Antidepressant mechanisms of deep brain stimulation for treatment resistant depression

by

Yesul Kim

Academic Year 2012-2015

Supervisors
Dr. Linda Byrne
Dr. Susannah Tye

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<tr>
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<td></td>
</tr>
<tr>
<td>Postal address: 5/31 Mercer Road, Armadale</td>
<td></td>
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Dedications

For my parents – you taught me how to find happiness from within.

&

To those who suffer from despair beyond despair.
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Abstract

Over the past decade, deep brain stimulation (DBS) has emerged as an alternative, reversible, and non-ablative neuromodulatory method to help individuals with treatment resistant depression (TRD). TRD patients typically fail to attain adequate benefits from traditional treatments, including several courses of pharmacotherapy, psychotherapy and electroconvulsive therapy. A proportion of these patients (~60%), however, reported therapeutic improvements in small scale clinical DBS trials. Unfortunately, the critical gap in our knowledgebase on the mechanisms of DBS may be delaying effective implementation of this promising therapy in a systematic manner. At this juncture, it is imperative to take a step back and study the antidepressant properties of DBS preclinically, so that these translational findings can shed light on the factors mediating treatment responsivity.

Investigations into the underlying effects of clinically relevant DBS targets included the nucleus accumbens (NAc), lateral habenula (LHb) and infralimbic cortex (IL; homologous to the human subgenual cingulate target) in an animal model of antidepressant resistance. To achieve this, male Wistar or Sprague Dawley rats received chronic administrations of adrenocorticotropic hormone (ACTH) for the induction of tricyclic resistance. The antidepressant-like effects of DBS were characterized through well-validated behavioral assay – the forced swim test (FST). The neurobiological alterations were quantified using immunoblotting, gene expression and mitochondrial function techniques in regions implicated in the pathophysiology of depression. Additionally, stimulation-evoked transient dopamine neurotransmission were explored as a potential common mechanism of NAc, IL and LHb DBS, using fast scan cyclic voltammetry.

Results collectively demonstrated that DBS to the NAc, LHb and IL is efficacious in otherwise, imipramine resistant animals. In the NAc DBS experiment, a proportion of the ACTH-treated animals bilaterally implanted with electrodes (DBS & Sham) exhibited mania-like heightened locomotor activity in the open field test, as well as exaggerated escape behaviors in the FST. Analyses of mitochondrial function in the prefrontal cortical tissue indicated a functional deficit
In energy generation in animals pretreated with ACTH. Interestingly, an over-compensative mitochondrial efficiency was observed for those animals with a hyperactive phenotype, presumably in response to the fluctuating energy demands.

In Chapter 6, sensors of energy demand, cell division/growth, apoptosis, protein synthesis and glucose/glycogen regulation were significantly altered in IL of ACTH treated animals relative to naïve controls. A combination of endogenous stress (i.e. ACTH) with environmental stress (FST) led to altered gene and protein expression. Phosphorylation of key proteins involved in energy regulation were decreased in ACTH/Stress animals compared to the ACTH/naïve group, however this was not reversed with IL DBS. In contrast, mRNA level of genes that respond to oxidative stress, hypoxia, ER stress, pro-inflammatory cytokines, nutrient deprivation and DNA damage were increased in ACTH/Stress compared with ACTH/naïve, and were restored following DBS. Data indicate that DBS may be reversing the maladaptation of genes involved in cellular stress rather than those involved in energy/glucose regulation.

For LHb DBS, the phosphorylation status of LHb Ca²⁺/calmodulin-dependent protein kinase (CaMKIIα/β), glycogen synthase kinase 3 (GSK3α/β) were significantly correlated with DBS-induced antidepressant-like behaviors. Concurrent with this, phosphorylated AMP-activated protein kinase (AMPK) in the IL was negatively associated with DBS-induced behavioral effects. The inverse relationship of CaMKII, GSK3 and AMPK phosphorylation in the LHb and IL, respectively, suggest differential roles of these regions in antidepressant processes. The distinctive relationships of protein expression found in these targets suggest that LHb DBS might engage in structural circuit changes that downregulate metabolic demand in the IL.

Lastly, an attenuation of stimulation-evoked transient NAc dopamine efflux was observed following NAc and IL DBS, whereas LHb DBS uniquely potentiated NAc dopamine release. A further examination of the LHb region found that DBS recovered dysregulated dopamine efflux in ACTH-treated animals. These findings suggest that animals with ACTH administration are unable maintain the DBS-mediated synaptic plasticity and induction of long-term potentiation.
In conclusion, antidepressant responsive versus resistant animals demonstrate disparate responses to stress. DBS was able to reinstate treatment response by modulating cellular stress, impaired synaptic plasticity, dysregulated dopamine transmission and energy metabolism. Hence, deficits in synaptic plasticity may be involved in the neuroprogression of depression. NAc dopamine dysregulation surfaced as another critical modulator of antidepressant responsivity. The implications from these results underscore the need for using appropriate animal models to gain valuable insight on the neurobiological state of the organism in health and disease.
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Chapter 1. Introduction

Current hypotheses of mood disorders and antidepressant activity: identifying key signaling pathways and molecules mediating treatment responsivity

According to the American Psychiatric Association (2013), affect reflects a pattern of observable behavioral expressions that reflects subjective emotional experience, whereas mood is a sustained emotion that influences one’s perception of the environment. Mood disorders are differentiated into unipolar depression and bipolar disorders by the type of mood fluctuations, severity, and its pattern of progression (Bowden, 2005). Since the publication of DSM-III in 1980, bipolar and unipolar depressive disorders have been regarded as a separate phenomenon. The position for their true separation –as against simply the presence or absence of manic/hypomanic episodes is challenged by differential (i) causes, (ii) episode-related and course of illness characteristics, (iii) biological underpinnings (driven by peripheral biomarkers including neuroimaging predictors) and (iv) patient-specific responses antidepressants and/or mood stabilizing medications. Many argue that limited distinction in the strict sense is a reflection of methodological limitations in study selection and clinical assessment (Cuellar, Johnson, & Winters, 2005). With such controversy around the diagnostic criteria, it has been difficult to deliver targeted intervention and establish corresponding mechanisms responsible for treatment response into remission. For this reason, it seems necessary to discretely examine the neurobiology of depression and antidepressant activity, rather than conflating these two. The given approach may help us to understand different pathophysiology’s that may underscore subtypes of depression and consequently, develop tailored treatments to those needs. Currently, no biomarkers for predicting treatment response in depression have been systematically assessed nor rigorously validated in clinical practice (Fu, Steiner, & Costafreda, 2013). The present review will focus on the underlying mechanisms of treatment response, namely, the various signaling pathways and their interactions
to facilitate or hinder neuronal plasticity, cell survival and growth. Resistance to treatments may indicate dysregulation in one or more pathways and/or networks that are not effectively modulated by available therapeutic agents.

**Defining unipolar and bipolar depression**

A rather broad range of physiological, neurovegetative, emotional characteristics exist for depression, including a prolonged (lasting 2 weeks or longer) depressed mood with changes in sleep, appetite, interest or energy. Depressive disorder can present as mild, chronic (captured in the definition of dysthymia) or to a more severe state known as treatment resistant depression (TRD). While a consensus on the definition is yet to exist for TRD, the field of psychiatric research has moved away from simply using a failure to remit after an adequate course of treatment as an indication of pure resistance. A staging method proposed by Thase and Rush (1997) has been developed for the clarification on treatment resistance, yet several methodological issues exist (please see Fava (2003) for details). It is important to recognize that an individuals’ experience of depression can be very chronic and debilitating, even when only a few symptoms are present (Fried & Nesse, 2014; Gotlib, Lewinsohn, & Seeley, 1995; Solomon, Haaga, & Arnow, 2001). For example, anhedonia, a failure to feel pleasure and loss of interest has been described as one of the core symptoms of depression. Due to the nature of these symptoms, it has detrimental implications when day-to-day activities and quality of life are concerned. Most definitions of treatment resistance are symptom and syndrome based where functional outcome is rarely considered. Treatment is usually chosen on an empirical basis, informed by clinical characteristics including depression severity, subtype, previous treatment responsivity and existing comorbid disorders (Levinstein & Samuels, 2014).

Bipolar disorder (BPD) is characterized by recurrent episodes of disturbed affect including mania and depression as well as changes in neurovegetative function, cognitive performance and general health. There are several types of BPD based upon specific duration and pattern of manic
(hypomania and with or without depressive symptoms) and depressive episodes (American Psychiatric Association, 2013). For the purpose of this review, BPD will be discussed broadly, encompassing the population as a whole. Criteria used for TRD would apply for BPD, with the proviso of a failure to respond to mood stabilizers as well as antidepressants (Gitlin, 2006). Some authors (Ghaemi, Sachs, Chiou, Pandurangi, & Goodwin, 1999; Sachs, 1996) additionally argued for the absence of antidepressants in defining both acute mania and maintenance stage, due to antidepressants possibly exacerbating manic or cycling mood fluctuations. Treatment strategies for BPD can also differ between non-compliance precipitated by episodes of mania vs. true breakthrough episodes. For example, if a manic episode occurs in the context of non-compliance, it is imperative to establish whether the patient discontinued treatment and the episode followed or whether the patient became hypomanic, discontinued treatment at that point (of hyped impulsivity) and then became even more symptomatic.

Four relatively consistent, differential symptoms have been reported for the two clinical categories of depression (Cuellar et al., 2005). Bipolar depressed patients were more like to report anhedonia than those with unipolar depression, with decreased anxiety, activity and somatization. In a more recent report examining the most severe lifetime depressive episodes, bipolar patients were more likely than those with unipolar depression to have higher rates of psychomotor disturbance, impaired concentration, early morning wakening, diurnal variation, psychotic symptoms and mixed features (Mitchell et al., 2011). Parker et al. (2012) further noted that bipolar depression corresponded closely to unipolar melancholic depression, in terms of its clinical features, but not with regards to a number of socio-demographic, illness course and correlate variables. Atypical features were common across bipolar and unipolar disorders within the mood-affected population, but somewhat more prevalent in bipolar disorder. The heterogeneity of the depressive syndrome is called to attention by Østergaard, Jensen, and Bech (2011) where 227
different combinations with ≥ symptoms fulfilling the DSM-IV criteria. This problem may be reflected in outcomes of two significant studies conducted in recent years:

i) With more than 4000 patients in the STAR*D study, only 30% of the patients remitted on treatment with a current first-line antidepressant drug (Citalopram) and other 30% failed to remit following four consecutive treatment trials of antidepressants from different pharmacological classes, even with rigorous dosing method (Gaynes et al., 2009).

ii) The hypothesis of increased risk and complication for depression caused by gene x environment interactions (e.g. the serotonin selective promoter gene polymorphism and adverse life events such as childhood maltreatment and medical conditions) has been subject to debate over the last few years. A recent meta-analysis by Karg, Burmeister, Shedden, and Sen (2011) found gene x environment interaction to increase the risk of depression, although it took more than 40,000 participants to establish this relationship. Uher (2011) further noted in his review how this interaction complicated treatment outcomes, yet the strength and generalizability of such influences are not sufficient (with available evidence) to justify personalized prescribing.

Apart from the criticisms on clinical classifications, variables such as treatment intolerance and adherence unfortunately inflates the TRD population statistics, although subgroups are arguably distinct. Other factors include misdiagnosis, severity of illness, comorbid psychiatric conditions, underlying medical condition (e.g. diabetes, cancer etc.), substance abuse, major life stressor, ineffective treatment, inadequate treatment duration and dose (Nemeroff, 2007). In terms of comorbid psychiatric conditions, BPD mis/under diagnosis is common, with approximately 40% of patients with BPD being misdiagnosed with unipolar depression (Ghaemi, Boiman, & Goodwin, 2000; Singh & Rajput, 2006; Smith & Ghaemi, 2010). The misdiagnosis of bipolar as unipolar
depressive disorder is important because of its clinically relevant consequences, including suicide. Most suicides in BPD occur during depressive episodes or mixed states with prominent depression (Bowden, 2005). In addition, a less obvious consequence of misdiagnosis is that the use of antidepressants for BPD patients (not on mood stabilizers) may increase the risk of manic switch, a mixed state or accelerate cycling (Bowden, 2005; Ghaemi et al., 2000).

Why use animal models of mood disorders?

Considering the degree of heterogeneity among patients with their representations of mood disturbances, is it not a major surprise that researchers are struggling to tease out clinically relevant effects and treatment and significant interactions between genetic and environment risk factors. Due to some of the difficulties aforementioned, rodent models are advantageous to study non-responsiveness to an initial line of treatment because molecular parameters such as proteins and gene expression can be manipulated and directly compared across multiple brain regions between responders and non-responders (Levinstein & Samuels, 2014). One goal of developing rodent models of TRD, is to better understand the neurobiological mechanisms that mediate treatment response. Naturally, a second goal is to provide a framework where those findings can translate to clinical trials. In so far, animal models have utilized three approaches: 1) separation of rodents into bimodal subpopulations that respond to or are resistant to traditional antidepressant drugs. These include the chronic mild stress (CMS) model (Jayatissa, Bisgaard, Tingström, Papp, & Wiborg, 2006), chronic social defeat (Der-Avakian, Mazei-Robison, Kesby, Nestler, & Markou, 2014) and the adrenocorticotropic hormone (ACTH) model (Walker et al., 2015); 2) Treatments that render rodents resistant to antidepressants (e.g. ACTH model (Kitamura et al., 2008; Walker et al., 2013) or inflammation (Rizzo et al., 2012)); 3) genetic models that show resistance to available pharmacotherapies (e.g. use of genetically modified mice targeting dopamine beta hydroxylase (Cryan & Mombereau, 2004), brain derived neurotrophic factor heterozygous null mice (Ibarguen-Vargas et al., 2009; Saarelainen et al., 2003)). Amongst many preclinical models of behavioral
depression, congenital learned helplessness (cLH) is a well-validated model of depression, originally developed by Overmier and Seligman (1967). The helplessness animals share symptoms of depression as seen in the clinical population including anhedonia, associative-cognitive deficits, weight loss, sleep disturbances, libido reduction and hypothalamic pituitary axis (HPA) axis disturbances (Enkel, Spanagel, Vollmayr, & Schneider, 2010). Furthermore, they display changes in monoamine receptors and respond to pharmacological interventions such as tricyclic antidepressants (Henn, Edwards, Anderson, & Vollmayr, 2002; Malberg & Duman, 2003; Sartorius et al., 2003). For greater detail on different animal models of depression (including a summary of validated antidepressant resistance), please refer to (Caldarone, Zachariou, & King, 2015).

Animal models using environmental and social context also exist for depression. The development of social defeat is accomplished by forcing a mouse to intrude into the space territorialized by a larger mouse of a more aggressive genetic strain, leading to an agonistic encounter that results in intruder subordination. Inbred population of susceptible mice to social defeat show increased social avoidance, anxiety-like behaviors and social hypothermia. These behavioral outcomes were accompanied by attenuated a.m. serum of corticosterone (CORT), upregulated brain-derived neurotropic factor (BDNF) levels in the nucleus accumbens (NAc) which subsequently led to the activation of downstream signaling molecules including glycogen synthase kinase 3 (GSK3) beta and mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK) 1 and 2 (Krishnan et al., 2007). Specific manipulations of early social environment in rats has been shown to foster chronic developmental changes and associated abnormalities in depression. Three months of social isolation did not affect animals’ locomotor activity but increased depression-like behaviors indicated by the forced swim test (FST). In comparison, environmental enrichment showed an antidepressant and anxiolytic-like effect, a potential “protective” role during the critical early life (Brenes, Rodriguez, & Fornaguera, 2008). Sáenz, Villagra, and Trías (2006) also reported three months of social isolation increased passive coping in rats where this was associated
with decrease norepinephrine in the ventral striatum. Conversely, increased serotonin (5-HT) and norepinephrine in prefrontal cortex (PFC) and ventral striatum correlated with more time using active coping strategies.

As mentioned in point 3 above, one approach to studying TRD is to use genetic models to achieve non-responsiveness to traditional antidepressants. Although these models show promise, the contribution of environmental factors to antidepressant resistance, such as stress, are not accounted for. Ongoing research of rat and mouse model of TRD exists, which uses the chronic administration of ACTH to induce pharmacological resistance to first line antidepressant treatment (Caldarone & Brunner, 2009; Kitamura, Araki, & Gomita, 2002; Walker et al., 2013; Walker et al., 2015). Interestingly, this model reflects and is consistent with human TRD population in terms of treatment efficacy. In ACTH pre-treated rats, augmentation of imipramine with carbamazepine and electroconvulsive therapy showed efficacy as well as bupropion and rapid acting agent ketamine (Kitamura et al., 2008; Li et al., 2006; Walker et al., 2015). The ACTH model is particularly invested in the concept that chronic stress conditions dysregulate the HPA axis leading to maladaptive responses associated with mood disturbances. Other models for TRD may arise that incorporates different physiological, cognitive and/or affective characteristics of depression where risk and comorbidity factors can be better studied. At this stage, however, this ACTH model offers a unique platform in which underlying biological factors mediating treatment response can be examined on.

In the upcoming sections, much of what is established on the mechanisms of antidepressant action has come from a combination of preclinical experimental designs as well as clinical observations and trials using different antidepressant drugs. According to many researchers, the lack of efficacy demonstrated by first and second generation antidepressants is partly due to the fact that the treatment for depression (especially for TRD) still has not progressed beyond the trial and error approach and that the neurobiology underlying how one arrives at remission remain largely unknown.
Hypotheses of mood regulation

While dysfunction within the monoaminergic neurotransmitter systems is likely to play an important role in the altered mood states, changes in monoamine levels probably represent secondary effects to a more primary abnormalities in signal transduction. For example, selective serotonin reuptake inhibitors' (SSRIs) failure to maintain a therapeutic response is evidenced by high rates of relapse if those drugs are too rapidly withdrawn following reduction of symptoms (Willner, Scheel-Krüger, & Belzung, 2013). Acute accumulation of monoamines following chronic antidepressant treatment may necessitate structural and functional changes by modulating downstream neurotrophic and neuroplasticity signaling cascades.

New theories on the pathophysiology of depression and antidepressant action proposes that mood disorders are caused by structural and/or functional changes in specific molecules and signaling pathways, and that therapeutic measures work by counteracting/compensating one or more of these maladaptation. Regional hyper/hypo-activity at different nodes within the network have been documented where metabolic disturbances are corrected with effective neuromodulation (Giacobbe, Mayberg, & Lozano, 2009). Structural and functional abnormalities in patients with mood disorders are associated (not consistently, but frequently) with low levels of BDNF, disturbances in HPA axis, activation of inflammatory and cell-mediated immune responses, increased oxidative and nitrosative stress, neurodegeneration and altered energy metabolism (Fišar & Hroudová, 2010; Gardner & Boles, 2011; Karege et al., 2005; Moylan et al., 2014; Stetler & Miller, 2011). Underlying mechanisms of pharmacotherapies and neuromodulatory methods may be targeting intermediary proteins or key molecules involved in one or more of the abovementioned signaling cascades, all working to facilitate neurogenesis, neuronal remodeling and rehabilitation.
**Monoamines hypothesis**

The classic monoamine hypothesis is an early milestone in the field of depression. It argues that depressive symptoms are manifested by 5-HT or noradrenaline (NE) deficiency at functionally important receptor sites in the brain, i.e. the brain monoamine systems have a primary role in the manifestation of mood disturbances. Discovery of the first effective antidepressants – monoamine oxidase inhibitors (MAOIs) and tricyclics, uncovered a significant role for 5-HT and NE in the etiopathogenesis of depression symptomatology. Today’s antidepressant agents offer a better therapeutic index with lower rates of side effects for most patients, but they are still designed to increase monoamine transmission acutely; either by inhibiting neuronal reuptake (e.g. SSRIs) or by inhibiting degradation by blocking mitochondrial enzymes (Milano et al., 2004). In one of the largest clinical trials, the STAR*D, only about one fourth of the patients achieved remission during the first treatment stage (Fried & Nesse, 2014). The ineffectiveness of first line treatments has been well-documented over the years, reflecting our limited understanding of the disease and lack of progress in novel drug discovery.

A series of human studies were also conducted to evaluate the effects of monoamine depletion on depressive symptoms in patients vs. healthy controls. Relapse following 5-HT depletion and catecholamine depletion was found to be specific to the type of antidepressant treatment and depletion target (Heninger, Delgado, & Charney, 1996). Interestingly, 5-HT or NE depletion did not decrease mood in healthy controls but slightly lowered mood in those with a family history of major depressive disorder (MDD) and a moderate mood decrease was found for acute tryptophan depletion only (Ruhé, Mason, & Schene, 2007). Acute tryptophan depletion, however, induced relapse in remitted patients on serotonergic antidepressants. Overall, depletion studies failed to demonstrate a causal relationship between 5-HT and NE with the manifestation as well as maintenance of depressive symptoms. Additionally, simultaneous disruption of indoleamine and catecholamine systems did not significantly alter mood in non-medicated depressed subjects.
(Berman et al., 2002). These outcomes may be explained from a sampling perspective where the diseased brain is unlike those healthy controls and there are distinctive variability’s in brain function and basal brain activity even within the depressed population. An antidepressant treated brain has been found to be in a different state from the non-depressed, post-mortem brain in terms of receptor densities of the monoamines system in the PFC and reduced grey-matter volume and glial density (Krishnan & Nestler, 2008; Muguruza et al., 2014; Rivero et al., 2014). It is important to remember that whilst studying mood alterations in healthy controls may be of value, the TRD population, due to its severe neuroprogressive state, represents a distinct biological entity. Altogether, conflicting preclinical and clinical outcomes led to major revisions on the classic monoamine hypothesis of depression.

Researchers began to investigate dopamine (DA) following the disappointing outcomes (or there lack of) of traditional antidepressants and serendipitous discoveries of its prominent role in mood in patients suffering from addiction and movement disorders. Supporting reports unveiled DA to critically dictate stress responses and core symptoms (e.g. hedonic activity, motivation, anticipation, mania) of mood disturbances. The existing body of knowledge is comprised of lesion studies (Katarzyna et al., 2011; Winter et al., 2007), delivery of DA agonists and antagonists into DA-rich regions of the forebrain, particularly in the striatum and the nucleus accumbens (Cipriani et al., 2009; Tohen et al., 2003; Vieta et al., 2005), effects of mood stabilizers (Tohen et al., 2003) and antipsychotics administration (Abler, Erk, & Walter, 2007), or measurement of DA metabolism in key brain regions (Conway et al., 2014; Martinot et al., 2001). This body of work has consistently showed that alterations to DA levels and activation led to reductions in depressive as well as manic symptom profiles. The DA system has been identified as a regulator of effort related processes (Phillips, Walton, & Jhou, 2007; Salamone, Correa, Mingote, & Weber, 2003). The effort related DA function of NAc and its associative forebrain regions are not only important for understanding the activational part of motivation, but also for its clinical implications related to natural motivation,
addictive behaviors as well as energy-related syndromes including psychomotor slowing, fatigue, or anergia in depression, hyperactivity and restlessness in mania (Salamone, Correa, Farrar, & Mingote, 2007).

In recent years, the modulation of ventral tegmental area (VTA)-NAc mesoaccumbens pathway has received much attention although the field has often used simplistic localization of function approach to examine limbic substrates (e.g. amygdala for fear, NAc for reward). West and Weiss (2011) found that chronic administration of antidepressant drugs, except for MAOIs, increased the spontaneous firing rate of VTA DA neurons. Moreover, animals receiving five electroconvulsive shocks increased both spontaneous firing rates and burst firing of VTA-DA neurons. Using chronic social defeat stress model in mice, in vivo firing rates and bursting properties of VTA DA neurons are dramatically increased in susceptible mice, but not in resilient mice and increased hyperpolarization-activated cation current in VTA DA neurons was normalized by chronic treatment with fluoxetine (Cao et al., 2010). It is suggested that these effects are consistent with accumulating reports of increased DA release in regions to which VTA neurons project after effective antidepressant intervention. One of the possible mechanisms by which the mesolimbic DA neurons bring about a positive effect is that they are largely responsible for determining adaptive vs. maladaptive responses to chronic stress (Cao et al., 2010). This further sensitizes or desensitizes the stress system to future insults on the organism.

Substantial evidence also indicates that risk for BPD is characterized by elevated activation in the fronto-striatal reward neural circuit involving those DA enriched ventral striatum and orbitofrontal cortex areas (Strakowski, Delbello, & Adler, 2005). It is proposed that individuals with abnormally elevated reward-related neural activation are at risk for experiencing excessive increase in approach-related motivation during life events involving rewards or goals attainment (Johnson, Ruggero, & Carver, 2005). In the extreme, this increase in motivation is reflected as hypomanic/manic behaviors. By contrast, MDD is characterized by decreased reward responsivity,
sensitive stress appraisal and decreased reward-related neural activation. Collectively, this suggests that risk for BPD and MDD is characterized by distinct and opposite profiles of reward processing and reward-related neural activation (Nusslock, Young, & Damme, 2014). The central role of DA in both disorders suggest that DA transmission could be a crucial modulator and possible pharmacological target for managing both manic and depressive symptoms.

Despite the progress and advancements made with optogenetics (Lammel, Tye, & Warden, 2014), the neurochemistry of DA is incompletely understood. The dopaminergic system is complex and its differential functioning depends on the neural population, brain region and the expressed receptor. Notably, recent evidence suggest that there are also differences in transcriptional neuroanatomy across different groups of DA neurons (Berk et al., 2007). Examining these differences in DA transcriptional neuroanatomy may be of use for developing a more selective and effective therapeutic agents (Nusslock, Young & Damme, 2014). A complex interaction also exists for the 5-HT, NE and DA systems where most of the activities exerted on 5-HT and NE affect DA release. For example, classic antidepressants have been found to result in phasic activation of DA (Friedman, 2007). Acute stress increases the amount of synaptic monoamines induced by antidepressants which in return produces secondary and perhaps long lasting neuroplastic changes on a transcriptional level affecting molecular and cellular plasticity. Changes that are reflective of stress, monoamines and plasticity are believe to be crucial in determining one’s treatment response.

**The stress system**

There is a crucial distinction between exposure to stress and the experience of stress. An intense stressor may cause little distress if coping mechanisms are adequate, while conversely, if coping mechanisms fail (or is maladaptive), a mild stressor may be perceived as highly stressful. The proposed neurobiology of stress and mood disorders must recognize that a failure to cope with
stress, rather than exposure to stress per se is a critical juncture during the prodromal stage (Walker et al., 2014; Willner et al., 2013). Depression diathesis include: early life experiences (Walker et al., 2014), genetic factors (Bosch, Seifritz, & Wetter, 2012) as well as personality factors (Rosenström et al. (2014), which may reflect early life experiences as well as heritable characteristics). The first episode of depression manifests against the level of vulnerability that is determined by genetic and experiential factors. It rises more commonly from a major adverse life event or from an accumulation of chronic minor stressors. These two types of precipitating events respectively form the basis of two of the major animal models of depression (learned helplessness and chronic mild stress). A person with a strong depressive diathesis may succumb to minor or trivial stressors. Over the course of persistent depressive episodes, the onset of clinically diagnosable depression becomes increasingly autonomous. This effect can be described both neurobiologically (an episode of depression sensitizes or ‘kindles’ the brain to respond to weaker and weaker precipitants); and physiologically (the depressed person relies increasingly on negative modes of information processing that come to be activated by increasingly minimal cues).

While recognizing that stress is a pathogenic factor in mood disorder etiology, it is important to remain mindful that the stress response itself is inherently adaptive and evolutionarily conserved to promote survival (Karatsoreos & McEwen, 2013). Appropriate coping mechanisms adopted by the individual in response to a stressor functions to coordinate system-wide reactions to perceived future threats (Cabib & Puglisi-Allegra, 2012). This process is initially beneficial, but can become dangerously pathogenic if followed by prolonged stress system activation and maladaptation (Cabib & Puglisi-Allegra, 2012; Krishnan et al., 2007; McEwen, 2007). The resistance phase has been suggested to be mediated by the HPA axis and coordinates adaptive stress responses over an extended period of time. As the individual’s resources become depleted, they enter into a third “exhaustion” stage wherein the physiologic systems begin to deteriorate, and
different stress-associated illnesses can emerge when defense or coping strategies are inappropriate (Selye, 1976).

Major physiological responses to stress occur through the activation of neuroendocrine systems – most notably the HPA axis. Hyperactivity of the HPA axis during depression is one of the most reliable findings in biological psychiatry (Pariante & Lightman, 2008). Furthermore, a high level of activity in the HPA axis has been able to predict treatment resistance (Willner et al., 2013). Antidepressants normalize HPA function and part of this effect involves a restoration of glucocorticoid receptor (GR) responsiveness. Conversely, chronic stress and long-term glucocorticoid treatment lead to the loss of dendritic spines and synaptic contacts in the hippocampus and the prefrontal cortex (Cerqueira, Mailliet, Almeida, Jay, & Sousa, 2007; Hajszan, MacLusky, & Leranth, 2005; McEwen, 1999).

In the neuroendocrine system, corticotrophin releasing factor (or hormone: CRF/CRH) is released from the paraventricular nucleus of the hypothalamus (PVN) to stimulate the anterior pituitary gland to produce ACTH. This in turn stimulates the release of glucocorticoids from the adrenal cortex into the blood circulation, which inter alia, exert negative feedback effects on the pituitary and hypothalamus that limit the degree of activation of the HPA axis (Willner et al., 2013). Signals of homeostatic imbalance in the brainstem leads to the activation of this axis, where ascending brain stem pathways project heavily to the parvocellular divisions of the PVN.

Importantly, there seems to be a reciprocal relationship between reward and stress appraisal in the brain. Exposure to natural rewards buffers the effect of stressor on HPA activity (Ulrich-Lai et al., 2010), and stress increases reward-seeking behaviors (Weiss, 2005). Experiences with rewarding stimuli generally evoke stress-like HPA responses, however the effects may be contingent on the type of rewards (Ulrich-Lai & Herman, 2009). The DA reward system demonstrates a range of complex responses to stressors, shown to critically mediate one's adaptive
and maladaptive states. A recent study indicates a GR-dependent neuronal dichotomy for the regulation of emotional and social behaviors in response to social stress, and clearly implicates glucocorticoids as a link between stress resiliency and dopaminergic tone (Barik et al., 2013). In turn, the dopamine system affects future appraisals and responses to stress. In support of this view, there is evidence that low levels of stress, or controllable stressors have been reported to immunize the system to subsequent stressors (Belda, Fuentes, Nadal, & Armario, 2008; Ma & Morilak, 2005).

All in all, it is well documented that stress interferes with processes responsible for bringing about antidepressant effects, namely neurogenesis (cell proliferation), neuronal survival and functionality. The cellular effects of stress lead to visible morphological changes (e.g. decrease in hippocampal volume –loss of dendrites) as well as functional consequences. The most likely mechanism by which stress suppresses adult neurogenesis in the hippocampus is via the activation of HPA axis and subsequent elevation of cortisol (glucocorticoids) levels. The hippocampus, in turn, provides negative feedback to the HPA axis. Stress also affects PFC functioning, where neurodegenerative changes include microglial activation, atrophy of pyramidal neurons, dendritic retraction and reduction of synaptic proteins. Close the PFC, the most stress-sensitive region is anterior cingulate cortex (Willner et al., 2013), whose functions are associated with changes in cognitive (dorsal) and emotion (ventral) characteristics of depression. While synaptic and morphological plasticity have been less extensively examined in the PFC compared to the hippocampus, it is clear that stress has similar deleterious effects on the mechanisms of neuroplasticity here (Pittenger & Duman, 2008).

Neuroplasticity

Stress can have a lasting impact on the structure and function of brain circuitry that translates into long lasting behavioral changes. Emerging data suggests that stress not only brings about neurochemical alterations, but also impairments of cellular plasticity and resilience (Carlson,
Singh, Zarate, Drevets, & Manji, 2006; Manji & Duman, 2000). Plasticity, the ability to undergo and sustain change, is essential for adaptive functioning of our nervous system. This capacity for change allows organisms to adapt to complex change they face in both their internal and external environments. Adaptive mechanisms in learning and memory as well as physiological homeostasis – all are dynamic processes that rely on plastic neural circuitry for appropriate regulation of mood and affect (Schloesser, Huang, Klein, & Manji, 2008). In recent years, mood disorders have been well documented with structural and functional impairments related to neuroplasticity in various regions of the CNS (Carlson et al., 2006; Manji & Duman, 2000). Furthermore, commonly prescribed psychotropic drugs target molecules and signaling cascades implicated in the regulation of neuroplasticity (Carlson et al., 2006).

