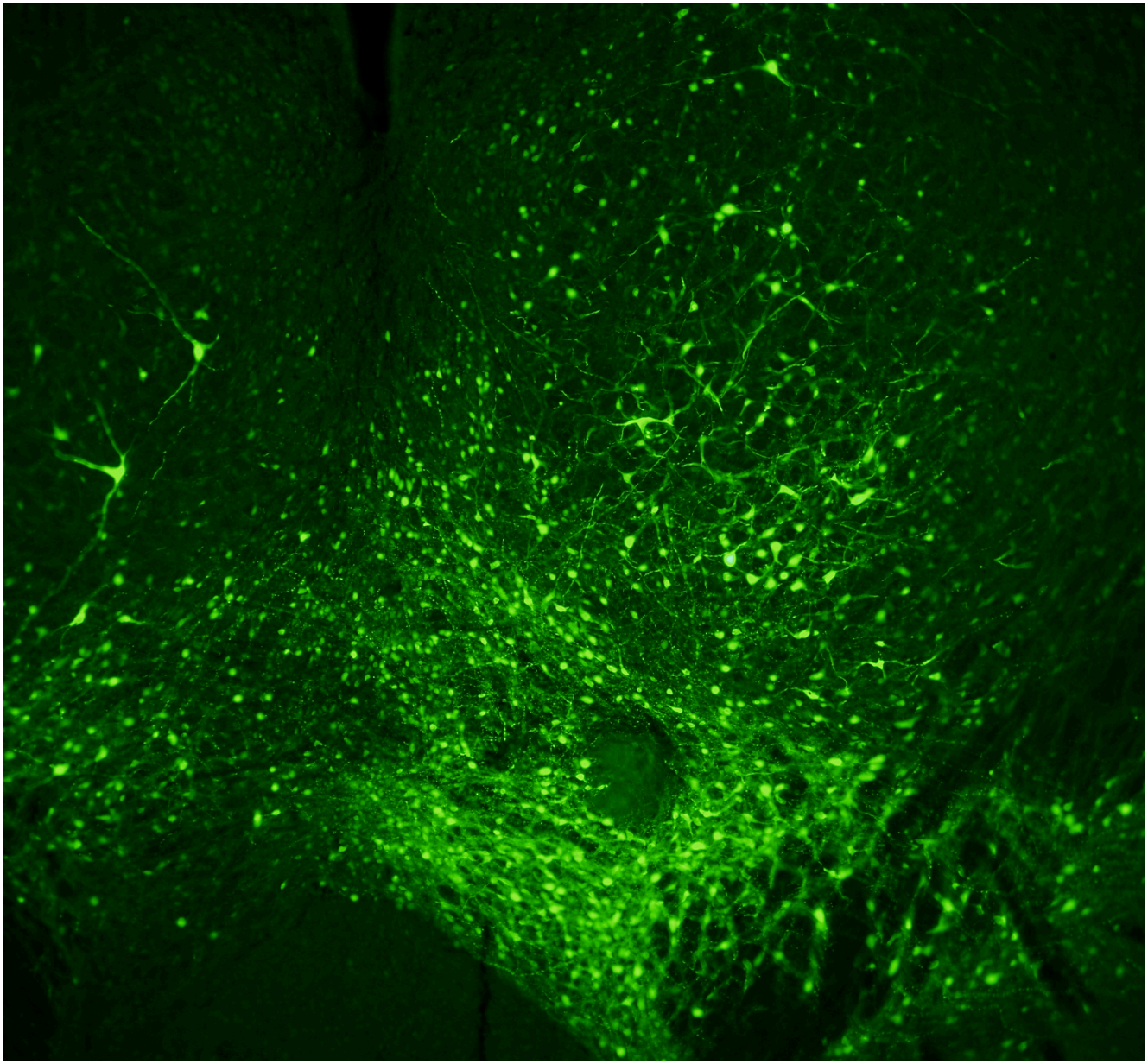


Figure 16. Schematic: In Susceptible mice, chronic social defeat increases the firing rate of VTA dopamine neurons, which subsequently gives rise to heightened BDNF signaling within the NAc. Unsusceptible mice display a resistance to this adverse cascade of events by upregulating various K^+ channels within the VTA. This figure was designed and illustrated by Kim Hoggatt (UTSW Biomedical Communications).

Together, these findings support a working model (Figure 16) by which elevations in BDNF within the NAc are both necessary and sufficient for the establishment of social avoidance in Susceptible mice. Long-lasting elevations in VTA excitability lead to enhanced rates of activity-dependent BDNF release from the VTA to the NAc, which appear to be absent in resilient/Unsusceptible mice, due to the upregulation of voltage-gated K^+ channels. Naturally occurring deficits in activity-dependent BDNF release, as well as experimental reductions in VTA excitability both promote resilient behavior, illustrating how NAc BDNF elevations are a type of "final common pathway" to the development of social avoidance. The precise

mechanisms by which dopamine neurons from Susceptible VTAs display enhanced firing rates are unknown at this stage; our gene expression arrays did not reveal clues to explain this finding. Since stress-induced avoidance behavior is vital for several aspects of species survival, this increase in VTA excitability is likely mediated via a number of redundant molecular pathways. One such operating mechanism is explored in greater detail in the next chapter.

Chapter IV: "AKTivating" the VTA



"Painting a Pretty Picture"

This representative micrograph depicts immunofluorescent dopaminergic neurons of the ventral tegmental area (VTA/A10), overexpressing green fluorescent protein (GFP) through adeno-associated viral (AAV) mediated overexpression. Stereotaxic surgery performed by Danielle Graham, PhD.

Chronic Social Defeat Stress Decreases AKT Phosphorylation in the VTA

Given that neuroadaptations within the VTA serve as the origin for vulnerability to social defeat, and since neurotrophic signaling in this region is a potent regulator of the behavioral and cellular response to stress and drugs of abuse (Bolanos and Nestler, 2004; Bolanos et al., 2003; Eisch et al., 2003; Grimm et al., 2003; Horger et al., 1999; Pierce and Bari, 2001; Russo et al., 2007), the purpose of this study was to explore stress-induced changes in neurotrophic signaling within the VTA. Figure 17A shows Day 11 behavioral data obtained for the first experiment: while non-defeated controls and Unsusceptible mice displayed a significant increase in interaction times, Susceptible mice (~60% of all defeated mice, interaction ratios <100) displayed a strong avoidance phenotype. 24 hours after social avoidance testing, VTA punches were obtained for immunoblotting studies, where we examined a panel of antibodies against molecules involved in BDNF signaling (Figure 17B). While social defeat did not produce significant changes in the levels of BDNF or TrkB.F (full length tropomyosin-related kinase B), VTAs from Susceptible mice displayed a significant reduction in AKT phosphorylation [Ser473, a site associated with catalytic activation (Manning and Cantley, 2007)], with no changes observed in total levels of AKT nor in the phosphorylated or total levels of several other signaling proteins within PI3K-AKT cascade (Figure 17C). This decrease in AKT activation was not observed in Unsusceptible mice, after a single defeat or after a prolonged period of social isolation, suggesting this change is unique to chronic and active forms of stress (Figure 17D). In contrast, chronic fluoxetine treatment significantly increased phospho-AKT levels (Figure 17D), and this effect was also observed in postmortem VTA samples from depressed humans chronically treated with antidepressants (Figure 17E). In these samples, levels of total BDNF protein were unchanged ($t_{16} = 0.22$, $p>0.5$).

Figure 17

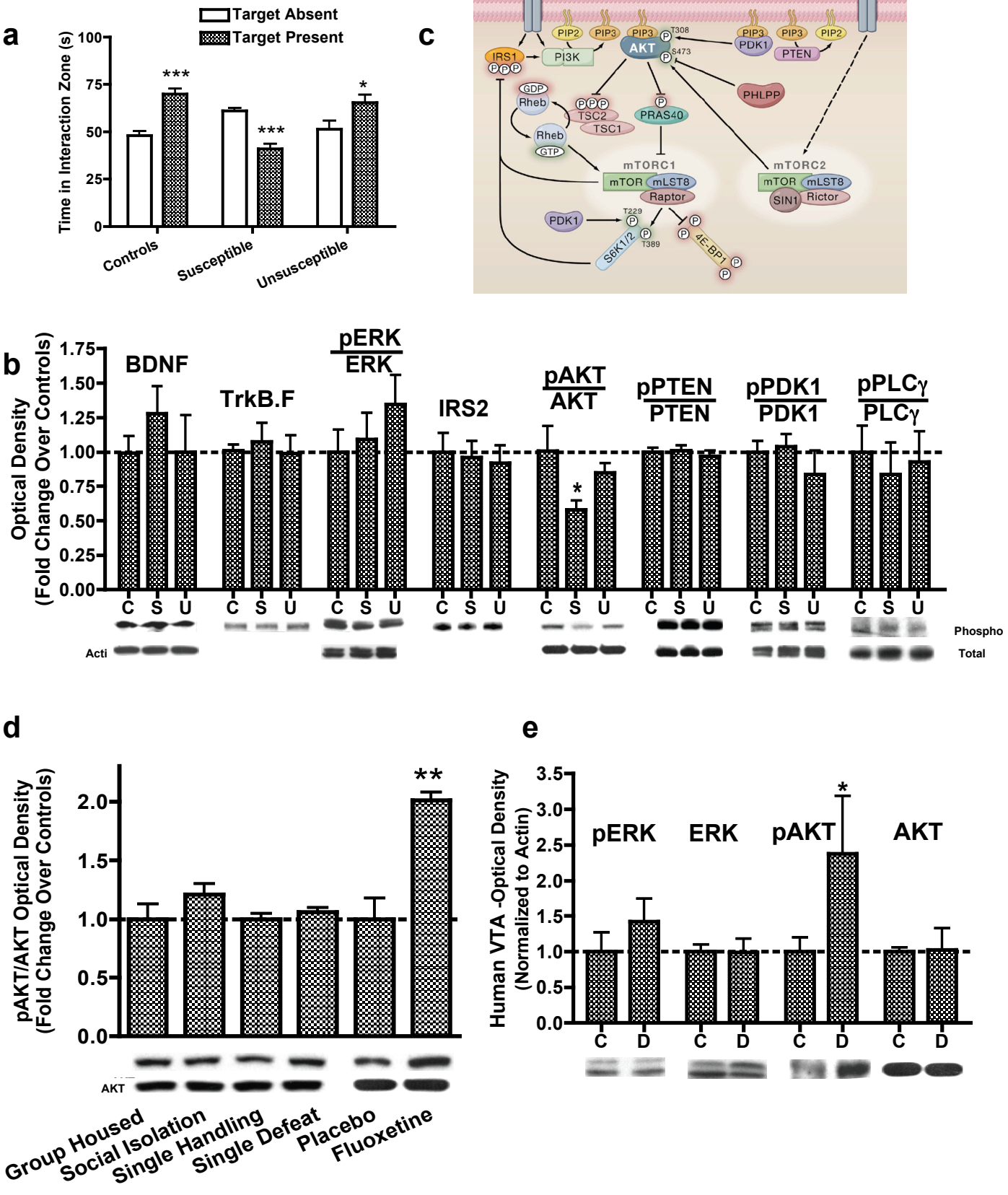

a
Target Absent
Target Present
Time in Interaction Zone (s)
Controls
Susceptible
Unsusceptible
c
RTK
PI3K
AKT
PDK1
PTEN
PHLPP
PRAS40
TSC2
TSC1
Rheb
mTORC1
mTORC2
mTOR
mLST8
Raptor
SIN1
Rictor
PDK1
S6K1/2
4E-BP1
b
Optical Density (Fold Change Over Controls)
BDNF
TrkB.F
pERK/ERK
IRS2
pAKT/AKT
pPTEN/PTEN
pPDK1/PDK1
pPLCγ/PLCγ
Phospho
Total
Acti
d
pAKT/AKT Optical Density (Fold Change Over Controls)
Group Housed
Social Isolation
Single Handling
Single Defeat
Placebo
Fluoxetine
e
Human VTA -Optical Density (Normalized to Actin)
pERK
ERK
pAKT
AKT