Neuroplasticity is a broad term that encapsulates dynamic changes in intracellular signaling cascades and gene regulation whereas synaptic plasticity results in long lasting changes in the strength and efficacy of neurotransmission. Specifically, synaptic plasticity is the mechanism through which information is stored and consolidated within individual synapses, neurons and neuronal circuits to guide the behavior of an organism. Long term potentiation (LTP) refers to a lasting (hours to days) increase in synaptic strength induced by brief high frequency electrical stimulation of afferent fibers or by coincident activation of pre and postsynaptic neurons. Long term depression (LTD), on the other hand, is involved in activity-dependent reduction in synaptic plasticity at both excitatory and inhibitory synapses (Marsden, 2013). Considering their role in learning and memory (e.g. emotional events, aversive stimuli etc.), several independent studies show modifications to LTP and LTD in the hippocampus from stressful experiences (Artola et al., 2006; Diamond, Park, Campbell, & Woodson, 2005; Kim, Foy, & Thompson, 1996). Further, restoring the balance of LTP/LTD contribute to maintaining synaptic efficacy that may otherwise represent as a factor of vulnerability to the consequences of stress (Popoli, Gennarelli, & Racagni, 2002).
LTP and LTD has been used to study the mechanisms of synaptogenesis where increased neuronal activity leads to insertion of glutamate receptors and maturation of spine synapses (Kessels & Malinow, 2009). The glutamate hypothesis of depression has recently evolved into a larger neuroplasticity hypothesis which proposes that chronic stress disrupts both structural (e.g. dendritic spines) and functional glutamatergic synaptic plasticity and that antidepressants at least work in part by reversing these disruptions. The formation of spine synapses or synaptogenesis is a key form of neuroplasticity, and represents a fundamental characteristic of neurons. Synaptogenesis is a structural change at a subcellular level that takes place in response to synaptic activity and provides a mechanism for processing and incorporating new information that can be used to make appropriate and adaptive responses (Duman & Li, 2012).

Evidence from animal models of anxiety and depression indicate that neurogenesis is necessary but does not occur for all antidepressants (David et al., 2009; Karpova et al., 2011; Santarelli et al., 2003). Scopolamine increases synaptic connections and functions in the PFC and also increase levels of extracellular glutamate in the PFC (Voleti et al., 2013). Furthermore, a single dose of scopolamine stimulates mammalian target of rapamycin complex 1 (mTORC1) signaling similar to the rapid antidepressant mechanisms of ketamine. Ketamine alone rapidly increases synaptic plasticity, with a primary role of glutamate and mTORC1 modulation synaptic remodeling, plasticity and synchronization (Duman, 2014). Lithium has been found to enhance this synaptogenic and anti-depressive behavioral effects when it is concomitantly administered with ketamine (Liu et al., 2013). Tianeptine prevents or reverses stress-associated structural and cellular changes and normalizes disrupted glutamatergic transmission in the hippocampus, the amygdala and the cortex in animal models of depression (Kasper & McEwen, 2008; McEwen et al., 2010). An inhibition of an excessive release of glutamate appears to be important to lamotrigine and riluzole mechanisms of action (Zarate et al., 2006). Common to those above mentioned antidepressants seem to be the actions of glutamate and an intimate relationship exists between regulation of
monoaminergic, glutamatergic systems and treatment response (Krishnan & Nestler, 2008; Paul & Skolnick, 2003). Further, these processes have been observed to take place in brain regions heavily implicated in antidepressant actions, namely the PFC and the hippocampus.

The down-regulation of neuroplasticity via neurotrophins pathways account for reduced cognitive as well as anxiety strengthening symptomatology of depressive disorders. Under-expressed neurotrophic factors have been associated with diminished volume of specific brain regions in MDD patients and antidepressants work therapeutically by reinstating such imbalance of growth factors. Nevertheless, many researchers now argue that this as an oversimplified representation on the topic of neurotrophins and their role in antidepressant action (Porcelli, Drago, Fabbri, & Serretti, 2011). As an example, the increased function of noradrenergic or serotonergic system, activates transcription factor CREB, BDNF and its receptor tropomyosin receptor kinase B (TrkB). Consequently, neuronal plasticity is enhanced and leads to a resumption of cellular functions. Not only neurotrophic factors facilitate molecular signaling for neuronal development earlier in life, they continue to be an essential driver of neuroplasticity in the adult brain (Duric & Duman, 2013; Greenberg, Xu, Lu, & Hempstead, 2009; Thoenen, 1995). According to the neurotrophic hypothesis, mood abnormalities arise from decreased neurotrophic support, leading to neuronal atrophy and decreased neurogenesis (Duman & Li, 2012). Vulnerability to depression can escalate as a result of neuronal damage, e.g. after chronic stress, long-term increased levels of glucocorticoids, hypoglycemia, ischemia, effects of neurotoxins or certain viral infections. This hypothesis links changes in stress-induced vulnerability and the therapeutic actions owing to the modification of intracellular mechanisms that modulate neurotropic factors necessary for neuronal survival and functioning. Heightened HPA activity and excessive glucocorticoids have been found to interfere with neurotrophin signaling. Conversely, neurotrophic factors can stimulate the HPA axis (Givalois et al., 2004; Naert, Ixart, Tapia-Arancibia, & Givalois, 2006). These observations identify a lack of sufficient neurotrophic support as a key contributor to the
neuroprogressive nature of depressive disorders as well as plasticity-induced antidepressant effects (Berk et al., 2011; Duman, 2004).

**Energy regulation**

As discussed earlier, chronic stress affects the progression of MDD by reducing synaptic plasticity, inducing structural changes in dendrites, activating inflammatory pathways, interfering with monoamine transmission and impairing neurogenesis (Pittenger & Duman, 2008). Emerging proteomics studies found stress and pharmacotherapies to modulate energy metabolism as well as cellular remodeling pathways in animal models of depression (Mallei et al., 2011; Piubelli, Carboni, Becchi, Mathe, & Domenici, 2011). Specifically, in a rat gene x environment interaction stress model, a number of mitochondrial proteins involved in energy production as well as associative metabolic enzymes were dysregulated. In organs demanding most energy (brain, liver and muscles), the highest number of mitochondria resides. It is well known that mitochondria strongly affect many intracellular processes coupled to signal transduction, neuron survival and plasticity (Hroudová, Fišar, & Raboch, 2013). To reiterate, mitochondria may be primary regulators of not only neuronal survival and death, but also plasticity (Fišar & Hroudová, 2010). Mitochondrial hypothesis directly corresponds to the above mentioned, neurotropic hypothesis because of an important role of calcium signaling pathway in both synaptic plasticity regulation. Mitochondrial dysfunctions (leading to decreased adenosine triphosphate (ATP) production, oxidative stress and induction of apoptosis) occur in the early stages of different neurodegenerative diseases, often being associated with mood disorders and antidepressant activity (Carboni et al., 2006; Gardner et al., 2003; Madrigal et al., 2001; Quiroz, Gray, Kato, & Manji, 2008; Rezin et al., 2008).

The role of mitochondria in BPD is supported both by observation in altered human brain metabolism in disease state and by the effects of mood stabilizers (lithium and valproate) on mitochondrial functions. Magnetic resonance studies in bipolar patients suggest mitochondrial
dysfunction is indicated by impaired oxidative phosphorylation, a resultant shift toward glycolytic energy production, attenuated total energy production and/or substrate availability and altered phospholipid metabolism (Stork & Renshaw, 2005). These conclusions are drawn from abnormal brain metabolism measured by $^{31}$P-magnetic resonance spectroscopy ($^{31}$P MRS), in other words decreased intracellular pH, decreased phosphocreatine and enhanced response of phosphocreatine (PCr) to photic stimulation. Also other authors (Kato & Kato, 2000) have studied brain phosphorus metabolism in autopsied brains in patients with BPD looking at mitochondrial DNA (mtDNA) using such a technique. According to bioenergetics hypothesis, mtDNA polymorphisms/mutations or mtRNA deletions caused by nuclear gene mutations can cause mitochondrial dysregulation of calcium leading to symptoms of cycling mood states that defines BPD. Recently, (Yuksel et al., 2015) reported $^{31}$P MRS pattern indicative of a disease related failure to replenish ATP from Pcr through creatine kinase enzyme catalysis during tissue activation. PCr/ATP ratio is a measure of the energetic reserve status of tissues with high energy utilization (including neurons) and lower ratio represents diminished energetic reserves at rest. A compensated bioenergetics were observed for BPD patients at resting state, possibly due to high-energy requiring processes implicated in BPD, such as neuroplasticity and glutamate turnover.

Effect of mood stabilizers on mitochondria has mostly been conducted in isolated brain regions of rodents and larger mammals in vitro. Lithium, the most commonly prescribed drug to BPD patients, caused desensitization to calcium, antagonized permeability transition and diminished cytochrome c release in isolated brain mitochondria. In isolated rat liver mitochondria, valproate inhibited oxidative phosphorylation (OXPHOS). Both lithium and valproate inhibited respiratory chain complexes I and IV in larger mammals (Brand & Nicholls, 2011). According to a study performed in rats, valproate reversed the inhibited activity of citrate synthase following amphetamine administration, whereas lithium prevented the amphetamine induced enzyme prevention (Corrêa et al., 2007). Chronic treatment with lithium, valproate and carbamazepine
prevented NMDA mediated toxicity. In one recent study performed with children suffering from epilepsy, carbamazepine and lamotrigine both influenced respiratory chain complexes and significantly affected ATP production (Berger, Segal, Shmueli, & Saada, 2010). These results (alongside other investigation in human blastoma and glioma cells, rat brain mitochondria and human white blood cells, please refer to Brand and Nicholls (2011) for detail) suggest that mood stabilizers exert neuroprotective effects to lessen the existing vulnerability of mitochondria from oxidative stress.

In contrast to substantial evidence on BPD and mood stabilizers, a small but growing body of research from animal studies, muscle biopsy and imaging data supports the role of mitochondria in MDD (Chang, Jou, Lin, Lai, & Liu, 2015). In rats, mitochondrial respiratory chain was inhibited following 21 days of chronic, repeated stress (Madrigal et al., 2001). Spatial distribution of mitochondria is abnormal in depressed patients. This facilitates the neuroprogressive nature of MDD by inefficient recruitment and trafficking of mitochondria to meet metabolic requirements under certain stress conditions or when their integrity is impaired. Additionally, aged and damaged mitochondria is not readily removed, and are thus unable to replenish healthy ones at distal terminals (Sheng, 2014). A significant decrease of mitochondrial ATP production rates and mitochondrial enzyme ratios in muscle were found in MDD patients compared to healthy controls (Gardner et al., 2003). Further, PET studies of cerebral blood flow or glucose metabolism indicates reduced blood flow and metabolic rate in PFC, anterior cingulate gyrus and basal ganglia (Videbech, 2000). Nevertheless, there is no conclusive in vivo outcome on the relationship between mtDNA variations and oxidative damage for MDD population.

There is relatively little data about the effect of antidepressants on mitochondrial functions. To date, most examine the influence of antidepressants (imipramine, desimipramine, amitriptyline, citalopram and mirtazapine) on activity of both mitochondrial monoamine oxidase and respiratory chain complexes in animals. Compared to classic uncouplers, imipramine and clomipramine
enhanced ATP synthase activity, hindered ATP synthesis and released respiratory control (Weinbach, Costa, & Wieder, 1985). Fluoxetine inhibited oxidative phosphorylation and decreased the activity of ATP synthase in the rat brain mitochondria (Curti et al., 1999) whereas desipramine induced apoptosis through the activation of caspases without any changes of mitochondrial action potential (Ma et al., 2011). Lastly, nortriptyline was identified as a protector of isolated mitochondria against programmed cell death, inhibited release of apoptotic mitochondrial factors and caspases, increased Ca\(^{2+}\) retention in mitochondria and delayed the Ca\(^{2+}\) induced loss of mitochondrial action potential, ultimately leading to further neuronal cell death (Wang et al., 2007; Zhang et al., 2008). With regard to the number and volume of mitochondria in the hippocampus, rats genetically susceptible to depressive behavior showed a significant increase in the number of mitochondria compared to control group (Chen, Wegener, Madsen, & Nyengaard, 2013).

Antidepressants may counteract with the structural impairments following changes to mitochondrial morphology and number that are a consistent feature of neuroplasticity.

Both direct and indirect effects of pharmacotherapies on mitochondrial function have been studied. Tricyclic antidepressants have anti-inflammatory and neuroprotective effects by modulating glial activation due to decreased production of nitric oxide and proinflammatory cytokines (Hwang et al., 2008) and mitochondria are well-known targets of nitric oxide. Recently, very different effects of three antidepressants, nefazodone, trazodone, and buspirone, on the induction of mitochondrial dysfunction and cytotoxicity were described (Dykens et al., 2008). Mitochondrial complex I, and to a lesser amount complex IV, were identified as the targets of nefazodone toxicity (a drug that was discontinued for hepatotoxicity). No inhibition to mitochondrial respiration was found for trazodone, and buspirone showed much lesser inhibition than nefazodone (Fišar & Hroudová, 2010).

Lastly, recent findings also suggest roles for mitochondria as mediators of at least some effects of glutamate and BDNF on synaptic plasticity. BDNF promotes synaptic plasticity, in part, by
enhancing mitochondrial energy production. Specifically, it increases glucose utilization and increases mitochondrial respiratory coupling at complex (Hroudová et al., 2013). The findings on mitochondrial changes to date are mixed and may not be a primary contributor, however is a secondary factor that facilitates the neuroprogression of the disease.

**Key molecules underlying treatment response**

The chance of finding sensitive and specific biomarkers for treatment response has increased, due to the introduction of new methods of investigating cellular and molecular biology. These methods have enabled us to better study the underlying antidepressant activities in different brain regions and/or networks. The choice of parameters have been approached from the perspective of identifying biological markers of treatment responsivity, derived first of all from various signaling pathways involved in the aforementioned hypotheses of mood disorders. In consideration of these parameters, activities of key molecules such as BDNF, glycogen synthase kinase 3 (GSK3), mammalian target of rapamycin (mTOR), Ca\(^{2+}\)/ calmodulin-dependent kinase II (CaMKII) and amp-activated protein kinase (AMPK) can be put forward as principle players in mediating responsiveness or resistance to certain pharmacological interventions. Given the complexity and intimate connectivity of signaling pathways involved in treatment responsivity, the number of chosen molecules discussed herein are not final.

**BDNF**

Among different neurotrophic factors, BDNF is the most widely distributed neurotrophic factor in the brain. BDNF is a critical mediator of activity-dependent plasticity in the developing and adult CNS (Thoenen, 1995). Recent evidence suggests a complex scenario for BDNF driven neuronal plasticity where two active forms of BDNF have been described. Signaling of mature-BDNF through TrkB receptor and pro-BDNF through the low affinity p75 neurotrophin receptor (p75NTR), which can exert opposing actions on the expression of these factors in limbic regions involved in the regulation of mood and cognition (Duman & Monteggia, 2006).
Basic research shows that stress decreases the expression of BDNF and causes alterations of hippocampal structure and function (Coe et al., 2003; Czéh et al., 2001). Given the functions of hippocampal circuitry and its connections to regions that are more directly involved in emotion and cognition (amygdala & prefrontal cortex), investigations of BDNF expressions in the hippocampus have attracted much attention. Antidepressant treatments increase the activated forms of upstream activators (TrkB and CREB) of BDNF in cortical and limbic areas (Saarelainen et al., 2003). Alterations of BDNF as well as upstream targets, i.e. TrkB, indicate that stress and appropriate treatment results in cellular changes, notably regulation of neurogenesis and synaptogenic responses. The formation of spine synapses or synaptogenesis is a key form of neuroplasticity and represents structural changes at a subcellular level that enables incorporation of new information to be used to make adaptive future responses to stress (Duman & Li, 2012).

The upregulation of BDNF is observed with different classes of antidepressants, including selective SSRI and norepinephrine selective reuptake inhibitors (NESRI), MAOIs, atypical antidepressants, and electroconvulsive therapy (Duman & Monteggia, 2006). The induction of BDNF is also dependent on chronic antidepressant treatment, consistent with the time course for therapeutic action of antidepressants to take place. It is worth noting that other treatments known to have antidepressant efficacy also increase the expression of BDNF in the hippocampus. These include the administration of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)kines, NMDA receptor antagonists, transcranial magnetic stimulation and exercise (Duman & Monteggia, 2006). In humans, LHb DBS has been related to increased peripheral BDNF (Hoyer et al., 2013) whereas in rats, with mPFC DBS, therapeutic behavioral results were correlated to partially increased BDNF in the hippocampus (Hamani et al., 2012).

Interestingly, differential roles of BDNF have been reported, contingent on the brain region where it is expressed. Increased BDNF production in the midbrain, hippocampus and the lateral ventricles results in an antidepressant effect (Shirayama, Chen, Nakagawa, Russell, & Duman, 2013).
2002), whereas overexpression of BDNF in the VTA (or NAc, in the mesolimbic DA system) resulted in pro-depressive symptoms (Berton & Nestler, 2006; Eisch et al., 2003). Specific deletion or knockout of BDNF in mesolimbic DA neurons in the VTA yields antidepressant-like response (Autry & Monteggia, 2012). An additional confounding factor is the dependence of BDNF gene expression on an enormous number of other genes, and their polymorphisms in the neuroplasticity pathway. For example, in rat models, SSRI treatment could not increase BDNF levels and exert an antidepressant-like behavioral effect in the absence of the CREB gene expression (Mattson, Maudsley, & Martin, 2004). Nonetheless, a large scale mRNA expression study measuring changes in SSRI-treated mice found that activation of BDNF-TrkB-CREB1 signaling pathway showed the best association with successful SSRI treatment (Kovacs et al., 2014). In stark contrast to the antidepressant effects of CREB-BDNF in the hippocampal-prefrontal circuit, activation of CREB-BDNF cascade in the VTA-NAc pathway results in pro-depressive like behavior (Yu & Chen, 2011). While dysfunction of the VTA-NAc circuit is thought to be associated with depression, antidepressants have been postulated to reverse this function.

Although preclinical research demonstrates a substantive evidence on the relationship between BDNF, stress and antidepressant activity, it is difficult to extrapolate these findings to humans due to the origin and function of serum-derived BDNF being unclear. Analysis of postmortem hippocampus tissue demonstrates that the expression of BDNF is decreased in depressed suicide patients and increased in patients receiving antidepressant medication at the time of death (Chen, Dowlatshahi, MacQueen, Wang, & Young, 2001; Karege et al., 2005). Similarly, decreased BDNF and TrkB levels have been correlated with decreased volume of the PFC (Dwivedi et al., 2003; Pandey et al., 2008). It is intriguing that similar to preclinical studies, BDNF expression is enhanced by MDD in the NAc tissue from patients (Krishnan et al., 2007). There are several reports demonstrating the possibility that peripheral BDNF may also influence central nervous system function, yet these findings have been dismissed after a meta-analysis. The serum levels of
BDNF are significantly decreased in depressed patients whereas successful antidepressant treatment has shown to reverse this state (Gonul et al., 2005; Shimizu et al., 2003).

Together, these findings suggest that behavioral outcome of drug treatment do not appear to directly reflect the levels of BDNF, but perhaps the functional role of BDNF within a particular network necessitates treatment responses (Pilar-Cuellar et al., 2014). BDNF release is necessary for increasing synaptogenesis and reverse the effects of chronic stress on atrophy of pyramidal neurons (Hoeffer & Klann, 2010; Jourdi et al., 2009).

**GSK3**

A metabolic enzyme, GSK3, is a serine/threonine kinase known to regulate cellular functions such as structure, gene expression, mobility and apoptosis. Recently, it has been linked to inflammatory pathways (an important positive regulator) where its activity has been found to be necessary for full stimulation of the production of several pro-inflammatory cytokines (e.g. IL6, IL1β, TNF). Furthermore, GSK3 is another crucial kinase that acts as an intermediary member of many intracellular signaling pathways, specifically in insulin, Wnt and MAPK pathways. Additionally, growth factors may exert many of their neurotrophic and neuroprotective effects in part by inhibiting GSK3. GSK3 phosphorylates and thereby inactivates many transcription factors and further modulates the function of cytoskeletal proteins such as protein tau.

More recently, an accumulation of reports discovered GSK3 inhibition as a basis for lithium’s mood stabilizing effects in BPD. The current concept supposes that lithium-induced serine-phosphorylation of GSK3 amplifies the direct inhibitory effect to allow for appropriate therapeutic levels of lithium to moderately down-regulate GSK3 activity. In other words, complete inhibition of GSK3 would have widespread consequences on many cellular functions, therefore the therapeutic action of lithium comes from not completely inhibiting GSK3, but only reducing its maximal activity (Jope, Yuskaitis, & Beurel, 2007).
Inhibition of GSK3 have been reported to reduce inflammation and apoptosis. Taken in conjunction with the evidence that inflammation too, is associated with mood disorders, it raises a new possibility that the therapeutic role of lithium-induced inhibition of GSK3 may result from reduction of inflammation, as reported to occur in mice treated with a clinically relevant doses of lithium (Martin, Rehani, Jope, & Michalek, 2005). Firstly, increased levels of inflammatory cytokines in patients with major depression or BPD has been well documented (Brietzke, Stabellini, Grassi-Oliveira, & Lafer, 2011; Brietzke et al., 2009; Dowlati et al., 2010). Furthermore, administration or increased production of cytokines in humans or animals can induce biochemical and behavioral changes that mirror those characteristic of deleterious mood states. Although it is not known whether the inflammatory condition contributes to the manifestation or is an effect of affective disorders, investigators argue that controlling inflammation may contribute to positive results on the following grounds: a) the depressive effects that cytokines can produce (Raison, Capuron, & Miller, 2006); b) documented results that classical antidepressants have anti-inflammatory effects (Hannestad, DellaGioia, & Bloch, 2011) as well as c) deep brain stimulation induced benefits are improved by adjunctive anti-inflammatory drugs (Perez-Caballero et al., 2014). Thus, findings of anti-inflammatory properties following lithium and other GSK3 inhibitors administration, reinforce the possibility where inflammatory processes likely contribute to the therapeutic responses.

Sodium valproate has recently become widely used as a mood stabilizer to treat BPD (Li, Ketter, & Frye, 2002). Although its mechanism of actions has been deemed similar to that of lithium, the underlying therapeutics of valproate needs further exploration. It is of considerable interest that valproate also shares with lithium the ability to reduce inflammation and ameliorate inflammation-associated conditions. Thus, the two most widely used mood stabilizers may have in common the capacity to reduce inflammation. However, other anti-inflammatory drugs do not share lithium’s mood stabilizing action, so it is clear that reducing inflammation alone is not enough to achieve mood stabilization. To demonstrate this, we can turn to studies examining the rapid
acting antidepressant effects of ketamine. The mechanisms of this drug have been shown to be dependent on mTOR and GSK3, which corresponds to rapid upregulation of structural and functional plasticity (Beurel, Song, & Jope, 2011). mTOR inhibition in vivo (TLR-stimulated immune cell line) induced a potent adaptive and innate inflammatory response that was abrogated by preventing rapamycin from increasing GSK3 activity (Wang et al., 2011). A direct interaction between mTOR signaling and GSK3 has been further reported in the regulation of cell growth (Buller et al., 2008; Inoki et al., 2006). An inhibition of GSK3 was found sufficient to activate mTOR-dependent protein translation for axonogenesis (Hur & Zhou, 2010). Nonetheless, reducing inflammation by inhibiting GSK3 may contribute some part of lithium’s actions (similar to that of ketamine) in controlling mood fluctuations and its long-term prophylactic action in preventing recurrences of episodes (Jope et al., 2007).

Since impaired serotonergic activity has long been implicated in antidepressant mechanisms, findings pinpointing enhanced serotonergic activity or antidepressants to subsequently inhibit GSK3 in mouse brain in vivo, lends further support to the possibility that impaired control of GSK3 is a component of failed treatment response (Li et al., 2004). Interestingly, Beaulieu et al. (2004) reported in mice that increases in dopamine-dependent activity are mediated via a GSK-dependent mechanism and that lithium plus other inhibitors of GSK3 attenuate the hyperactivity in mice that lack the dopamine transporter. Using the forced swim test, a paradigm relevant to DA mesolimbic pathway, to assess behavioral despair and the efficacy of novel drugs, administration of a peptide inhibitor of GSK3 rapidly induced antidepressant-like behavioral effects (Beaulieu et al., 2004; Gould, Einat, Bhat, & Manji, 2004). Together, the normalization of GSK3 and monoamines activity seem to attribute to antidepressant mechanisms.

An integration between GSK3 and an energy signals, AMPK, has been found to regulate cell growth in coordination (Inoki, Kim, & Guan, 2012). Disruption in GSK3 function within the AMPK complex promotes higher AMPK and cellular catabolic processes even under anabolic conditions.
indicating GSK3 acts as a critical sensor for anabolic signaling to regulate AMPK. Facilitating GSK3 dissociation from the AMPK complex may be a novel therapeutic approach for stimulating AMPK activity without suppression cellular energy levels (Suzuki et al., 2013).

**mTOR**

The induction of synaptogenesis, which is crucial for long lasting treatment response, requires protein synthesis and the activation of mTOR (Duman & Li, 2012). mTOR, a highly conserved nutrient-responsive regulator requires signals from both nutrients (glucose, amino acids) and growth factors. Activation occurs via a number of pathways, through GSK3β and most notably the release of BDNF, stimulation of its receptor TrkB and downstream signaling cascades Akt and MEK-ERK. mTOR signaling regulates local protein synthesis during long-term synaptic plasticity (Tang et al., 2002). Recently, the mTOR signaling pathway has been implicated in the therapeutic effects of mood stabilizers such as lithium and rapid acting antidepressant, ketamine. It has been postulated that the inhibition of mTOR following lithium treatment, has the capacity to increase autophagy and is related to one’s ability to withstand a variety of insults following sub-chronic administration of rapamycin in rats (Cleary et al., 2008). This inhibition of mTOR has been corresponded to the antidepressant-like behaviors observed in the FST, induced by lithium administration. Activation of mTOR with ketamine in rodent chronic unpredictable stress models however, increased synaptogenesis in the prefrontal cortex which is crucial for mediating the antidepressant effects of this therapy (Dwyer & Duman, 2013). The synaptic actions of ketamine allows for rapid recovery from deleterious effects of repeated stress and prevents further neuronal atrophy and loss of synaptic connections. Recent observations highlight the role of N-methyl-D-aspartate receptors (NMDAR) in the antidepressant action of ketamine. Similar to NMDA receptor antagonist Ketamine, the antidepressant actions of scopolamine require mTORC1 signaling and are associated with increased glutamate transmission and synaptogenesis (Voleti et al., 2013). In contrast to NMDAR antagonists, typical antidepressants (e.g. SSRI or tricyclics) that requires
several weeks to produce antidepressant effects fail to activate mTOR signaling (Li et al., 2010), indicating that these agents have differential mechanisms to achieve treatment effects and possibly reflect patients with a specific depression symptomatology.

Preclinical studies were conducted to determine whether behavioral changes brought on by ketamine are also dependent on mTOR signaling. Rapamycin pretreatment completely blocked the antidepressant effects of ketamine in the FST, learned helplessness and novelty suppressed feeding tests (Li et al., 2010). As an example, Duman and Li (2012) demonstrated the rapid antidepressant action of ketamine from a single dose which completely reversed the deficits previously seen in sucrose consumption test in chronic unpredictable stress (CUS) exposure for three weeks. These ketamine induced antidepressant-like behaviors paralleled the rapid reversal of the atrophy of PFC pyramidal neuron spine density with CUS exposure (Li et al., 2011). Together, the results provide support for the hypothesis that the rapid synaptogenic effects of ketamine underlie the therapeutic response to this agent.

In clinical trials, decreased blood AKT 1 (upstream of mTOR) and mTOR RNA expression were observed in BPD patients during episodes, relative to that of healthy controls. Interestingly, after lithium treatment, alterations in AKT1 expression were positively correlated with improved Hamilton Rating Scale for depression scores, yet these associations were not found for mTOR (Machado-Vieira, Soeiro-De-Souza, Richards, Teixeira, & Zarate Jr, 2014). These findings may reflect the beginning changes where they are involved in the regulation of many downstream signaling. One plausible hypothesis is that the down-regulation of this pathway observed in BPD impacts cellular energy production through its action in the mitochondria. (Cunningham et al., 2007) demonstrated that the inhibition of mTOR decreases the genetic expression of mitochondrial transcriptional regulators, resulting in decreased mitochondrial gene expression and oxygen consumption.
Typical antidepressants have been reported to influence synaptic plasticity and related proteins to achieve a therapeutic response. As an exemplar, chronic fluoxetine administration is reported to increase dendritic spine density in the restrosplenial granular and prelimbic cortical regions (Ampuero et al., 2010). Chronic treatment with imipramine and fluoxetine can also reverse the neuronal atrophy in the hippocampus by typical antidepressants but this effect did not take place with atypical antidepressant tianeptine (Bessa et al., 2009). These outcomes highlight the unique ability of mTOR signaling (in the PFC) in increasing synaptic protein synthesis and synaptogenesis to rapidly reverse the synaptic deficits caused by chronic stress. The site of action is important in promoting positive versus negative effects.

CaMKII

Mechanisms of neuroplasticity and cellular resilience are involved in complex antidepressant drug action. There is overwhelming attention paid to, and evidence mounting for antidepressants harnessing signaling pathways related to neuroplasticity through an up-regulation of cAMP/PKA/CREB cascade, modulation of CaMKII activity and upregulation of MAPK cascade (Pittenger & Duman, 2008). CaMKII is an abundant brain kinase regulating neuronal responses to Ca\(^{2+}\) fluxes and represents an essential effector in neuroplasticity. Alpha and beta isoforms are most abundant in the brain. Appropriate Ca\(^{2+}\) signals induce autophosphorylation of the kinase at residue Thr286, generating a persistently activated form of the kinase that shows Ca\(^{2+}\)-independent enzymatic activity, which is crucial for various forms of neuroplasticity (Tiraboschi et al., 2004). CaMKII has been demonstrated to translocate and potentiate inhibitory synapses in response to moderate NMDA receptor activation. Activity-dependent induction, stabilization and expansion of spines require NMDA receptor activation and incorporation of AMPA receptor subsets—a direct connection to CaMKII activity in both of these processes (Robison, 2014). Once CaMKII is bound to the BDNF receptor, it may organize additional anchoring sites for AMPA receptors at the synapse (Du, Gray, et al., 2004). It is intriguing that activation of synaptic NMDA receptors versus non-
synaptic receptors has an opposite effect on cell survival via differential regulation of CREB function, a key molecule implicated in antidepressant activity. Calcium enters through synaptic NMDA receptors, which in turn induces CREB activity and BDNF gene expression as strongly as did stimulation of L-type receptors, triggered by glutamate exposure or hypoxic/ischemic conditions. This activates a general and dominant CREB shut-off pathway that blocked induction of BDNF expression. Synaptic NMDA receptors have antiapoptotic activity, whereas stimulation of extrasynaptic NMDA receptors caused loss of mitochondrial membrane potential (an early marker for glutamate-induced neuronal damage) and cell death (Du, Gray, et al., 2004; Hardingham, Fukunaga, & Bading, 2002). Most importantly for this present discussion, these processes involving CaMKII, NMDA and AMPA receptors are the very same signaling cascades that mood stabilizers and antidepressants exert their major effects on (Du et al., 2003; Gould, Chen, & Manji, 2004). These observations have led to an extensive series of studies, which have clearly demonstrated that AMPA receptor trafficking is highly regulated by antidepressants and mood stabilizers (Gray, Du, Falke, Yuan, & Manji, 2003).

A growing body of literature suggests that CaMKII is regulated by stress and can be a target for antidepressant drugs. GR recruit the plasticity and survival pathways activated via CaMKIIα, BDNF, TrkB and CREB in the hippocampus (Chen, Bambah-Mukku, Pollonini, & Alberini, 2012). GRs mediate long term memory formation of emotionally important events by recruiting CaMKIIα-BDNF-CREB dependent neural plasticity pathway in rats (Chen et al., 2012). Acute and repeated, but not chronic stress exposure significantly increased phosphorylated CaMKII levels without affecting the levels of CaMKII (Suenaga, Morinobu, Kawano, Sawada, & Yamawaki, 2004). The increase in the intracellular Ca^{2+} concentration by the activation of AMPA receptors may play a role in the stress-induced phosphorylation seen in the rat hippocampus (Suenaga et al., 2004). Long term treatment with SSRIs or a dual inhibitor of 5-HT and NA reuptake increased autophosphorylation and activity of CaMKII in hippocampal subcellular fraction enriched with
synaptic vesicles and synaptic cytosolic fraction (Consogno, Racagni, & Popoli, 2001; Popoli, Vocaturo, Perez, Smeraldi, & Racagni, 1995). Additionally, repeated electroconvulsive seizures or imipramine both induced a large increase in the activity of this kinase contained in the total particulate fraction, but decreased the activity of soluble kinase. Although concomitant behavioral studies were not undertaken, the fact that different types of drugs and electroconvulsive therapy resulted in similar effects on CaMKII activity, suggested CaMKII activity to play an important role in the process of alleviating depression-related symptoms (Du, Szabo, Gray, & Manji, 2004).