Figure 17. Social Defeat Reduces AKT Phosphorylation in the VTA. **a,** Social interaction times for non-defeated controls (t_{54} = 5.82, $p<0.0001$, n=28), Susceptible (IR<100, t_{52} = 6.52, $p<0.0001$, n=27) and Unsusceptible mice (IR≥100, t_{32} = 2.30, $p<0.05$, n=17) mice. **b,** VTA punches one day after interaction testing showed that chronic social defeat causes a ~40% reduction in phospho-AKT levels ($F_{2,42}$=3.41, $p<0.05$, n=11-18), without changes in the total level of AKT or in the total or phosphorylated levels of several other signaling molecules. **c,** Schematic signaling diagram reproduced from Manning and Cantley (2007), depicting signaling molecules upstream and downstream of AKT activation. **d,** While prolonged social isolation (10 weeks, t_{12}=1.37, $p>0.1$) or a single episode of defeat (t_{12}=0.94, $p>0.1$) did not alter levels of phospho-AKT in the VTA, chronic fluoxetine administration significantly increased phospho-AKT levels (t_7 = 3.77, $p<0.05$, n=4). **e,** Postmortem VTA samples from depressed humans treated chronically with antidepressants also displayed a significant increase in phospho-AKT levels (t_{15} = 2.16, $p<0.05$, n=6-12/group) without altering ERK activation.Abbreviations: C (Controls), S (Susceptible), U (Unsusceptible), BDNF (brain-derived neurotrophic factor), TrkB.F (full-length tropomyosin-related kinase B), ERK (extracellular-signal regulated kinase), IRS2 (insulin-receptor substrate 2), PTEN (phosphatase and tensin homolog), PDK1 (phospho-inositol dependent kinase 1), PLCγ (phospholipase Cγ).

Social Defeat Reduces the Soma Surface Area of VTA Dopamine Neurons

Chronic morphine administration induces a significant reduction in the soma surface area (SSA) of VTA dopamine neurons (Chu et al., 2007; Sklair-Tavron et al., 1996), and this morphological adaptation mediates tolerance to opiate reward through decrements in IRS2-AKT signaling pathway (Russo et al., 2007). While significant reductions in AKT activity were only observed in Susceptible mice, VTA dopaminergic neurons from Susceptible and Unsusceptible mice displayed a surprisingly equivalent reduction in soma surface area (Figure 18A). To directly explore the relationship between AKT signaling and VTA SSA, we employed validated herpes simplex viral vectors encoding a constitutively active (HSV-AKTca) and dominant negative (HSV-AKTdn) forms of AKT (Russo et al., 2007). Three days after stereotaxically infusing these viral vectors into the VTA, at a time of maximal transgene expression (Barrot et al., 2002), these manipulations produced significant changes ($F_{2,64}$=37.23, $p<0.001$, Figure 18 B): AKTca and AKTdn produced a significant increase and decrease, respectively, in VTA SSA. These data demonstrate a critical role for AKT tone in the control of soma morphology (Kumar et al., 2005; Russo et al., 2007), but do point towards other molecular pathways in mediating this

effect. Figure 18C shows a representative confocal image of the VTA from an HSV-GFP infected animal, demonstrating the localization of GFP to TH positive neurons.

Figure 18

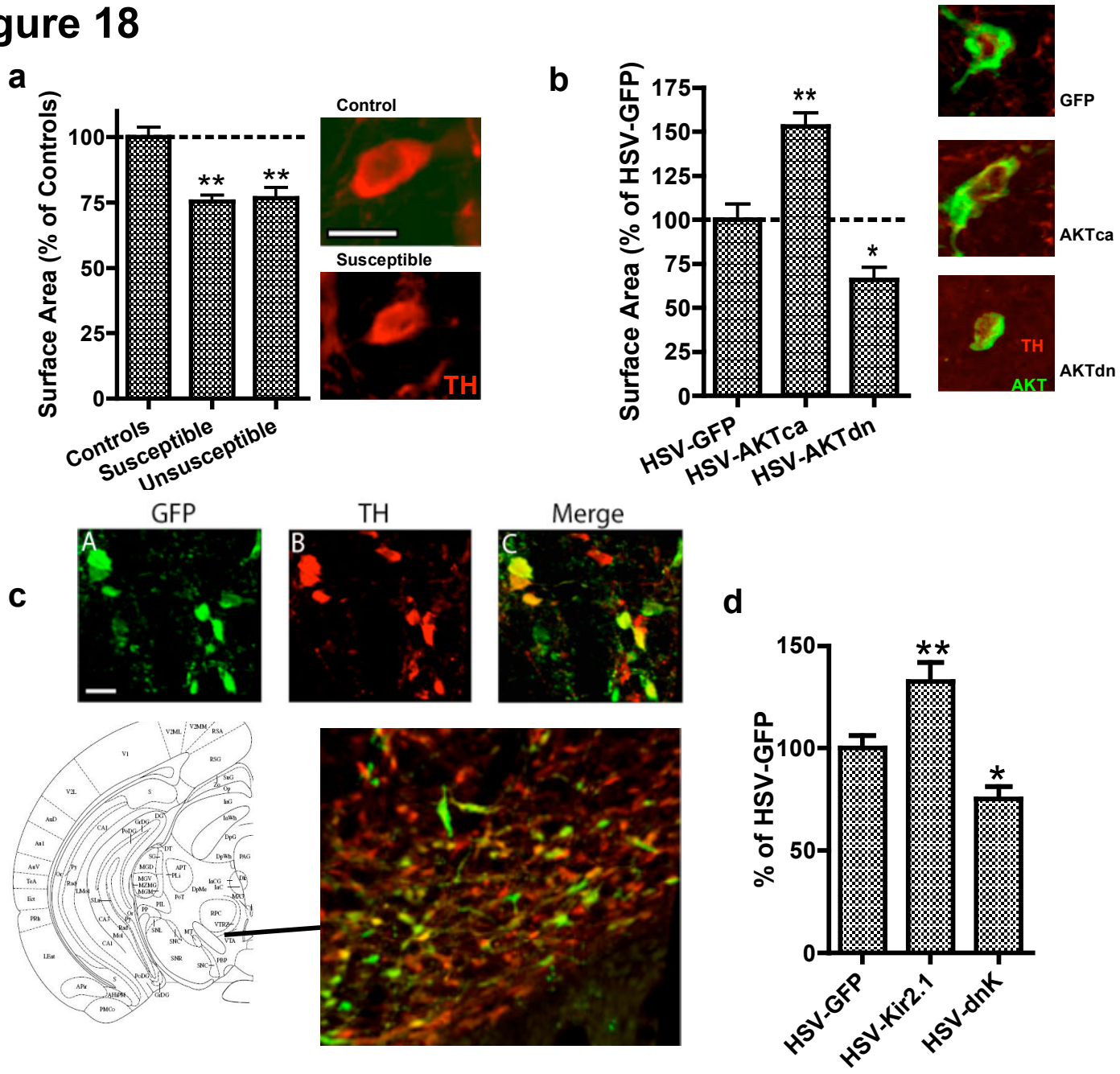


Figure 18. Social Defeat Produces A Significant Reduction in Soma Surface Area of VTA Dopaminergic Neurons. **a,** Both Susceptible and Unsusceptible VTA slices displayed significant reductions in VTA soma surface area ($F_{2,42}$=18.17, p<0.0001). **b,** HSV-AKTca increases and HSV-AKTdn decreases the soma surface area of VTA dopamine neurons. Representative virally infected neurons are shown on right. **c,** Validation of viral-mediated gene transfer in VTA (scale bar = 20 μm), showing cells expressing virally encoded GFP (a, Cy2), TH positive cells (b, Cy3), and merged confocal image of A and B showing double labeling (c) in region of VTA as shown in schematic (Paxinos and Watson, 2002). **d,** Altering excitability of VTA dopamine neurons affect soma morphology: Kir2.1 overepxression (which reduces spontaneous activity) produced a significant increase in soma surface area, whereas the opposite change was observed in HSV-dnK overexpressing neurons ($F_{2,26}$=12.19, p<0.001). Data obtained in collaboration with Scott Russo, PhD and Michelle Mazei-Robison, PhD.

In our previous studies (Krishnan et al., 2007), we demonstrated that increased VTA firing rates were responsible for mediating the maladaptive molecular and behavioral consequences of susceptibility to social defeat. To examine whether our morphological changes represent a direct consequence of intrinsic increases in cellular excitability, we tested the ability of K^+ channel vectors to alter VTA SSA. The stereotaxic overexpression of HSV-Kir2.1 and HSV-dnK (validated in Figure 13A) produced significant changes in VTA soma morphology: as shown in Figure 18D, HSV-Kir2.1 produced a significant increment in VTA SSA, while HSV-dnK (*kcnab2* rendered dominant negative) resulted in a significant SSA decrement. These bidirectional data are consistent with a model where increases in neuronal excitability directly produce changes in soma morphology, and this model is explored further in the discussion.

Reductions in AKT Activity Recapitulate Susceptibility

To causally implicate decreased AKT phosphorylation as a mediator of defeat-induced social avoidance, we tested the ability of HSV-AKTca and HSV-AKTdn, infused into the VTA, to alter behavior in the social defeat model. Since activated IRS2 binds to PI3K (phosphatidylinositol-3-kinase) and functions upstream of AKT (Yamada et al., 1997), we also examined the effects of wildtype IRS2 (HSV-IRS2wt) and a dominant negative mutant of IRS2 (HSV-IRS2dn) which have previously been shown to increase and decrease, respectively, AKT phosphorylation (Russo et al., 2007). This approach enabled us to experimentally manipulate levels of AKT activity without affecting the total amount of the enzyme, and hence better mimic the effects of chronic stress. In the first experiment, we injected HSV-GFP, HSV-IRS2dn, or HSV-AKTdn into the VTA of a group of age- and weight-matched naïve c57BL/6 mice (n=6-8). Three days later, these groups were exposed to a submaximal defeat paradigm, consisting of 3

short defeats interspersed over one hour (Krishnan et al., 2007). On the fifth day, GFP expressing mice displayed levels of social interaction comparable to non-defeated controls (Figure 19A). In contrast, IRS2dn and AKTdn expressing mice did not significantly increase their interaction rates in this way, indicating an increased vulnerability. To obtain the converse type of information, we obtained three groups of Susceptible mice matched for Day 11 interaction times. On Day 12, we stereotaxically infused HSV-GFP, HSV-IRS2wt, or HSV-AKTca into the VTA of these three groups, respectively, and measured social interaction behavior three days later. While GFP expressing mice demonstrated the expected reduction in interaction times in response to a CD1 social cue, IRS2wt and AKTca expressing mice did not display social avoidance (Figure 19B), showing that restoring AKT function in the VTA ameliorates the effects of chronic social defeat.

Interestingly, when NAc samples from these groups were analyzed for levels of BDNF, HSV-AKTca infused mice displayed significantly lower levels of BDNF (Figure 19C), suggesting that the stress-resistant properties of AKT restoration are associated with blockade of stress-induced increases in BDNF signaling to the NAc, which we have previously shown is important for the Susceptible phenotype (Berton et al., 2006; Krishnan et al., 2007). As a control, separate groups of naïve c57BL/6 mice were infused with these viral vectors and examined for changes in locomotor activity. Modulating IRS2 or AKT function in this way had no effect on overall locomotor activity (Figure 19D), the motivation to socially interact with an unfamiliar CD1 mouse (Figure 19E), or the accumulation of BDNF protein within the NAc (Figure 19F). Collectively, these data show that a defeat-induced decrease in AKT function is both necessary and sufficient for the display of social avoidance, in a manner that cannot be simply explained by alterations in locomotor activity or sociability.

Figure 19

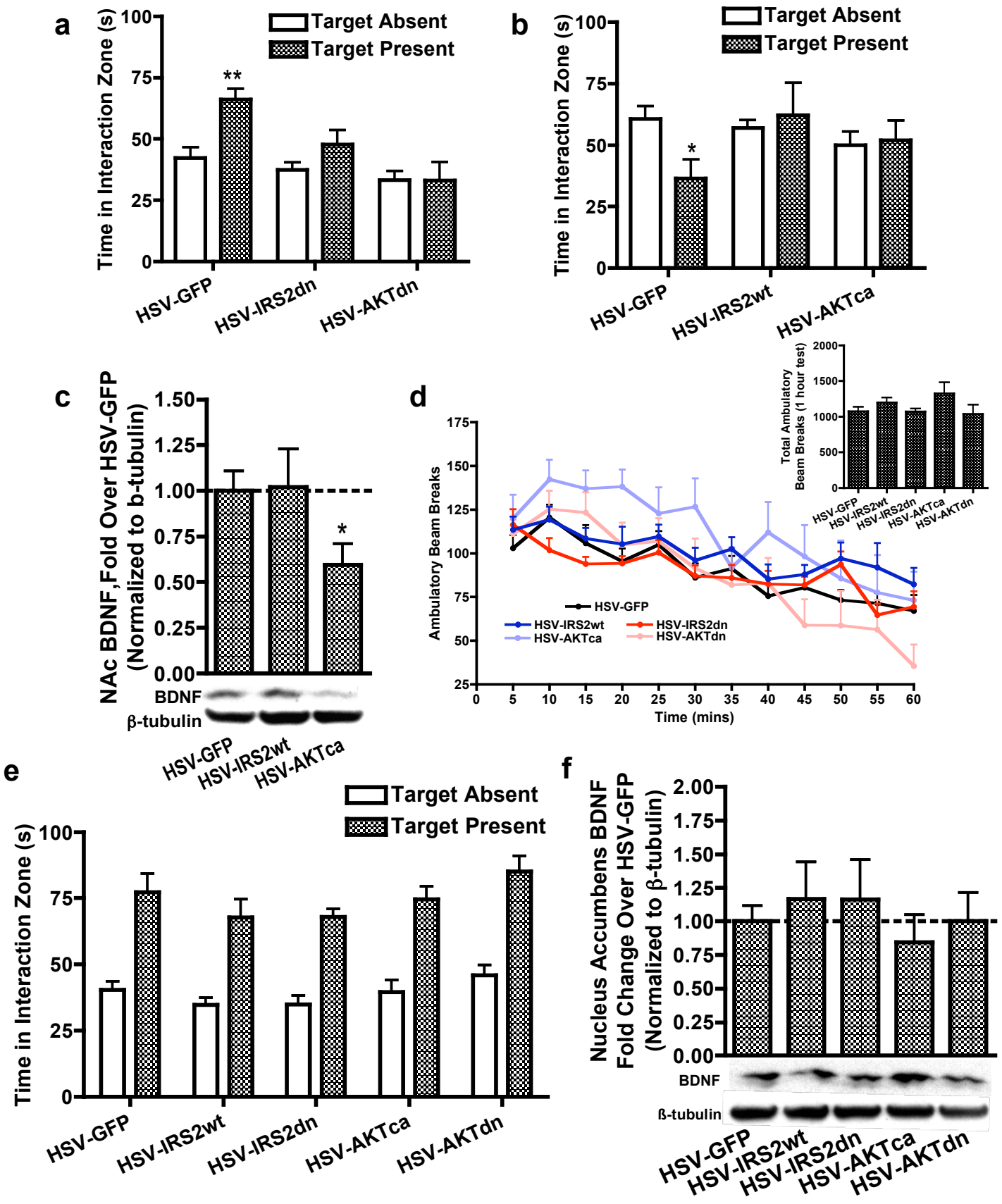

a
Time in Interaction Zone (s)
Target Absent
Target Present
HSV-GFP
HSV-IRS2dn
HSV-AKTdn
b
Time in Interaction Zone (s)
Target Absent
Target Present
HSV-GFP
HSV-IRS2wt
HSV-AKTca
c
NAc BDNF,Fold Over HSV-GFP
(Normalized to b-tubulin)
BDNF
β-tubulin
HSV-GFP
HSV-IRS2wt
HSV-AKTca
d
Ambulatory Beam Breaks
Time (mins)
HSV-GFP
HSV-IRS2wt
HSV-IRS2dn
HSV-AKTca
HSV-AKTdn
Total Ambulatory
Beam Breaks (1 hour test)
e
Time in Interaction Zone (s)
Target Absent
Target Present
HSV-GFP
HSV-IRS2wt
HSV-IRS2dn
HSV-AKTca
HSV-AKTdn
f
Nucleus Accumbens BDNF
Fold Change Over HSV-GFP
(Normalized to β-tubulin)
BDNF
ß-tubulin
HSV-GFP
HSV-IRS2wt
HSV-IRS2dn
HSV-AKTca
HSV-AKTdn

Figure 19. Decreased VTA AKT activity mediates social avoidance following chronic social defeat stress. **a,** Naïve mice infused with HSV-GFP and subjected to sub-maximal social defeat (see Materials and Methods) showed high levels of interaction (t_{10}=3.83, p<0.01), whereas IRS2dn or AKTdn overexpression in the VTA decreased interaction levels (p>0.1 in both cases). **b,** In the converse experiment, we examined the effects of viral manipulation on three matched groups of Susceptible mice (30.2±7.8 s, 36.5±5.4 s and 35.0±6.2 s, $F_{2,28}$=0.23, p>0.5, n=7-11). While a clear avoidance phenotype was observed after an intra-VTA injection of HSV-GFP (t_{19}=2.65, p<0.05), HSV-IRS2wt or HSV-AKTca alleviated this form of avoidance (p>0.5). **c,** One day after the avoidance test in C, NAc BDNF protein levels in HSV-AKTca infected mice are significantly lower than those in control animals (t_{19}=2.52, p<0.05). **d,** VTA overexpression of GFP, IRS2 or AKT viruses in non-stressed mice does not significantly alter ambulatory locomotor hyperactivity or habituation over a 1-hr beam break locomotor test (*virus main effect,* $F_{4,43}$=1.44, p>0.2). Overall locomotor activity was also comparable across viral groups (inset). **e,** VTA overexpression of GFP, IRS2 or AKT in non-stressed mice does not alter baseline social interaction rates (*virus x target interaction*, $F_{4,43}$=0.98, p>0.3), as well as (**f**) levels of BDNF in the nucleus accumbens measured three days after viral overexpression ($F_{4,33}$=0.32, p>0.5).

Modulating AKT Activity in the VTA Regulates Depressive Behaviors in Rats

To further explore a possible role for AKT activity in the VTA in regulating depression-like behavior, we carried out a series of experiments in rats. In the forced swim test (FST), rats placed in a cylinder of water initially struggle, but soon display immobile posturing, which is taken to be an analog of the behavioral despair and helplessness often observed in stress-related disorders (Cryan et al., 2002; Lucki et al., 2001). Three days following intra-VTA stereotaxic infusions (FST Day 2), we found that the latency to immobility was dependent on viral treatment (Figure 20A): IRS2dn and AKTdn expressing rats displayed significantly lower latencies to immobility, a pro-depression-like effect. During this test, viral groups were not different on counts of swimming, but displayed significant differences on counts of climbing and immobility (Figure 20B). These effects were unrelated to total locomotor activity, which was unchanged by viral treatment ($F_{4,95}$= 1.01, p>0.3, n=16-23). Similar results were obtained on the sucrose preference test. Various forms of chronic stress produce sucrose preference deficits (Willner, 2005) that are often reversed by chronic antidepressant treatment (Papp et al., 1996). When tested three days after viral infusions, sucrose preference varied as a function of viral treatment

(Figure 20C): IRS2dn and AKTdn expressing rats displayed significantly lower preference scores, without clear differences in fluid intake (Figure 20D).