CaMKII associated changes extend to altered expressions of other plasticity-related proteins. CaMKII mediated phosphorylation of CREB in the PFC elicits anti-depressive and cognition enhancing effects in olfactory bulbectomized mice (Han et al., 2009). This is of particular interest as CREB activity in the PFC following an antidepressant administration is crucial for treatment response. Reduced prefrontal cortical expression of CaMKIIα mRNA was found in the postmortem brains of individuals suffering from severe bipolar and unipolar depression (Xing et al., 2002). These findings highlight and reinforce PFC as a key region of interest, containing antidepressant properties in the clinical population, and that CaMKII is involved in the underlying molecular mechanisms.

CaMKII is also associated with and phosphorylates GSK3α/β, another key component in the acquisition of treatment efficacy. The pro-survival effect of CaMKII was mediated by GSK3 phosphorylation and inactivation. This is the first study to identify a novel CaMKII-GSK3 pathway that couples depolarization to neuronal survival with implications for the neurodegenerative population (Song et al., 2010). Potential substrates of GSK3 that mediate the pro-apoptotic effects of potassium withdrawal is likely to include key mitochondrial components of the apoptotic signaling pathway. The novel cellular mechanisms, observed in these studies are not specific to depression. Therefore, a direct examination is warranted in animal models of depression to establish these activity-dependent cellular processes for their applicability in the treatment of mood disorders.
Recently, an evidence of CaMKII activity can be found in learned helplessness animal model of depression. Overexpression of CaMKIIβ in the lateral habenula caused depressive-like behaviors in both rats and mice whereas a knockdown of CaMKIIβ yielded antidepressant effects (Li et al., 2013). It is possible that stress modulation of habenula CaMKII expression increases glutamate inputs onto VTA GABAergic interneurons, thereby inhibiting VTA dopaminergic neurons. CaMKII expression in NAc also regulates mood and stress responses via the associative reward circuitry (Robison, 2014). Chronic exposure to fluoxetine down-regulates NAc expression of CaMKIIα in mice and this reduction in CaMKII is both necessary and sufficient for the behavioral effects of the drug in a social defeat stress model. There is the possibility that chronic antidepressant treatment leads to down-regulation of NAc CaMKII expression to compensate for acute synaptic potentiation. Basolateral amygdala CaMKII expression has been suggested to play an important role in mood and emotional memory (McReynolds & McIntyre, 2012). A robust up-regulation of kinase activity was found in synaptic vesicles from both hippocampus and prefrontal/frontal cortex, following therapeutic responses achieved via different antidepressant drugs and electroconvulsive shocks.

From these findings, CaMKII function in depression and antidepressant action is likely to differ across regions, nature of stressors and disease progression. The reported up-regulation of CaMKII in cell bodies, as opposed to presynaptic terminals, could lead to other types of synaptic plasticity and neuroprotection that also contribute to the actions of antidepressant treatments. Thus, future therapeutic approaches involving CaMKII must achieve regional specificity either intrinsically or through the targeting of region-specific downstream molecules (Robison, 2014).

**AMPK**

AMPK is a fuel-sensing enzyme activated by cell's energy demand, and its activation is required for mitochondrial biogenesis and function. AMPK, as well as coordinating cellular metabolism, has the ability to trigger stress pathways, regulate cell division, autophagy and
apoptosis. Of note, it was shown that AMPK activity is important in maintaining protein synthesis-dependent forms of synaptic plasticity (Potter et al., 2010). Its activation subsequently leads to an up-regulation of ATP producing catabolic pathway and a down-regulation of ATP consuming anabolic process (Ruderman et al., 2010). AMPK improves survival under metabolic stress by integrating nutritional and hormonal signals in the peripheral tissues and the hypothalamus (Marques et al., 2012). AMPK is highly expressed in the hypothalamus and the activation of AMPK in vivo has demonstrated complex responses according to the magnitude and type of the stress (Pacak, 2000). Previously described restraint and surgical stress studies purport a notion of marked heterogeneity in neuroendocrine stress responses (Gaillet, Lachuer, Malaval, Assenmacher, & Szafarczyk, 1991; Reis, Guerra, Reis, & Coimbra, 1995). Likewise, hypothalamic AMPK activity has been shown to respond differentially to various stressors (Marques et al., 2012). The PVN has been found to be specifically associated with changes in AMPK activity (Kola, 2008). Glucocorticoids, among other hormones and its interactions with AMPK has been explored extensively in the PVN with regards to appetitive control. Excessive or chronic exposure to glucocorticoids not only results in insulin resistance but also increase hypothalamic AMPK activity either directly or via endocannabinoid synthesis (Lim, Kola, & Korbonits, 2010; Nakken, Jacobs, Thomson, Fillmore, & Winder, 2010). Recently, a pharmacological activator of AMPK was able to acquire positive treatment effects in a mice model of depression-like and insulin-resistant state induced by co-treatment of CORT and high fat diet. Concurrently, significantly higher levels of CORT were observed alongside blood glucose (Liu, Zhai, Li, & Ji, 2014). These outcomes, put AMPK forward as a potential antidepressant compound targeting CORT-induced HPA dysregulation. These findings, together with related studies of the stress system and AMPK introduces AMPK as a putative target for enhancing patients' treatment response.

Another important function of AMPK is through the phosphorylation of raptor for the inhibition of mTOR and cell-cycle arrest arising from energetic stress (Gwinn et al., 2008). In an
inverse manner to AMPK, mTOR is activated when there is abundant energy source and efficiency. To add further complexity to mTOR function, mTORC1 is rapidly inactivated by a wide variety of cell stress to ensure that cells do not grow under unfavorable conditions (Gwinn et al., 2008). There are multiple mechanisms in which AMPK inhibits mTORC1, thereby maintaining cellular energy homeostasis. Linear signaling pathway involving the phosphorylation of tuberous sclerosis complex 2 gene (TSC2) to activate tuberous sclerosis complex (TSC), subsequently attenuates the TORC1 pathway. Cells can also inhibit mTORC1 through AMPK dependent direct regulation of mTORC1 involving raptor (Gwinn et al., 2008; Inoki et al., 2012). A third mechanism involves a pathway in the down-regulation of Rheb activity through a phosphorylation-dependent mechanism (Zheng et al., 2011). In so far, their interactions have been better studied for neurodegenerative disorders such as Alzheimer’s disease, diabetes, appetitive control and cancer. Given that the engagement of mTORC1 and AMPK mediates cell growth or the induction of autophagy, further research is warranted to apply these findings for mood disorders. Appropriate control over cell growth and survival is crucial in the context of adaptive responses to and restorative processes after stress.

Deep brain stimulation and its mechanisms in altering one’s treatment response

This review presented current knowledge on mood disorders and factors mediating the frontiers of treatment responsivity. All in all, these hypotheses on their own, may not be so relevant in looking to deepen our understanding on the mechanisms that moderate resistance to a given intervention. More likely, each of them (through interactions) have their unique function in enhancing adaptive neuronal remodeling, restructuring and plasticity. It seems probable that an effective intervention modulates pathological cellular and molecular networks in severely refractory patients, in an attempt to reinstate their responsivity. DBS has emerged as an alternative, final method that may exert its antidepressant power by changing the structure (hardware) as well as the function of the brain, not only producing a physiologic effect. Constant, chronic and long-term stimulation of regions that are heavily implicated in the pathophysiology of depression and
antidepressant activity is necessary for reversing resistance in TRD population (Lozano et al., 2008; Schlaepfer, 2015). In so far, findings from preclinical and clinical research suggest that different DBS targets may be acting upon particular symptom profiles that may be specific to the individuals’ depression subtype, severity and progressive stage (Hamani et al., 2014; Schlaepfer, 2015). Evidence has been building to expand our limited knowledge on the effects of DBS. A detailed discussion of these findings can be found in the upcoming Chapter 2. To summarize, metabolic changes have been reported in the frontostrial, amygdala and the SCG region following DBS on and off (Riva-Posse et al., 2014). Early responses to SCG deep brain stimulation in severely depressed patients were poorer when they received anti-inflammatory drugs (Perez Caballero et al., 2013). Additionally, a role for serotonin and BDNF has been associated to the antidepressant-like behaviors following mPFC DBS (Hamani et al., 2012). Findings from subthalamic stimulation for Parkinson’s disease support casual changes in human neural plasticity after long-term stimulation (Van Hartevelt et al., 2014). Mitochondrial function and antidepressant actions of DBS, however, are not known.

Conclusions

Stress, allostatic overload and neuroinflammation all impair synaptic plasticity and cellular resilience. Disrupted plasticity together with increased cellular vulnerability contribute significantly to the pathophysiology of mood disorders and directly accelerate neuroprogression of the disease course (Machado-Vieira, 2013; Berk et al., 2007). Some of the signature characteristics of disease progression include oxidative stress, decreased neurotrophic factor expression, reduced neurogenesis, impaired regulation of calcium together with alterations in endoplasmic reticulum and mitochondrial function, energy metabolism and insulin signaling (For detailed reviews see Berk et al., 2011; Brietzke et al., 2011, Machado-Vieira et al., 2013). This review emphasizes the complex processes underlying neuronal plasticity, whereby structural increases, such as neurogenesis, neurite aborization and synaptogenesis cannot alone account for plasticity, but these
responses need to be balanced by programmed neuronal death, neurite retraction and synaptic pruning for healthy, adaptive functioning. Functional activity within the neuronal network determines which connections should be maintained and strengthened as opposed to ones eliminated (Castren & Rantamaki, 2010; Katz & Shatz, 1996). In other words, the adaptive formations are sustained for resilience and the deleterious functions abandoned for optimal responses to future threats (i.e. stressors).

Biological markers and predictors of response to drug administration as well as molecular targets of novel antidepressants are searched on the basis of emerging and evolving hypothesis around neurogenesis and plasticity. According to these hypothesis, the leading role in the pathophysiology of mood disorders and antidepressant efficacy are destined for changes in cellular energy metabolism. Mitochondrial dysfunctions and consequently, impaired neuronal metabolism can lead to disturbances in neuronal function, plasticity, network circuitry and further systems-wide degeneration. Together, these ideas highlight the importance and need for preclinical research. They can help us to develop a framework for understanding the neurobiology of depression and treatment response for targeted, systematic and efficacious antidepressant discovery.
Figure 1. Neuroprogression in depressive disorders. Psychosocial and physical stressors combined with pre-existing vulnerabilities precipitate a first depressive episode. Biochemical factors that may accelerate the neuroprogressive nature of MDD and BDP include inflammation, monoamine alterations, HPA axis dysregulation, and disturbance to neurotrophic function, which interact to cause cellular damage, stimulate apoptosis and decrease neuronal growth and survival. These effects makes the individual more susceptible to further episodes and pushes them into an increasingly treatment resistant state. Each episode corresponds to progressive cognitive and functional decline and associated structural brain changes. Image adapted from Moylan, Maes, Wray, and Berk (2013) with permission.
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Chapter 2. Deep brain stimulation for treatment resistant depression

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CHAPTER 11
DEEP BRAIN STIMULATION FOR TREATMENT RESISTANT DEPRESSION

Yesul Kim¹, Katheryn M. O’Connor², Susannah J. Tye³

¹ BA, GdipPsych, PhD Candidate, School of Psychology, Faculty of Health, Deakin University, Melbourne, Australia.
² BS, Department of Psychiatry & Psychology, Mayo Clinic, Rochester MN, United States
³ PhD, Department of Psychiatry & Psychology, Mayo Clinic, Rochester MN, United States

INTRODUCTION

Deep brain stimulation (DBS) has emerged as an alternative, reversible, non-ablative neuromodulatory treatment for severely refractory Major Depressive Disorder (MDD) and Bipolar Depression (BD). In the past decade, DBS for mood disorders has evolved with clinical studies of modest case series, yet it remains largely investigational. For those patients who do not receive therapeutic benefit from other available treatment options, including pharmacotherapy, psychotherapy and electroconvulsive therapy, DBS holds great promise (Bewernick et al., 2012; Fava & Davidson, 1996). In general, DBS is believed to work by modulating the cortico-striato-thalamo-cortical (CSTC) circuits (see figure 1). The CSTC and its associative limbic and motor circuits have been implicated in the pathogenesis of MDD and BD. Various DBS targets have been examined for Treatment Resistant Depression (TRD) including the subgenual cingulate gyrus, ventral capsule/ventral striatum, nucleus accumbens, inferior thalamic peduncle, lateral habenula and the medial forebrain bundle. Still, many important clinical questions remain, including: 1) what is the most appropriate, effective and therapeutically consistent target; 2) what are the optimal stimulation parameters, and 3) what are the progressive mechanisms of action and how can recovery be optimized over time? This chapter will review reported clinical
trials of DBS for MDD and BD, describing rationale for target selection, reported efficacy, clinical applicability and hypothesized mechanisms of action.

**Figure 1. Various DBS targets and modulation of the CSTC circuits**

Abbreviations –vmPFC: ventromedial prefrontal cortex; SCG: subcallosal cingulate gyrus; ITP: inferior thalamic peduncle; VTA: ventral tegmental area; dorsal SNR: dorsal substantia nigra pars reticulate; LHb: lateral habenula; VP: ventral pallidum; DMmc: dorsomedial motor cortex; GPi: globus pallidus; MFB: medial forebrain bundle; NAcc: nucleus accumbens core; NAcsh: nucleus accumbens shell.

**DBS Targets and Outcomes**

**Subcallosal Cingulate Gyrus (SCG): Area 25**

To date, the subcallosal cingulate gyrus (SCG) is the most extensively researched DBS target. Reversal of SCG hypermetabolism in TRD is the primary rationale for targeting this region with high-frequency stimulation DBS (Lozano et al., 2012; Lozano et al., 2008; Mayberg et al., 1999; Mayberg et al., 2005). Clinical trials of SCG stimulation were originally described by Helen
Mayberg and colleagues in 2005, in a trial of 6 patients and subsequently in a larger study of 20 patients (Lozano et al., 2008). Approximately two thirds of patients in these studies showed improvement of MDD symptoms, and 35% had complete remission. Blinded, sequential intraoperative stimulation at 130Hz was applied to determine the threshold for safe and effective stimulation. At final follow-up (3-6 years), 42.9% of patients demonstrated complete resolution of symptoms (Kennedy et al., 2011).

In an effort to replicate and extend these findings, Puigdemont et al. (2012) investigated the short and long-term outcomes and safety of SCG in an independent sample of 6 patients. Response rates in this group were similar or greater than those reported previously. Seven patients displayed marked improvement after 6 months of chronic stimulation, and 50% remitted after 1 year of DBS. During the follow-ups, antidepressant drugs were not changed but were reduced in parallel with signs of clinical improvement. Interestingly, Puigdemont et al. (2012) found a significant relationship between long-term response and electrode location. Another earlier study, analyzing responders vs. nonresponders in SCG DBS, found robust white matter tract connections from the SCG target to the orbitofrontal cortex, amygdala/hippocampus, hypothalamus, and NAcc to be critical for mediating therapeutic effects of DBS (Johansen-berg et al., 2008). Riva-Posse et al. (2014) further defined the combination and location of specific white matter tracts necessary to achieve clinical response. Tractography maps revealed a fiber bundle template involving bilateral forceps minor, cingulum, and medial frontal striatal/subcortical fibers activation was responsible for response and remission.

More recently, Holtzheimer et al. (2012) presented an open-label, sham-stimulation study of 17 patients with treatment resistant unipolar and bipolar depression undergoing DBS of the SCG. In this single-center study, the majority of patients (92%) responded, and over a half (58%)
were in remission following two years of stimulation. However, for some patients, improvements in depression severity took a longer time course without any clear reasons for the delay. It seems likely that premorbid functioning, psychosocial support and temperament/personality contributes to the rate of recovery and responsiveness in patients with TRD. Interestingly, overall these findings argued against a clinically significant sham effect over time. Although depression severity was significantly lower after 4 weeks of sham stimulation, the mean reduction was not clinically significant.

SCG DBS may work via normalizing CSTC neural networks through a reduction of SCG activity. Given the strong reciprocal connectivity between the prefrontal cortex and brainstem monoaminergic nuclei, SCG DBS may normalize a putative monoaminergic hypofunction secondary to abnormal inputs from the prefrontal cortex (Puigdemont et al., 2011).

**Ventral Capsule/ Ventral Striatum**

Stimulation of the ventral capsule/ ventral striatum (VC/VS) was first performed in patients with Obsessive Compulsive Disorder (OCD) and it was found that Improvements not only occurred in OCD symptoms but also in depression scales. These findings of improved mood led to the exploration of this target for TRD (Aouizerate et al., 2004; Greenberg et al., 2006). Specifically, Greenberg et al (2006) reported that 50% of the eight patients with comorbid depression experienced improved mood and affect. These observations, and the implications they had for with the involvement of CSTC circuits, opened up further clinical trials and preclinical investigations targeting VC/VS for the treatment of severe MDD patients.

Malone et al. (2009) presented results of 15 patients with VC/VS stimulation and according to the Hamilton Depression Rating Scale (HDRS), patients demonstrated a 20%
remission rate after 6 months of treatment and a 40% responder rate at the last follow-up.
Corresponding remission rates assessed by Montgomery-Asberg Depression Rating Scale (MADRS) were 33.3% at 3 months, 26.6% at 6 months and 33.3% at last observation. DBS was reported to be well tolerated, with minimal complications. Most notable complications included pain at implantation site, lead fracture, transient hypomania, increased depression, transient dysphagia and syncopal episodes (Malone et al., 2009). Hypomania was resolved by adjustment of stimulation parameters, where one incident of worsening depression was addressed with changes in medication and a close monitoring of neurostimulator battery function. Hence, the authors stressed the importance of post-operative care as patients felt quite distressed by return of depressive symptoms after accidental battery depletion or failure. Relative to cognitive functioning, Kubu and colleagues (2013) observed no decline across individuals, and neuropsychological improvements were reported on 10 OCD and 11 MDD patients. Similarly, Malone et al. (2009) failed to find deleterious effects on measures of general intellectual ability, language, processing speed, executive function, learning and memory.

Taken together, the reported clinical findings VC/VS as a viable target for DBS provide encouraging evidence of sustained therapeutic effects. With close postoperative monitoring and successful electrode implantation, this region offers therapeutic potential for individuals suffering TRD who are unresponsive to conventional treatments.

**Nucleus Accumbens**

The reward circuitry of the nucleus accumbens (NAc) has been associated with depression for many years. The NAc is located within the most ventromedial portion of the VS. Considering its intimate connections to areas involved in depression phenotypes, NAc emerged as a practical candidate for neuromodulation.
In a small initial study of three patients undergoing DBS of the NAcc experienced improvements in anhedonia (i.e., inability to feel pleasure; Schlaepfer et al., 2008) but did not experience complete remission. The changes brought about were also reflective of positive affect and metabolic changes in brain networks associated with depression. After one week of DBS, it was found that metabolic activity increased in the NAc, amygdala, dorsolateral and dorsomedial prefrontal cortex and decreased in the ventral and ventrolateral medial prefrontal cortex, which have been described as hypermetabolic in depressed states (Schlaepfer et al., 2008). These observations support the hypothesis that NAc DBS can modulate the CSTC circuit.

Preliminary positive effects of NAc DBS on depression were thus established, yet there was limited information on its impact on cognitive functioning. At one year after bilateral NAcc DBS, Grubert et al. (2011) found no cognitive decline and a trend towards improved cognitive performance, generally improving from below average to average performance in 10 patients. These precognitive effects were independent of the antidepressant effects or changes in NAcc DBS parameters. This study addresses limited information regarding the impact of NAcc DBS on TRD.

Recently, in a larger scale study, 5 of 11 (45%) patients responded, and the treatment effect seen at 12 months was sustained at 4 years (Bewernick et al., 2012). Ratings of both depression (HDRS, MARSD) and anxiety (Hamilton Anxiety Scale) were significantly reduced from the first month of DBS on. Clinically equally important, improved hedonic activities were also reported in those responders. Notably, all 11 patients improved on quality of life measures. However, one nonresponder committed suicide. The authors judged this suicide as unrelated to the DBS. Other than this adverse event, the only adverse effects were weak symptoms of
hypomania (elevated mood, fewer hours of sleep), which were observed in two patients after a stimulation parameter change and which disappeared within 24 hours.

In patients who responded well to NAc DBS, the effects have been sustained and have not adversely affected cognitive function. As is true of other DBS at other sites, there is surgical risk of the procedure itself. However, given the NAc role in reward, motivation, aggression and impulsivity, there are also psychiatric risks in targeting NAc, including the possibility of aggravating depression or generating other adverse effects such as mania and suicide. This is in light of the high risk of suicide (approximately 15% in TRD; Wulsin et al., 1999), and compared to suicide rate found in SCG DBS (Kennedy et al., 2011).

**Inferior Thalamic Peduncle**

The inferior thalamic peduncle (ITP) is a system of fibers that connect the midline and intralaminar thalamic nuclei of the nonspecific thalamic system (NSTS) with the orbitofrontal cortex, which is involved in the physiopathology of MDD. Subcortical orbitofrontal cortex (OFC) connections include the temporal lobe amygdala, ventral striatum, hypothalamus, substantia nigra, raphe nuclei and ventral tegmental area. The physiological and anatomical significance, together with postmortem imaging observations highlight ITP and its related circuits as engaged in mood and behavior control (Jimenez et al., 2005).

The first and only case to describe targeting the ITP was a 2005 case report by Jimenez et al. described a 49 year-old patient with recurrent episodes of unipolar depression of 20 years duration. Bilateral 8 contact stimulating electrodes were stereotactically in areas at and around the ITP. Depression relapsed partially at the end of first week. Continued electrical stimulation
improved depression as measured by the HDRS without antidepressant medication (Jimenez et al., 2005).

Preclinical experiments of the effects of DBS on ITP, present particular challenges. ITP is not identified in the rat stereotactic atlas, thus lesions and electrical stimulation of the reticular thalamic nucleus and OFC (structures connected by the ITP) have been used as an alternative. This may explain the lack of preclinical and clinical research on ITP DBS for TRD. However, the ITP remains an interesting exploratory target. Previously mentioned DBS sites in this chapter as well as regions implicated in MDD (e.g. temporal lobe amygdala, hypothalamus) all have anatomical connections with the OFC. Moreover, the anatomical proximity of the internal capsule, NAc and ITP opens up the possibility that the stimulated area may involve more than one target.

**Lateral Habenula (LHb)**

Studies have reported increased activation of the lateral habenula (LHb) in humans with depression (Morris et al., 1999; Smith et al., 1999) as well as in certain animal models of depression (Caldecott-Hazard, Mazziotta & Phelps, 1988; Shumake et al., 2003). Increased activation of this region leads to the down regulation of the serotonergic, noradrenergic, dopaminergic systems and stimulation of the hypothalamic-pituitary-axis.

Recently, LHb DBS has yielded successful results and is thought to be a mediating mechanism for antidepressive actions (Sartorius & Henn, 2007; Sartorius et al., 2010). The first patient showed initial symptom remission, but experienced two full relapses when the stimulation device malfunctioned, although the patient was unaware of the malfunction (Sartorius et al., 2010). The second improved more than 50% as measured by the Hamilton
Depression Scale, but suffered from a comparable relapse during an off period when the stimulation device was changed following infection.

Strikingly, the DBS-OFF relapses in the abovementioned cases were rapid in onset, occurring approximately within one week, and the remission times were slow, taking over 8-12 weeks to occur in the DBS-ON phase. Similar patterns were also noticeable in patients treated with DBS of the SCG. Of note, the SCG as well as the medial forebrain bundle is part of the midline neurocircuitry that potentially affects the LHb inhibitory projections. For this reason, the investigators suggest that the uneven time course observed in those 2 patients (e.g., rapid relapses followed by slow remissions) is likely linked to alterations of neuroplasticity and perturbations of monoaminergic systems that are pertinent to theories of depression (Sartorius et al., 2010).

One of the major surgical challenges of DBS in this area is the prevention of intracranial bleeding when introducing the electrode. Careful and precise anatomical planning is required. Schneider and colleagues (2013) recently conducted a feasibility and safety study of LHb DBS in 27 patients. They found that a standard frontal trajectory (>40 degrees relative to anterior commissure) was safe for bilateral stimulation in 48% of the patients and that a steeper frontal trajectory (that is <40 degrees relative to the anterior commissure) was required for bilateral stimulation in 96% of the cases. They concluded that DBS of this small midline structure or rather, its major afferent bundle which is adjacent to the third ventricle and thalamus related veins, presents predictable, but solvable challenges to neurosurgeons (Schneider et al., 2013).

**Medial Forebrain Bundle**

In 2013 Schlaepfer et al. explored supero-lateral branch of the medial forebrain bundle (slMFB) DBS in a pilot study of 6 patients. The modulation of a network of forebrain structures
via the medial forebrain bundle (MFB) was inadvertently discovered by activation of myelinated fibers descending from the frontal lobe into the ventral tegmentum area (VTA). It was thought that upstream dysfunctional cortical input into the network might thus be ameliorated by modulating cortical regions, whereas downstream dopaminergic VTA neurons could be recruited and activated directly. As a result, the VTA might exert its activating effects on forebrain structures and the prefrontal cortex by way of DBS to the sMFB.

In a pilot study for the safety and efficacy of sMFB DBS to 7 patients with TRD showed similar intraoperative effects of increased appetitive motivation to one another (Schlaepfer et al., 2013). At last observation in week 12, 6 out of 7 patients were responders; among them, four were classified as remitters (MADRS <10). Unexpectedly, the positive effects of sMFB were rapid. The mean MADRS was reduced by >50% at day 7 after onset of stimulation, and 6 of the 7 patients had significant effects over only 2 days of stimulation. Comparable acute and rapid effects in depression have only been previously observed in studies with the use of ketamine (Zarate et al., 2006) or sleep deprivation (Ibrahim et al., 2011). Nevertheless, clinical studies reports the clinical effect of ketamine limited to 2 weeks (Murrough, 2012) and the antidepressant effects of sleep deprivation alone are generally reversed after the next recovery sleep (Wu & Bunney, 1990). In contrast, the effects of sMFB DBS reported by Schlaepfer et al. (2013) seems to be enduring. It is important to note that sMFB stimulation was linked only with appetitive motivation, however was not associated with a direct reinforcing, hedonic or liking effects.

This recent data raises questions about the degree to which the various stimulation sites can be conceptualized as part of or an entry point into a single dysregulated neurocircuit or circuits. Recent preclinical study (Hamani et al., 2014) showed that despite similar
antidepressant effects following stimulation to homologous SCG, NAc and MFB regions, they induce distinct changes in regional brain activity and functional connectivity. The MFB may act as an integrative extension of other associative circuits underlying the pathophysiology of MDD to frontal structures (Kiening & Sartorius, 2013). Direct MFB stimulation thus may provide a shorter route to the prefrontal cortex and a speed access toward therapeutic reaction. The rapid onset of positive MFB DBS effects, if replicated, could not only be of great benefit for patients suffering from TRD but may also be a factor in the search for the underlying mechanisms of fast-working antidepressant strategies (Kiening & Sartorius, 2013).

**CONCULSION AND FUTURE DIRECTIONS** Each of the various DBS sites studied in the treatment of depression have been found to have some positive effects, and each appears to be associated with surrounding anatomic regions implicated in psychomotor, cognitive, mood, emotional and neurovegetative symptoms of depression (see figure 2). The differences in methods used to evaluate DBS efficacy among the studies reported to date prevents a consensus on the efficacy of a given target at this time. Larger controlled, DBS-ON/OFF clinical trials are needed to identify optimal targets, patient selection criteria, and the long-term effects of DBS for depression, augmentation of social functioning, and normalization of brain metabolism. Such studies should also include the effects of DBS on standard measures of attention, learning, and memory. The potential for adverse events such as infection, suicide, and hypomania, also should be carefully monitored in the post-operative period. At this point in time, it appears that DBS for depression holds great promise for patients with treatment-refractory depression.
Figure 2. DBS sites and its surrounding regions’ function in psychomotor, cognition, mood, emotions and neurovegetation

**Therapeutic mechanisms of DBS**

The therapeutic mechanisms of DBS in TRD are not well understood. Although research on the efficacy of DBS for TRD have been underway for approximately 10 years, they are often uncontrolled clinical trials and the mechanisms mediating therapeutic effects are only more recently becoming apparent. Unlike movement disorders, therapeutic responses occur via slow, progressive improvements in symptoms. While patients will describe an immediate “insertional”
effect, this is often followed by a sharp decline before the progressive and enduring improvement occurs (Taghva et al., 2013). Further complicating matters, neuronal effects of DBS vary depending not only on the location of stimulation (brain area, cell bodies, or white matter fibers), but also on the various parameters of stimulation (voltage, pulse width, and frequency), and the overall activity state of the regions to be modulated. (Butson et al., 2007; Holtzheimer and Mayberg, 2011; Perlmutter and Mink, 2006). Regardless, patients have reported a common reduction of depressive symptoms and restoration of function with all of the aforementioned DBS target trials.

With the exception of several imaging studies conducted during clinical trials, the majority of investigations of mechanism of action in high-frequency stimulation research has been conducted in small animal models of depression and treatment resistant depression. The main mechanism is thought to facilitate a “resetting” of faulty circuitry in the CTSC pathway (Hammond et al., 2007; Kopell and Greenberg, 2008; Taghva et al, 2013). This requires restoration of synaptic plasticity and facilitation of neurogenesis, known to be important mechanisms of antidepressant action. In addition to neurotransmitters, cytokines and growth actors have been implicated in the DBS-mediated antidepressant effects (Gersner et al., 2010; Hamani et al., 2012; Perez-Caballero et al., 2013), occurring via gradual synaptic adaptations (plasticity) (Falowski et al., 2011).

**Cytokines**

Inflammation and glial-mediated responses have been shown to play a key role in the therapeutic mechanism of DBS in a number of disease states, including Parkinson’s disease, however little is known about their role TRD. Perez-Cabellero et al. (2013) have shed light on this issue using a rodent model of depression, and demonstrating that implantation of the electrode in the
infralimbic cortex (homologous to human SGC) was sufficient to induce an antidepressant effect in rodents as measured in the forced swim test (FST; a preclinical behavioral screen for antidepressant response). This effect was maintained for up to two weeks post-implantation, after which active DBS was necessary to sustain a therapeutic response. These researchers were able to effectively block this therapeutic effect with anti-inflammatory pre-treatment and further demonstrated that an increase in astrocytes and inflammatory mediators up to two weeks after implantation correlated with antidepressant effects. Moreover, this inflammatory-mediated response was shown to enhance synaptic efficacy at serotonin synapses through upregulation of serotonin receptors and facilitation of serotonin signaling.