Figure 20

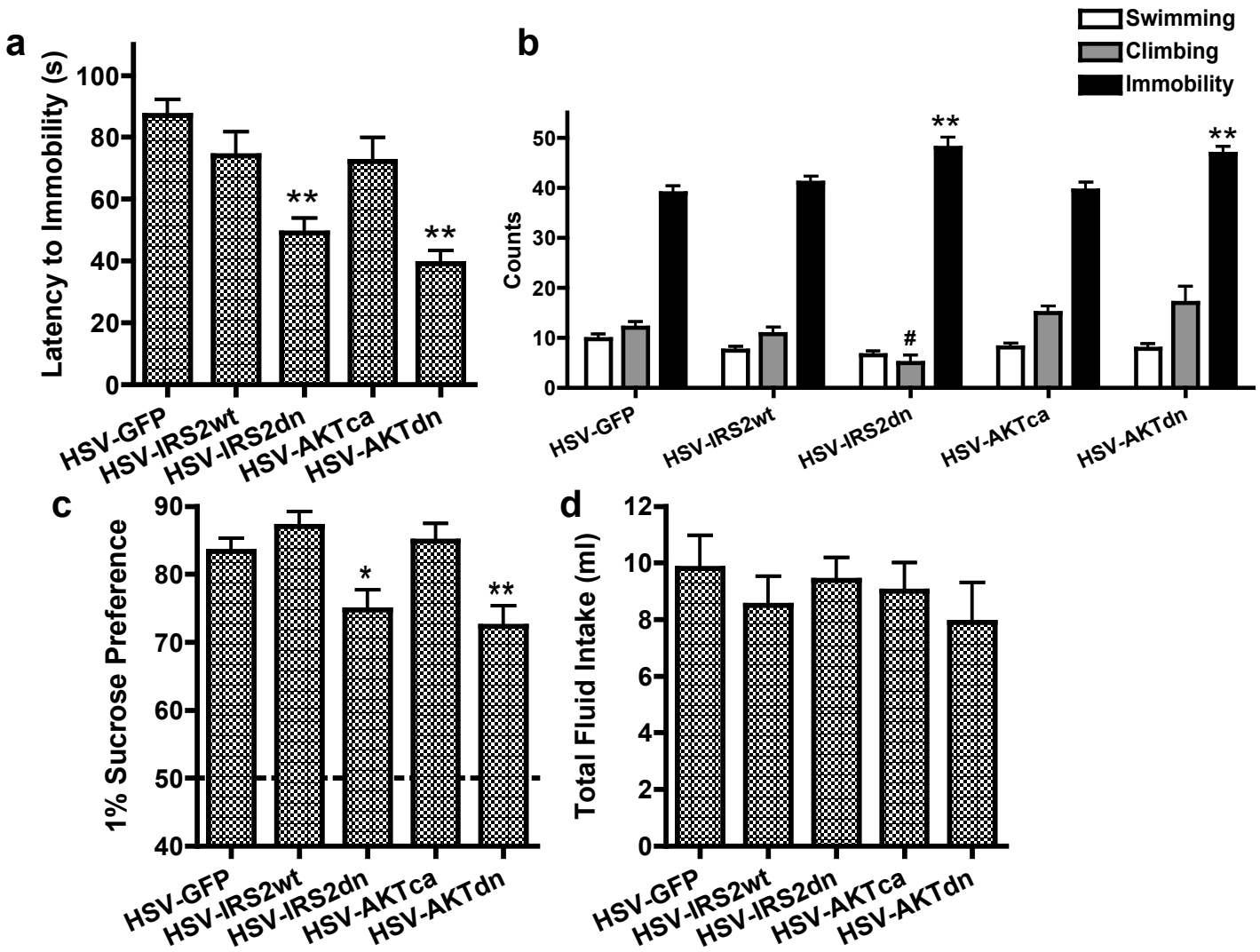


Figure 20. Decreases in IRS2 and AKT signaling in the rat VTA enhance depression-related behaviors in rats. **a,**VTA-specific reductions in IRS2 or AKT function significantly decreased the latency to immobility on Day 2 of the rat forced swim test ($F_{4,98}$=9.99, p<0.0001, n=13-28). **b,** On this day, while viral groups were no different on counts of swimming ($F_{4,90}$ = 1.78, p>0.1), IRS2dn overexpressing rats displayed significantly lower climbing counts ($F_{4,90}$ = 5.57, p<0.0001), and both IRS2dn and AKTdn overexpressing rats displayed significantly enhanced total immobility counts ($F_{4,90}$ = 6.84, p<0.0001, n=13-28). **c,** We tested the effects of these manipulations on five groups of rats matched for equal "pre-test" sucrose preference scores ($F_{4,92}$ = 0.33, p>0.5, n=13-28). Three days post-surgery, IRS2dn and AKTdn overexpression produced significantly lower sucrose preference scores ($F_{4,92}$ = 6.00, p<0.001). **d,** The total fluid consumed during the sucrose preference test was not changed by viral manipulation ($F_{4,92}$=0.47, p>0.5). Data obtained in collaboration with Sergio Iniguez and Carlos Bolanos, PhD (Florida State University).

PI3K Inhibition Results in an Increase in VTA Spontaneous Firing Frequency

Long-lasting increases in the excitability of VTA dopamine neurons mediate defeat-induced increases in NAc BDNF levels as well as several behaviors associated with susceptibility (Krishnan et al., 2007). However, VTA-intrinsic mechanisms that underlie this electrophysiological adaptation are unknown. Since AKTca overexpression in socially defeated mice was able to significantly reduce levels of BDNF protein levels in the NAc (Figure 19C), we sought to explore how modulating AKT function would alter the spontaneous firing frequency of VTA dopamine neurons. LY294002 is a reversible PI3K inhibitor capable of blocking AKT phosphorylation (Kuzman et al., 2005) and100 μM LY294002 blocks the expression of long-term potentiation in the hippocampal CA1 region (Sanna et al., 2002). Extracellular recordings of VTA dopamine neurons showed that 100 μM LY294002 caused a significant increase in the pacemaker frequency of these neurons (Figure 21A), and this effect was reversed by washout. This finding indicates that inhibition of AKT is sufficient to increase VTA neuronal excitability. One of AKT's known targets is the β_2 subunit of the $GABA_A$ receptor: AKT phosphorylates Ser410 of the β_2 subunit, which increases membrane insertion and GABAergic transmission (Wang et al., 2003). Through whole-cell recording techniques, we observed that LY294002 bath application caused a significant reduction in both the amplitude and frequency of spontaneous GABA-mediated inhibitory postsynaptic currents (IPSCs) recorded in dopaminergic neurons (Figure 21B).

In summation, data presented in this chapter allow us to propose the working model depicted in Figure 22. Susceptibility to defeat-induced avoidance reduces AKT activity in VTA dopaminergic neurons (through as yet unknown mechanisms). The subsequent decreases in GABAergic transmission result in heightened firing rates of VTA dopamine neurons, and

resultant increases in activity-dependent BDNF release onto the NAc. Enhanced VTA firing rates simultaneously produce reductions in the soma surface area of dopaminergic neurons, a morphological change that is capable of altering responses to natural and drug rewards (Russo et al., 2007).

Figure 21

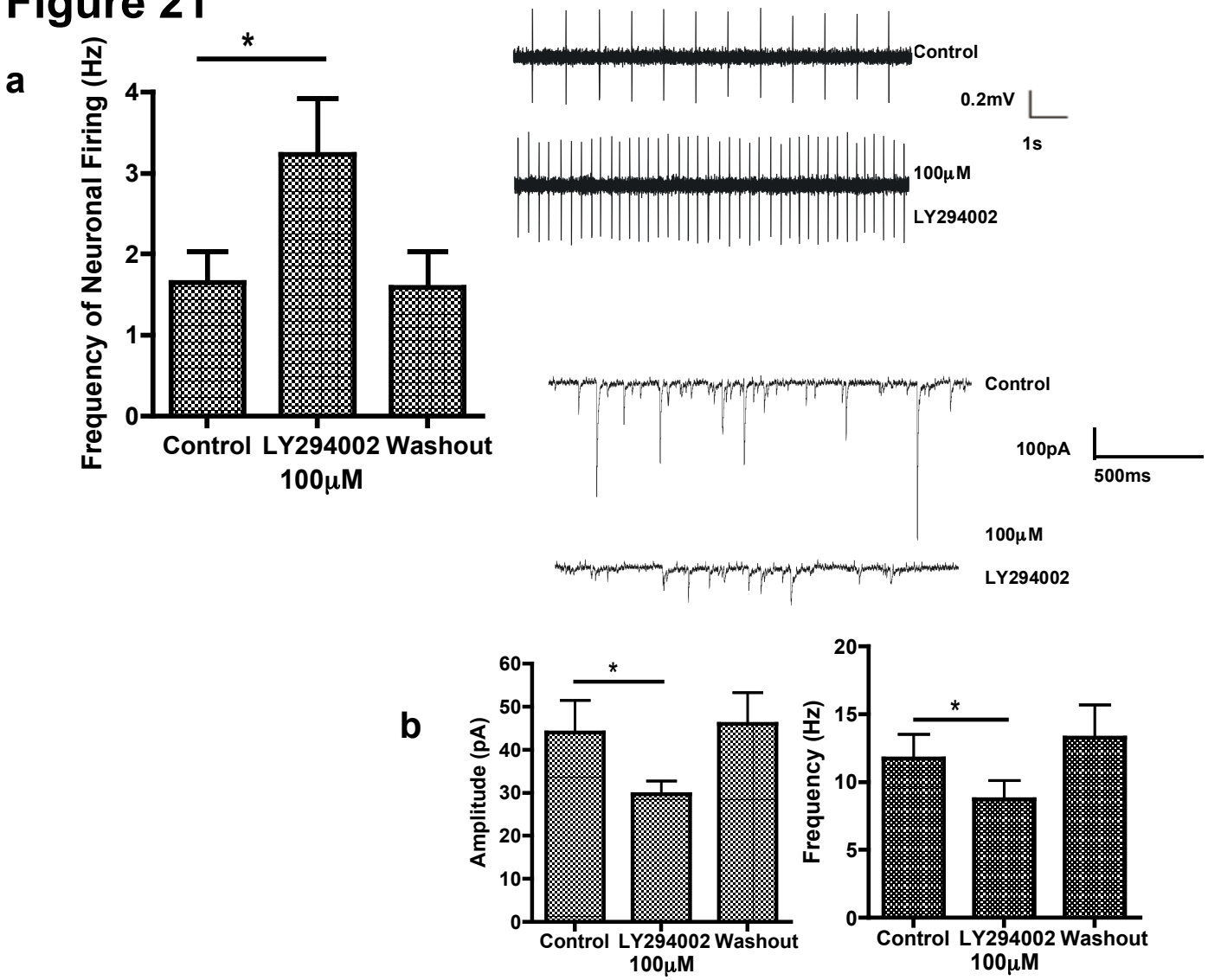


Figure 21. Decreased AKT signaling increases the excitability of VTA dopamine neurons. **a,** Extracellular recordings from VTA dopamine neurons reveal that 100 μM LY294002, an inhibitor of PI3K which reduces AKT phosphorylation and activity, significantly increases spontaneous pacemaker firing frequency (t_{10}=2.0, $p<0.05$, n=5-6, 3-4 mice/group, paired *t* test), which is reversed by washout (representative traces shown on right). **b,** In whole-cell voltage-clamp recording experiments, 100 μM LY294002 also produced significant decrements in the amplitude (t_6 = 3.09, $p<0.05$) and frequency (t_6 = 3.16, $p<0.05$, n=7 neurons/group, 3-4 mice/group, paired *t* tests) of spontaneous GABA-mediated inhibitory post-synaptic currents (IPSCs) onto VTA dopamine neurons (representative IPSC traces shown above). Data obtained in collaboration with Ming-Hu Han, PhD.

Figure 22

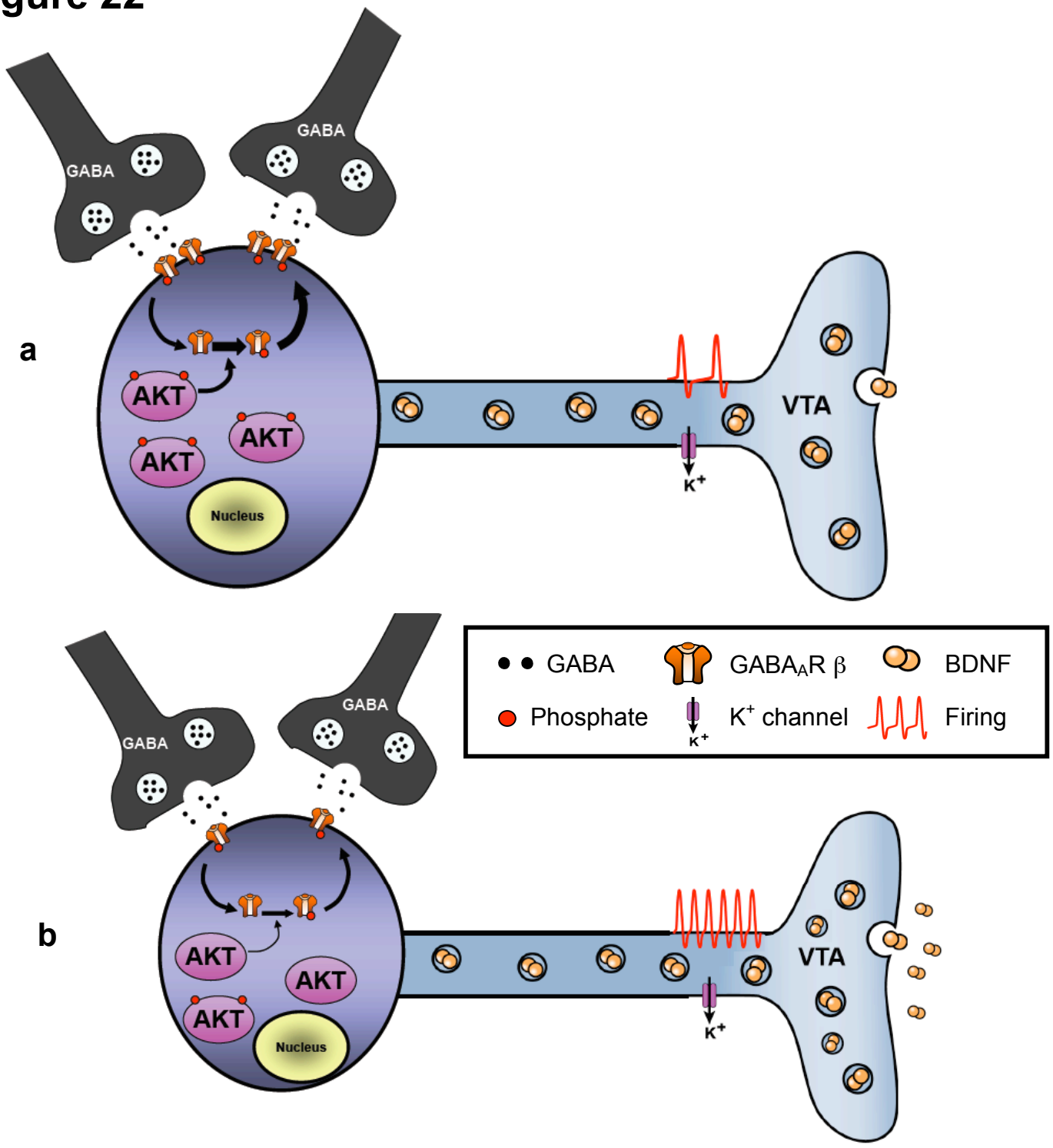


Figure 22. Working model of the role of AKT in the VTA after chronic stress. **a,** At baseline, VTA dopamine neurons receive tonic inhibition from surrounding GABAergic interneurons as well as projection neurons. High basal levels of AKT activation support the membrane insertion of $GABA_A$ receptors ($GABA_AR$) via the AKT-mediated phosphorylation of $GABA_AR$ β_2 subunits. **b,** Following chronic exposure to social defeat stress, reduced AKT tone leads to the reduced phosphorylation of $GABA_AR$ β_2 subunits and consequently reduced membrane expression of the receptors. This leads to enhanced excitability (which appears to reduce soma volume), and subsequent increased rates of activity-dependent BDNF release in targets regions of the VTA neurons (e.g., NAc). Abbreviations: GABA (γ-amino butyric acid), BDNF (brain-derived neurotrophic factor).

Chapter V: Discussion

"Southwestern Sunflowers"

These flowers were captured on the South campus of The University of Texas Southwestern Medical Center, adjacent to the McDermott Administration Building ("B Building"). It was obtained in April of 2005, a few months before I officially joined the Nestler laboratory as a graduate student. While they may superficially resemble sunflowers, this particular flower is the *Rudbeckia hirta* ("Black-eyed Susan"). Photograph by Vaishnav Krishnan.

A Model of Resilience

Upon exposure to psychological stress, why do some individuals succumb to debilitating psychiatric disease whereas others progress normally? Retrospective epidemiological studies reveal that the most significant non-genetic risk factors for depression and PTSD include i) adverse childhood experiences, ii) neuroticism, and iii) female gender (Fava and Kendler, 2000; Yehuda, 2002; Yehuda and Ledoux, 2007). On the other hand, resilient behavior is associated with a number of key psychological attributes including positivism, the ability to gather social support, a resilient explanatory style and humor (Southwick et al., 2005). These empirically obtained data help identify at-risk and resilient populations, but offer little insight into the neurobiological bases for variations in susceptibility. The goal of these experiments was to identify molecular mechanisms underlying vulnerability to stress-induced psychopathology, as well as molecular adaptations that promote resistance to those changes.

To achieve this goal, we required a model of chronic stress that would possess the following characteristics. First, the stressor must be coupled with a valid task that provides an objective/quantitative output, and must be of relatively high throughput so as to allow for the screening of a large number of animals. This stressor would have to be severe enough to produce long-lasting and objectively quantifiable depression-related impairments. At the same time, the magnitude of the stressor must be mild enough as to observe considerable phenotypic heterogeneity, such that only a subset of mice can be categorized as being "pathologically" impaired. The social defeat paradigm obeyed these characteristics, and allowed us to segregate socially defeated c57BL/6 mice into Susceptible and Unsusceptible subgroups based on the avoidance scores, which were used as the **primary** means to assign an animal's susceptibility. While measuring and classifying susceptibility in this way greatly simplifies subsequent

behavioral and molecular analyses, it may give the false impression that stress-responses are a dichotomous variable. This was not our intention, and at each stage in our experiments, we complement our statistical analysis of "group averages" with correlational data (Tables 2 and 4).

A defeated mouse's susceptibility assignment also remained relatively stable over time: interaction scores obtained on Day 11 correlated significantly with those obtained on Day 39. Through a series of behavioral characterization experiments, we demonstrated that while Unsusceptible mice were immune to several depression-like changes (e.g., anhedonia, enhanced cocaine reward 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.

Predicting Susceptible and Resilient Mice: "Krishnan's Conundrum"

We hypothesized that since stress-related disorders are often observed in individuals who are driven towards engaging in activities that possess some inherent risk ("sensation seekers/ risk seekers", Charney and Manji, 2004), susceptibility to social defeat would be related to elevations in exploratory behavior, or perhaps to other indices of depression-related behavior. We reasoned that such behavioral changes may provide clues about neuromolecular signatures of vulnerability and, having established a reliable animal model, we would be able to directly test hypotheses related to *in vivo* biomarkers of vulnerability. To this end, we measured a series of behavioral traits on naïve mice (prior to social defeat), and correlated these indices with Day 11 interaction

scores. Thus far, these attempts have revealed an amazing lack of correlations, leading us to the conclusion that resilience (at least in this paradigm) appears to function as a *latent* trait, requiring a stressful exposure for it to be revealed (which is often the case for human resilience). Importantly, susceptibility in this paradigm is not simply the consequence of a pre-existing subordinate status, possessing lower body weight, lower motivation to socially interact or enhanced anxiety-related behavior. Greater insights into this phenomenon may be obtained through socially defeating cohorts of outbred mice, which possess greater inherent variability along a variety of behavioral measures. In addition, pre-defeat behavioral testing should be expanded to include standard laboratory assays of spatial and fear memory, which as described below, are clearly relevant to the manifestation of social avoidance.

Non-Environmental Non-Genetic Sources of Variability: Epigenetic Contributions

In this work, we demonstrate that genetically identical (inbred) mice can display phenotypic differences after exposure to chronic stress, which are mediated through specific molecular differences within the mesolimbic dopamine circuit. 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 dominance hierarchies established within group housing (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 or 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 both environmentally mediated and transgenerationally inherited chromatin alterations such as histone acetylation or methylation (Tsankova et al., 2006; Wong et al., 2005). We hypothesize that such epigenetic chromatin modifications are an important alternative to ensuring natural variation, particularly in populations such as the c57BL/6J strain of mice, where genomic variation is artificially "constrained" due to inbreeding and a relative lack of chemical mutagens.

A recent study examining the genomics of inbred mice suggest that mice obtained from commercially available inbred strains may in fact display significant genomic heterogeneity, i.e., these mice are truly not "isogenic". One report documented the presence of *copy number variations* (CNVs) within members of the c57BL/6J line of mice (Watkins-Chow and Pavan, 2008). CNVs are defined as deletions or duplications of >1kb of genomic DNA, and naturally segregating CNVs have been identified in at least two genes on chromosome 19 in c57BL6/J mice (Watkins-Chow and Pavan, 2008). Since such CNVs in genes affect transcript and protein levels, they are aptly positioned to contribute significantly to the phenotypic variability described here. Future work is clearly required to delineate the relative contribution of genomic, epigenetic, genetic, and environmental factors that may together explain variations in susceptibility.

A Role for Neural Reward Substrates

Susceptible mice showed a deficit in natural reward (sucrose preference), coincident with an increase in drug reward (cocaine place conditioning), 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 within the NAc, 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). While we demonstrate that an important source for these BDNF elevations are afferent dopaminergic neurons of the VTA, it is clear that BDNF's pro-vulnerability effects are not exerted through actions on the VTA. Direct evidence for this comes from our experiments where we selectively ablated TrkB (BDNF's receptor) within the VTA; such conditional knockouts of TrkB performed comparable to wild type controls on the social defeat paradigm. 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.

Gene expression profiling studies provided important insights into the nature of molecular neuroplasticity occurring within these regions. It should be noted that microarray data can be analyzed in several different ways: a variety of algorithms have been developed for each step of microarray data analysis including standardization, normalization and statistical analysis. Specific lists of genes are often quite different when re-analyzed using these different algorithms. Aside from the gene lists we obtained, our microarray experiment provided three main results independent of the method of analysis we employed ("*meta-results*"). First, the absolute number of genes upregulated or downregulated was significantly higher within NAc samples, indicating that medium spiny neurons of the NAc may possess the ability to regulate the expression levels of a greater number of genes. Second, the Unsusceptible phenotype was also 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. Finally, the number of genes identically regulated between Susceptible and Unsusceptible mice was significantly smaller within the VTA, indicating that within this circuit, the VTA perhaps served as a type of "master regulator" to *decide* between Susceptible and Unsusceptible behavioral (and molecular) profiles.

The selective upregulation of three 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. The ionic mechanisms by which Susceptible VTA dopamine neurons increased their excitability were not immediately revealed from our microarray gene experiments. 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. This model is consistent with the observation that VTA neurons are strongly activated during the threat of social subordination (Tidey and Miczek, 1996), and the phenomenon of anterograde axonal BDNF transport that is reported to occur within this circuit (Altar and DiStefano, 1998). The BDNF field has known about anterograde axonal transport within this circuit for several years (Altar et al., 1997): our data suggest an important physiological function for this type of transport, i.e., serving to strengthen emotionally salient associations. 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.

BDNF G196A/Val66Met: A "Pro-resilient" SNP

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., 2006; Chen et al., 2004). 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 and activity, Met/Met mice displayed a striking Unsusceptible phenotype in the social defeat paradigm, which was associated with a ~50% reduction in levels of BDNF protein in the NAc. On Day 11, measures of VTA firing were similar between socially defeated Val/Val and Met/Met mice suggesting a dissociation between the effects of dopamine and BDNF: BDNF release (as a consequence of enhanced VTA activity) serves a pathological role, whereas increases in dopamine release (similar in Val/Val and Met/Met mice) appears to play a benign role.

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). Since the publication of our findings, Weinberger and colleagues have demonstrated evidence for biological epistasis between the BDNF Val/Met allele and a serotonin transporter (5HTT) polymorphism: while carriers of the 5HTT *short* promoter allele display lower cg25 (subgenual cingulate cortex) volumes and impaired limbic connectivity, this effect is absent in individuals heterozygous for the Met allele (Pezawas et al., 2008). Clearly, further studies are required to examine the influence of the BDNF Val/Met polymorphism on human psychopathology in light of our findings.

Relationship to Learning: "Resilient" or "Retarded"?