The clinical relevance of this finding is supported by the reported effects of anti-inflammatories on clinical antidepressant responses to DBS. Perez-Cabellero et al. (2013) also reported differences in patient responses to bilateral DBS of SGC, which segregated according to anti-inflammatory treatment. Although early responses to DBS were similar for those patients receiving non-steroidal anti-inflammatory drugs (NSAID) and those who were not, antidepressant response began deteriorating after ~two weeks and was lost by one month post-DBS surgery for NSAID-treated patients. This rapid antidepressant response generated by DBS has been described as occurring prior to stimulation (Jimenez et al., 2005; Hotzheiner et al., 2012), and has been attributed to the generation of a microlesion. However, the Perez-Cabellero et al. study was the first to measure an insertional effect, the impact of anti-inflammatories, and long-term therapeutic responses to DBS.
Growth factors such as brain derived neurotrophic factor (BDNF) are well established as reduced in patients with depression, a finding similarly documented in rodent models of depression (Shimizu et al., 2003; Smith et al., 1995; Nibuya et al., 1995; Kiening and Sartorius, 2013). A subsequent increase in BDNF levels, in addition to changes in neuroplasticity, has been observed following successful antidepressant treatment and is thought to be a critical mechanism through which antidepressants exert their therapeutic effects (Nibuya et al., 1995; Alter et al., 2003). Preclinical data has shown similar results in rodent models of depression following therapeutic DBS (Gershner et al., 2010; Hamani et al., 2012; Friedman et al., 2009). Not only are levels of BDNF increased, but morphological changes have been observed in the dendrites of prefrontal cortex (PFC) pyramidal neurons (Falowski et al., 2011). Interestingly, Hamani et al. (2012) found a possible interaction between BDNF and serotonin, demonstrating that depletion of serotonin prevented BDNF reduction normally seen in rats undergoing chronic mild unpredictable stress to induce depression-like behavior. This concept has previously been suggested and attributed to impairment of stress adaption by the suppression of hippocampal glucocorticoid and BDNF responses (Zhou et al., 2008). However, Hamani et al. (2012) also demonstrated that an animal’s preference for sucrose, a measure of antidepressant efficacy, did not improve following DBS in serotonin-depleted rats with normal hippocampal BDNF. This suggests that although decreases in BDNF are seen in depression, and subsequent increases occur following antidepressant treatment, it is the interaction of BDNF with serotonin systems, possibly mediated in part by inflammatory responses, that is critical to therapeutic response.
Figure 3. Proposed therapeutic mechanisms of DBS action in treatment resistant depression. These mechanisms include, inhibition of local neuronal cells, activation of axons of passage and facilitation of neurotransmitter efflux, as is well established in the field of DBS for movement disorders. In addition, DBS activation of inflammatory responses and upregulation of neurotrophic factors is known to critically contribute to the progressive antidepressant actions of DBS for depression.
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Chapter 3. Methods

General Scope and Aims

This thesis encompasses various techniques on a molecular and behavioral level in order to understand the mechanisms of action of deep brain stimulation (DBS) within the research context and resource availability. Clinically relevant DBS targets, the nucleus accumbens (NAc), infralimbic (IL) and lateral habenula (LHb) were under examination. These brain regions are not only implicated in disease pathophysiology, but modulation of their function and associative circuits have been observed to bring about therapeutic treatment response. Our general aim is, therefore, to examine the antidepressant properties of different DBS targets. On the other hand, DBS is used as a probe to gain insight on cellular, molecular and systematic factors that mediate treatment responsivity in tricyclic resistant rats.

In the first 3 experimental chapters, antidepressant-like behavioral effects of DBS were investigated in conjunction with significant molecular adaptations. This approach was taken in the hopes to characterize key proteins, signaling pathways and gene expression responsible for achieving antidepressant effects in otherwise, treatment resistant animals. These complex biological processes that mediate responsivity include neuroplasticity, mitochondrial function, regulation of cell death and growth as well as monoamine transmission. The final chapter explores the mesolimbic dopamine (DA) system as a potential common mechanism resulting from DBS.

Theories and Rationale

Animals

The effects of DBS were assessed in the living brain of rats which are standard species utilized for determining basic neurochemical mechanisms in mammals. Their neuroanatomy has
been extensively studied where stereotactic targeting of specific brain nuclei can be achieved with high accuracy. As the structure and function of neuronal systems under investigation are well-conserved across mammalian species, findings observed herein are translational to higher order mammals, including humans.

In the four upcoming experimental chapters, Wistar or Sprague Dawley rats were used. These rodent strains have been consistently and systematically used in preclinical research to investigate antidepressant efficacy of novel interventions such as ketamine and electroconvulsive therapy (Logan & McClung, 2015). Furthermore, modelling specific phenotypes of depression has been valuable in providing insight on the neurobiology of disease. From the viewpoint of animal models in helping us to better understand the neurobiology of mood disorders, it has proven useful. As an example, stress based models gave insight on the cellular alterations that may contribute to reduce hippocampal volume found in severely depressed population (Czéh, Fuchs, Wiborg, & Simon, 2015). More recently using Sprague Dawley rats, the inherent issues in the cause-and-effect were addressed on the exact role of inflammation as a risk factor in complex and interactive pathways of stress to depression (Wood et al., 2014). Advanced techniques such as optogenetics in freely moving rats, identified that DA neurons potently alters neural encoding of depression-related behaviors in limbic circuitry (Tye et al., 2013).

**Animal Model of Antidepressant Treatment Resistance**

Activation of the hypothalamic pituitary adrenal (HPA) axis is a major neuroendocrinological response of the organism to stress. In response to either emotionally salient or physiological stress, responsiveness of glucocorticoid production is essential, achieved via multiple feedback loops involving the corticotrophin releasing factor (CRF) and vasopressin (AVP) from the hypothalamus. In turn, adrenocorticotropic hormone (ACTH) secretion is activated from the pituitary, which stimulates the culmination of glucocorticoids (cortisol in humans and
corticosterone in rodents) from the adrenal cortex (Pariante & Lightman, 2008). Collectively, HPA axis activity is governed by a complex set of feedback interactions among three endocrine glands (Figure 1).

We and others have previously established an animal model of treatment resistant depression (TRD) in which chronic ACTH treatment blocks the effects of tricyclic antidepressant imipramine and desipramine (Kitamura, Araki, & Gomita, 2002; Kitamura et al., 2008; Tokita, Fujita, Yamaji, & Hashimoto, 2012; Walker et al., 2013). Rats treated with ACTH (100μg/day) for 14 days exhibited significantly higher plasma corticosterone levels, producing a desensitization of somatodendritic serotonin (5-HT) 1A autoreceptors (Kitamura et al., 2007). Our preliminary studies have also shown alterations in PFC monoamine levels and irregular proinflammatory cytokines (Walker et al., 2013; Walker et al., 2015).

In clinical settings, a dysfunctional HPA axis has been associated with mood disturbances and treatment response (Willner, Scheel-Krüger, & Belzung, 2013). Aberrant activation of the HPA axis correlates with treatment resistant depression population. A recent meta-analysis found that ACTH levels were elevated to a similar degree during depression and this effect was robust to controls for methodological quality (Stetler & Miller, 2011).

In our animal model, daily doses of ACTH were administered in an attempt to disrupt the HPA feedback system, in which a heightened basal activity of HPA will enable us to investigate the underlying processes that takes place when the animals are faced with a stressor. Using the above mentioned preclinical protocol, we aimed to specifically examine the antidepressant properties of chronic and acute high frequency stimulation of clinically relevant neuromodulatory targets. Overall, it was of great interest to elucidate the mechanisms by which DBS is restoring adaptive brain function and those cellular process that mediates stress and treatment response.
Figure 1. The activation of HPA axis stress system under perceived stressor. Please note here that corticosterone (cortisol in humans) applies to rats. ACTH is secreted from the pituitary gland, and in turn, stimulates the release of glucocorticoids in the adrenal glands.


Social Isolation

Early life environmental and social context are not only critical for healthy brain development but for reducing vulnerabilities or strengthening resilience to pathology. It has been demonstrated that social isolation produces several long-term adaptations that are phenotypic of depressive symptoms. These include increased anxiety in many behavioral paradigms (Weiss, Pryce, Jongen-Rêlo, Nanz-Bahr, & Feldon, 2004), impairments in learning and memory tasks (Larsson, Winblad, & Mohammed, 2002; Schrijver, Bahr, Weiss, & Würbel, 2002) as well as behavioral despair (Brenes, Rodríguez, & Fornaguera, 2008; Lapiz et al., 2003; Sáenz, Villagra, & Trías, 2006). Isolation reared rats also have abnormalities in monoamine transmission in the NAc, PFC and ventral striatum (Brenes et al., 2008; Lapiz et al., 2003). Given that we wanted to induce treatment resistance in rats, complimentary social isolation during acclimatization (during early adolescent life ~5 weeks after birth) and throughout the experiment into adulthood was
implemented. Studies show social isolation alone can achieve depressive phenotypes and we hope to further bring treatment resistance in our animal model.

**Deep Brain Stimulation principles**

DBS first made significant advancements for 1) treating patients suffering from neurological motor disorders such as Parkinson’s, essential tremor and dystonia; 2) controlling epileptic seizures; and 3) providing relief for people with chronic pain. In the last decade, DBS has been operationalized for the treatment of refractory neuropsychiatric disorders including Tourette’s syndrome, obsessive compulsive disorder and TRD. More recently, it has been suggested as a potential treatment for eating disorders and severe substance abuse addiction. Despite such advancements, there remains a gap in the existing experimental data investigating the mechanisms of chronic DBS in preclinical research as well as clinical trials.

The main biological, electrochemical and neurochemical effects of DBS are a result of electrical currents that flow into and out of neurological substrates, including cells, axons, dendrites and glial cells, leading to polarization of these elements. As it can be seen in Figure 2, the current is created by a pulse generator and is delivered to the targeted tissue via electrodes implanted in the brain.
Figure 2. An example of DBS device targeting the Thalamus. The pulse generator (or Neurostimulator) is similar to a pacemaker device where a battery is connected to an extension circuit to generate electrical signals that are delivered by the leads to the target regions. The clinician programs and adjusts the settings of the pulse generator externally. The lead is made up of a thin, insulated and coiled wires (which varies between manufacturer in its composition, size and design) that deliver stimulation to DBS regions.

Image sourced from WebMD, url: http://www.webmd.com/parkinsons-disease/guide/dbs-parkinsons

In an electrical circuit, stimulation current is delivered by means of at least one anode (positive) and cathode (negative) stimulation electrode. A negative stimulus depolarizes nearby neurons, whereas a positive stimulus causes nearby neurons to hyperpolarize (Gielen & Molnar, 2012). Stimulation current distributions that occur as a result of at least one cathode and at least one anode that do not interact physiologically are referred to as "monopolar". With bipolar stimulating electrodes, on the other hand, one tip is the anode and another, cathode, where the electric current is passed between the two electrodes that are close together. As a result, a current pathway is concentrated between these two electrodes leading to localized stimulation in the tissue immediately surrounding the electrodes. In applications designed to target tissue that is located further away, larger in size or protected from high-impedance of the dura, monopolar stimulation is implemented to
increase the stimulation distance (Figure 3.). To date, the field is moving towards optimizing closed-loop electrodes for personalized care in which the stimulation parameters can be adapted in response to physiological changes (Chang et al., 2013).

![Figure 3. Monopolar and bipolar electrode configuration. Image sourced from Hung, Goldberg, and Judy (2010) with permission.](image)

Despite its history of use, it is still unclear how electrical stimulation works to alleviate symptoms of treatment resistant disorders. Traditionally, DBS has been compared to the neural mechanism that result from neurosurgical lesions (Kringelbach, Jenkinson, Owen, & Aziz, 2007). Since then, the general question has evolved to whether DBS inhibits or excites neurons. Specifically, the properties of normal and diseased brain tissue depend on the presence of different types of neurons and supporting glial cells that all use different types of ion channels with variable voltage sensitivity (Kringelbach et al., 2007). As mentioned previously, many of the known neural processing principles are preserved across species and therefore, findings from DBS studies of movement disorders in rodents as well as nonhuman primates are highly relevant for elucidating the effects of DBS in humans. Altogether, preclinical evidence from neurophysiological/chemical recordings and neuroimaging data suggest that DBS helps to modulate oscillatory activity, the activity of postsynaptic neurons, neurotransmitter release and elicits changes associated with blood flow, blood oxygenation and consumption (Kringelbach et al., 2007).
Overall, previous literature pinpoints the most probable mechanistic model of DBS to a 'stimulation-induced modulation of pathological network activity'. Results are difficult to interpret, given varying experimental circumstances in animal models and between clinical trials, alongside our lack of knowledge on the neurobiology of depression. Yet, the weight of evidence suggest that DBS works by modulating an existing (dysfunctional) network of interacting brain regions. In other words, DBS may start exerting its antidepressant properties through the local effects of the DBS electrode on the neural activity in the DBS target, which is passed on to mono-and poly-synaptic network connections (Kringelbach et al., 2007). Together, DBS may achieve therapeutic responses through different mechanisms, depending on the physiological and geometric properties of the DBS target, the stimulation parameters and the connectivity of the affected region of the individual (specified to the neuroprogressive state of the patient).

**Fast Scan Cyclic Voltammetry principles**

As mentioned, DBS has been successful in testing a variety of neuropsychiatric disorders including TRD and there is a growing body of literature indicating that the therapeutic mechanisms of DBS include the regulation of monoamine transmission. Thus, the utilization of tools to measure neurotransmitter release in real-time, during DBS, is crucial. Fast scan cyclic voltammetry (FSCV) is an electrochemical technique that is used to monitor the release and uptake dynamics of endogenous monoamine levels in vitro and in vivo (Figure 4A &B). The background-subtracted cyclic voltammogram serves as a chemical signature to identity the neurochemical of interest. More recently, FSCV has been used to characterize naturally occurring DA signals. Phasic DA refers to transient DA release produced by the activation of DA neuron firing in response to behaviorally relevant stimuli. This large amplitude, but brief pulse of DA is proposed to activate postsynaptic DA receptors, but is rapidly removed from the synaptic space by fast, low-affinity/ high-capacity re-uptake systems before it can trigger homeostatic responses (Grace, 1991). These so-called “dopamine transients” typically exhibit an amplitude within the nanomolar range with a duration of
a few hundred milliseconds and occur with a baseline frequency of ~1 to 2 per minute (Robinson & Wightman, 2006). Their signals are considerably more difficult to record. DA oxidizes at + 0.6V which can be measured with FSCV by using carbon-fiber microelectrode. In order to mimic transient DA efflux in the NAc, we electrically evoked the Ventral Tegmental Area (VTA). Studies in animal models of depression have demonstrated that stress (physiologically relevant stimuli) potently activates mesoaccumbens DA neurons originating in the VTA and stimulates dopaminergic transmission to limbic brain regions.

Figure 4. A) oxidation and reduction of dopamine; B) quantification of evoked dopamine levels and confirmation of neurochemical specificity.

Wireless instantaneous neurotransmitter concentration sensing system (WINCS) is a small, battery-powered, wireless device which supports the electrochemical in vivo recording technique of FSCV, which is able to measure dopamine, glutamate, adenosine and serotonin. This device consists of 1) front-end analog circuit for FSCV (i.e. current-to-voltage transducer); 2) Bluetooth transceiver; 3) microprocessor; and 4) direct-current battery (Figure 5 A&B). WINCs is electrically connected to a brain-implanted microsensor and wireless linked to a home-based computer via Bluetooth. The temporal specificity provided by the use of WINCS is highlighted in Figure 5 C-E.
Figure 5. A) conceptual view of WINCS principles; B) WINCS circuit board (a: microprocessor, b: Bluetooth transceiver, c: leads for working electrode and reference electrode) with attached Ultralife battery; C) from top to bottom - applied triangle waveform, background current and DA, background-subtracted current; D) colour-plot of DA produced at homebased computer using WINCS analyzer; E) background-subtracted cyclic voltammogram and current measured at the peak oxidative potential for DA, plotted with time. Image sourced from Bledsoe et al. (2009) with permission.
Behavioral Paradigms

**Forced Swim Test (FST).** FST, also known as the behavioral despair test, was developed using rats to test for antidepressant screening. Rodents are forced to swim in a narrow space from which there is no escape route – an inescapable stressful condition. After an initial period of vigorous activity, a characteristic of immobile posture is evident where the rats move only when necessary to keep their heads above the water. As such, the FST has been established to have high predictive validity. To date, this behavioral paradigm has become a useful tool for screening novel antidepressants as well as alternative treatment methods (e.g. DBS and optogenetics).

The animals’ immobility is interpreted as indicating they had learned that escape was impossible and had to adopt an immobile position to conserve energy, viewed anthropomorphically as hopelessness from escaping this stressful situation (Castagné, Moser, Roux, & Porsolt, 2010). This immobile posture was therefore given the name “behavioral despair” and it was subsequently found that immobility time could be reduced by a wide range of clinically active antidepressants (Porsolt, Bertin, & Jalfre, 1977). However, the validity of this assumption has been heavily criticized. For example, a rat swimming in a cylinder is considered a non-ethologically relevant stressor and thus it may be inappropriate to relate results from this test to human depressive symptoms. Furthermore, considering immobility in terms of a shift from active coping to passivity is anthropomorphic. In fact, it has been suggested that energy conservation achieved by immobility may actually be a more efficient form of coping (West, 1990) and that immobility represents a switch from an active to passive coping strategy. Therefore, the FST may have some face validity but low construct validity (Powell, Fernandes, & Schalkwyk, 2012). Despite these limitations the FST has been regarded as an effective screening tool for novel drug discovery. Testing antidepressant-like behavioral effects using the FST therefore, gives an opportunity for our measures to be comparable to that of pre-existing DBS and pharmacotherapy outcomes.
**Open Field Test (OFT).** The OFT is a common measure of exploratory behavior and general activity in rats, where both the quality and quantity of the activity can be measured. Principally, the open field is an enclosure, generally square, rectangular or circular in shape with surrounding walls that prevent escape. The most basic and common outcome of interest is "movement"; however, this can be influenced by motor output, approach response to novelty, exploratory drive, freezing or other fear-related behavior, grooming, sickness, relative time in circadian cycle, among many other variables. Distance moved, time spent moving, rearing and change in activity over time are among many measures that can be tabulated and reported (Gould, Dao, & Kovacsics, 2009).

It is important to note that for experiments involving rodents, observers are not measuring the effects of treatments on exploration, but the effects on the reaction of the subjects to a stressful event. In other words, anxiolytic treatments do not themselves increase exploration in the open field but they decrease the stress-induced inhibition of exploration behavior.

The Open Field Test cannot claim predictive validity for anxiety in general, but this model seems to have good face and construct validity. Face validity implies that the anxiety response observed in the animal is identical to the one observed in humans (Prut & Belzung, 2003). In the open field, the observed behavior is avoidance of threatening places, which can also be observed in humans. In rodents, forced confrontation with novelty is stressful (Misslin & Cigrang, 1986). Stress induces anxiety-like behaviors, as it does in humans. So the model also fit construct validity (i.e. similar etiology).

**Western Blot Analysis**

Western blotting technique has made significant advances in both cell and molecular biology in the last three decades. It is used in research to separate and identify specific proteins from a complex mixture of proteins extracted from cells/tissues. In this technique, a mixture of
protein is separated based on a molecular weight and type through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. This membrane is then incubated with labels antibodies specific to the protein of interest. Once detected, the target protein will be visualized as a band on a blotting membrane, X-ray film or an imaging system. The aforementioned processes of the western blot technique is summarized in Figure 6.

Despite its overall simplicity, western blot analysis is a powerful tool that confirms the presence of proteins of interest. It can further be used to associate protein expression to corresponding behavior of animals in specific brain regions. The results achieved are also easy to analyze, interpret and unambiguous.

Figure 6. Overview of Western Blotting – separation of protein mixtures by electrophoresis; transfer to a blotting membrane; and detection of target protein, which only becomes visible in the final stage as a band similar to that shown in the far right. (‘Introduction of Western Blotting’ from www.abdserotec.com)

**Gene expression – microarray analysis**

Gene expression is a process by which information, the nucleotide sequence, of a gene is used in the protein synthesis (genetic transcription and translation). Therefore, gene expression analysis determines the pattern of gene expression changes at the level of genetic transcription in health and disease. Microarray analysis allow simultaneous measure of the expression level of thousands of genes within a particular mRNA sample (Tarca, Romero, & Draghici, 2006). Biological interpretations of large gene lists often presents many difficulties, of which, summarizing which
genes are associated with specific biological processes and ranking these processes by over-representation analysis; condensing repetitive or redundant annotation data; identifying functional biological modules consisting of related genes and terms; and viewing inter-relationships between groups of genes and groups of biologically relevant terms, are some major challenges (Alvord et al., 2007).

The database for visualization and annotation, visualization and integrated discovery (DAVID) is a clustering tool, providing a module centric approach for functional analysis of large gene lists to give relevant biological context. The advantages of this method of classifying groups of genes and terms into biological modules are: it largely reduces redundant results into manageable size; it is much easier to understand and visualize gene-to-gene, term-to-term where it is much easier to relate biological modules of interest to research questions (Alvord et al., 2007).

Specifically, it adopts kappa statistics, a chance-corrected measure of co-occurrence between two sets of categorized data, to statistically measure the annotation profile in a binary categorical scale. Kappa is more suitable than Pearson correlation, which is typically used for continuous variables.

**Mitochondrial Function Analysis**

The predominant physiological function of mitochondria is the generation of adenosine triphosphate (ATP) by oxidative phosphorylation, but additional functions include the generation and detoxification of reactive oxygen species, involvement in some forms of apoptosis, regulation of cytoplasmic and mitochondrial matrix calcium, synthesis and catabolism of metabolites and the transport of the organelles themselves to correct locations within the cell. Abnormality in any of these processes can be termed mitochondrial dysfunction. “Healthy” mitochondria under correctly designed incubation conditions show high respiratory control: a large increase in respiration rate with adenosine diphosphate (ADP) followed by a return to state 4. Mitochondrial respiratory control encapsulates the main function of mitochondria: their ability to idle at a low rate yet
respond to ADP by making ATP at a high rate. A lower respiratory control ratio (RCR) would indicate some sort of mitochondrial dysfunction that is either impeding ATP production, increasing uncoupled respiration, or both. A high RCR implies that the mitochondria have a high capacity for substrate oxidation and ATP turnover and a low proton leak. However, there is no absolute RCR value that is diagnostic of dysfunctional mitochondria, because values are substrate and tissue-dependent. Mitochondrial respiratory control is a complex function whose value depends on numerous factors, and this complexity is its main strength: a change in almost any aspect of oxidative phosphorylation will change RCR (Brand & Nicholls, 2011).

State 3 respiration refers to peak respiration following addition of excess DP and this is the best measure of mitochondrial capacity. State 4 respiration refers to the point where ADP becomes exhausted and the residual respiration at this point is largely uncoupled respiration. The mitochondrial RCR, defines as the respiration in state 3 \( \frac{ADP}{} \) divided by that in state 4 (state 3/state 4).

The Seahorse XF-24 Extracellular Flux Analyzer simultaneously interrogate the two major energy producing pathways of the cell—mitochondrial respiration and glycolysis in a microplate and analyzed it real-time. It is able assess oxidative phosphorylation (state 3, state 4 and RCR) by determining oxygen consumption rate and extracellular acidification rate \textit{in vitro}. As XF measures cellular bioenergetics on live cells, it enables time-relevant testing of multiple conditions per assays. Testing of more conditions with the same amount of sample called for this equipment, with increased throughput all the while using less sample compared to conventional respiratory techniques, together maximizes the value of the experiment.
Materials & Procedure

Animals

Male Wistar and Sprague Dawley rats arrived at 5 weeks of age for acclimatization prior to experimental procedures. These animals were transported to either the Mayo Clinic animal facility (from Harlan rodent colonies) or transported from the Howard Florey Laboratories facility to the Mental Health Research Institute (MHRI) Oak Street for subsequent experimental protocols. At both facilities animals had *ad libitum* access to rodent chow and tap water. The 12 hour light period of the circadian cycle was from 06:00 a.m. to 06:00 p.m., and all procedures were carried out during the light period. Additionally, humidity and temperature were also actively controlled. At the Mayo Clinic animal holding facility, rats were housed individually whereas at MHRI, rats were housed in groups of 4.

All experiments were conducted in accordance with the NIH guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee and the NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes for Chapter 7.

Four experimental cohorts were used in studies described in this thesis (Figure 7). The first three experimental cohorts were treated and tested at the Mayo Clinic animal facility where as the last cohort was treated and tested at the MHRI. The number of rats used in each cohort was based on availability of animals at the time of experiments and the number of animals per group required to detect significant group differences based on our previous experience with various paradigms, with n = 10-12 per group generally considered optimal to sufficiently power the analysis.
Figure 7. Experimental timelines of a). Lateral habenula deep brain stimulation (DBS) in an animal model of antidepressant resistance: a role for CaMKII, GSK3 and AMPK in stress response; b). Global transcriptional profiling of infralimbic deep brain stimulation in an animal model of antidepressant treatment resistance; c). Antidepressant and mania-like effects of nucleus accumbens deep brain stimulation in an animal model of treatment resistance; d). Region-and frequency-specific transient dopamine release as a mechanism of deep brain stimulation and Potentiation of accumbens dopamine is a therapeutic mechanism of lateral habenula deep brain stimulation in the antidepressant-resistant brain.

Acronyms: FST –the Forced Swim Test; OFT –Open Field Test; HF –High Frequency; ACTH – adrenocorticotrophic hormone; FSCV –Fast Scan Cyclic Voltammetry

**ACTH protocol**

The drugs used for ACTH-induced treatment resistance included: ACTH (AnaSpec, San Jose, California, USA) 100μg/day dissolved in distilled water and control vehicle consisting of 0.9% saline (Fisher Healthcare, Hanover Park, Illinois, USA). Animals were randomly selected to receive chronic administration of ACTH or Saline for 14 days (21 for Chapter 4) via intraperitoneal
injection. Antidepressant resistance was validated by imipramine hydrochloride (Sigma–Aldrich, St Louis, Missouri, USA; 10 mg/kg dissolved in 0.9% saline) administration prior to behavioral tests.

**Antibodies**

List of antibodies used for immunoblotting and in the validation process of the gene expression analysis are included in the following Table 1.

**Table 1. Optimised antibody details in rat cerebellum by the Translational Neuroscience lab**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Antibody</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaMKII</td>
<td>CaMKII (pan) #3362</td>
<td>Cell Signalling, MA, USA</td>
</tr>
<tr>
<td></td>
<td>phosphor-CaMKII (Thr286) #3361</td>
<td>Cell Signalling, MA, USA</td>
</tr>
<tr>
<td>GSK3</td>
<td>GSK3 α/β #5676</td>
<td>Cell Signalling, MA, USA</td>
</tr>
<tr>
<td></td>
<td>phosphor-GSK3 α/β #9331</td>
<td>Cell Signalling, MA, USA</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMPK #2532</td>
<td>Cell Signalling, MA, USA</td>
</tr>
<tr>
<td>mTOR</td>
<td>mTOR #2983</td>
<td>Cell Signalling, MA, USA</td>
</tr>
<tr>
<td>phosphor-mTOR</td>
<td>phosphor-mTOR #8238</td>
<td>Cell Signalling, MA, USA</td>
</tr>
<tr>
<td>TrkB</td>
<td>TrkB #sc-8316</td>
<td>Santa Cruz, TX, USA</td>
</tr>
<tr>
<td>phosphor-TrkB</td>
<td>phosphor-TrkB #sc8058</td>
<td>Santa Cruz, TX, USA</td>
</tr>
<tr>
<td>AKT</td>
<td>AKT (pan) #4685</td>
<td>Cell Signalling, MA, USA</td>
</tr>
<tr>
<td>phosphor-AKT</td>
<td>p-AKT (S473) #4060</td>
<td>Cell Signalling, MA, USA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAPDH #ab9484</td>
<td>Abcam, MA, USA</td>
</tr>
<tr>
<td>b-Actin</td>
<td>b-Actin #A5441</td>
<td>Sigma-Aldrich, MO, USA</td>
</tr>
</tbody>
</table>

Abbreviations include –phosphor: phosphorylated; CaMKII: Ca²⁺/calmodulin-dependent kinase II; GSK3: glycogen synthase kinase 3; AMPK: AMP-activated protein kinase; mTOR: mammalian target of rapamycin; BDNF: brain-derived neurotrophic factor; TrkB: tropomyosin receptor kinase B; AKT: protein kinase B; CREB: cAMP-response element binding protein; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; b-Actin: beta-actin.
Anesthesia

For experimental Chapters 4-6, animals were anesthetized with isoflurane (~1.5%, dosage range recommended to be between 0.25-2.5%; Baxter, IL, USA) inhalation via a nose cone that is connected to a vaporizer (Harvard Apparatus, MA, USA). General urethane anesthetic (1.5g/kg) given via intraperitoneal injection and local lidocaine analgesia (1cc -20% W/V dissolved in distilled water) injected on the scalp were used for long duration FSCV experiments.

Stereotactic surgery

Stereotaxic atlases are empirically based on a large group of rats of a specified sex, weight and strain. For this reason, we can confidently determine the stereotaxic position of each structure of interest. In rodent stereotaxy, this coordinate system originates from the point called the bregma, classically defined as the midpoint of the curve of best fit along the coronal suture (Paxinos & Watson, 2007). The anteroposterior (AP) axis is parallel to the midline plane of the skull and crosses the point called the lambda, defined as the midpoint of the curve of best fit along the lambdoid suture (Paxinos & Watson, 2007; Paxinos, Watson, Pennisi, & Topple, 1985). The mediolateral (ML) axis of the coordinate system is parallel to the interaural line, and the dorsoventral (DV) axis is perpendicular to the AP and ML axes. Figure 8 illustrates the bregma and lambda in rats.
The stereotaxic instrument was set up with all the materials needed. The area and instruments were clean and sterilized. Animals were weighed for their pre-surgery weight and prepared for surgery. Animals were placed on a heating pad to maintain optimal body temperature whilst being mounted onto the Kopf stereotaxic frame (Kopf Instruments, CA, USA). An anterior/posterior 1.5-2cm incision was made on the scalp with a sterile scalpel extending from the lambda to just in-between the eyes of the animal. From bregma, the correct coordinates needed for the placement of the probe (i.e. electrodes) was located with the aid of the stereotaxic atlas (Figure 9).
A pencil mark was made with a sterile pencil at this location on the skull; this is where the drill penetrated the skull. Drilling continued until the cannula can clear in a straight path. This step was repeated for all other electrode targets. Next, for stereotaxic surgery for deep brain stimulation, a drill was required to make three or four surface holes for skull screws using tweezers (example shown in Figure 10.). The twisted bipolar platinum iridium electrodes (5.6mm, 7mm or 10mm long depending on the DBS target, .075mm in diameter; Plastics One, Virginia, USA) were lowered according to the calculations made from the bregma. Bipolar electrode configuration was
used for the upcoming experimental chapters in order to control for current spread given small rat
brain regions. A small amount of liquid dental cement (~1ml) was mixed to cover the screws while
the bottom of the electrodes were held in place. A thicker batch (~2ml) was then made and covered
the whole area, enough to secure it. Once the dental cement was completely dry, the cannula that
was holding the electrodes in place were removed and the animal was also freed from the
apparatus. Antibacterial cream was applied all the way around the cement cap and animal were
placed in its own cage and monitored until it became conscious.

Figure 10. Jewelry screws were places on a scalp cap in order to firmly hold the
dental cement. This is also an example of 'surgery' animal conditions where
animals only undergoes surgery without electrodes placement.

**Deep Brain Stimulation device and application in animals**

Animals received electrical stimulation via either the ISO-Flex Master-8 (AMPI, Jerusalem,
Israel) or back-mounted DBS device (Deakin University, Geelong, AUS). ISO-Flex, for Chapter 5 and
6, were used for 3 day (130 Hz; 200μA; 90μSec) where animals were connected to a signal
generator through a constant current bipolar stimulus isolator unit with a stimulator cable. These
cables were routed to their home cage with enough room for the animals to move freely during the
stimulation phase.

In order to better reflect the longer duration of stimulation applied to the targeted brain
regions in clinical trials, the laboratory animal should ideally receive this brain stimulation
continuously without interruption. The devices that are connected to the implanted electrode through long insulated wires that run from the device to the animal’s head can be interrupted at times. For example, the stimulator can get disconnected from the electrode over the course of the study. To improve the quality of preclinical work and prevent possible mishap during the course of the experiment, a back-mountable, battery powered, micro DBS device was developed by (Kouzani, Abulseoud, Tye, Hosain, & Berk, 2013). A real-life picture of this device is included in Figure 11, alongside an example of animal in DBS group. Micro DBS was only used in Chapter 4 for NAc DBS, as it doubled the length in stimulation time (7 days).

![Sample of the single piece, back-mountable micro DBS device and an animal with 2 pieces in its backpack. Image sourced from Kouzani et al. (2013) with permission (left picture).](image)

**Fast Scan Cyclic Voltammetry experiments**

For the animals in Chapter 7, the same surgical procedure were implemented as described for DBS behavioral experiments. Adjustments for FSCV procedure includes, multiple bar holes drilled for implantation of the reference, stimulating and the carbon-fiber microelectrodes. Furthermore, jewelry screws were unnecessary as the animals were anesthetized and they were non-survival surgeries. Similar to DBS stereotaxic surgeries, the coordinates were derived from Paxinos and Watson (2007) rat atlas. The stimulating electrodes were inserted in DBS targets, whereas the carbon-fiber micro electrode was position in the NAc to obtain DA recordings. The
reference electrode was placed into superficial cortical tissue contralateral to the CFM and stimulating electrode. Stimulation was delivered via an optical isolator and programmable pulse generator (ISO-Flex/Master-8, AMPI). An example experimental set-up of FSCV is included below (Figure 12.)

Figure 12. IL, NAc and LHb DBS is connected to an ISo-Flex device. Carbon-fiber microelectrode (red) was placed in the NAc neurochemical dopamine recording.

**Behavioral Tests**

**FST.** In the FST, rats were placed in a 200 mm diameter, 600 mm high plexiglass cylinder (Cleversys Inc, Virginia, USA) filled with 25°C water to a depth of 18 cm for 6 min. The behavior of the animals was monitored and recorded with a video camera. The predominant behavior over each 2 sec interval was scored over 6 min. The animals’ behavior was recorded as immobile (passive coping) or mobile (active coping including swimming, escape and climbing patterns).
Figure 13 captures animals presenting with different coping strategies under this inescapable environmental stressor.