It is 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 suggests that naturally occurring variations in episodic memory (or other forms of memory) may alter responses to social defeat. This raises an important question: does a memory deficit serve as confounding factor when interpreting the results of a social defeat experiment on a knockout/transgenic mouse? If so, what type of memory test must one perform so as to rule out a memory impairment? Spatial memory? Fear memory? We hypothesize that the relationship between alterations in memory and socially resilient behavior 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. Support for this model comes from studies examining the dynamics of social defeat's regulation of hippocampal neurogenesis, a key cellular substrate for learning and memory. These studies demonstrate that while chronic social defeat acutely produces a reduction in hippocampal proliferation (as measured through bromodeoxyuridine labeling), the persistence of a Susceptible phenotype is associated with enhanced neuronal survival (Lagace et al., 2008). X-irradiation mediated ablation of hippocampal neurogenesis was sufficient to produce a resilient phenotype (Lagace et al., 2008), consistent with a mal<u></u>adaptive role for learning-related processes. These data warrant a more detailed exploration of the relationship between learning impairments and resilient behavior. As just a few examples, it would be important to demonstrate similar relationships in other transgenic/knockout lines of mice that possess memory phenotypes, and also to examine whether experimental manipulations

that produce memory impairments (e.g., electroconvulsive shock, hippocampal and amygdalar lesions) also result in resilient behavior.

Reduced VTA "AKTivation": A Second Molecular Signature of Vulnerability

By coupling a chronic stress paradigm to a quantitative measure of social withdrawal, we showed that vulnerability to defeat-induced social avoidance was mediated through enhanced firing of VTA dopamine neurons and a consequent increase in the activity-dependent release of BDNF from the VTA to the NAc (Krishnan et al., 2007). While the site of BDNF's pro-vulnerability effects appears to be the NAc, it is clear that primary neuroadaptations within the VTA that modulate dopamine neuron excitability serve as an origin for the manifestation of vulnerability. Here, we illustrate how one such adaptation in Susceptible mice, decreased AKT phosphorylation, is both necessary and sufficient recapitulate the behavioral, morphological and electrophysiological signatures of vulnerability to social defeat stress.

Repeated morphine exposure decreases the soma surface area of VTA dopamine neurons (Chu et al., 2007; Sklair-Tavron et al., 1996) through reductions in the activity of the IRS2-AKT signaling cascade (Russo et al., 2007). In our study, Susceptible mice displayed an identical VTA phenotype. We provide evidence that the downregulation of IRS2-AKT signaling mediates the decreased size of VTA dopamine neurons through the use of viral vectors, which demonstrate that inhibition of this pathway decreases dopamine cell size in the VTA, while increased activity causes the opposite effect. Postmortem studies of cortical regions obtained from depressed humans (Hsiung et al., 2003; Karege et al., 2007) have also described reductions in AKT function. In our study, we observed a significant increase in AKT phosphorylation in depressed VTA postmortem samples, without a change in total AKT levels. This effect was recapitulated in

mice chronically treated with the selective serotonin reuptake inhibitor (SSRI), fluoxetine, which suggests that the changes we observed in postmortem VTA are likely a consequence of chronic antidepressant exposure. However, given our small sample size, further studies are obviously warranted to dissect the contribution of depression-related disease processes versus antidepressant-induced changes in the VTA and other brain regions.

Reductions in Phospho-AKT: Potential Mechanisms and Consequences

It remains unclear whether reduced AKT function represents a pre-existing risk factor or the result of an interaction between social defeat stress and other, as yet undefined, mechanisms. In particular, the upstream mechanisms underlying the stress-induced downregulation in AKT signaling in Susceptible mice are currently unknown, as well as those mechanisms that allostatically maintain AKT activity within normal levels in VTAs from Unsusceptible mice. Our immunoblotting studies did not reveal changes in the activity of BDNF, IRS2, PDK1 or PTEN, all of which are upstream nodes capable of altering AKT activity. Indeed, there was a trend for increased BDNF levels within Susceptible VTAs, which is consistent with the marked pro-depresssant effect of BDNF within the mesolimbic dopamine circuit (Berton et al., 2006; Eisch et al., 2003; Krishnan et al., 2007). BDNF and its receptor are only one among a constellation of growth factor-receptor tyrosine kinase systems linked to IRS-PI3K-AKT signaling, and further studies are required to determine whether the defeat-induced downregulation of AKT signaling in the VTA is secondary to changes in any of these other systems. As just one example, psychological stress is thought to play a key role in the development of metabolic syndrome (Rosmond, 2005), a constellation of symptoms that includes abdominal obesity, insulin resistance, and hypertension. Insulin receptors are expressed on VTA dopamine neurons

(Figlewicz et al., 2003), and decreased AKT signaling within the VTA (and other brain regions) may be a central correlate of system-wide insulin resistance brought about by exposure to chronic stress.

Through viral-mediated gene transfer, we showed that decreased AKT activation within the VTA is necessary and sufficient to confer vulnerability to defeat-induced social avoidance. We also extended our findings to the rat model, which allows one to more accurately target the VTA through stereotaxic surgery (Bolanos et al., 2003). While HSV-IRS2dn and HSV-AKTdn significantly enhanced depression-related behaviors, overexpression of IRS2wt or AKTca had no measurable effects. These viruses *did* promote a resilient phenotype when overexpressed in the background of abnormally low levels of AKT phosphorylation, i.e., following social defeat. In addition, the overexpression of AKTca significantly reduced stress-induced increases in NAc BDNF protein levels. These data suggest that, at baseline, the activity of this pathway may be sufficiently high in the VTA to maximally drive downstream effects. Thus, small decrements in the activity of this cascade may have significant physiological (and behavioral) ramifications, such as in our observation that 100 μM LY294002, a PI3K inhibitor which is known to reduce AKT phosphorylation, significantly increased the pacemaker frequency of VTA dopamine neurons. Since heightened rates of VTA firing act as a crucial mediator of the vulnerability to social defeat stress (Krishnan et al., 2007), these data suggest that the robust pro-depressant effects of HSV-IRS2dn and HSV-AKTdn may be mediated through their ability to potentiate VTA dopamine neuron excitability. AKT has a diverse array of known substrates (Manning and Cantley, 2007), including the β_2 subunit of the $GABA_A$ receptor: phosphorylation of Ser410 of the $GABA_A\beta_2$ subunit by AKT has been shown to increase the insertion of $GABA_A$ receptors into the plasma membrane, thereby enhancing GABA-mediated inhibition (Wang et al., 2003).

Whole cell-recording experiments allowed us to observe a decrease in the magnitude of postsynaptic $GABA_A$ receptor mediated IPSCs, leading us to deduce that reduced AKT activity in the VTA of socially defeated mice may increase VTA excitability through reduced GABAergic neurotransmission. PI3K inhibition achieved in this manner was sufficient to reduce both the amplitude and frequency of GABA IPSCs, suggesting the involvement of both presynaptic and postsynaptic mechanisms in reducing GABA transmission. The effects of PI3K inhibition on the excitability of these GABAergic neurons currently remain unexplored.

To explore the relationship between the electrophysiological and morphological changes within the VTA occurring on Day 11, we examined how our validated potassium channel vectors would affect the soma surface area of VTA dopamine neurons. In these experiments, we observed that enhancing the excitability of VTA neurons (through HSV-dnK) produced a significant decrease in VTA SSA, and the opposite effect was observed upon Kir2.1 overexpression (which effectively hyperpolarizes neurons). Since the magnitude of a neuronal membrane's exposed surface area is intricately linked to membrane capacitance and other measures of excitability, our data suggest that reductions in soma volume observed following social defeat may represent a compensatory mechanism against increases in excitability. Further experiments are required to examine the downstream molecular events responsible for cytoskeletal reorganization events involved in such cell volume reductions, and whether morphine and social stress trigger these pathways through common mechanisms.

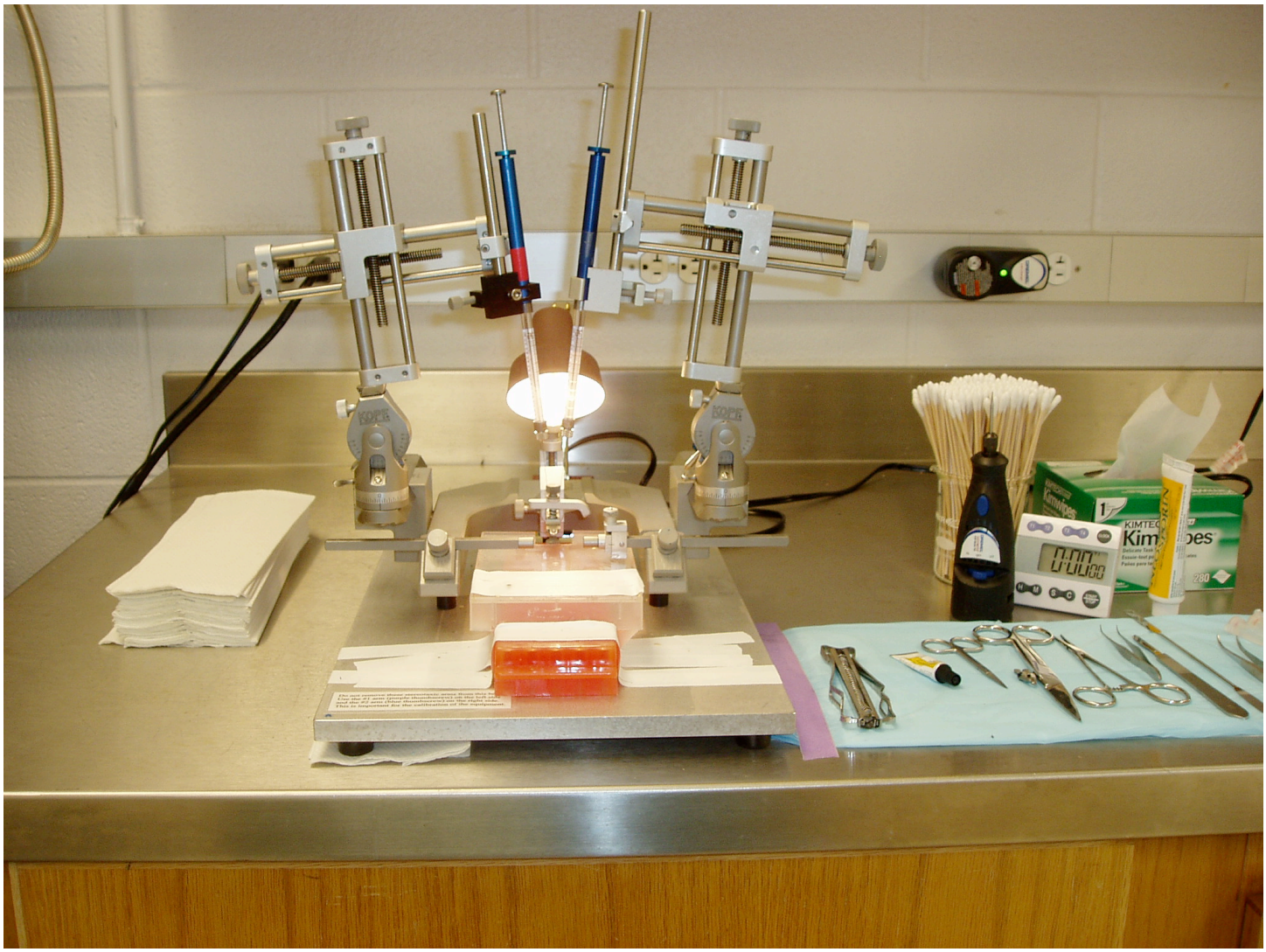
Conclusions

In conclusion, we propose that resistance to social defeat serves as an excellent model for studying the more general phenomenon of resilience in humans. The resistance described here

constitutes a long-lasting and 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). The ethological validity of subordination stress combined with the highly quantitative social interaction test very elegantly position this paradigm to provide insights into the molecular bases of both vulnerability and resilient behavior. Resilient individuals display a striking ability to preserve optimism in the face of adverse situations, a characteristic that may reflect reward substrates that are especially plastic, or that are insensitive to change (Charney, 2004; Curtis and Cicchetti, 2003). 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. Stress-induced downregulations of AKT activity in the VTA appear to be a molecular initiator of a "vulnerability cascade": reductions in phosphorylated AKT decrease inhibitory GABAergic transmission, which in turn leads to enhanced VTA excitability and BDNF release onto the nucleus accumbens, and this enhanced electrical activity appears to produce reductions in the soma surface area of VTA dopamine neurons. Our studies further substantiate the important role of neuroadaptations within the mesolimbic dopamine circuit in the control of emotional homeostasis and resilience (Berton et al., 2006; Charney, 2004). In addition, these results illustrate the potential of a *two-pronged* approach to developing novel therapeutic agents to combat stress-related disorders, i.e., those designed to reverse vulnerability-related

molecular events, as well as pharmacological tools that may enhance pro-resilient homeostatic mechanisms.