Figure 13. Rats placed in escapable FST apparatus. Example of swimming, indicative of mobility and antidepressant-like behaviors as opposed immobility which is interpreted as ‘passive’ coping mechanism. Image sourced from Abelaira, Reus, and Quevedo (2013) with permission.

**OFT.** For the purposes of our experiments, the OFT were used not only to observe animals’ anxiolytic behavior but to also remove any possibility of motor deficits that may confound the results of FST. The OFT apparatus, represented in Figure 14, consisted of a blue plexiglass box, 622.3mm x 622.3mm (CleverSys Inc.). Behavior was recorded with a video camera for 6 min where time spent in the box, ambulation (number of squares crossed), defecation, rearing and time spent grooming provide the measures of depression-related behavior.
Brain Harvest and Dissection

For Chapters 4-6, animals were euthanized by using FatalPlus (Vortech Pharmaceuticals, Dearborn, Michigan, USA; constituents: pentobarbital sodium 390 mg/ml; propylene glycol 0.01 mg/ml; ethyl alcohol 0.29 mg/ml; benzyl alcohol (preservative) 0.20 mg/ml) at ~0.70 cc. For Chapter 9, animals were culled through urethane overdose, delivered via intra-cardiac injections.

In the case for LHb and NAc DBS studies, brains were harvested 30 minutes following the FST, and slowly frozen on dry ice. Each brain regions of interest were then dissected out on an ice plate with dry ice. For NAc study, the medial prefrontal cortex was sliced and prepared for mitochondrial analysis at the time of brain harvest procedures. With IL DBS, brains were harvested 1 hr post FST. Brains were quick frozen by liquid nitrogen.

Lastly, brains were harvested and stored in formalin for histological verification of electrodes for the FSCV study in the last experimental chapter.
Western Blot Analysis

The unbound antibody is washed off leaving only the bound antibody to the protein of interest. These bound antibodies (and its corresponding bands) are then detected by developing a film or developed by using the ChemiDoc MP system, a full feature instrument for gel or western blot imaging. The thickness of the band corresponds to the amount of protein present.

After the proteins were extracted from different brain regions of interest (obtained from dissection and subsequently homogenized), the volume of the protein were prepared to 50 μg in each well. The samples are then diluted into a loading buffer with a tracking dye to allow identification of separation process. This is then heated in order to denature the higher order structure, which ensures that the negative charge of amino acids is not neutralized, enabling the protein to move in an electric field during the electrotransfer. Once the sample and gel preparation steps are completed, electrophoresis begins. Electrophoresis process allows protein to separate by their size through stacking and resolving gel. When proteins are loaded on the gel, they have a negative charge but they will travel toward the positive electrode when voltage (30mA/gel) is applied. Approximately after an hour of power supply, the dye front runs off to the bottom of the gel, leaving defined bands. These bands then need to be transferred to a membrane for quantification. The transfer is done using an electric field oriented perpendicular to the surface of the gels, causing the gels to migrate and appear on the membrane. Such blotting process includes a fiber pad (sponge) at each end and filter papers to protect the gel and the blotting membrane. Lastly, before quantification, blocking is a very important step for western blotting as it prevents antibodies from binding to the membrane nonspecifically. Blocking with a primary antibody is made with 5% skim milk in tri-buffered saline with tween 20 or 5% bovine serum albumin (BSA) for 1 hour and incubated overnight on a shaker in 4 degrees. The membrane is then washed and blocked with a secondary antibody. In order to visualize the membrane, prepared ECL mix is
incubated with the membrane for 1-2 minutes. For quantification, the membranes were visualized in the dark room or by using ChemiDoc™ touch imaging system (Bio-Rad, CA, USA).

**Microarray Analysis**

**RNA extractions and Microarrays.** Total RNA was extracted from brain tissue using TRIzol reagent (Invitrogen, Melbourne, Australia), and purified using RNeasy-Mini Kit (Qiagen, Mannheim, Germany). RNA quality and quantity was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and RNA6000 NanoAssay Kit (Agilent, Melbourne, Australia). Fluorescently-labelled cDNA was prepared from 800ng RNA using Agilent’s Quick-Amp Labelling and One-Color RNA Spike-In kits. Cyanine 3-CTP-labelled cDNA was hybridized for 17h to Agilent Whole Rat Genome (4x44k) Oligonucleotide Microarray Slides using Agilent Gene Expression Hybridization kit. Microarray fluorescent images were acquired using GenePix 4000B scanner, with data extraction performed via GenePix 5.1 software (Molecular Devices, Melbourne, VIC, Australia). Normalization and primary analysis of microarray data was performed using Aqutiy 4 software (Molecular Devices), as previously described (Carey et al., 2006). Fluorescent reading for duplicate genes were averaged. Each array dataset sample was normalized so that the median expression value in each array was 1.0.

**Gene Set Enrichment Analysis using DAVID.** Unpaired t-tests were used to identify genes, from the normalized microarray data, with evidence of differential expression between treatment groups. The complete gene lists of putative differentially expressed genes were imported into the online DAVID software package (version 6.7; National Institute of Allergy and Infectious Diseases, MD, USA). Using the functional annotation tools, the data was analyzed to determine enrichment values for defined Kyoto Encyclopedia of Genes and Genomes pathways. Pathways were selected based upon significant enrichment using Fisher’s exact statistical analysis (p <0.05) and fold enrichment score ≥1.5.

**Gene Validation.** Validation of pivotal genes within pathways was performed with
alternative sets of treated animals by semi-quantitative real-time PCR and/or immunoblotting.

**RT-PCR.** Total RNA was extracted from the dissected brain region using the RNeasy-Plus Mini Kit, as per manufacturer’s directions. Amount of RNA was quantified using Nanodrop and 1μg was used for cDNA synthesis using iScript reverse transcriptase, as per manufacturer’s instructions. cDNA was aliquoted and sealed in plates and frozen at -20°C immediately. iQSYBR Green supermix was used for RT-PCR per manufacturer’s directions. For the RT-PCR reaction, 1ml cDNA and 300nM final concentration of each of the relevant Forward and Reverse primers were utilized. The PCR conditions were 95°C for 3 min and 39 cycles of 95°C for 10 sec and 58°C for 30 sec, followed by melt curve 65°C for 5 sec and 95°C for 5 sec. RT-PCR results were normalised to reference gene Hprt2. Relative gene expression was calculated as 2-ΔCt, where Ct is threshold cycle. All sequences were designed using Beacon Designer 7.2 (Premier Biosoft International, CA, USA).

**Mitochondrial Function Analysis**

**Freezing of tissue for mitochondrial isolation.** The PFC tissue was removed immediately and immersed into 4mls of cold isolation buffer (Mannitol 200mM, Sucrose 50mM, Potassium Phosphate 5mM, EGTA 1mM, MOPS 5mM, and Bovine Serum Albumin 0.10% pH 7.2). The tissue was then minced (while in solution) with a pair of scissors. The buffer was tipped to decant the original buffer and 2mls of fresh cold isolation buffer containing 20% DMSO was added. Samples were then frozen on dry ice. Samples were stored at -80°C for later isolation of mitochondria.

**Tissue analysis.** To isolate mitochondria from PFC samples, the tissue was rapidly thawed before being homogenized first with a Teflon homogenizer and then with a handheld homogenizer for 2 x 10 sec at the lowest speed setting. Homogenates were then spun in a centrifuge at 800g for 5 min at 4°C. The supernatants were then spun in a centrifuge at 10,000g for 10 min at 4°C. The mitochondrial enriched fraction was then re-suspended in mitochondrial assay solution (MAS; 70mM sucrose, 220 mM mannitol, 5mM KH2PO4, 5mM MgCl₂, 2mM HEPES, 1mM EGTA, 0.2% fatty
acid-free BSA) and protein content was determined by the BCA method. 2.5µg of mitochondrial protein in 50µL was added to 24-well Seahorse V7 plates in triplicate. Mitochondrial function was assessed in MAS supplemented with 5.5mM succinate and 2.2mM rotenone using the Seahorse XF24 Analyzer at 37°C. Multiple cycles of 60 sec mix and 4 min measure were used to establish state III and state IV respiration following injection of 2mM ADP. Respiration rates were determined in point by point mode.

Statistical Analysis

All statistics presented herein, were analyzed using IBM SPSS Statistics 22 and illustrated as figures using GraphPad Prism 6. An entire animal was removed from analysis when one (or more) data point was ± 2 standard deviations. Independent samples t-tests were conducted to examine significant differences between 2 variables. For more than 2 means, one-way analysis of variance or multivariate analysis of variance were conducted. In order to observe meaning association between variables, Pearson correlation was used. Data were tested for normality and homogeneity of variance prior to subsequent inferential statistics.
References


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Chapter 4. Nucleus accumbens deep brain stimulation efficacy in ACTH pre-treated rats: alterations in mitochondrial function relate to antidepressant rats

Manuscript under review in Translational Psychiatry
Nucleus accumbens deep brain stimulation efficacy in ACTH pre-treated rats: alterations in mitochondrial function relate to antidepressant-like effects

Running title: NAc DBS, Mitochondria and Mood

Yesul Kim 1,2, Sean McGee 3,4, Juliane K. Czeczor 3, Adam J Walker 1,2, Rajas Kale 2,5, Abbas Kouzani 5, Ken Walder 3, Michael Berk 6 & Susannah J Tye 1, 1,2,7,8.

1 Deakin University, School of Psychology, Faculty of Health, Melbourne Burwood Campus, Victoria, Australia
2 Department of Psychiatry & Psychology, Mayo Clinic, Minnesota, USA
3 Deakin University, Centre for Molecular and Medical Research, School of Medicine, Faculty of Health, Geelong Waurn Ponds Campus, Victoria, Australia
4 Metabolism and Inflammation Program, Baker IDI Heart and Diabetes Institute, Melbourne, Victoria, Australia
5 Deakin University, School of Engineering, Faculty of Science Engineering & Built Environment, Geelong Waurn Ponds Campus, Victoria, Australia
6 Deakin University, IMPACT Strategic Research Centre, School of Medicine, Faculty of Health, Geelong, Victoria, Australia
7 Department of Molecular Pharmacology & Experimental Therapeutics, Mayo Clinic, Minnesota, USA
8 Department of Psychiatry, University of Minnesota, Minnesota, USA

Present Address:

Corresponding Author 1: Susannah J. Tye
Corresponding Address 1: Department of Psychiatry & Psychology
Mayo Clinic
200 First St SW
Rochester MN 55905
USA
Corresponding Phone 1: +1 507-255-4322
Corresponding Fax 1: +1 507-284-3933
Corresponding E-mail 1: tye.susannah@mayo.edu
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Abstract

Mitochondrial dysfunction plays a critical role in the pathophysiology of mood disorders and treatment response. To investigate this, we established an animal model exhibiting a state of antidepressant treatment resistance in male Wistar rats using 21 days of adrenocorticotropic hormone (ACTH) administration (100μg /d). Firstly, the effect of ACTH treatment on the efficacy of imipramine (10mg/kg) was investigated alongside its effect on prefrontal cortex (PFC) mitochondrial function. Secondly, we examined the mood regulatory actions of chronic (7 day) high frequency nucleus accumbens (NAc) deep brain stimulation (DBS; 130 Hz, 100μA, 90μSec) and concomitant PFC mitochondrial function. Antidepressant-like responses were assessed in the open field test (OFT) and forced swim test (FST) for both conditions. ACTH pre-treatment prevented imipramine-mediated improvement in mobility during the FST ($p < 0.05$). NAc DBS effectively improved FST mobility in ACTH-treated animals ($p < 0.05$). No improvements in mobility was observed for sham control animals ($p > 0.05$). Analyses of PFC mitochondrial function revealed that ACTH-treated animals had decreased capacity for ATP production compared to controls. In contrast, ACTH animals following NAc DBS demonstrated greater mitochondrial function relative to controls. Interestingly, a proportion (30%) of the ACTH-treated animals exhibited heightened locomotor activity in the OFT and exaggerated escape behaviors during the FST, together with general hyperactivity in their home-cage settings. More importantly, the induction of this mania-like phenotype was accompanied by over-compensative increased mitochondrial respiration. Manifestation of a DBS induced mania-like phenotype in imipramine resistant animals highlights the potential use of this model in elucidating mechanisms of mood dysregulation.
Symptomatically, depressive periods are characterized by decreased energy, paralleled by reduced brain energy generation (1, 2). This is highlighted by clinical studies examining glucose utilization, blood flow and energy metabolites in relevant depressive cohorts (3-5). Mitochondrial function plays a central role in energy regulation, where it is directly influenced by glucocorticoids, oxidative stress, inflammation and antidepressants (6-9). In turn, mitochondrial function regulates not only catabolic processes, but also production of free radicals and apoptotic processes for cellular homeostasis (10). Mitochondrial dysfunction may therefore lead to disruption of adaptive neural plasticity and an imbalanced cellular response to stressors. Notably, efficacious pharmacotherapies including antidepressants, lithium and electroconvulsive therapy all seemingly affect mitochondrial function (11-14). One of the most important functions of mitochondria is the generation of adenosine triphosphate (ATP), the molecular unit of intracellular energy, which powers numerous cellular processes and is needed to recover from insults including oxidative and nitrosative stress. This is essential for the maintenance of the above mentioned processes including cellular integrity and neural plasticity.

The link between major depressive disorder (MDD), bipolar disorder (BD) and mitochondrial dysfunction has been under-recognized. The brain, as an intensely energy-dependent tissue, is particularly vulnerable to the effects of mitochondrial dysfunction - primary mitochondrial disorders are evidenced by neurological symptoms (6). A small but growing body of preclinical and clinical research, including proteomic analysis (15, 16), mitochondrial DNA data (17) and imaging techniques (5) support a role for mitochondria in neuroprogressive processes that may occur in treatment resistant depression (TRD).

The heterogeneity of MDD and the lack of animal models displaying a treatment resistance phenotype is a challenge (18). Our preliminary model of TRD (19, 20) includes typical physiological characteristics of the clinical MDD population, namely disturbances in the hypothalamus-pituitary-
adrenal axis, brain mitochondrial function, dopamine transmission and antidepressant resistance. The nucleus accumbens (NAc), a key dopaminergic terminal region of the limbic system, is a neurosurgical target for antidepressant modulation (21, 22). In our earlier work, NAc deep brain stimulation (DBS) has been shown to increase dopamine efflux in animals resistant to imipramine (23-26). Direct electrical stimulation of the NAc region altered depression-related behaviors and restored an antidepressant response (21, 24, 27-29). Leveraging off this, investigation of the antidepressant and mania inducing effects of NAc DBS in relevant animal models with face validity at the levels of neuronal circuitry, biochemistry and phenomenology, has the potential to increase our understanding of the role of the underlying neurobiology, particularly mitochondrial function, in antidepressant treatment response.

In this study, we used chronic adrenocorticotropic hormone (ACTH) treatment to investigate the efficacy of NAc DBS in a cohort of tricyclic antidepressant-resistant animals with the behavioral changes assessed using the forced swim test and open field test. The aim of this study was to investigate mitochondrial function in an animal model with face validity and its potential association with treatment efficacy.

**Materials and Methods**

All experiments were conducted in accordance with the NIH guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC).

**Animals**

This study utilized male albino Wistar rats weighing 200-400g. Animals were housed individually, in controlled temperatures (20-22°C) on a 12h light-dark cycle. Food and water were available *ad libitum*. Animals entered the study at approximately six weeks of age following a week-long
acclimatization period, and completed testing in their tenth week, at which point they were
sacrificed. Animals were randomly assigned into one of 8 groups, under 2 conditions ‘Vehicle’ and
‘ACTH’ using simple randomization. Group sizes were justified using software G*Power 3.1 for an
estimated medium effect, with a power of 0.95. Control (CTRL) groups under each condition had $n$ of
10 whereas surgery groups sized $n = 15$ to account for potential ‘drop-outs’. Some animals had to be
removed over the course of study for health reasons (e.g. head-cap failure) and were thus removed
from the analysis. As a result, final group numbers at the end of phase 2 were as follows: for ‘Vehicle
($N = 41$)’ condition including CTRL ($n = 10$), SURG ($n = 12$), SHAM ($n = 10$), DBS ($n = 9$); ‘ACTH ($N = 44$)’
including CTRL ($n = 10$), SURG ($n = 11$), SHAM ($n = 9$), DBS ($n = 14$). All procedures were carried out in
accordance with the National Institute of Health Guide for care and use of laboratory animals (NIH
publications no.80-23) and were approved by the Institutional Animal Care and Use Committee of
the Mayo Clinic.

Drugs

The drugs used in this experiment included: isoflurane (~1.5%), adrenocorticotropic hormone
ACTH 1-24 (AnaSpec, San Jose, California, USA), 100μg/day dissolved in distilled water; imipramine
hydrochloride (Sigma–Aldrich, St Louis, Missouri, USA) 10 mg/kg dissolved in 0.9% saline; control
vehicle 0.9% saline (Fisher Healthcare, Hanover Park, Illinois, USA); and FatalPlus (Vortech
Pharmaceuticals, Dearborn, Michigan, USA), (constituents: pentobarbital sodium 390 mg/ml;
propylene glycol 0.01 mg/ml; ethyl alcohol 0.29 mg/ml; benzyl alcohol (preservative) 0.20 mg/ml)
0.70 cc. Drugs were all administered via intraperitoneal injection apart from isoflurane which was
delivered via inhalation.

Anesthesia and intra-operative monitoring

The animals were anaesthetized with isoflurane inhalation in an induction chamber and maintained
with a nose cone (World Precision Instruments, FL). The concentration of isoflurane ranged between
1-3% to ensure adequate sedation of the rat during surgery. Once anaesthetized, the animal was placed in a stereotaxic frame (Model 1430, David Kopf Instruments, CA). The skull was secured with a nose clamp, incisor bar and ear bars. Constant body temperature (36.5°C) was maintained using a heat pad, and the animal's body temperature was measured using a digital thermometer placed under the abdomen. While under general anesthesia, the animal was monitored by respiratory rate and hind-paw pinch.

**Stereotactic Surgery**

A midline 1.5-2cm incision was made starting just caudal to the eyes and ending just rostral to the ears to expose the two skull landmarks: bregma and lambda. A 2mm diameter trephine hole was broached in the skull at the site corresponding to the targets. The dura matter was opened using a fine needle. Then stimulating electrodes (twisted bipolar platinum iridum electrodes, 10mm long, .075mm in diameter; Plastics One, Virginia, USA) were placed bilaterally in the NAc core targets (antero-posterior +1.5, medial-lateral ±1.5, dorsal-ventral -7.0 from the bregma) using stereotactic coordinates (40).

The electrodes were secured in place using dental cement. Four small jeweler screws were also placed in the skull surface to prevent sliding of the dental cement over the surface of the skull during the post-operative period.

**Deep brain stimulation**

The electrodes were connected to a back-mounted DBS device and the battery connected (both the device and the battery were secured in a rodent jacket (Harvard Apparatus; Massachusetts, USA)) so that the animals could not disturb the device. Animals remained awake throughout the course of DBS and were closely observed for the first hour of stimulation. We had not previously observed any adverse effects of DBS of the NAc using the currents applied in this study (130 Hz, 90 μS, 100 μA).
DBS was continuously delivered for 7 days via the back-mounted DBS device. This occurred in the animal’s homecage and the animal was free to move about the cage without any restriction.

**Behavioral Testing**

*Open field test (OFT).* The arenas used were 622.3mm x 622.3mm (CleverSys Inc., Virginia, USA). Animals were each placed in the central zone of the arena, and allowed to move freely for 6 minutes. Their behavior was recorded by video camera. Data was then analyzed ‘blind’ using the behavioral analysis package CleverSys TopScan. Open field test was used to quantify locomotor and exploratory activities by observing the animals.

*Porsolt forced swim test (FST).* The forced swim apparatus (CleverSys Inc., Virginia, USA) was used in this experiment (dimensions: 600 mm height x 200 mm diameter) and filled with tap water (25±1°C) to a depth of 25 cm. Animals were exposed to a 15 minutes learning trial, conducted two hours after the OFT. A 6 minutes test session was then conducted on the subsequent day. Sessions were recorded by a video camera, and then analyzed ‘blind’ using the behavioral analysis package CleverSys ForcedSwimScan (CleverSys Inc., Virginia, USA) and validated with hand scoring. The behaviors of interest included ‘immobility’ (passive coping behavior), ‘swimming’ and ‘climbing’ (active coping behavior).

**Experimental procedure**

After 7 days of acclimatization, electrodes were implanted bilaterally into the NAc using stereotactic surgical procedures. Following recovery from surgery (3 days) animals first received 14 days of injections of either saline (0.9%) or ACTH-(1-24) (100μg/d). In order to test for antidepressant efficacy in the ACTH model, animals were challenged with imipramine (10mg/kg) on the 14th day for the OFT. Two hours post-test, animals received their initial forced swim stress exposure for 15
minutes. Then a FST was conducted on day 15 (6 minutes), 30 minutes after the second imipramine administration (Figure 1a).

DBS Animals received high-frequency NAc DBS (130Hz, 100μA, 90μs) after another 7 days of ACTH or saline treatment, using a back mounted DBS device (devices were off during behavior). Behavioral tests were recorded for subsequent analysis. Final OFT, FST training and FST were conducted on day 21 and 22 to investigate the efficacy of DBS (Figure 1a). Animals were sacrificed 30 minutes after FST, and brains were harvested and cardiac blood samples were collected. Isolated plasma aliquots of cardiac blood (centrifuged at 3300g for 10 minutes) were taken, and stored at -80°C.

Euthanasia and Dissection

Rats were euthanized by an IP overdose of pentobarbital (20mg/kg). The brain was then dissected out of the skull. The prefrontal cortex (PFC) was removed and prepared for mitochondrial isolation. The remaining portion of the brain was frozen by placing on dry ice, followed by storage at -80°C.

Immunohistochemistry

The remaining portion of brain was embedding using Cryo-M-Bed (A-M Systems 527738). 6mm coronal plane sections were taken of the NAc. The sections were fixed using ice-cold acetone for 10 minutes, followed by 10 minutes of drying time.

Hematoxylin and Eosin Staining. After fixation and drying, the slides were then incubated in phosphate buffered saline for 10 minutes, followed by 30-second incubation in hematoxylin stain, then rinsed under running water for 5 minutes. The following dips were done: Blueing 4 dips, water 10 dips, water 10 dips, 95% Ethanol 4 dips, Eosin 6 dips, 50% Ethanol 12 dips, 70% Ethanol 12 dips, 95% Ethanol 12 dips, Absolute Ethanol 12 dips, Xylene 12 dips. Slides were allowed to completely dry before preserving in Vecta-mount (Vector Laboratories LTD, CA, USA) and coverslipping. Refer to Figure 1b for electrode tract images.
Freezing of tissue for mitochondrial isolation

The PFC tissue was removed immediately and immersed into 4 ml of 4°C isolation buffer (Mannitol 200mM, Sucrose 50mM, Potassium Phosphate 5mM, EGTA 1mM, MOPS 5mM, and Bovine Serum Albumin 0.10% pH 7.2). The tissue was then minced (while in solution) with a pair of scissors. The buffer was tipped to decant the original buffer and 2 ml of 4°C isolation buffer containing 20% DMSO was added. Samples were then frozen on dry ice. Samples were stored at -80°C for later isolation of mitochondria.

Mitochondrial function analysis

To isolate mitochondria from PFC samples, the tissue was rapidly thawed before being homogenized first with a Teflon homogenizer and then with a handheld homogenizer for 2 x 10 sec at the lowest speed setting. Homogenates were then spun in a centrifuge at 800g for 5 min at 4°C. The supernatants were then spun in a centrifuge at 10,000g for 10 min at 4°C. The mitochondrial enriched fraction was then re-suspended in mitochondrial assay solution (MAS; 70mM sucrose, 220 mM mannitol, 5mM KH₂PO₄, 5mM MgCl₂, 2mM HEPES, 1mM EGTA, 0.2% fatty acid-free BSA) and protein content was determined by the BCA method. 2.5μg of mitochondrial protein in 50μL MAS was added to 24-well Seahorse V7 plates in triplicate. Mitochondrial function was assessed in MAS supplemented with 5.5mM succinate and 2.2mM rotenone using the Seahorse XF24 Analyzer at 37°C. Multiple cycles of 60 sec mix and 4 min measure were used to establish state III and state IV respiration following injection of 2mM ADP. Respiration rates were determined in point by point mode.

Statistical analyses

In all instances, the Shapiro-Wilk test of normality and Levene’s test for homogeneity of variance were utilized. Data points more than 2 standard deviations from the mean, were deemed ‘extreme’ outliers and were excluded from the analysis. When data were normally distributed, one-way
analysis of variance (ANOVA) was conducted, where it was followed by Sidak’s post-hoc tests. When these assumptions were violated however, the data were then analyzed by non-parametric Kruskal-Wallis one-way tests. For experimental questions with only two specific groups of interest, an independent $t$ test was used. Statistical significance was set at $\alpha = 0.05$. Analyses were performed using GraphPad Prism 6.0 and IBM SPSS package 22.0.

Results and Discussions

1 a). Validation of Antidepressant Resistance

The first phase of the experiment (Figure 1a) started with 2 groups of animals (with no surgery) receiving daily administration for 14 days of either ACTH (100μg/d, $n = 12$) or vehicle (water, $n = 8$). These animals were then further randomly allocated into 4 groups (2 under each condition where $n = 4-6$) for imipramine (10 μg/kg/d) or saline treatment in order to measure response to this tricyclic antidepressant.

Antidepressant resistance to imipramine was demonstrated in our ACTH-treated animal model. No differences in locomotor activity were detected between groups, precluding a motor component in the observed effects in the FST (S1 Figure 1).

With respect to immobility time in the FST, one way ANOVA revealed a statistically significant difference between the 4 treatment conditions ($p < 0.05$). ACTH treatment increased immobility time in the FST and this was not reversed by treatment with imipramine (Figure 2a). Therefore, administration of ACTH induced a model of treatment resistant depression in these rats.
b). Reduced mitochondrial function in ACTH treated animals

In order to quantify mitochondrial function, respiration analyses were performed on mitochondria isolated from the prefrontal cortex (PFC) using a Seahorse XF Analyzer.

State 3 respiration, the peak respiration achieved following addition of excess adenosine diphosphate, is a widely accepted measure of mitochondrial capacity. An unpaired t-test revealed a statistically significant difference in state 3 respiration, indicating that ACTH treatment reduced the capacity for ATP production \((p < 0.05; \text{Figure 2b}).\)

[Figure 2]

2) a). NAc DBS efficacy in imipramine resistant animals

The second phase of the experiment included animals selected to receive continuous DBS stimulation for 7 days. The SHAM group denotes animals with electrode placement in the NAc with no electrical stimulation, whereas animals within the DBS group had active stimulation to the NAc. The SURG group indicates animals with 4 jewelry screws placed on the skull cap. CTRL condition refers to animals with no surgical procedures and only receiving the drug (or vehicle) administration. In total, this phase included 8 groups, 4 (DBS, SHAM, SURG, CTRL) each belonging to either ACTH or vehicle treatment condition \((n = 6-12).\)

No differences in locomotion during the OFT between groups suggests that there was no motor impairment or damage to confound findings in the FST analyses \((p > 0.05; \text{S2}).\)

The FST was used to investigate the antidepressant efficacy of NAc DBS after 7 days of uninterrupted stimulation in animals presumed non-responsive to imipramine treatment. When compared to the
ACTH group's phase 1 immobility scores following imipramine administration, there was a 76% reduction in their immobility time after continuous high frequency stimulation. A one way ANOVA revealed a statistically significant difference between ACTH treated groups in their final FST ($p < 0.05$; Figure 3a). In ACTH treated rats, NAc DBS decreased immobility time in FST by 54% compared with controls ($p < 0.05$).

In contrast, saline animals with DBS did not display significant reductions in their immobility time compared with that of the saline control group (Figure 3a).

![Figure 3](image_url)

b). Mitochondrial function and antidepressant response

Respiratory control ratio (RCR) is the ratio of state 3 to state 4 respiration and measures the coupling efficiency of mitochondria. A lower RCR would indicate mitochondrial dysfunction that is either impeding ATP production, increasing uncoupled respiration, or both. NAc DBS increased RCR in ACTH treated rats compared with the SHAM and control groups ($p < 0.05$; Figure 3b). No differences were seen in the vehicle treated rats.

3. Heightened locomotor activity in ACTH treated animals following DBS electrode placement

When analyzing OFT data, it was evident that there was a bimodal distribution, with a subset of rats (in the DBS and SHAM groups, $n=7$) exhibiting markedly increased activity levels in terms of distance travelled. We selected this subset based on the distance travelled greater than mean + 1 standard deviation of the control (vehicle) animals ($M = 29014$, $SD = 5372$). Any animals with OFT trial performance beyond this point were considered hyperactive.
Given violations to a test of normality ($W = 0.701, p < 0.05$) and tests of homogeneity of variance ($F_{8,71} = 4.838, p < 0.05$), a Kruskal-Wallis test was used to confirm a significant group effect where hyperactive animals travelled the furthest during the OFT ($p < 0.05$; Figure 4a).

The OFT results together with representative trace image (Figure 4b) clearly distinguish animals with heightened locomotor behavioral patterns that were followed by extreme application of models of active coping strategies in the FST including persistent diving and escape actions ($p < 0.05$, Figure 4b). These behaviors persisted and were recognizable in their home-cage setting (see SI Video 1 & 2).

Interestingly, when examining the RCR (state3/state4), we observed an increase in mitochondrial function in the hyperactive rats relative to other groups pre-treated with ACTH ($p < 0.05$; Figure 4c).

This study aimed to examine the mechanisms underlying TRD and antidepressant activity in terms of mitochondrial function. Firstly, the data provide evidence for treatment resistance to imipramine, induced by a chronic administration of ACTH. Secondly, our current findings show reduced mitochondrial capacity in treatment resistant ACTH-treated animals which was restored by continuous NAc DBS for 7 days. Concurrently, NAc DBS reduced immobility time in the FST. Lastly, a subgroup of ACTH animals developed a mania-like phenotype, suggested by their adoption of increased behavioral patterns alongside heightened mitochondrial function. Together, these results support a biphasic role for mitochondrial function in regulation of both depressive and manic mood states and additionally reflecting treatment response.
Chronic administration of ACTH interfered with the treatment response to a tricyclic antidepressant and lowered mitochondrial state 3 respiration rate, indicative of reduced ATP production capacity. One possible mechanism whereby ACTH contributes to this dysfunction may be through adaptations of the HPA axis, which has consistently been shown to be hyperactive in patients suffering from depression (30). Daily administration of ACTH blocks antidepressant efficacy and alters key PFC monoamine concentration following stress, and down-regulates glucocorticoid responses (19). Mitochondria participate in stress responses in part by sensing levels of glucocorticoids. Receptors for glucocorticoids exist within the mitochondria, or translocate from the cytoplasm into mitochondria in the presence of their ligand, endowing these organelles with the ability to sense and readily respond during acute stress (31).

We were secondly able to reverse the imipramine resistant effects of ACTH using bilateral NAc DBS. The efficacy of NAc DBS in a model of treatment resistance was observed using well validated behavioral paradigms. These findings corroborate with previous literature on the potential therapeutic effects of NAc DBS for TRD (27, 29, 32). Following 1 week of DBS, animals previously resistant to tricyclic antidepressant responded, indicated by reduced utilization of passive coping strategies during the FST. The FST is established as a valid tool for screening antidepressant effects, specifically observing the animals' behavioral despair and helplessness under stress.

Mitochondrial dysfunction can be regarded as the inability of mitochondria to appropriately produce ATP in response to energy demands. 'Healthy' mitochondria under standard conditions have a high RCR; in other words, a large increase in respiration rate with ADP administration followed by a return to state 4. Mitochondrial respiratory control encapsulates one of the main functions of mitochondria, which is their ability to idle at a low rate yet respond to ADP triggered demand by generating ATP at a high rate. ACTH-treated rats had significantly lower RCR, suggestive of mitochondrial dysfunction. Notably, a high RCR was found in ACTH pretreated animals following DBS
treatment compared to ACTH SHAM and ACTH CTRL, suggesting that mitochondrial efficiency is increased with NAc DBS. As values are substrate and tissue dependent, there is no absolute RCR value that is diagnostic of dysfunctional mitochondria. Mitochondrial respiratory control is a complex function whose values respond to numerous factors and this complexity is its main strength: a change in almost any aspect of oxidative phosphorylation will change RCR (33).

Additional physiological functions of mitochondria include the generation and detoxification of reactive oxygen species, involvement in apoptosis, regulation of cytoplasmic and mitochondrial matrix calcium, synthesis and catabolism of metabolites and the transport of the organelles themselves to the correct location within the cell (33). Notably, many of these functions are altered in mood disorders, including dysregulated intracellular calcium, increased oxidative stress and apoptosis (34, 35). From these results, it seems likely that mitochondria play a role in the neuroprogression of MDD and in antidepressant treatment response.

Increasingly, emerging data show an inextricable interconnection between oxidative stress and inflammatory responses (Morris & Berk, 2015). Recent meta-analysis indicated globally increased cell mediated immunity and macrophage activities (Dowlati et al., 2010; Liu et al., 2010; Maes et al., 2012). In light of converging findings, the activation of immuno-inflammatory pathways, and oxidative stress coupled with mitochondrial dysfunction may be strong contributors to the progression and persistence of TRD.

Finally, following NAc DBS, we observed a subgroup of animals with markedly higher locomotor activity, exemplified by increased rates of active climbing and frequent diving. These animals firstly exhibited such a phenotype with electrode implantation and ACTH administration, which was further exacerbated by active DBS stimulation. Animals not receiving NAc DBS preferentially implemented passive coping strategies in the FST. Mania-like activity included persistent interest in novel stimuli and areas outside their provided enclosure as well as erratic
motor movements. In addition, these home cage behavioral patterns were also evident in their performance during behavioral tests. Notably, a high RCR was found for these animals, offering a possible over-compensatory mechanism where increased mitochondrial function is responsive to the great energy demands. Overall these observations, together with resistance to imipramine, are indicative of induction of a mania-like phenotype in a vulnerable subset of animals following HPA stimulation and a physical disruption to the dopaminergic mesoaccumbens pathway. Future studies will need to further quantify these mania-like characteristics, investigate the fine grained biological mechanisms mediating such an effect and determine factors predisposing to vulnerability for this behavioral phenotype. But this paradigm does suggest a putative animal model of mania with face validity from the perspective of neurocircuitry and neurochemistry.

It is important to emphasize that neither unipolar nor bipolar depression are necessarily classic (or primary) mitochondrial disorders. While noting that some people with primary mitochondrial disorders like MELAS have very high rates of bipolar like symptoms (36), by and large those with bipolar disorder do not exhibit the symptoms of classic mitochondrial disorders. Instead, emerging data suggest that upstream abnormalities (likely encoded in the nucleus) converge at mitochondrial function (37), leading to altered synaptic plasticity and impaired cellular resilience (38). Given the biphassic nature of bipolar disorder, it is hypothesized that the disorder represents a bi-directional state dependent alteration in mitochondrial regulation (39). Our data presented here suggest that it may be possible to develop a new model of bipolar disorder with a biphasic mitochondrial and behavioral response to ACTH administration.

The results of this study should be considered in light of limitations shared by many translational models of psychiatric disorders. Firstly, the models such as the FST alone may not be sufficient to provide strong predictive validity of antidepressant-like effects of DBS. Secondly, animals do not have genotype/phenotype features that may resemble all clinical depression-like and
bipolar states before testing, apart from their resistance to imipramine and heightened locomotor activities. Despite these aforementioned caveats, preclinical models are integral for the study of DBS efficacy, and offer mechanistic contributions to our understanding in the field not available in human studies. In humans, the appraisal of clinical effects of DBS to date is primarily carried out through the use of subjective measures. Hence, placebo/sham effects are difficult to incorporate and measure in clinical trials. Preclinical research can help characterize the biological mechanisms of DBS and DBS induced mania, as well as to further isolate the unique effects of chronic stimulation in antidepressant mechanisms.

All in all, mitochondrial dysfunction seems to play a role in the impairments of cellular plasticity and resilience manifested in the context of mood-related disorders. DBS of the NAc was effective in reducing immobility time in antidepressant resistant animals. Bearing in mind that PFC regions heavily project to the accumbens and some of the projections from the PFC to subcortical areas run through white matter fibers of the frontal cortex, these findings suggest that mitochondrial function within the PFC may impact motivated behaviors associated with NAc involvement. Possible factors that may underlie the altered bioenergetic phenotype observed in this preclinical model of tricyclic antidepressant-resistance include: dopamine dysregulation, mitochondrial dysfunction, oxidative stress, inflammation and glial cell abnormalities.

In the future, the field of neuromodulation must look to basic research to establish key factors influencing different sub-populations, driving mood states and affecting treatment responsivity. The challenges of developing an animal model of treatment resistant depression and/or mania are many, however, the potential benefits of such a feat are no doubt worthwhile to help unlock the neurobiology of these illness states.

Supplementary information is available at Translational Psychiatry’s website.
References


Figure legends

Figure 1. (a) A detailed outline of experimental timeline (b) H&E staining validation of electrode tracts in DBS animals treated with ACTH.

Figure 2. (a) Differential effects of imipramine on FST immobility time between ACTH pre-treated and vehicle animals (n = 8-12). Saline treated animals responded to the antidepressant-like effects following imipramine administration. Treatment resistance induced with a chronic treatment of ACTH for 14 days; (b) ACTH animals show lower capacity to generate ATP in response to the energy demand relative to the vehicle group. State 3 respiration point represents a maximal ADP stimulation respiration. The values are displayed as means and ±SEM. * p < 0.05 † p = 0.07

Figure 3. (a) Effects of DBS on the forced swim test in ACTH treated animals (n = 7-12). NAc DBS significantly decreased time spent immobile in animals previously shown to resist imipramine. In comparison, DBS did not yield significant antidepressant-like effects on vehicle animals; (b) Mitochondrial function in the PFC, represented as the respiratory control ratio (RCR). Animals following NAc DBS show greater mitochondrial function as opposed to attenuated ratio in ACTH-treated animals. The values are displayed as means and ±SEM. * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 4. (a) A subset of animals developed hyperactive motor activity over the course of ACTH treatment (only evident in DBS or sham surgery animals with ACTH on board). No such effects were observed in vehicle animals. Animals in this heightened locomotor activity subgroup significantly differed in their distance traveled compared to other ACTH groups (b) Representative trace image of control ACTH vs. hyperactive animal & Percentage of time engaged in each coping style during FST –Representation of coping behaviors for control ACTH and manic-like phenotype animals during the FST-training. Persistent dive, escape and active climbing behaviors indicate these animals had an exaggerated drive to escape the FST apparatus compared to controls. (c) Animals exhibiting mania-like behaviors (HYP) show heightened RCR relative to other ACTH pre-treated groups. The values are displayed as means and ±SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
Financial Disclosures

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Chapter 5. Antidepressant actions of lateral habenula deep brain stimulation differentially correlate with CaMKII/GSK3/AMPK signaling locally and in the infralimbic cortex

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Antidepressant actions of lateral habenula deep brain stimulation differentially correlate with CaMKII/GSK3/AMPK signaling locally and in the infralimbic cortex

Authors: Yesul Kim a,b, Chunling Hu a, Linda K. Byrne b, Mark A. Frye a, Susannah J. Tye I, a,b,c,d
a – Department of Psychiatry and Psychology, Mayo Clinic, Rochester, Minnesota 55905, USA
b – School of Psychology, Deakin University, Burwood, Victoria 3125, Australia
c – Department of Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Rochester, Minnesota 55905, USA
d – Department of Psychiatry, University of Minnesota, Minneapolis, MN 55455, USA.

Present address:
Corresponding Author I: Susannah J. Tye
Corresponding Address I: Department of Psychiatry & Psychology
Mayo Clinic
200 First St SW
Rochester MN 55905
USA
Corresponding Phone I: +1 507-255-4322
Corresponding Fax I: +1 507-284-3933
Corresponding E-mail I: tye.susannah@mayo.edu

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Abbreviations: TRD – Treatment Resistant Depression; HPA – Hypothalamic Pituitary Axis; LHb – Lateral Habenula; IL – Infralimbic; DBS – Deep Brain Stimulation; CaMKII – Calcium/calmodulin Dependent Kinase Type II; GSK3 – Glycogen Synthase Kinase 3; AMPK – AMP-activated Protein Kinase
**Abstract**

High frequency deep brain stimulation (DBS) of the lateral habenula (LHb) reduces symptoms of depression in severely treatment-resistant individuals. Despite the observed therapeutic effects, the molecular underpinnings of DBS are poorly understood. This study investigated the efficacy of high frequency LHb DBS (130 Hz; 200μA; 90μSec) in an animal model of tricyclic antidepressant resistance. Further, we reported DBS mediated changes in Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaMKII\(\alpha/\beta\)), glycogen synthase kinase 3 (GSK3\(\alpha/\beta\)) and AMP-activated protein kinase (AMPK) both locally and in the infralimbic cortex (IL). Protein expressions were then correlated to immobility time during the forced swim test (FST). Antidepressant actions were quantified via FST. Treatment groups comprised of animals treated with adrenocorticotropic hormone alone (ACTH; 100μg/day, 14 days, n =7), ACTH with DBS, sham, surg or control (n =7, n =8, n =8 and: n =8 respectively). DBS significantly reduced immobility in ACTH-treated animals (p < 0.05), an effect that required active stimulation rather than electrode implnataions. For DBS group, western blot results demonstrated phosphorylation status of LHb CaMKII\(\alpha/\beta\) and GSK3\(\alpha/\beta\) significantly correlated to immobility time in the FST. Concurrently, we observed phosphorylation status of CaMKII\(\alpha/\beta\), GSK3\(\alpha/\beta\), and AMPK in the IL to be positively associated with antidepressant actions of DBS. These findings suggest that activity dependent phosphorylation of CaMKII\(\alpha/\beta\), and GSK3\(\alpha/\beta\) in the LHb together with the downregulation of AMPK in the IL, contribute to the antidepressant actions of DBS.

**Key words** Lateral Habenula, Deep Brain Stimulation, Treatment Resistant Depression, CaMKII, GSK3, AMPK
Introduction

The lateral habenula (LHb) plays a critical role in mood, reward, incentive motivation and stress responses [1-5]. Patients suffering from severe depression [6] and animal depression models of alpha-methyl-para-tyrosine administration, amphetamine withdrawal, stress exposures, genetic manipulation or selective breeding [5, 7-10], show elevated activity in this region. Local deactivation of the LHb via lesioning elicits antidepressant responses, promoting escape behavior in the congenital learned helplessness (cLH) rats during inescapable paradigm [11]. These behavioral changes were mediated, in part, by the regulation of dorsal raphe serotonin levels and ventral tegmentum dopamine transmission [11-14]. Recently, deep brain stimulation (DBS) of the LHb has been trialed for treatment resistant depression (TRD), with therapeutic effects reported to coincide with periods of active stimulation [15]. Similarly, LHb DBS effectively achieved an antidepressant response in the cLH model of depression [16, 17]. Nevertheless, the mechanisms through which LHb activity is up- or downregulated and whether it yields pro- or antidepressant actions, respectively, are not well understood.

Stress-induced molecular adaptations in the LHb region have important implications for modulating associative networks and depression-like behaviors [17-20]. In particular, recent attention has been given to the role calcium/calmodulin-dependent protein kinase type II (CaMKII) in learning and synaptic plasticity. Both exposure to stress and administration of the antidepressant escitalopram alter the expression of CaMKII in the LHb [19]. In cLH model, CaMKIIα and CaMKIIβ levels were shown to be significantly up-regulated in this region, prior to receiving effective antidepressant treatment [17]. Conversely, the down-regulation or blockade of CaMKIIβ activity reversed the depressive phenotype in these animals in standardized behavioral paradigms [17]. CaMKII is involved in many signaling cascades critical to cellular homeostasis, growth and plasticity, and functions together with glycogen synthase kinase 3 (GSK3) to regulate synaptic vesicle recycling in mature synapses [21, 22]. This activity-dependent interaction poses a possible mechanistic link.
between high frequency DBS and local CaMKII/GSK3 modulation. GSK3 also serves as a mediator in cellular metabolism and plasticity. Upstream of this, AMP-activated protein kinase (AMPK), a cellular energy sensor, is activated when cellular energy reserves are low and coordinates intracellular functions mediating plasticity [23]. Stress-induced changes in energy metabolism and substrate utilization via this cell signaling system is implicated in the pathophysiology of depression [24, 25]. Plasticity within this intracellular pathway may be an important contributor to the pathophysiological underpinnings of TRD.

Like the LHb, hypermetabolism of the subgenual cingulate gyrus (SCG) is well documented in the TRD population also, and the down regulation of this activity follows a successful antidepressant response, regardless of treatment method [26-30]. The LHb and SCG regulate affective and cognitive aspects of depressive symptoms [31-34]. The link between their hyperactivity and depressive states has been well-documented in patients [27, 35, 36]. Based on the existing literature, LHb function is important for alleviating depression symptomatology. Therefore, the present study further aims to validate this using LHb DBS in an animal model of antidepressant resistance, induced via chronic adrenocorticotropic hormone (ACTH) treatment [37-43]. In addition, we aimed to quantify antidepressant-like behavioral effects and its associated metabolic signaling in LHb and IL. DBS effects on CaMKIIα/β, GSK3α/β, and AMPK expressions locally as well as in the infralimbic cortex (IL; rodent homologue of SCG) specifically, will be examined in terms of the correlative relationship between behavior x protein signal.

Materials and Methods

All experiments were approved by Mayo Clinic Institutional Animal Care and Use Committee and conducted in accordance with The Code of Ethics of the EU Directive 2010/63/EU for animal experiments. All animals were housed individually, with food and water available ad libitum.
**Surgical procedures and post-operative care**

Male Wistar rats (190-230g) were anesthetized with isoflurane (1-3%) for stereotactic surgery. A 1.5-2cm incision was made and small holes were broached in the skull at the site corresponding to the targets. Twisted bipolar platinum iridium electrodes (5.6mm long, .075mm in diameter; Plastics One, Virginia, USA) were bilaterally implanted into the LHb (from bregma: antero-posterior (AP) -3.7mm, medial-lateral (ML), ±0.8mm, dorsal-ventral (DV) -5.4mm; Paxinos & Watson, 2007). Stimulating electrodes and screws were mounted with dental cement. Animals in the sham-surgery group were anesthetized and had four screws placed in the skull and secured with dental cement. Control animals did not undergo any surgical procedures.

**Experimental procedure**

After 3 days of acclimatization, rats received 14 days of either saline (0.9%) or ACTH (100μg/day) treatment as described previously (38). On day 7, DBS electrodes were implanted as described above. Continuous high frequency DBS stimulation (130 Hz, 90 μS, 200 μA) was delivered for 3 days prior to the FST (Iso-Flex /Master-8; AMPI, Jerusalem, Israel).

**Behavioral testing**

**Open field test (OFT)** was used to quantify animals’ general locomotor activity in a novel environment. A plexiglass box with a 622.3mm x 622.3mm base, and camera oriented with a top-down view was used for this test (CleverSys Inc., Virginia, USA). Animals were each placed in the central zone of the arena, and allowed to move freely for 6 min and behavior recorded by a video camera unit.

**Porsolt forced swim test (FST)** was used to quantify antidepressant response. Animals were placed into a narrow cylinder filled with water on the training day and consecutive test day. The apparatus consisted of clear plexiglass cylindrical tanks (60 cm height x 20 cm diameter;
CleverSys Inc., Virginia, USA) filled with tap water (23-25°C) to a depth of 25 cm. Animals were initially exposed to 15 min learning trial on day 14, conducted 2 hr after the OFT. A 6 min test session was then conducted on the subsequent day. For this test, the animals’ behavior was quantified to assess either active (swim, climb, escape) vs. passive (immobility, pass-dives) coping mechanisms. Behavioral data were analyzed using the cleversys topscan and forced swim software (CleverSys Inc., Virginia, USA) and validated by hand scoring.

*Tissue collection and western blot analysis*

Animals were injected with 100mg/kg of Fatal Plus (Vortech, Michigan, USA) 30 min following the FST test. Brains were rapidly removed and frozen on dry ice. Tissue samples were dissected over dry ice and homogenized in lysis RIPA buffer. Electrode location was confirmed during dissections. Samples containing equivalent amounts of protein were applied to 15% acrylamide denaturing gels and transferred to an Immobulin P for 1.5hrs at 90V. Each membrane was blocked for 15 min with 10mL of tris-buffered saline with tween 20 buffer (TBST), +5% milk to block (5% bovine serum albumin (BSA)+TBST for phosphor-antibodies). After washing, membranes were incubated with primary antibodies: CaMKII (pan) antibody (1:1000) #3362, phospho-CaMKII (Thr286, 1:3000) antibody #3361, GSK3α/β (1:2000) #5676, phospho-GSK3α/β (Ser21/9, 1:1000) #9331, AMPK (1:1000) #2532, phospho-AMPK (1:2000) #2535, (all from Cell Signaling Technology, Beverley, MA) for overnight with 1x phosphate buffered saline (PBST) + 5% milk (5%BSA+TBST for phospho-antibodies). Membranes were washed and incubated the following day with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody (1:2000-4000). Immunoreactive proteins were visualized using the enhanced chemiluminescence detection system (Biorad; location). Images were scanned and analyzed using Bio-Rad Chemi Doc (Hercules, CA). β-actin antibody #A5441 (Sigma, St Louis, MO) was quantified as loading control for each gel.
Statistical analyses

Firstly, the Shapiro-Wilk test of normality and Levene’s test for homogeneity of variance were also conducted. One-way multivariate analysis of variance (MANOVA), followed by Tukey’s test for multiple comparisons were used where appropriate for statistical analyses on behavioral and molecular observations. Statistical significance was set at $p = 0.05$. When assumptions for normality and homogeneity of variances were violated, the data were then analyzed by non-parametric Kruskal-Wallis one-way tests. Correlations between antidepressant-like effects in the FST and protein expressions were assessed using linear regression analyses. Calculations were performed using the IBM SPSS Statistics 22.0 software package and graphically represented using GraphPad Prism 6.0.

Results

Electrode localization

Electrode location was confirmed during tissue dissections and representative traces recorded diagrammatically (39). Bipolar stimulating electrode tips were localized to the LHb (in the range of -3.7 mm posterior to bregma, ±3.7 mm lateral to midline, and -5.7 mm ventral from dura; fig 1). No histological damage was observed for animals receiving DBS treatment.

[Insert Figure 1]

OFT & FST behavioral outcomes following LHb DBS

No statistically significant differences were found for distance nor ambulation across treatment groups ($p > 0.05$); and in the number of bouts taken, ($p > 0.05$; fig 2a-c). These behaviors
during the OFT indicate that findings in the FST are not due to any motor impairment or hyperactivity.

Animals when faced with an inescapable forced swim paradigm differed in their coping strategies, specifically the duration of immobile posture, $F(4, 32) = 17.92, p < 0.0001, \eta^2 = 0.69$ (fig 2d). LHb DBS animals, overall, displayed 75% reduction in immobility time compared to ACTH pretreated animals ($p = 0.02$). Further, DBS animals displayed attenuated immobility when compared to the surg group, indicative of distinct treatment effect ($p = 0.07$; fig 2d).

In terms of active coping strategies, animals demonstrated differences in climbing behavior, $F(4, 32) = 87.06, p < 0.0001, \eta^2 = 0.91$. Specifically, animals receiving active stimulation of the LHb used climbing significantly more than ACTH ($p = 0.001$) and sham ($p = 0.0001$; fig 2d) groups. Significant differences were found for time spent swimming ($F(4, 32) = 26.36, p < 0.0001, \eta^2 = 0.76$, fig 2e, f), prominent in ACTH sham animals.

[Insert Figure 2]

**Western blots**

i) Protein expression in the lateral habenula

Changes in p/CaMKIIα levels were observed between treatment groups, $\chi^2 (3) = 8.81, p = .032$, with a mean rank of 18.17 for ACTH, 13.50 for ACTH DBS, 8.08 for ACTH SURG and 8.50 for CTRL. A trend was observed in p/CaMKIIβ, $\chi^2 (3) = 7.01, p = .076$ (with a mean rank of 17.33 for ACTH, 13.70 for ACTH DBS, 8.42 for ACTH SURG, 8.83 for CTRL) and in p/AMPK, $\chi^2 (3) = 7.44, p = .059$ (with a mean rank of 18.00 for ACTH, 11.80 for ACTH DBS, 7.67 for ACTH SURG and 10.50 for CTRL). Phosphorylated GSK3α and GSK3β changed in parallel, but in short exposure to stress (or relay time
between stress and decapitation), the signals of other phosphorylated proteins were too weak to be detected.

The most interesting and significant findings lie in the relationship between immobility time during the FST and protein expression (fig 3 a-e). Negative and moderate to strong correlations were observed for phosphorylated/total protein levels of CaMKIIα/β, GSK3α/β and AMPK in DBS ACTH animals. No significant correlations were observed for other groups. A table containing the results of linear regressions for all conditions is presented in Supplementary Materials.

[Insert Figure 3]

ii) Protein expression in the infralimbic cortex.

Protein expression in the IL region did not significantly differ across treatment groups following an inescapable stress exposure. Nevertheless, trends were observed for CaMKIIα, $F(3, 27) = 2.75, p = .06$ and CaMKIIβ, $F(3, 27) = 2.57, p = .07$.

More importantly, significant correlations were similarly observed for immobility in the FST and phosphorylated/total protein levels in the IL, however in an inverse direction to that observed in the LHb region. Linear regression analyses identified significant positive and moderate to strong relationships between immobility time and phosphorylated proteins and/or phosphorylated/total protein levels. CaMKIIα, CaMKIIβ, GSK3α, GSK3β, and AMPK were consistently found to be phosphorylated 30 min post stress exposure in for DBS treated animals (fig 4a-e). A linear regression results table for all conditions can be found in the Supplementary Materials.

[Insert Figure 4]
Discussion

This study demonstrated antidepressant-like effects of LHb DBS in animals pretreated with ACTH and further presents data to suggest these behavioral responses relate to local as well as IL CaMKIIα/β, GSK3α/β and/or AMPK activity. The DBS group showed significantly reduced immobility behavior during the FST following active stimulation, compared to other conditions. With ACTH sham animals gaining no therapeutic benefit from electrodes insertion, it seems that active stimulation is necessary to bringing about antidepressant-like effects. These results corroborate with other animal models of depression where LHb DBS has also reduced immobility in the FST [9, 17, 44]. This study further aimed to quantify the effects of ACTH and DBS treatment x inescapable stress on the expressions of CaMKIIα/β, GSK3α/β, and AMPK in the LHb and IL. Significantly higher levels of LHb p/CaMKIIα were observed for ACTH-treated animals relative to other groups. A similar, near significant, increase was observed for ACTH-treated animals in p/CaMKIIβ and p/AMPK. Linear regression analyses further demonstrated that DBS-mediated reductions in immobility negatively correlated with phosphorylated CaMKII, GSK3 and AMPK in the LHb. Conversely, phosphorylation of these proteins in the IL, positively correlated with DBS-induced antidepressant response quantified as time spent immobile.

The outcomes of this study suggest the antidepressant action of LHb DBS may involve local activation of CaMKII, GSK3 and AMPK signaling, concurrent with opposing effects in the IL. It is possible that high frequency stimulation triggered activity dependent activation of this pathway in the LHb, which in turn facilitated a correlative downregulation of activity and energy demand in the IL supportive of increased active/reduced passive coping responses in the FST. As mentioned, a reversal of SCG hypermetabolism has been observed for patients in remission following efficacious antidepressant treatments [29, 45]. Decreased IL metabolism following fluoxetine administration has been reported by preclinical studies using optogenetic manipulation [31, 32, 46, 47]. CaMKII has been found to phosphorylate GSK3 during chronic depolarization and is the best possible candidate...
for mediating acute activity-dependent phosphorylation of GSK3 activity [21]. Activity-dependent consumption of ATP, likewise, is associated with phosphorylation of AMPK and mediates structural plasticity as well as mitochondrial transport [48]. Taken together, the phosphorylation status of these proteins couples depolarization to encourage neuronal survival and synaptic plasticity [22, 49], a necessary process in achieving antidepressant responses [50].

Existing literature uses deficits in energy metabolism to explain the cellular damage, atrophy and reduced capacity for synaptic plasticity observed in mood disorders [51]. We have previously shown that mitochondrial function is reduced in ACTH-treated animals (Kim et al, under review). This work demonstrated that mitochondrial capacity to create ATP was significantly attenuated by pretreatment with ACTH. This metabolic deficit may contribute to the neuroprogressive nature of depression [52], and contribute to the antidepressant resistance observed in these animals [43]. AMPK functions as an energy checkpoint within a cell, coordinating cellular functions for survival when reserves run low. This includes limiting cell cycle and growth processes as well as long-term plasticity responses to high frequency stimulation [53]. A role for efficacious antidepressant treatments in mediating these cellular growth and plasticity processes in TRD is emerging. Inhibition of GSK3 via lithium treatment has long been an established augmentation therapy in TRD, believed to exert neuroprotective actions and increase neuroplasticity, neurogenesis and cell resilience [54]. Ketamine has also been shown to recruit mTOR and GSK3 to exert its rapid antidepressant actions, which coincides with rapid upregulation of structural and functional plasticity [55]. Further work is needed to better understand the role of cellular metabolism in treatment resistance and responsivity, particularly to DBS.

Based on our results, one of many antidepressant actions of LHb stimulation may arise from an inverse relationship between DBS-mediated alterations in the metabolic function of local cells as well as those in the IL region, together coordinating therapeutic behavioral responses to stress. Chronic DBS stimulation has been found to promote structural and synaptic plasticity, changes in
metabolic activity, whereas acute stimulation results in antidromic and/or orthodromic activation [56]. The differential association of phosphorylated protein status of CaMKIIα/β, GSK3α/β, and AMPK in these two regions suggest LHb DBS may downregulate metabolic demand in the IL. Functional activation of LHb and IL are necessary for different processes involved in stress appraisal, response and antidepressant activity [57, 58]. Moreover, they both play a key role in mediating the psychological as well as physiological reactions to emotionally negative or stressful events [35, 59]. The interplay between local DBS effects and functional changes needs to be better understood, particularly with respect to the effects of chronic stimulation. The present study provides interesting new data to suggest DBS of the LHb directly modulates metabolic cell signaling cascades, both locally and in the IL region in ACTH pretreated animals. It will be important for future work to further investigate these mechanisms within the specific context of the neurobiological underpinnings of disease progression, and antidepressant-resistance. DBS therapeutics holds much potential for the treatment of severe, refractory depression. To do so, it is important that we elucidate its disease specific mechanisms so that we can reliably extend this promising neuromodulation therapy to those patients most in need.

There are two major limitations that were beyond the scope of the present paper. Firstly, further investigation on downstream or upstream targets and intermediary proteins would be of great value. Our exploratory approach placed particular interest on studying the role of specific proteins known to be abundant in the LHb (CaMKII), antidepressant response (GSK3) and energy regulation (AMPK). Future studies may be able to provide a more comprehensive picture of the molecular mechanisms mediating LHb antidepressant processes. In addition, given the size of LHb structure, the surrounding structures may have been modulated by DBS intervention, thereby possibly contributing to the observed antidepressant-like effects. Best efforts were made in order to reduce such confounding variables by implementing optimal DBS parameter setting (see Hamani et
al., 2010), as well as including two positive controls. Future studies may want to explore different frequency, current intensity and stimulated hemisphere to further investigate the relationship between the LHb and IL.
References


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Conflicts of interest

Authors report no conflicts of interest.
Figure legends

**Figure 1.** Schematic illustration of the anatomical placement of stimulating electrodes in the LHB.

**Figure 2a, b & c.** Animals’ locomotor activity during the 6 minutes OFT for speed and total distance travelled (n = 7-8). No apparent differences in motor ability is observed across the treatment groups; **d.** Animals who received DBS show reduced immobility time compared to that of ACTH and saline controls (n = 7-8); **e & f.** For active coping strategies, including climbing and swimming, significant differences were only exhibited in animals’ climbing behavior (n = 7-8). All values are represented as means and standard error of mean.

**Figure 3.** Activation of three proteins of interest in the LHB region of DBS animals (n = 5) where they received active high frequency stimulation. Higher expression of CaMKII, GSK3 an AMPK was associated with lower immobility time. Linear regression results are displayed with corresponding \( p \) and \( r^2 \) values.

**Figure 4.** Phosphorylated/expression proteins for IL region in DBS animals (n = 7). Lower phosphorylation and activation was moderate to strongly correlated with reduced time spent immobile following 3 days of chronic stimulation. Linear regression results are displayed with corresponding \( p \) and \( r^2 \) values.
Chapter 6. Global transcriptional profiling of infralimbic deep brain stimulation in an animal model of antidepressant treatment resistance

Manuscript prepared for submission in Molecular Psychiatry.
Abstract

Treatment resistant depression is a major unmet need for which deep brain stimulation (DBS) is an emerging therapy. However, the physiological mechanisms giving rise to antidepressant treatment resistance in depressive illness and the mechanisms underlying responses to DBS remain poorly understood. Using a genome-wide transcriptomics approach, we aimed to identify molecular pathways contributing to both antidepressant resistance and DBS efficacy in a preclinical model of antidepressant-resistance induced via chronic adrenocorticotropic hormone (ACTH; 100μg; i.p.; 14 days). Animals were allocated to stress-naïve or forced swim test (FST) stress conditions and received either ACTH or saline (0.9%) control treatments. Additional groups received either imipramine (to validate antidepressant treatment non-response) or infralimbic (IL) DBS (n=7-8 per group). The infralimbic area was dissected and global gene expression profiles obtained for saline, ACTH and ACTH-DBS groups (Agilent). Gene set enrichment analysis (DAVID) was performed following Bonferroni correction and KEGG pathways were identified (Fisher exact score p<0.05). Differential expression of pivotal genes and proteins were confirmed in independent groups (n=4-5) by RT-PCR and/or immunoblotting. Significant alterations were observed in key sensors of energy demand, cell division/growth, apoptosis, inflammation, protein synthesis and glucose/glycogen regulation following stress. Cellular distress molecular patterns indicative of oxidative stress, hypoxia, endoplasmic reticulum stress, pro-inflammatory cytokines, nutrient deprivation and DNA damage, such as Gadd45β, Gadd45γ, HIF1α, CHOP and p53, were increased in the treatment-resistant group and attenuated by DBS (p<0.05). This suggests that development of antidepressant resistance may relate to reduced capacity for cellular adaptation to stress, and that DBS helps to restore this capacity and overcome mechanisms contributing to cellular distress. Such actions may be critical in initiating longer-term neural adaptations that contribute to treatment efficacy in DBS therapeutics.
for depression. This has potential for augmentation to further optimize DBS mechanism(s) of action and identifies novel specific therapeutic targets.

KEYWORDS: Deep Brain Stimulation, depression, treatment resistance, antidepressant,
Introduction

Depression is a heterogeneous disorder with some forms of the disease highly resistant to available treatments. For these most severe and refractory manifestations of the disease, emerging neuromodulation therapeutic strategies such as deep brain stimulation (DBS) hold promise. However, the therapeutic mechanisms remain poorly understood. This is especially true of the molecular mechanisms, and this gap may contribute to the limited success of multisite studies, while uncovering these may identify new treatment targets. Preclinical rodent studies provide a unique opportunity for elucidating these molecular mechanisms. Given the inherent paucity of data in this area, we have utilized a ‘hypothesis generating’ genome-wide transcriptomics approach to systems biology in order to identify molecular mechanisms of DBS in a preclinical model of antidepressant treatment-resistance (Wang et al., 2015). The first and most thoroughly studied DBS target for treatment resistant depression (TRD) is subcallosal cingulate (SCC) white matter (Riva-Posse, et al., 2014; Holtzheimer et al., 2012; Lozano et al., 2008; Mayberg et al., 2005). Through careful, blinded investigational studies, this work has identified critical neural tracts that mediate SCC DBS antidepressant responses (Riva-Posse, et al., 2014). This work has demonstrated that there is an acute ‘reset’ response following initiation of DBS, followed by a more chronic recovery suggestive of longer-term adaptations mediated by synaptic plasticity. This concept was first put forward to conceptualize drug-induced neural plasticity occurring in response to antidepressant and other psychotropic drug (Hyman & Nestler, 1996), however may have relevance to the mechanism(s) of DBS action. Currently, we understand so little about the neurobiological mechanisms mediating acute and progressive responses to DBS in the treatment of depression, and why some patients respond quickly, while others take much longer to achieve the same level of therapeutic response. Elucidation of early phase molecular responses of neural tissue to DBS in antidepressant-resistant brains may help to uncover molecular mechanism and systems critical to initiation of synaptic plasticity.
processes underlying longer term adaptive responses; and in turn, targets for optimization and enhanced responsivity.