Chapter VI: Materials and Methods



"Mouse Stereotaxis"

During my thesis work, I spent several long hours in the noticeably *window-less* room, NC4.204 (the "Mouse Stereotaxic Surgery Room"). Here, I employed this particular small animal stereotaxic frame (shown above) in combination with a variety of dissecting tools, a hand-held drill, Hamilton syringes and needles, and a virus of interest to carry out local viral-mediated gene transfer, a technique highly popularized by the Nestler laboratory. Photograph by Vaishnav Krishnan.

Subjects and Drugs

Male 7-week old c57BL/6 (Jackson), CD1 retired breeders (Charles River), 9-13 week old floxed BDNF mice (Berton et al., 2006), male floxed TrkB mice (Luikart et al., 2003), 10-14 week old BDNF Met/Met and Val/Val mice (Chen et al., 2006) and 350-375g Sprague-Dawley rats (Charles River) were used. For all experiments, animals were i) male, ii) fed *ad libitum*, ii) allowed a one week habituation period before experimental manipulation and iv) housed at 23-25°C on a 12-hr light/dark cycle (lights ON at 0700 hrs). Behavioral testing was conducted during the light phase, and was performed under conditions of dim lighting except for the social avoidance task, which was conducted in darkness. In experiments where testing apparatuses were reused for multiple animals, surfaces were wiped with a solution of 70% ethanol in water to remove olfactory cues. To avoid retesting confounds, mice were never tested twice on any of the below-mentioned behavioral tests. All experiments were performed in accordance with the Institutional Animal Care and Use Committee (UTSouthwestern and Florida State University) and the Institutional Review Board (UTSW).

Cocaine (5 mg/kg) and imipramine (20 mg/kg) were administered through intraperitoneal injections. For stereotaxic surgery, mice were anesthetized with a "cocktail" of ketamine (100 mg/kg/10 ml) and xylazine (10 mg/kg/10 ml) (Henry Schein) in sterile saline. For implantation of subcutaneous temperature transponders, mice underwent isofluorane anesthesia (Henry Schein) delivered via a gaseous anesthetic dispenser. Isofluorane anesthesia was also utilized for the implantation of subcutaneous fluoxetine pellets (Innovative Research), which were implanted in the dorsal interscapular region and designed to administer 20 mg/kg/day of fluoxetine (or placebo) over a 20-day interval.

Social Defeat

Social defeat and avoidance testing were performed according to published protocols (Berton et al., 2006; Tsankova et al., 2006). CD1 retired breeder mice used for all defeat experiments were screened for consistent aggression (attack latencies <30 seconds on 3 consecutive screening sessions with a c57BL/6 intruder). Mice screened in this way were then housed in cages fitted with perforated plexiglas separators (Ace Acrylics, Irving, TX), which allow sensory contact without permitting physical contact. For 10 min a day for 10 days, mice were introduced into the aggressor's half of the cage and physically interacted, and were then separated for the remainder of the day. Instances of severe fighting were immediately interrupted, and topical Neosporin was applied to minor wounds. Non-defeated controls were housed in identical cages opposite each other and were rotated similarly. Immediately after the first defeat (for single defeat experiments) and the tenth defeat (for chronic social defeat experiments) all mice were singly housed. "Day 11" measures refer to tests conducted approximately 24 hrs after the last defeat episode, while "Day 39" measures correspond to tests performed 28 days later; during this interim period socially defeated and control mice remained individually housed. To examine the effects of "pro-vulnerable" manipulations, mice were subjected to a "sub-threshold" defeat paradigm consisting of three defeats within one day. These mice received stereotaxic infusions of HSV vectors three days prior, OR had received an infusion of BDNF on the day of this defeat set. Each episode lasted 5 min and was separated by a 15-min rest period. Avoidance testing was measured on the following morning. For chronic social isolation stress, c57BL/6 mice were individually housed for a period of 10 weeks (between 3 weeks and 13 weeks of age); control mice were housed in groups of 4-6 mice. For the social

interaction test, we measured the time spent in the interaction zone 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). Social isolation stress was applied by singly housing c57BL/6 mice for a period of 10 weeks (between 10 and 20 weeks of age); controls for this experiment were housed in groups of 4.

Injury Ratings

24 hrs after the cessation of social defeat stress, all control and socially defeated mice were examined by an observer blind to condition. This individual was instructed to provide a single injury rating score for each mouse between 1 and 10 (where 10 represents a total lack of injury). Wounds obtained during social defeat were minor, and included small tail nicks or small wounds near the tail. These wounds were observed to heal very quickly after cessation of social stress, and by Day 39, socially defeated mice could only be distinguished from non-defeated controls by the quality of fur on the belly surface (slightly ruffled).

Assessing Social Dominance

To explore the relationship between "pre-defeat" social hierarchical status and "post-defeat" interaction ratios, we devised a rudimentary 3-day task to distinguish the alpha male within a cage of two mice ("dyad"). On the first day, we measured the weights of all mice, and subjected pair housed mice to total caloric deprivation for 24 hours. On Day 2, cages were provided with enough standard chow to feed one mouse/day (0.4gms). On Day 3, cages were then provided with 0.2gms of chow ($1/4^{th}$ the amount of food consumed by an average mouse per day). On the morning of Day 5, cages were provided with adequate food, and weights were once

again measured. During this paradigm, the mouse that that lost the least amount of weight was assigned the alpha male (dominant), and the other, the omega male (subordinate). Social defeat was initiated 7 days later, when body weights were restored to normal levels.

Sucrose Preference

For sucrose preference testing in mice, 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 approximately 1600 hrs, the fluid level was noted and the position of the tubes were interchanged. Sucrose preference was calculated as a percentage [100 x volume of sucrose consumed (in bottle A) / total volume consumed (bottles A and B)], and was averaged over at least three days of testing. In the case of rats, a shortened sucrose preference test was employed (Bolanos et al., 2003), where animals were first habituated to a 1% sucrose solution. On "Pretest day", rats were singly housed and given access to a two-bottle choice for 30 min; the position of the bottles was balanced between the groups. Rats with a preference ≥60% were randomly assigned to viral conditions such that average pretest scores were similar, and three days following stereotaxic surgeries, sucrose preference "post-test" scores were obtained using this same protocol.

Anxiety and Locomotor Measures

Open-field testing was performed in arenas similar to those used for the social interaction test (without target enclosures). Videotracking-based methods were employed to record the distance moved and time spent in the total arena and a delineated "center zone" (34cm x 34cm).

The elevated plus maze was designed in black Plexiglass fitted with white surfaces to provide contrast. Mice were positioned in the center of the maze, and behavior was recorded by videotracking for a five min period (Monteggia et al., 2007). Locomotor activity was measured in a novel cage (Monteggia et al., 2007) fitted within a photocell grid device that counted the number of ambulatory photo beam breaks within 5 mi blocks during an hr-long period.

Immobility Measures (FST and TST)

The time spent immobile during a 6 min tail suspension test (TST) was obtained as previously described (Liu 2003) using an automated TST device (Med Associates Inc). Similarly, the forced swim test (FST) was conducted as previously described (Monteggia et al., 2007), by placing mice in a 4L beaker containing approximately 3L of tap water at a temperature of 24±1^{o}C. All sessions were videotaped and scored by an independent experienced observer who recorded the duration of immobility during the last 4 min of the FST. To examine the swim-stress induced corticosterone response, mice were sacrificed approximately 10 min after the cessation of forced swimming. For rats, a two-day forced swim test protocol was employed to obtain measures of immobility (Eisch et al., 2003). Three days after surgery, rats were placed in plastic cylinders (30 x 45cm) filled to 30 cm depth with 25ºC tap water and were forced to swim for 15 min. Rats were retested the next day under identical conditions for 5 min, and all sessions were videotaped and scored by blinded observers who measured counts of climbing, swimming and immobile behavior (Cryan et al., 2005).

Cocaine Conditioned Place Preference (CPP)

Conditioned place preference to cocaine was obtained by using an established conditioning protocol (McClung et al., 2005). We utilized a low dose of cocaine (5 mg/kg) to best observe cross-sensitization. On day 1 of CPP (corresponding to Day 12 of the social defeat paradigm) mice were tested for 30 min in the CPP apparatus to measure pre-conditioning side bias ("Preconditioning"). On days 2 and 4, mice were given a cocaine injection and provided restricted access to only side of the apparatus for 20 min, and on days 3 and 5, mice were given a saline injection and paired with the other side. On day 6, preference to either side was measured during a 30 min test session ("Postconditioning").

Cardiac Hypertrophy

Upon cervical dislocation, the heart was rapidly excised, dissected clean of all connecting tissue and great vessels, rinsed in cold phosphate buffered saline, blotted dry, and then weighed (Hill et al., 2000). Data are reported as heart mass (mg) normalized to body mass (g) [HW/BW, measured on day of sacrifice].

Measurements of Core Body Temperature

Wildtype c57BL/6 mice were implanted with IPTT-300 subcutaneous temperature transponders (Biomedic Data Systems) upon arrival to the UTSW vivarium (Liu et al., 2003a). Transponders were implanted in aseptic conditions under isofluorane anesthesia, and each mouse was fitted with a new transponder in the superior dorsal/interscapular region. Measurements

were obtained with a DAS-5002 electronic transponder reader (BMDS). This approach allows one to obtain core body temperature values (accurate to one decimal point) by avoiding the stress associated with measuring core rectal temperatures. Transponders once positioned do not display significant tissue migration and cause negligible tissue damage. "Anticipatory hyperthermia" was measured by recording body temperatures approximately 30 min prior to defeat on days 4, 6, 8 and 10 of the social defeat paradigm. Since there were negligible differences between these time points, we averaged these values together for each mouse. "Social hyperthermia" was measured during the social interaction test on either Day 11 or Day 39, by recording temperatures immediately before and after the completion of social avoidance measurements. On Day 11 and Day 39, we also obtained temperature measurements at 0400, 0800, 1200, 1600, 2000, 0000hrs over one complete circadian cycle. The circadian amplitude for each mouse was obtained by calculating the difference between the maximum and minimum temperatures during this period.