One approach for studying the molecular mechanisms of MDD is through animal models with chronic stress diathesis. Hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis during depression is a reliable clinical finding (Pariante & Lightman, 2008) and has been found to predict treatment resistance (Willner et al., 2013). In rodents, chronic administration of ACTH blocks the antidepressant effects of many pharmacologic interventions (Kitamura et al., 2002; Kitamura et al., 2008; Tokita et al., 2012; Walker et al., 2013; 2015). This model, although not as robust in its face validity for certain aspect of depressive illness as other chronic stress models is inherently more powerful for elucidation of mechanisms mediating treatment response and non-response. The simplicity of its design – chronic ACTH treatment – provides a consistent and standardized approach to modulating physiological processes that are critical to facilitation or blockade of antidepressant response. What’s more, it addresses an important and often undiscussed factor contributing to antidepressant resistance in TRD, the progressive loss of response over time (Moylan et al., 2013). This is not an uncommon phenomenon and is often observed in patients at the extreme end of the ‘TRD’ spectrum who present for DBS. These patients may have been responsive to a particular treatment and then over time their therapeutic responsivity is lost. We need to better understand both this neuroprogressive phenomenon, and the mechanisms of DBS that might reverse it. Several studies demonstrated the advantage of using animal models of disease to inform clinical research by providing hypothesis free candidate gene selection that are affected in MDD and altered consequent to treatment response (Barreto et al., 2012; Malki et al., 2011; Molteni, Macchi & Riva, 2013). DBS can also serve as an effective tool for probing mechanisms of antidepressant response in TRD, thought to have a distinct pathophysiology compared with more treatment responsive forms of depressive illness.
Based on anatomical connections and cytoarchitectural features, the ventral aspect of the medial prefrontal cortex, particularly the infralimbic (IL) cortex, is the region most commonly suggested as the anatomical correlate of the SCC. In rodents, the IL has been implicated in mechanisms of stress coping (Diorio, 1993; Ostrander, 2003; Amat, 2005), autonomic (Hardy, 1988; Resstel, 2004, Tavares, 2004), and conditioned response extinction (Milad, 2002; Milad, 2004; Quirk, 2000). Due to the complexity of clinical depression and somatic symptoms that cannot be reproduced in animal model, an endophenotype assay in response to antidepressant-like behavior can be a useful tool for elucidating mechanisms of response and non-response. The stress coping forced swim test (FST) assay, first introduced by Porsolt et al. (1977), has been implemented as an effective tool for screening antidepressant responses and better understanding the underlying mechanisms (Nestler et al. 2002). The present study utilized this behavioral screening assay, and aimed to interpret changes in endogenous gene expression induced by this test in treatment resistant and responsive rodents. Further, it assessed the impact of IL DBS on gene expression responses in ACTH-treated antidepressant-resistant animals, which may provide clues on acute local neural responses to DBS in antidepressant-resistant and DBS responsive animals. Findings from our study suggest the ACTH pretreatment has reduced the capacity of cells in the IL region to ‘cope’ with the supplementary stress of the FST. Cellular distress molecular patterns were identified, together with differential expression of critical cellular metabolism and growth/plasticity pathways in ACTH-treated animals post-stress relative to saline-treated control. DBS was effective in reducing cellular distress patterns within the IL and we propose such cellular effects contribute to the early cellular responses that function to initiate longer term adaptations that confer plasticity and treatment response clinically.
Methods

We first confirmed the antidepressant actions of IL DBS in ACTH-treated animals non-responsive to the tricyclic antidepressant imipramine, indexed by the forced swim test. In brief, male Sprague-Dawley rats were treated with daily injections of either saline (0.9%) or ACTH-(1-24) (100μg/d) for 14 days and randomly assigned to the stress-naïve or forced swim test (FST) groups. Imipramine (10mg/kg), high frequency IL DBS (1 hour, 130 Hz) or sham IL DBS (no stimulation) were administered to assess treatment efficacy. We then investigated how chronic ACTH treatment affects gene expression in the rodent IL following FST stress, relative to saline treated controls, as well as effects of IL DBS on local gene expression in ACTH-treated animals.

Animal experiments:

Male Sprague-Dawley rats (ARC, Australia and Harlan Laboratories, USA) were housed in pairs in a temperature controlled room. Food and water were available ad libitum. Room temperature was maintained at 21±2°C, humidity 40-70%, with a 12h light/dark cycle. Animals, weighing 200-350g at the time of testing, were treated with daily injections of saline (0.9%) or ACTH (100 μg) for 14 days and randomly allocated to the stress-naïve or forced swim test (FST) groups. Experimental groups included: saline/stress naïve, saline/FST, saline-imipramine/FST, ACTH/naïve, ACTH/FST, ACTH-imipramine/FST and ACTH-DBS/FST (n=7-8 per group). The open-field test (OFT) was used to assess locomotor, anxiety and exploratory behaviors and FST was used to assess antidepressant response (reduced immobility). Application of DBS was stopped immediately prior to the forced swim test and animals were euthanized 1 hour following FST. Brains were snap frozen in liquid nitrogen and stored at -80°C. All procedures were carried out in accordance to institutional guidelines for ethical animal care and use.
Drugs

The drugs used in this study were: ACTH-(1-24) (AnaSpec, California, USA), 100μg/day dissolved in distilled water; imipramine hydrochloride (Sigma-Aldrich, New South Wales, Australia), 10mg/kg dissolved in 0.9% saline; Saline (Baxter, New South Wales, Australia), 0.9%. Drugs were all delivered via intraperitoneal (i.p.) injection at ~10am each day.

Stereotactic Surgery

Animals were anaesthetized with isoflurane inhalation in an induction chamber (World Precision Instruments). Once anaesthetized, the animal was placed in a stereotaxic frame (David Kopf Instruments, CA) and maintained with isoflurane. The skull was secured with a nose clamp, incisor bar and ear bars. Constant body temperature (36.5°C) was maintained using an isothermal heat pad (Braintree Scientific). A midline 1.5cm incision was made in the skin overlying the skull to expose bregma. A 2mm diameter trephine hole was broached in the skull at the site corresponding to the target. The dura matter was opened using a fine needle and a stimulating electrode (Plastics One) was placed in the IL target (stereotactic coordinates: AP: +3.0; ML: ±0.4; DV: -5.6) (Paxinos & Watson, 2007). Three small jewelry screws were also placed in the skull surface and stimulating electrodes were secured in place with dental cement.

Porsolt Forced Swim Test (FST). The FST is a well-established screening tool for antidepressant efficacy, with strong predictive validity (Nestler & Hyman, 2010). The apparatus consisted of a clear plexiglass cylindrical tank (60 cm height × 20 cm diameter) filled with tap water (23–25 °C) to a height of 25 cm. Animals were exposed to 10 min learning trial, conducted two hours after the OFT. A 6 min test session was then conducted on the subsequent day. Sessions were recorded by video camera, and then reviewed for analyses. On completion of the FST, each test session was de-identified, and then manually scored in 2 s intervals using the criteria outlined by Porsolt et al. (1977). The behaviors of interest included ‘immobility’ (passive behavior), ‘swimming’ and
'climbing' (active behaviors). The effect of treatment on behavior was compared using separate one-way analysis of variance (ANOVA) (GraphPad Prism 6.0).

**Open Field Test (OFT).** The OFT was used to quantify locomotor, exploratory and anxiety-like behavior. The apparatus comprised a clear plexiglass box (36 cm × 50 cm × 50 cm) with 16 homologous squares marked on the base. Animals were each placed in the central zone of the arena, and allowed to move freely for 6 min and behavior was recorded by video camera. Each test session was de-identified, and then manually scored in 2 s intervals. The primary behavior of interest was ‘ambulation’, an indication of locomotor activity. Other behaviors of interest included ‘rearing’, time spent in central regions of the arena, and ‘grooming’ counts. These behaviors were used as indications of exploratory and anxious behavior. The effect of treatment on behavior was compared using separate one-way analysis of variance (ANOVA) (GraphPad Prism 6.0).

**Tissue Collection**

Following behavioral testing on day 15, animals were humanely euthanized by anaesthetic overdose 1 h after completion of the FST (sodium pentobarbitone 'Lethabarb'0.4cc, Virbac, New South Wales, Australia). Animals in the stress naïve groups were euthanized at the same time of day via identical methodology. Brain samples were extracted and flash frozen in liquid nitrogen and stored at −80°C for later analysis of tissue.

**RNA Extractions and Microarrays:**
Total RNA was extracted from IL PFC using TRIzol reagent (Invitrogen, Melbourne, Australia), and purified using RNeasy-Mini Kit (Qiagen, Mannheim, Germany). RNA quality and quantity was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and RNA6000 NanoAssay Kit (Agilent, Melbourne, Australia). Fluorescently-labelled cDNA was prepared from 800ng RNA using Agilent's Quick-Amp Labelling and One-Color RNA Spike-In kits. Cyanine 3-CTP-labelled cDNA was hybridized for 17h to Agilent Whole Rat Genome (4x44k)
Oligonucleotide Microarray Slides using Agilent Gene Expression Hybridization kit. Microarray fluorescent images were acquired using GenePix 4000B scanner, with data extraction performed via GenePix 5.1 software (Molecular Devices, Melbourne, VIC, Australia). Normalization and primary analysis of microarray data was performed using Aqutiy 4 software (Molecular Devices), as previously described (Carey KA, Segal D, Klein R, Sanigorski A, Walder K, Collier GR, and Cameron-Smith D. Identification of novel genes expressed during rhabdomyosarcoma differentiation using cDNA microarrays. Pathology International 56: 246-255, 2006.). Fluorescent reading for duplicate genes were averaged. Each array dataset sample was normalized so that the median expression value in each array was 1.0. The microarray dataset generated conforms to MIAME guidelines and is available at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi) series record GSEXXXX.

Gene Set Enrichment Analysis (GSEA) using DAVID:

Unpaired t-tests were used to identify genes, from the normalized microarray data, with evidence of differential expression between treatment groups (nominal p<0.05) (refer to Error! Reference source not found.). The complete gene lists of putative differentially expressed genes were imported into the online Database for Annotation, Visualization and Integrated Discovery (DAVID) software package (version 6.7) (Huang et al., 2009). Using the functional annotation tools, the data was analyzed to determine enrichment values for defined Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways. Pathways were selected based upon significant enrichment using Fisher’s exact statistical analysis (p<0.05) and fold enrichment score ≥1.5.

Gene Validation:

Validation of pivotal genes and proteins within pathways was performed with alternative sets of treated animals by semi-quantitative real-time PCR (RT-PCR) and/or immunoblotting (n=4-5 per treatment group).
RT-PCR - Total RNA was extracted from the dissected brain region using the RNeasy-Plus Mini Kit (Qiagen, Valencia, CA), as per the manufacturer’s directions. RNA was quantified using Nanodrop 2000 (Thermoscientific, Wilmington, DE) and 1μg was used for cDNA synthesis using iScript reverse transcriptase (BioRad), as per the manufacturer’s instructions. cDNA was aliquoted and sealed in plates and frozen at -20°C immediately. iQSYBR Green supermix (BioRad) was used for RT-PCR as per the manufacturer’s directions. For the RT-PCR reaction, 1ml cDNA and 300nM final concentration of each of the relevant Forward and Reverse primers were utilized. The PCR conditions were 95°C for 3 min and 39 cycles of 95°C for 10 sec and 58°C for 30 sec, followed by melt curve 65°C for 5 sec and 95°C for 5 sec. RT-PCR results were normalized to the reference gene *Hprt1* (*expression of Hprt1 did not differ significantly between treatment groups*). Relative gene expression was calculated as 2-ΔCt, where Ct is threshold cycle. Supplementary Table 1 displays the list of primer sequences. All sequences were designed using The National Center for Biotechnology Information (NCBI) Primer-Blast and oligos were ordered from Integrated DNA Technologies (IDT; Coralville, IA).

Western Blotting - Each dissected brain region was solubilized on ice by homogenization in ice-cold RIPA lysis buffer (50mM Tris at pH 7.4 containing 150mM NaCl, 1mM EDTA, 0.5% Deoxycholic Acid (SDC), 1% Triton X-100 and 0.1% SDS) with freshly added phosphatase and protease inhibitors (1mM Na3VO4, 2mM PMSF, 10μg/ml leupeptin, 10μg/ml aprotinin and 10μg/ml pepstatin A) using individually wrapped sterile 1.5ml pestles (Argos #P7339-901). After solubilization, lysates were incubated on ice for 15 min and were then cleared of cellular debris by centrifugation at 930xg for 20 min at 4°C. Supernatants were collected into pre-chilled 1.5ml microfuge tubes. Protein quantification was determined using BioRad’s Bradford assay kit with BSA as the standard. Samples were denatured in Reducing Sample buffer followed by incubation at 100°C for 5 min before being loaded onto and resolved by either 7.5 or 15% SDS-PAGE at 20μg per lane. Proteins were separated at 140 V for ~45 min and subsequently transferred onto polyvinylidene difluoride
(PVDF) membranes (Millipore) using 1x transfer buffer (25 mM Tris, pH 8.2 containing 192 mM glycine, 20% methanol, 0.1% SDS) at 4°C, 100 V for 100 min. PVDF membranes were blocked in 1x Tris-buffered saline, pH 7.4 and 0.1% (v/v) Tween20 (TBST) containing either 5% (w/v) BSA or 5% (w/v) skim milk for 1 h on a rocking platform at room temperature (22°C). The membranes were then incubated with the relevant primary antibody (TrkB; truncated and full length), Santa Cruz, p-Trk, Cell Signaling; AMPK, Cell Signaling; p-AMPK, Cell Signaling; GSK3, Cell Signaling; p-GSK3, Cell Signaling; p21, Santa Cruz; p-53, R&D Systems; mTOR, Cell Signaling and p-mTOR, Cell Signaling) as per the manufacturer’s methods for each antibody. Protein bands were visualised using enhanced chemiluminescent (ECL) detection system reagents (Invitrogen, USA) and exposed to X-ray film (Kodac, Ultra blue sensitive). The films were then scanned on to the Chemidoc MP Imaging System (BioRad) and quantitated using Analysis Software Image Lab version 4.0 (Bio-Rad, Hercules, CA).

Statistical Analysis - Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS version 20.0, Fullerton, CA, USA). Normality of data distribution was determined using a one-sample Kolmogorov-Smirnov test, and then analyzed using independent samples T-test or one-way ANOVA. Homogeneity of variance of group mean differences was determined using Levene’s Test, and post-hoc analysis of ANOVA used either Fisher’s least significant difference (LSD, homogeneous variance) or Games-Howell (non-homogeneous variance). For data not normally distributed, group mean differences were determined using the Kruskal-Wallis test followed by post-hoc analysis with Dunn’s multiple comparison test using GraphPad statistical software (version 6.0). Data were considered statistically different at p<0.05 and each data point presented as the mean of ≥4 animals ± SE unless otherwise stated.
Results

Tricyclic antidepressant resistance and response to DBS

We established antidepressant resistance in male Sprague-Dawley rats utilizing chronic pretreatment with ACTH that was sufficient to alter behavioral response to the tricyclic antidepressant imipramine, as measured by the forced swim test. Imipramine (10mg/kg) significantly reduced immobility time in saline-treated rats only (p<0.05). In contrast, high frequency IL DBS effectively reduced immobility time in ACTH-treated animals (p<0.05) (Figure 1). No differences were observed for locomotor activity (Figure 1).

Differential gene expression profile in the infralimbic cortex following forced swim stress

Differential gene expression (P < 0.05 and fold enrichment score ≥1.5) in the IL was determined as outlined in Table 1. The complete data set is shown in Supplementary Table S2. Our primary comparisons of interest focused on stress naïve versus stress exposure states for saline- and ACTH-treated animals. Additionally, we assessed the impact of DBS on these differential expression patterns for ACTH-treated animals.

Table 2 Number of genes and pathways with evidence of differential expression between treatment groups.

<table>
<thead>
<tr>
<th>Number of</th>
<th>Saline v ACTH</th>
<th>Saline v Saline/FST</th>
<th>Saline/FST v ACTH/FST</th>
<th>ACTH v ACTH/FST</th>
<th>ACTH/FST v ACTH-DBS/FST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes with differential</td>
<td>5376</td>
<td>4590</td>
<td>381</td>
<td>1390</td>
<td>1245</td>
</tr>
<tr>
<td>expression (p&lt;0.05)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genes with mapped IDs to</td>
<td>2039</td>
<td>1969</td>
<td>148</td>
<td>519</td>
<td>473</td>
</tr>
<tr>
<td>rat genome</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genes that could not be</td>
<td>3337</td>
<td>2621</td>
<td>218</td>
<td>738</td>
<td>627</td>
</tr>
<tr>
<td>mapped</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Molecular pathways differentially expressed under forced swim stress in the IL of saline-treated antidepressant-responsive animals.

The lists of genes with evidence of differential expression were subjected to functional enrichment analysis using the KEGG and PANTHER systems. We found 43 pathways in which differentially expressed genes were overrepresented in the IL of saline pretreated animals under conditions of forced swim stress relative to the stress naïve state. 24 such pathways were identified using Panther software.

Molecular pathways differentially expressed under forced swim stress in the IL of ACTH-treated antidepressant-resistant animals.

The list of differentially expressed genes in the IL of ACTH-treated stress-naïve and ACTH-treated forced swim test stress exposed was subjected to functional enrichment analysis using the KEGG and PANTHER systems. Using KEGG software, we found 17 pathways in which differentially expressed genes were overrepresented (Table 2), while only 4 pathways were identified using Panther software (Table 3).
Table 2 KEGG pathways identified as significantly overrepresented in the differentially expressed genes between ACTH and ACTH-FST rat IL.

<table>
<thead>
<tr>
<th>KEGG Pathway</th>
<th>Number of differentially expressed genes in pathway</th>
<th>Fold Enrichment</th>
<th>Fisher Exact p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I diabetes mellitus</td>
<td>8/</td>
<td>4.8</td>
<td>2.0E-4</td>
</tr>
<tr>
<td>p53 signaling pathway</td>
<td>9/</td>
<td>4.4</td>
<td>1.7E-4</td>
</tr>
<tr>
<td>Graft-versus-host disease</td>
<td>7/</td>
<td>5.3</td>
<td>2.8E-4</td>
</tr>
<tr>
<td>Allograft rejection</td>
<td>7/</td>
<td>4.9</td>
<td>4.5E-4</td>
</tr>
<tr>
<td>Antigen processing and presentation</td>
<td>10/</td>
<td>4.2</td>
<td>1.1E-4</td>
</tr>
<tr>
<td>Endocytosis</td>
<td>15/</td>
<td>2.4</td>
<td>1.2E-3</td>
</tr>
<tr>
<td>Autoimmune thyroid disease</td>
<td>7/</td>
<td>4.1</td>
<td>1.3E-3</td>
</tr>
<tr>
<td>Adipocyte signaling pathway</td>
<td>8/</td>
<td>3.6</td>
<td>1.5E-3</td>
</tr>
<tr>
<td>mTOR signaling pathway</td>
<td>6/</td>
<td>3.6</td>
<td>6.3E-3</td>
</tr>
<tr>
<td>MAPK signaling pathway</td>
<td>16/</td>
<td>1.9</td>
<td>1.2E-2</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>9/</td>
<td>2.3</td>
<td>1.6E-2</td>
</tr>
<tr>
<td>Pathways in cancer</td>
<td>17/</td>
<td>1.7</td>
<td>2.2E-2</td>
</tr>
<tr>
<td>Viral myocarditis</td>
<td>7/</td>
<td>2.7</td>
<td>1.3E-2</td>
</tr>
<tr>
<td>Insulin signaling pathway</td>
<td>9/</td>
<td>2.2</td>
<td>2.3E-2</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>7/</td>
<td>2.6</td>
<td>1.8E-2</td>
</tr>
<tr>
<td>ErbB signaling pathway</td>
<td>7/</td>
<td>2.6</td>
<td>1.8E-2</td>
</tr>
<tr>
<td>Chronic myeloid leukemia</td>
<td>6/</td>
<td>2.6</td>
<td>2.7E-2</td>
</tr>
</tbody>
</table>
Molecular pathways differentially expressed in the IL of antidepressant-resistant animals with efficacious DBS.

The differentially expressed IL gene list of ACTH-treated forced swim test stress exposed animals with and without DBS was subjected to functional enrichment analysis using the KEGG and PANTHER systems. We found 21 pathways in which differentially expressed genes were overrepresented using KEGG (Table 4), and 4 pathways using Panther software (Table 5).
Table 4 KEGG pathways identified as significantly overrepresented in the differentially expressed genes between ACTH-FST and ACTH-FST-DBS rat IL.

<table>
<thead>
<tr>
<th>KEGG Pathway</th>
<th>Number of differentially expressed genes in pathway</th>
<th>Fold Enrichment</th>
<th>Fisher Exact p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathways in cancer</td>
<td>19/</td>
<td>2.0</td>
<td>3.3E-3</td>
</tr>
<tr>
<td>Oocyte meiosis</td>
<td>10/</td>
<td>3.0</td>
<td>1.8E-3</td>
</tr>
<tr>
<td>Thyroid cancer</td>
<td>6/</td>
<td>6.8</td>
<td>2.0E-4</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>6/</td>
<td>5.3</td>
<td>8.3E-4</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>11/</td>
<td>2.9</td>
<td>1.3E-3</td>
</tr>
<tr>
<td>Progesterone-mediated oocyte maturation</td>
<td>8/</td>
<td>3.2</td>
<td>3.6E-3</td>
</tr>
<tr>
<td>Acute myeloid leukemia</td>
<td>6/</td>
<td>3.6</td>
<td>6.2E-3</td>
</tr>
<tr>
<td>Chronic myeloid leukemia</td>
<td>7/</td>
<td>3.2</td>
<td>6.4E-3</td>
</tr>
<tr>
<td>Melanoma</td>
<td>7/</td>
<td>3.2</td>
<td>5.4E-3</td>
</tr>
<tr>
<td>Chemokine signalling pathway</td>
<td>10/</td>
<td>1.9</td>
<td>3.5E-2</td>
</tr>
<tr>
<td>Gap junction</td>
<td>7/</td>
<td>2.8</td>
<td>1.2E-2</td>
</tr>
<tr>
<td>Type I diabetes mellitus</td>
<td>5/</td>
<td>3.2</td>
<td>2.0E-2</td>
</tr>
<tr>
<td>Endocytosis</td>
<td>11/</td>
<td>1.9</td>
<td>3.5E-2</td>
</tr>
<tr>
<td>Natural killer cell mediated cytotoxicity</td>
<td>7/</td>
<td>2.4</td>
<td>2.6E-2</td>
</tr>
<tr>
<td>Melanogenesis</td>
<td>7/</td>
<td>2.5</td>
<td>2.1E-2</td>
</tr>
<tr>
<td>Regulation of actin cytoskeleton</td>
<td>12/</td>
<td>1.9</td>
<td>2.4E-2</td>
</tr>
<tr>
<td>T cell receptor signaling pathway</td>
<td>8/</td>
<td>2.4</td>
<td>1.8E-2</td>
</tr>
<tr>
<td>Complement and coagulation cascades</td>
<td>6/</td>
<td>2.7</td>
<td>2.3E-2</td>
</tr>
<tr>
<td>Cell adhesion molecules</td>
<td>9/</td>
<td>2.1</td>
<td>2.5E-2</td>
</tr>
<tr>
<td>Edometrial cancer</td>
<td>5/</td>
<td>3.2</td>
<td>1.9E-2</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>6/</td>
<td>2.4</td>
<td>3.7E-2</td>
</tr>
</tbody>
</table>
Table 5 PANTHER pathways identified as significantly overrepresented in the differentially expressed genes between ACTH-FST and ACTH-FST-DBS rat IL.

<table>
<thead>
<tr>
<th>PANTHER Pathway</th>
<th>Number of differentially expressed genes in pathway</th>
<th>Fold Enrichment</th>
<th>Fisher Exact p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 pathway feedback loops 2</td>
<td>6/</td>
<td>2.7</td>
<td>2.3E-2</td>
</tr>
<tr>
<td>PDGF signaling pathway</td>
<td>11/</td>
<td>1.7</td>
<td>5.0E-2</td>
</tr>
<tr>
<td>Inflammation mediated by chemokine and cytokine signaling pathway</td>
<td>17/</td>
<td>1.6</td>
<td>3.8E-2</td>
</tr>
<tr>
<td>Ras pathway</td>
<td>8/</td>
<td>2.5</td>
<td>1.4E-2</td>
</tr>
</tbody>
</table>

Validation of IL molecular pathways differentially regulated under stress in treatment resistance and DBS treatment response

Significant alterations were observed in key pathways of energy demand, cell division/growth, apoptosis, protein synthesis and glucose/glycogen regulation across groups.
Figure 1. Critical pathways of interest – targeted for validation
Figure 2 – Cellular metabolism pathway genes and proteins

Figure 3 – Cellular stress pathway genes and proteins
Figure 4 – Cell survival pathway genes and proteins
Discussion:

Novel molecular signatures of resistance and response were identified in the IL of TCA treatment resistant and responsive animals. Additionally, the impact of the IL DBS on these local molecular mechanisms in TCA-resistant animals was determined. The IL, as the rodent homologue of the human subgenual cingulate, is an area of particular interest for elucidating mechanisms associated with treatment response and non-response (Mayberg ref). It is now well established that this region is hyperactive in depressive states and that activity is consistently reduced by efficacious treatment response, regardless of mechanism. This is true for response to placebo, cognitive behavioral therapy, pharmacotherapies such as SSRI, ECT as well as DBS therapeutics in the most treatment resistant of patients (McCormick et al., 2009; Mayberg, Henn).

This study demonstrated that induction of treatment resistance with chronic ACTH treatments results in a substantially distinct gene expression profile in many pathways and systems in the IL of ACTH and saline-treated animals exposed to the FST stress relative to their stress naïve counterparts. Functional pathway analysis revealed that inflammatory, metabolic, cell stress and cell survival molecular pathways were differentially up- or down-regulated in the IL of these animals that foundationally distinguished these treatment-resistant and responsive groups. Moreover, application of IL DBS served to partially reverse the metabolic gene expression profile and nearly fully reversed the cellular stress signaling profile of the ACTH treated TCA-resistant animals.

Exposure to the forced swim behavioral stressor significantly elevated p53 expression in the IL of ACTH-treated TCA-resistant animals. p53 is a critical molecular mediator of cellular stress and survival, and for this reason, plays a critical role in cancer proliferation. p53 interacts with other regulatory components of cellular metabolism, growth and survival, including Akt1, Wnt, and GSK3β. Akt signaling plays a critical role in cellular growth and metabolism via interaction with
JNK, MAPK, mTOR and GSK3β. An important functional outcome of this is regulation of glucose/glycogen production, cell growth and neural plasticity. These changes in term alter downstream pathways associated with cell stress including β-oxidation and proteasomal degradation. This finding highlights a potentially compensatory mechanism occurring with the IL under stress – upregulation of cellular metabolism as a potentially compensatory mechanism for the elevated energetic demands on this region under duress. As noted above, the IL and subgenual cingulate are well established to be metabolically hyperactive during depressive states, with this metabolic overactivity normalized with efficacious treatment. Collectively, our data suggests this metabolic shift (upregulation in depression) may functionally occur as cells in this region attempt to keep up with demand and counter the damaging and potentially lethal effects of cellular stress, to in turn support cellular survival and growth processes. That is, ACTH-treated animals have significant demand on this region due to chronic stimulation of the HPA-axis. In turn, with supplemental forced swim stress, this region has limited capacity to respond to additional demands and cells in the region demonstrate a molecular profile reflective of allostatic overload / cellular stress (Walker et al., 2014). It is interesting to note that DBS reverses this profile in ACTH-treated animals, with differential signaling identified for the p53 feedback loop in these animals.

Hypoxia is also induced under states of cellular stress, and this was observed in our ACTH-treated stressed animals. An increase in HIF gene expression levels, observed herein, has been associated with oxygen deprivation. Increased hypoxia due to stress, decreases VEGF and activates tight junction and vesicular transport by activating SNAP and SNARES, an important mechanism to compensate for energy deficits. Exposure to the stress of the FST activated caspase, WNT, insulin and HIF, may serve to compensate for glucose and oxygen deprivation.

The role of inflammatory signaling in this model is of particular interest, particularly given recent data emerging in the field as to the effects of acute inflammation in chronically stressed animals and the potential of anti-inflammatory antidepressive strategies (Kim et al., 2015; Köhler et al., 2014).
Herein we demonstrate that inflammatory signaling cascades were upregulated in the IL of treatment-responsive saline pretreated animals following exposure to the FST. Conversely, these same pathways were downregulated in the IL following forced swim exposure in the ACTH-treated antidepressant resistant animals. This differential expression profile may reflect the impact of chronic ACTH-pretreatment on inflammation in the brains of these animals, such that subsequent stress exposure attenuated rather than enhanced these responses. DBS, not surprisingly, resulted in upregulation of these inflammatory pathways relative to ACTH-FST animals not receiving DBS. Of note, levels of monocyte chemoattractant protein-1 (MCP-1), a potent chemokine synthesized by several cell types, were 400 fold elevated in DBS animals relative to all other groups. This was the most robust difference observed across groups for all genes and proteins quantified, and may reflect and important initiating or contributing factor to the other molecular responses observed with DBS. Altering the microglial milieu within the brain may be an important contributing factor to other observed metabolic and cellular stress and cell growth adaptations occurring therein. Given glial cells play a particularly important role in maintaining neuronal energetic capacity at the tripartite synapse, this potential interaction warrants further investigation as a potentially important mechanism in both antidepressant treatment resistance and response. Specifically, its potential to impact response to DBS in the most refractory of patients needs to be explored.

Overall, treatment with DBS in the IL cortex has a mechanism of action which reverses or stops the cellular cascade initiated by stress. IL DBs decreases GADD45B, GADD45G and Hifα, which were all increased in our ACTH stress model. Moreover, HSP27 activity is increased with DBS, this could be due to the introduction of a foreign substance such as electrode. A higher level of p53 and CHOP is associated with DNA damage and apoptosis. In our ACTH stress model, levels of p53, p21 and CHOP were elevated, whereas the IL DBS group showed a reduction in the expression of these genes. Inflammatory markers such as MCP-1 show increased activity with DBS treatment. MCP-1 is the primary response to any foreign substance that could be elevated due to electrode placement.
However, this inflammatory response seems to revert to baseline indexed by other inflammatory markers such as TNFα and interleukins (IL). Moreover, cell check cycle is another critical cycle that is regulated with DBS treatment, shown by decreased levels of CREB2/ATF4 and cyclin B levels. Lastly, IL DBS suppresses major contributors involved in growth hormone and cell signaling, which is correlated with suppression of apoptotic markers.

Identifying the unique and independent pathway responsible in subtypes of depression could be beneficial to diagnose patient’s depression subtype. This supports systems biology based approaches and can also lead to further research into depression drug therapy to better assist the patients.
Figures for discussion

1.

2.
Acknowledgements:

MB is supported by a NHMRC Senior Principal Research Fellowship 1059660.

Conflicts of Interest:

MB and has received Grant/Research Support from Bristol Myers Squibb, Eli Lilly, Glaxo SmithKline, Meat and Livestock Board, Organon, Novartis, Mayne Pharma, Servier and Woolworths, has been a speaker for Astra Zeneca, Bristol Myers Squibb, Eli Lilly, Glaxo SmithKline, Janssen Cilag, Lundbeck, Merck, Pfizer, Sanofi Synthelabo, Servier, Solvay and Wyeth, and served as a consultant to Astra Zeneca, Bioadvantex, Bristol Myers Squibb, Eli Lilly, Glaxo SmithKline, Janssen Cilag, Lundbeck Merck and Servier.
References


Chapter 7. Region- and frequency-specific transient dopamine release as a mechanism of deep brain stimulation in anesthetized rats

Yesul Kim

Deakin University

Contributing authors: Rodney J. Anderson¹, Kyoko Hasebe², Kevin Bennet³, Kendall H. Lee⁴, Mark A. Frye⁵ and Susannah J. Tye⁵

¹ Faculty of Medicine, Nursing and Health Sciences, Monash University, Australia.

² Faculty of Health, Deakin University, Australia.

³ Division of Engineering, Mayo Clinic, USA.

⁴ Department of Neurosurgery, Mayo Clinic, USA.

⁵ Department of Psychology and Psychiatry, Mayo Clinic, USA.