Stereotaxic Surgery

33 gauge needles (Hamilton) were employed for all surgeries, during which 0.5 μl of purified high-titer virus was bilaterally infused over a 5 min period of time, followed by an additional 5 min post-infusion rest period. All distances are measured relative to bregma. Mouse NAc: 10º angle, AP = +1.6 mm, Lat = +1.5 mm, DV = -4.4 mm (Barrot et al., 2002) Mouse VTA: 7º angle, AP = - 3.2 mm, Lat = +1.0 mm, DV = -4.6 mm (Berton et al., 2006) Rats were anesthetized with a ketamine (90 mg/kg) and xylazine (10 mg/kg). We employed established rat VTA coordinates (AP: -4.9mm, Lat: +2.2mm, DV: -7.6mm, 10º angle (Bolanos et al., 2003), and unilaterally infused 2 μl over 10 min. Injection sites were confirmed in all animals

by standard histological methods. All behavioral experiments were performed on day 3 after viral surgery, when maximal transgene expression is observed (Barrot et al., 2002).

The following viruses were used:

AAV-CreGFP, AAV-GFP (Berton et al., 2006)

HSV-Kir2.1, HSV-GFP (Dong et al., 2006)

HSV-dnERK [K52R mutant] (Robinson et al., 1996; Russo et al., 2007)

HSV-dnK [*Kcnab2* S188A, R189L] (Campomanes et al., 2002; Gulbis et al., 1999)

Recombinant BDNF (a generous gift from Regeneron Pharmaceuticals (Graham et al., 2007)

HSV-AKTca, HSV-AKTdn, HSV-IRS2wt, HSV-IRS2dn (Russo et al., 2007)

Histology

Animals were anesthetized with an overdose of chloral hydrate and perfused transcardially with 0.9% saline followed by cold 4% paraformaldehyde. Brains post-fixed overnight in 4% paraformaldehyde, stored in 20% glycerol solution, and sectioned on a frozen microtome (rat: 45 μm, mice: 30 μm). Sections were blocked in 3% normal donkey serum (NDS), 0.3% Triton X [PBS], and incubated overnight in primary antibody along with 0.3% Tween-20 [anti-GFP (Abcam). Sections were incubated with a biotinylated anti-rabbit secondary antibody (1:200; Jackson) for 2 hr at room temperature. Stained sections were then slide-mounted, dehydrated in ethanol and citrosolv, and cover-slipped with clear DPX. To detect AKT, mounted sections were dried overnight prior to immunohistochemistry. Antigen retrieval was performed on slide-mounted sections using 0.01M citric acid, pH 6.0, at 95°C for 15 min followed by quenching of endogenous peroxidases using 0.3% H_2O_2 in 1X PBS for 30 min. Sections were blocked with 3% normal donkey serum (Jackson Immunolaboratories) and 0.3% Triton-X 100 in PBS for 30 min

and then incubated with primary antibody (1:500, 3% serum and 0.3% Tween-20) overnight. Immunolabeling was detected using either fluorescent-tagged donkey anti-mouse IgG (1:200, 1 hr, Jackson) or biotinylated donkey anti-rabbit IgG secondary antibodies (1:200, 1 hr, Jackson). Following incubation with biotinylated secondary, slides were incubated in avidin-biotin complex (ABC Elite, Vector Laboratories) for 1 hr and staining was visualized with tyramide signal amplification (PerkinElmer). Dehydrated and coverslipped slides were then visualized and photographed using a Zeiss 560LSM confocal microscope using a 63X water objective and a scan speed of 8 dpi.

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 was 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 3 biological replicates for each group, each consisting of pools of mRNA from 4 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 $p<0.05$). Microarray data can be accessed through NCBI's Gene Expression Omnibus (GEO

Accession Number: GSE8870). The following primer sequences were used: mouse *bdnf* [5'CCATAAAGG-ACGCGGACTTGTACA-3' & 5'-AGACATGTTTGCGGCATCCAG-3'], mouse *gapdh*[5'-AACGACCCCTTCATTGAC-3' & 5'-TCCACGACATACTCAGCAC-3'], mouse *avp* [5'-GCCAGGATGCTCAACACTACG-3' & 5'-TCTCAGCTCCATGTCAGAATG-3'], human *bdnf* [5'-AGTGCCGAACTACCCAGTCGTA-3' & 5'-TATGAATCGCCA GCCAATTCT-3'], and human *gapdh*: 5'-ATGGGGAAGGTGAAGGTCG-3' & 5'GGGGTCATTGATGGCA ACAATA-3'].

Immunoblotting and Immunoassays

Tissue punches of VTA (1.25 mm diameter) and NAc (core and shell, 1 mm diameter) from c57BL/6 mice were sonicated in an EMSA lysis buffer [20 mM HEPES, 0.4 M NaCl, 20% glycerol, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP40, 50 mM DTT, 0.1% protease and phosphatase inhibitors (Sigma)], and then centrifuged at 14,000 rpm for 15 min. 20-40μg samples (estimated through the Bradford assay) were treated with ß-mercaptoethanol and subsequently electrophoresed on precast 4-20% SDS gradient gels (Biorad). Proteins were transferred to a PVDF membrane, washed thoroughly in 1x Tris-buffered saline with 0.1% Tween-20 (TBS-T), and blocked in a solution of 5% w/v milk [TBS-T] for 1 hr at 25^{o}C. Primary antibody incubations were performed overnight: anti-$BDNF_{N\text{-}20}$ (mature BDNF, 1:200; *Santa Cruz*), anti-actin (1:10,000; *MP Biomedicals*), anti-β-tubulin (1:40,000; *Upstate*), anti-TrkB (TrkB.F & TrkB.T, 1:5,000; *Upstate*), anti-AKT (AKT1,2 and 3), anti-phosphoAKT_{S473} (AKT1,2 and 3), anti-ERK1/2, anti-phospho$ERK1/2_{T202\ Y204}$, anti-PLCγ, anti-phospho$PLC\gamma_{Y783}$, anti-PTEN, anti-phospho$PTEN_{S380}$, anti-PDK1, anti-phospho$PDK1_{S241}$, anti-phospho$GSK3\beta_{S9}$ and anti-GSK3β, anti-phospho$mTOR_{S2481}$, and anti-mTOR (1:500; *Cell Signaling*) and anti-IRS2

(1:1000, *Upstate*). After further washes, membranes were incubated with peroxidase labeled goat anti-rabbit IgG or horse anti-mouse IgG (1:40,000; Vector). Bands were visualized with SuperSignal West Dura substrate (Pierce) and quantified with Scion Image (NIH).

Enzyme Immuno-Assays (EIAs)

The quantitative determination of serum steroid levels was performed with the aid of commercially available kits for corticosterone (Immunodiagnostic Systems) and dehydroepiandrosterone-sulfate (ALPCO Diagnostics). Total trunk blood was obtained during the light phase (~1000-1200hrs) and centrifuged at 3000rpm for 10 min, following which the supernatant (serum) was stored at -20ºC for until time of assay. Appropriate dilutions of serum samples were employed [corticosterone (1:50), DHEA-S (1:10)], and grossly hemolytic serum samples were excluded. The BDNF E_{max} ImmunoAssay System (Promega) was employed for detection of total BDNF in lysates of human nucleus accumbens (Chen et al., 2006; Durany et al., 2000). EIAs were performed with strict adherence to manufacturer's guidelines (all samples were assayed in duplicate, and absorbance values outside the linear portion of standard curve were excluded from analysis).

Human Postmortem Studies

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, & 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 1-2cm blocks. Dissected NAc and VTA 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 8000g at 4 °C. The pH of this supernatant was measured in duplicate. Each sample's RIN (RNA integrity number) was determined by isolating total RNA using Trizol (Invitrogen) followed by analysis with an Agilent 2100 Bioanalyzer. For protein studies, approximately 100 mg of NAc tissue was homogenized in 1ml of lysis buffer (100μg/ml PMSF, 2μg/ml aprotinin of leupeptin, aprotinin and pepstain in PBS) with a Polytron homogenizer (900 rpm x 12 times). Samples were then sonicated and by using the Bradford assays, protein concentrations were found to be between 2 and 5μg/μl.

Electrophysiology

Brains removed from anesthetized mice were placed in sucrose-artificial CSF (aCSF), which was derived by fully replacing NaCl with 254 mM sucrose in aCSF [128mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 10 mM D-glucose, 24 mM $NaHCO_3$, 2 mM $CaCl_2$, and 2 mM $MgCl_2$ (oxygenated with 95% O_2 and 5% CO_2, pH 7.35, 295-305 mOsm). Coronal brain slices containing VTA were cut using a microslicer (Ted Pella) in cold sucrose-aCSF. Slices were kept for recovery in a holding chamber with aCSF for one hr at 37°C, and then at room temperature.

VTA slices were transferred into a recording chamber, in which a constant flow of aCSF (~2.5 ml/min) was maintained throughout the experiment at 34°C. Single-unit extracellular potentials were recorded by the use of glass microelectrodes filled with 2.0 M NaCl and monitored through high-input impedance amplifier (axoclamp 2B, Axon Instruments). VTA dopamine neurons in these experiments were identified by location and well-established electrophysiological criteria: regular spontaneous firing, action potential (AP) with triphasic waveforms (positive, negative, positive) (Krishnan et al., 2007; Ungless et al., 2004; Werkman et al., 2004), and AP width (from start to trough) >1.1 ms under the filter conditions of 300Hz-0.8 KHz. The waveform of AP for measuring AP width was obtained by averaging 30-40 AP episodes. The firing rate of dopamine neurons were recorded in the bridge mode of amplifier AxoClamp 2B, and data acquisition and on-line analysis of firing rate were realized with Digidata 1322A digitizer and pClamp 8.2 (Axon Instruments). Data acquisition and on-line analysis of firing rate were realized with Digidata 1322A digitizer and pClamp 8.2 (Axon Instruments). LY294002 (Sigma) was first dissolved in DMSO (Sigma) to prepare a stock solution. Final concentrations in bath solutions were DMSO: 0.2%, and LY294002: 100 μM (Sanna et al., 2002). For whole-cell voltage-clamp recording experiments, cells were visualized with an upright microscope using infrared differential interference contrast (IR-DIC) illumination, and recordings were made under continuous single-electrode voltage clamp mode. Recording electrodes (2-4MΩ) were filled with pipette solution containing 120mM CsCl, 10mM phosphocreatine-Na, 10mM HEPES, 10mM EGTA, 2mM ATP-Mg and 0.3mM GTP (pH 7.2-CsOH, 288mOsm). Putative dopamine neurons in the VTA were identified in whole-cell recordings by the presence of a large hyperpolarization-activated current (I_h) (Saal et al., 2003; Ungless et al., 2003), which was evoked by holding cells at −60 mV and stepping to −120 mV in 10 mV increments. 1mM kynurenic acid was added to the aCSF

to block NMDA and AMPA/kainate receptor-mediated excitatory postsynaptic responses. Spontaneous inhibitory postsynaptic currents (sIPSCs) were obtained at the holding potential -60mV, and confirmed to be GABA-mediated in each recording by adding the selective $GABA_A$ receptor antagonist SR95531 (2 μM) after testing the effects of 100 μM LY294002.

VTA slice cultures were prepared as described previously (Han et al., 2006). Acutely obtained VTA slices (250 μm) were maintained in an incubator for ~60 min, following which HSV vectors were pipetted onto the VTA area of the slice surface. Slices were maintained overnight at 34°C in culture medium [MEM (Gibco) containing 30 mM HEPES, 20 mM D-glucose, 5% B27, 5 mM L-glutamine, 25 unit/ml streptomycin/penicillin], and single-unit recordings were obtained the following day 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. Unless otherwise specified, data are presented as *mean + sem*, with significant post-hoc differences indicated with * (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

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