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Abstract

Over the last decade, deep brain stimulation (DBS) has demonstrated potential as a therapeutic intervention for treatment resistance depression (TRD). However, its mechanisms of action remain unknown and further experimental and clinical studies are needed to determine the central actions of DBS. Each of the clinical targets for DBS modulate the mesoaccumbens dopamine (DA) system; a system extensively studied for its involvement in normal reward-related function. Dysfunction of the mesoaccumbens dopamine system also plays a critical role in depressive pathophysiology. The influence of DBS at neurologic targets used to treat TRD on mesoaccumbens dopamine neurotransmission has not been investigated. We have addressed this issue by quantifying the effects of DBS of the nucleus accumbens (NAc), infralimbic cortex (IL) and lateral habenula (LHb) on transient NAc DA release in urethane-anesthetized male Sprague Dawley rats. Real-time ventral tegmental area-evoked (125μA, 60Hz, 2 ms pulse width for 23 pulses) NAc DA efflux was measured with fast-scan cyclic voltammetry during the pre-DBS phase (baseline; 60 min), high (130Hz) or low (10 Hz) frequency (both at 100μA, 90μs pulse width) DBS phase (90 min) and post-DBS phase (120 min). Data demonstrated that low frequency DBS elicited a negligible response in all three regions, while high frequency DBS to IL and NAc significantly attenuated DA release in the NAc. In contrast, LHb high frequency DBS induced a significantly potentiated DA-efflux in the NAc. The IL- and LHb-DBS-induced effects were sustained for >2hrs post-DSB, indicative of long-term depression and potentiation, respectively. These findings demonstrate a regional- and frequency-specific effect of DBS on NAc DA release and long-term plasticity, therefore suggesting that modulation of synaptic plasticity may be a possible mechanisms of action.

Keywords: dopamine, nucleus accumbens, infralimbic, lateral habenula, deep brain stimulation
Deep brain stimulation (DBS) for treatment resistant depression (TRD) is a promising clinical neuromodulation intervention (Bewernick et al., 2010; Holtzheimer et al., 2012; Lozano et al., 2008; Mayberg et al., 2005; Puigdemont et al., 2012; Sartorius et al., 2010; Schlaepfer et al., 2007). Neuromodulatory targets include the subgenual cingulate gyrus (SCG), nucleus accumbens (NAc) and lateral habenula (LHb) for patients with TRD (Holtzheimer et al., 2012; Sartorius et al., 2010; Schlaepfer et al., 2007). These regions were targeted due to converging evidence that shows their prominent role in stress appraisal (Amat et al., 2005), emotion regulation (Damasio et al., 2000; Liotti et al., 2000; Mayberg et al., 2014), dysfunction in emotion processing (Drevets, Bogers, & Raichle, 2002; Mayberg et al., 2005; Wu et al., 1999) and involvement in the successful amelioration of depressive symptomatology via various treatment modalities (Dougherty et al., 2003; Goldapple et al., 2004; Kennedy, Foy, Sherazi, McDonough, & McKeon, 2007; Mayberg, 2002; Mayberg et al., 2000; Mottaghy et al., 2002). Despite encouraging results from early single site trials, where a large number of patients experienced relief and remitted from TRD, the mechanisms of action underlying the therapeutic effects are unresolved. Our lack of understanding of these mechanisms may have contributed to the failure or discontinuation of larger, randomized, double-blind studies that have attempted to extend these techniques (Cavuoto, 2013; Dougherty et al., 2014; Morishita, Fayad, Higuchi, Nestor, & Foote, 2014). It is therefore, imperative that we investigate the antidepressant actions of DBS so that the translation of early clinical success is not stalled unnecessarily.

The goal of this paper is to quantify the effects of high frequency (HF) and low frequency (LF) DBS on transient stimulation-evoked NAc dopamine (DA) efflux in the rodent homologue of the human SCG, the infralimbic (IL) cortex (Gabbott, Warner, Jays, & Bacon, 2003; Shumake & Gonzalez-Lima, 2003; Takagishi & Chiba, 1991), NAc and LHb. Norepinephrine and serotonin have traditionally been the focus of treatment efficacy and disease pathophysiology in major depressive disorder (MDD). However, an increasing body of literature indicates the involvement of mesolimbic
DA pathway in mood regulation and its dysfunction in MDD symptomatology, which is particularly more pronounced in the treatment resistant population (Dunlop & Nemeroff, 2007; Malhi & Berk, 2007; Nestler & Carlezon, 2006; Tremblay et al., 2005). Transient DA is a critical gating mechanism for glutamatergic information flow through the NAc (See Figure 1.) wherein it effectively selects whether cortical or limbic inputs activate GABAergic output to subcortical regions (Chaudhury et al., 2013; Goto & Grace, 2005). Recent findings from diffusion tensor magnetic resonance imaging tractography (Coenen, Schlaepfer, Allert, & Mädler, 2012; Schoene-Bake et al., 2010), optogenetics (Lammel, Tye, & Warden, 2014; Warden et al., 2012) and fast scan cyclic voltammetry (Stuber, Roitman, Phillips, Carelli, & Wightman, 2005) further reinforce the important role of the reward-related system in TRD. These studies conceptualize depressive symptoms as arising from dysregulated networks that are responsible for stimulus appraisal, reward/aversion processing and reward/punishment anticipation. Consequently, patients suffering from TRD may achieve therapeutic effects by manipulating these systems via pharmacological or DBS intervention (Schlaepfer, Bewernick, Kayser, Hurlemann, & Coenen, 2014). DA neurotransmission in the NAc, which arises from DA producing neurons in the ventral tegmental area (VTA), plays an important neuromodulatory role in regulation of plasticity thought to be a key mechanism of antidepressant response (Bonci & Malenka, 1999; Friedman, Friedman, Dremencov, & Yadid, 2008).
Figure 1. Major dopaminergic, glutamatergic and GABAergic connections to and from the VTA and NAc in the rodent brain. Dopaminergic projections from the VTA to the NAc releases DA in response to reward-related (or aversion-related) stimuli. The NAc also receives densely innervated glutamatergic circuits from the medial prefrontal cortex (mPFC), hippocampus (HIP) and the amygdala (Amyg). Lastly, there are GABAergic projections from the NAc to the lateral hypothalamus (LH) and the VTA. These circuits control aspects reward-related perception, memory and learning.

Neuroplasticity within the mesolimbic DA pathway has been shown to be involved in the maintenance of emotional homeostasis (Krishnan et al., 2007), with disruption of neuroplasticity potentially contributing to the neuroprogressive stage of treatment resistance (Pittenger & Duman, 2008). The requirement of DA for the induction of striatal long term potentiation (LTP) and long term depression (LTD) makes these two forms of synaptic plasticity unique in comparison to long term synaptic plasticity changes in other brain regions (Calabresi, Picconi, Tozzi, & Di Filippo, 2007). Dysregulation of DA signaling may thus negatively impact long-term plasticity and an individual’s capacity for adaptive responses to antidepressant action over time.

Here, we examined VTA stimulation-evoked transient NAc DA neurotransmission during DBS of the IL, NAc or LHB regions of male wistar rats. We hypothesized that HF stimulation but not LF stimulation, would modulate phasic DA neurotransmission. Further, given that Walker et al. (2013) observed attenuation of transient DA levels in the ACTH preclinical model, we hypothesized
that DBS may function to recover this signal. Correcting the altered DA transmission, together with disrupted hypothalamic pituitary axis stress system may be potential pharmacological and/or neuromodulatory targets for reinstating treatment response. We also monitored DA neurotransmission after discontinuing stimulation to these regions in order to evaluate neuroplasticity as a possible mechanism underlying the therapeutic effects of DBS. Lastly, we hypothesized that HF stimulation would induce lasting effects on neurotransmitter efflux, consistent with long term plasticity observed in animals’ response to tetanic (i.e. HF) stimulation.

Methods

Animals

Male Sprague-Dawley rats were purchased through Deakin University Animal House (Geelong, Victoria, Australia) and Animal Resources Centre (Perth, WA, Australia). Experiments were carried out between 9:00 a.m. and 6:00 p.m. on animals weighing 200-350g. Animals were housed in pairs, in a temperature controlled environment with 12-hour light/dark cycles and access to food and water ad libitum. Animal care and experimental procedures were conducted in accordance with the Prevention of Cruelty to Animals Act 1986 (2009), and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004). Ethics approval was received for the project from the Animal Welfare Committee, Deakin University (Geelong, Australia) and the Florey Institute of Neuroscience and Mental Health Animal Experimental Ethics committee, University of Melbourne (Melbourne, Australia).

Surgical Procedure

Rats were anaesthetized with urethane (1.5g/kg) intraperitoneally, placed in a stereotaxic frame (David Kopf Instruments, Tujunga, California, USA) and body temperature maintained at
37°C with an isothermal heat pad (Delta Phase Isothermal Pad, Braintree Scientific, Braintree, Massachusetts). An incision was made along the midline of the scalp and the skin retracted to expose the skull. Four burr holes were drilled in the skull for placement of the reference, recording, and two stimulating electrodes. Concentric bipolar stimulating electrodes (SNE-100, d = 0.25mm, exposed surface = 0.75mm; Rhodes Medical Instruments, Summerland, CA, USA) were placed in the VTA or DBS target region (IL, NAc or LHb). A recording electrode (carbon-fiber microelectrode) was placed in the core of the NAc. Both the stimulating and recording electrodes were placed in the left hemisphere, with the reference electrode positioned in superficial cortex in the contralateral hemisphere. A chloridized silver wire (Ag/AgCl) was used as a reference electrode. Coordinates for all electrodes were calculated using the atlas of Paxinos and Watson (2007). Coordinates for the recording electrode in the NAc, in millimetres, (referenced to bregma) were: anteroposterior (AP) = +1.2, mediolateral (ML) = +1.5, dorsoventral (DV) = -6.5 to 8.0; the stimulating electrode in the IL (referenced to bregma) AP= +3.0, ML=0.1, DV= -5.8, NAc AP +1.5, ML ±1.5, DV -7.0 or LHb AP -3.7, ML, ±0.8, DV -5.4 and the stimulating electrode in the VTA (interaural coordinates) AP= -2.16, ML= -0.6, DV= -2.0 to 1.2. Due to space constraints, the stimulating electrode in the IL was lowered at an angle of 60° from the contralateral hemisphere to its final coordinates. Once a robust voltammetric response was obtained (determined by the size of the current at the peak oxidation potential of DA) the electrodes were maintained at these coordinates throughout the experiment.

**Electrochemistry**

Fast scan cyclic voltammetry was performed using 5μm-diameter carbon fiber electrodes. Voltammetric scans, stimulus waveform generation and timing, data collection were performed using Wireless Instantaneous Neurotransmitter Concentration System (Bledsoe et al., 2009). All carbon fiber electrodes were tested for stable background currents and responsiveness prior to each experiment sitting. Voltammetric scans from -0.4V to 1.3V and from 1.3V to -0.4 were performed at 300V/s in a triangle waveform which was repeated every 100ms, with the potential
held at -0.4V between scans. The high scan-rates used produce a large background current (Heien & Wightman, 2006), which is removed by subtracting a series of cyclic voltammograms prior to stimulation. The resultant background-subtracted voltammogram provides a signature for the species oxidised or reduced at the CFM. DA at the carbon-fiber surface is oxidised (at ~ +0.6V versus Ag/AgCl) and the electroformed DA-o-quinone is then reduced back to DA (at ~ -0.3V versus Ag/AgCl). Changes in the resulting oxidative current are proportional to changes in DA concentration at the carbon fiber surface (Robinson & Wightman, 2006).

**Electrical Stimulation**

DA release was evoked with monophasic stimulation pulses at 125μA, 60Hz, 2ms pulse width for 23 pulses, (Master 9 Pulse Stimulator and Iso-Flex Stimulation Pulse Isolator; AMPI, Israel). These parameters were chosen to mimic endogenous transient DA release (Heien & Wightman, 2006). During the DBS phase of the experiments the brain regions were stimulated for 90 minutes at 100μA, 90μs pulse width, and 130Hz for the HF DBS condition and 10Hz for the LF DBS condition. The stimulation parameters for the HF condition were chosen to approximate the charge density of that experienced by patients receiving DBS for TRD (Hamani, Diwan, Isabella, Lozano, & Nobrega, 2010) that takes into account the smaller volume of the rat brain and the size of the electrodes.

**Histological Verification of Electrode Placement**

At the conclusion of each experiment, while all electrodes were still in place, lesions were created by applying a DC current to the stimulating electrodes (100μA for 5sec). Animals were euthanized using a 1.0 ml urethane intracardial injection, the brain removed immediately and fixed in a 10% formalin solution. Brain slices were prepared (40μm coronal sections) using a cryostat microtome (Cryostat HM500 Series) at -23°C, mounted on glass slides and viewed under a light
microscope. The location of the recording electrode in the NAc was determined from the tract left by the electrode shaft (see Figure 2 for histology schematics).

Figure 2. Target locations of stimulating electrodes and recording carbon-fiber electrode (left to right). A, B & C is in reference to IL DBS; D, E & F is a schematic representation of NAc DBS; LHb electrodes tracts are G, H & I. Specifically, first column represents VTA-evoked stimulation sites, second column indicates HF DBS sites and lastly, the third column shows recording sites. Green dots refer to LF DBS whereas HF is illustrated with red dots. Figures adapted from Paxinos and Watson (2007).
DBS Experimental Design and Statistical Analysis

Each DBS experiment consisted of three phases: a baseline phase, DBS phase, and a post-DBS phase. For each experiment, baseline was calculated by averaging all responses subsequent to final coordinates being reached. Each animal was assigned to either a HF condition \( n = 5 \) or a LF condition \( n = 4-5 \). DA release, measured as current at the maximum oxidation potential of DA, was the measured variable. An independent samples \( t \)-test was used to compare the mean change in DA release from baseline during HF to that of LF stimulation. The statistical significance level was set at \( p < .05 \). The homogeneity of variance assumption was confirmed using Levene’s test for equality of variance.

Results

Differential DA efflux with NAc, IL or LHb DBS in healthy control animals

Results show differential changes to VTA-evoked DA release in the NAc, depending on the DBS target. Further, HF stimulation brought on greater attenuation of DA following IL (Figure 3.) and NAc DBS, whereas HF LHb DBS potentiated DA release in the NAc. Table 1 summarizes the associated percent change in DA efflux prior to stimulation (baseline), during stimulation and following DBS. Mean percentage changes from baseline to the effects of HF or LF is graphically represented for all targets in Figure 5.
Figure 3. A decrease in DA efflux from baseline levels recorded in the NAc following HF stimulation to the IL. Representative current time plots for baseline (top left) versus during HF stimulation (top right). Note that the colorplot corresponds to the above current representative figure. The purple spots represent VTA evoked DA release.
Table 1. A summary table of percentage change observed for VTA-evoked DA release in the NAc prior to, following and post NAc, IL and LHb DBS.

<table>
<thead>
<tr>
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<th>Percent Change</th>
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<tr>
<td></td>
<td>PreDBS vs. DBS</td>
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<tr>
<td>Lhbf HF</td>
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<tr>
<td>M</td>
<td>33.09</td>
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<td>SD</td>
<td>12.40</td>
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<tr>
<td>SEM</td>
<td>5.54</td>
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<tr>
<td>Lhbf LF</td>
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<tr>
<td>M</td>
<td>-1.98</td>
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<tr>
<td>SD</td>
<td>2.32</td>
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<tr>
<td>SEM</td>
<td>1.16</td>
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<tr>
<td>IlHF</td>
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<td>M</td>
<td>-27.60</td>
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<td>SD</td>
<td>10.77</td>
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<tr>
<td>SEM</td>
<td>6.22</td>
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<td>IlLF</td>
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<td>M</td>
<td>6.08</td>
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<td>SD</td>
<td>11.78</td>
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<tr>
<td>SEM</td>
<td>5.89</td>
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<tr>
<td>NAc HF</td>
<td></td>
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<tr>
<td>M</td>
<td>-10.30</td>
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<tr>
<td>SD</td>
<td>20.89</td>
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<tr>
<td>SEM</td>
<td>9.34</td>
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<tr>
<td>NAc LF</td>
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<tr>
<td>SD</td>
<td>19.77</td>
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<td>SEM</td>
<td>8.84</td>
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LHb –lateral habenula; NAc –nucleus accumbens, IL –infralimbic, DBS –deep brain stimulation, HF –high frequency, LF –low frequency; n =3-6 for each group. Late DBS refers to second half of active DBS whereas Post DBS refers to recordings made with no active DBS (once stimulation stopped).

**IL region.** During 90 min of HF DBS of the infralimbic cortex DA release in the core of the NAc decreased by a mean of -27.60% of baseline (SEM = 6.22) to a significantly greater degree than the low frequency stimulation group that showed a mean decrease of 5.61% of baseline (SEM = 3.96), t(10) = 5.87, p < .01, d = 4.48 (the homogeneity of variance assumption was satisfied, with Levene’s test showing no significant difference between variances). Note the gradual decrease in the relative concentration of DA over time in both the HF and LF conditions; this may be due to decreasing sensitivity of the recording electrode. DA release did no return to baseline levels after stimulation was discontinued for period of at least 2 hr.
**NAc region.** An independent samples t-test showed that the magnitude of DA signal during HF DBS was significantly attenuated in comparison to pre HF DBS DA signal evoked by VTA 23 pulses stimulation, $t(4) = 6.58, p < .01$. A Cohen’s $d (d = 5.37)$ indicated that the magnitude of transient DA release between pre HF DBS and HF DBS was considerably differed by 5.37 standard deviations. Also, the magnitude of phasic DA release after HF DBS was significantly smaller than those before HF DBS, $t(4) = 6.20, p < .01$, and the effect size ($d = 5.07$) revealed the substantial mean difference (approximately 5 SD) in the amount of phasic DA release between pre and post HF DBS (Figure 4.). The magnitude of post HF DBS DA signal did not significantly differ from those of during HF DBS, $t(4) = 1.81, p > .05$.

![Figure 4. A comparison of VTA-evoked transient DA release between baseline (left) and post HF DBS (right).](image)
**LHb region.** An independent samples t-test was conducted to examine differences in mean percentage changes between HF and LF condition in rats. This analysis revealed that only HF DBS potentiates VTA-evokes NAc DA efflux in control animals, \( t(9) = 6.917, p < 0.001 \). Interestingly, in the LHb, HF DBS potentiates NAc DA release in controls (pre-DBS vs. DBS), \( t(10) = 1.288, p > 0.05 \). VTA-evoked NAc DA efflux 2 hours post-DBS relative to during DBS in control animals show long term plasticity evident for healthy controls, \( t(10) = 4.331, p < 0.01 \).

![Figure 5. Mean percent change (± SEM) in evoked dopamine efflux from baseline in the NAc after 90 minutes of high-frequency stimulation (n = 3-6) and low-frequency stimulation (n = 4-6). Error bars represent the standard error of the mean. Dopamine was evoked with 23 pulses, 60Hz, 125μA, 2ms pulse width, monophasic stimulation of the VTA.](image-url)
Discussion

We found that HF stimulation of the IL and NAc in the rat attenuated VTA evoked phasic DA neurotransmission in the core of the NAc, whereas LF stimulation elicited a negligible response. Clinically, HF stimulation is necessary for the induction of antidepressant properties of DBS in the TRD population. Consistent with these clinical findings, we demonstrated that HF stimulation, but not LF stimulation, of these regions modulated DA transmission. Interestingly, a potentiation NAc DA was observed for animals with LHb HF DBS. Mechanisms underlying changes of phasic DA efflux in the NAc with DBS may have occurred via direct (monosynaptic) and indirect (multisynaptic) pathways.

The monosynaptic pathway for IL DBS involves excitation of glutamatergic efferents via orthodromic activation from DBS, which projects directly to the NAc (Takagishi & Chiba, 1991; Vertes, 2004). In the NAc, the excitation of GABAergic medium spiny neurons may inhibit DA neurotransmission. It has been shown experimentally that glutamate input to the NAc can affect DA neurotransmission independent of the action on DA neurons in the VTA (Howland, Taepavarapruk, & Phillips, 2002). A number of indirect pathways may be involved in the attenuation of phasic DA in the NAc glutamatergic neurons project to the VTA (Carr & Sesack, 2000; Geisler, Derst, Veh, & Zahm, 2007; Geisler & Zahm, 2005; Takagishi & Chiba, 1991; Vertes, 2004) and form synapses with GABAergic interneurons (Adell & Artigas, 2004; Carr & Sesack, 2000) as well as forming minor connections with GABAergic neurons of the ventral pallidum (Takagishi & Chiba, 1991; Vertes, 2004). Importantly, both of these regions project to and synapse with DA neurons within the VTA (Adell & Artigas, 2004; Bayer & Pickel, 1991; Geisler & Zahm, 2005; Kalivas, 1993). This is remarkable as excitation of GABAergic neurons from both these sources can lead to inhibition of the DA neurons in the VTA which project to NAc (Adell & Artigas, 2004; Björklund & Dunnett, 2007; Carr & Sesack, 2000; Ikemoto & Panksepp, 1999; Moore & Bloom, 1978; Swanson, 1982). IL glutamatergic neurons also synapse with GABAergic projection neurons in the VTA that project
directly to the NAc (Carr & Sesack, 2000). Support for an indirect route in modulating NAc DA, comes from studies that use pharmacological manipulation of the rat mPFC to increase or decrease VTA DA neuron firing rates, depending on the drug administered, which subsequently increase or decrease DA concentration in the NAc (Murase, Grenhoff, Chouvet, Gonon, & Svensson, 1993).

Cellular mechanisms underlying DBS of the accumbens, or the nearby structure ventral capsule/ventral striatum, have not been as extensively studied (Veerakumar & Berton, 2015). The attenuating DA efflux observed herein may arise from the innervation of local DA cell bodies and astrocytic adenosine triphosphate release. Furthermore, NAc receives strong serotonergic and dopaminergic input from the dorsal raphe nucleus and VTA respectively, whose axons all travel via the medial forebrain bundle (Lammel et al., 2014). This is important as the dorsal raphe nucleus is an important source of serotonergic and glutamatergic synaptic inputs to the VTA (Qi et al., 2014). Entrainment of these local and distal circuits to stimulation frequency may also explain the alterations in DA efflux via NAc DBS.

Given that NAc receives inputs from both cortical and limbic regions which in turn sends outputs to relevant motor regions, the NAc is in an ideal position to translate cognitive and emotional input to behavioral responses (Floresco, 2007). Here, DA neurotransmission acts a key facilitator of information processing, selecting which neural input will successfully activate post-synaptic outputs to the basal ganglia (Goto & Grace, 2005). It follows that dysfunction of DA neurotransmission in the NAc critically influences the anhedonic and amotivational symptom characteristics of MDD and that appropriate modulation of the mesolimbic DA pathway may be involved in the amelioration of depressive symptoms using DBS.

Striatal dependent learning and memory consolidation has been linked to anhedonia and other cognitive impairments in MDD (Bressan & Crippa, 2005; Dunlop & Nemeroff, 2007; Nestler & Carlezon, 2006). LTD, a form of synaptic plasticity, of phasic DA release in the NAc was observed for
two hours following the discontinuation of HF stimulation of the IL. After LHb HF stimulation, LTP was observed, whereas NAc HF stimulation produced mixed results. The mechanisms underlying LTD in the NAc involve interactions between the neurotransmitters DA and primarily glutamate (Calabresi et al., 2007). N-methyl-D-aspartate receptors (NMDARs) are glutamatergic receptors critical to NAc LTD induction (Thomas, Malenka, & Bonci, 2000) where NMDAR activation enables calcium influx to the neuron, triggering intracellular CaMKII-dependent mechanism (Huang & Hsu, 2012). Our findings in Chapter 5 show the modulation of CaMKII expression in the NAc to be associated with antidepressant actions in treatment resistance animals. These findings provide a potential link between DA transmission and CaMKII dependent NMDAR function.

In terms of IL DBS, the fact that LTD was induced following HF stimulation rather than LTP, could indicate the presence of tonic concentration levels of DA prior to stimulation, which is thought to be critical in determining the polarity of long term synaptic plasticity (Goto & Grace, 2005). It has been previously suggested that the bi-directional (increases or decreases) modulation of DA release can have markedly different effects on brain function dependent on the state of the organism. Goto, Otani, and Grace (2007), using in vitro slice preparations of PFC, found HF tetanic stimulation that is normally sufficient to induce LTP in vivo, instead resulted in the induction of LTD. However, when a low concentration of DA was applied into the bath solution to mimic tonic background, HF stimulation instead resulted in the induction of LTP, suggesting that the level or tonic DA could determine transient DA release in the PFC. Our differential LTP and LTD findings in the LHb and IL, respectively, may be due to differential influence of tonic DA levels on these projections.

Considering the gating influence hypothesized to be a critical function of phasic NAc DA in the regulation of cortical and limbic processes, DBS may function to restore balance to a dysregulated mesolimbic DA pathway in TRD. In other words, restoring adaptive appraisal and behavioral responses dependent on optimal responsivity of the mesocorticolumbic DA system.
Unfortunately, a major caveat of this study is that we did not examine DA function in our animal model of antidepressant resistance for all DBS regions due to availability of resources. Uniquely, LHb DBS findings herein show LTP in comparison to LTD in NAc and IL. Previous literature on LHb stimulation, both clinical and preclinical, suggests a likely inhibitory effect of DA. As LHb is largely glutamatergic, an inhibition of excitatory synapses to the VTA may explain the increase of DA release in the NAc in response to VTA-evoked transients. Therefore, we have decided to pursue our interest in LHb further and use this site to compare the effects DBS on NAc DA transmission in adrenocorticotrophic treated animals vs. controls in the next Chapter 8.
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depressive disorder revealed by a dopaminergic probe. *Archives of general psychiatry, 62*(11), 1228-1236.


Chapter 8. Potentiation of accumbens dopamine is a therapeutic mechanism of lateral habenula deep brain stimulation in antidepressant resistant rats

Yesul Kim

Deakin University

Contributing authors: Adam J Walker¹, Kevin Bennet², Kendall H. Lee³, Susannah J. Tye⁴

¹ Faculty of Health, Deakin University, Australia.

² Division of Engineering, Mayo Clinic, USA.

³ Department of Neurosurgery, Mayo Clinic, USA.

⁴ Department of Psychology and Psychiatry, Mayo Clinic, USA.

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Abstract

Impaired synaptic plasticity has been implicated in major depressive disorder. Recently, several studies examining the therapeutic effects of DBS postulates the possibility of plasticity as an important mechanism of this neuromodulatory treatment. In order to investigate this, adrenocorticotropic hormone (ACTH)-induced antidepressant resistant animals were subjected to high frequency lateral habenula (LHb) deep brain stimulation (DBS; 90 minutes at 100μA, 90μs pulse width, and 130Hz). The experiment involved real-time measurements of ventral tegmental area-evoked (125μA, 60Hz, 2ms pulse width for 23 pulses) dopamine (DA) efflux in the nucleus accumbens for induction of striatal-dependent plasticity. Results show that ACTH-treated animals failed to maintain recovered DA release once DBS was discontinued. Saline control animals, however, was able to induct long term potentiation of DA transmission. Together, current findings suggest a role for synaptic plasticity in treatment resistance and response in major depression.

Keywords: dopamine, lateral habenula, deep brain stimulation, synaptic plasticity
The habenula is uniquely positioned to regulate many of the deficits in emotion, motivation, reward and cognitive processing implicated in mood disorders. Interest in the lateral habenula (LHb) has recently demonstrated that such behaviors are mediated by LHb regulation of dopamine (DA) and serotonin systems. Within the brainstem, LHb efferents mainly target the nuclei containing monoamine neurons: the dopaminergic ventral tegmental area (VTA) and substantia nigra pars compacta, serotonergic dorsal and median raphe, and cholinergic laterodorsal tegmentum (Hikosaka, Sesack, Lecourtier, & Shepard, 2008). The LHb forms a node of connection between the cortex and brainstem monoamine neurons that operates in parallel to the medial forebrain bundle. As a result, the LHb is known to be involved in a variety of physiological responses like reward (Hong & Hikosaka, 2008), reward error processing (Matsumoto & Hikosaka, 2009), suppression of escape related behavior (Nieh, Kim, Namburi, & Tye, 2013) and stress (Kazi, Mori, Kuchiïwa, & Nakagawa, 2004). LHb cells exert a relatively short latency and potent inhibitory influence (Hikosaka et al., 2008) where the LHb neurons are heterogeneous in their neurochemical expression patterns at rest (Kim & Chang, 2005). Studies using electrophysiology and anatomical tools indicate the majority to have an excitatory, glutamatergic phenotype (Geisler & Trimble, 2008; Kalén, Karlson, & Wiklund, 1985; Weiss & Veh, 2011; Yang, Hu, Xia, Zhang, & Zhao, 2008).

The inhibition of dopaminergic neurons in the VTA is less well understood. Accumulating evidence suggests that the LHb is a promising candidate to exert an inhibitory control over the VTA dopaminergic system. Supporting information dates back to observations that lesions of the habenula complexes result in activation (Nishikawa, Fage, & Scatton, 1986) and that electrical stimulation of the LHb provoked inhibition of mesencephalic DA neurons (Christoph, Leonzio, & Wilcox, 1986). In fact, in a more recent study on primates (Matsumoto & Hikosaka, 2009), it was demonstrated that the inhibitory input from the LHb, elicited by weak electrical stimulation, plays an important role in determining the reward-related activity of DA neurons. Ji and Shepard (2007) found that single pulse stimulation of the LHb completely suppressed the dopaminergic activity in
the VTA for close to 100 ms. More importantly, their findings suggest that LHB-induced suppression of DA cell activity is mediated indirectly by orthodromic activation of putative GABAergic neurons in the ventral midbrain.

The infralimbic (IL; subgenual cingulate gyrus homologous) and LHB have significantly increased metabolism in a genetic rat model of depression (Mirrione et al., 2014; Shumake, Poremba, Edwards, & Gonzalez-Lima, 2000) and this is also consistently observed in the clinical population (Mayberg et al., 2014). Such hyperactivity is then reversed with effective antidepressant, including DBS, treatment. Previously, stimulation in rats under animal models of depression show that DBS to the IL, nucleus accumbens (NAc) and LHB decreases depressive-like phenotype in various behavioral assays (Hamani et al., 2014). Our preliminary studies using the adrenocorticotropic hormone (ACTH) model have also shown effectiveness of LHB (Kim et al., 2015, under review), NAc (Kim et al., 2015 under review) and IL (Tye et al., 2015; Reker et al., 2015 –both manuscripts in preparation) DBS in imipramine resistant animals.

The aim of this chapter is to examine the VTA stimulation-evoked transient DA neurotransmission in NAc during high frequency DBS of the LHB in ACTH-treated animals compared to that of healthy controls. Similar to the previous chapter, we monitored DA release after discontinuing the stimulation of LHB in order to evaluate neuroplasticity as a possible mechanism underlying the therapeutic effects of DBS. We hypothesize that high frequency stimulation would induce lasting changes on neurotransmitter efflux in healthy rats whereas this function would be impaired in treatment resistant animals.

Methods

Animals

Male Sprague-Dawley rats were purchased through Deakin University Animal House (Geelong, Victoria, Australia) and Animal Resources Centre (Perth, WA, Australia). Animals were
housed in pairs, in a temperature controlled environment with 12-hour light/dark cycles and access to food and water ad libitum. Fast Scan Cyclic Voltammetry recordings were conducted between 9:00 a.m. and 6:00 p.m. on animals weighing between 200-350g. Animal care and experimental procedures were carried out in accordance with the Prevention of Cruelty to Animals Act 1986 (2009), and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004). Ethics approval was received for the project from the Animal Welfare Committee, Deakin University (Geelong, Australia) and the Florey Institute of Neuroscience and Mental Health Animal Experimental Ethics committee, University of Melbourne (Melbourne, Australia).

Animal model of tricycle-resistance

Similar to that of published papers (Walker et al., 2013; Walker et al., 2015), a separate cohort of animals were administered either 14 days of adrenocorticotrophic hormone (ACTH, 100μg/day, n = 6) or saline (0.9%, n = 7) intraperitoneally, prior to FSCV experiments.

Surgical Procedure

Rats were anaesthetized with urethane (1.5g/kg) via an intraperitoneal injection, then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, California, USA) where body temperature was maintained at 37°C with an isothermal heat pad (Delta Phase Isothermal Pad, Braintree Scientific, Braintree, Massachusetts). An incision was made along the midline of the scalp and the skin was retracted to expose the skull surface. Four burr holes were drilled in the skull for placement of the reference, recording, and two stimulating electrodes. Concentric bipolar stimulating electrodes (SNE-100, d = 0.25mm, exposed surface = 0.75mm; Rhodes Medical Instruments, Summerland, CA, USA) were placed in the VTA or LHb. A recording electrode (carbon-fiber microelectrode) was placed in the core of the NAc. Both the stimulating and recording electrodes were placed in the left hemisphere, with the reference electrode positioned in superficial cortex in the contralateral hemisphere. A chloridized silver wire (Ag/AgCl) was used as a reference
Coordinates for all electrodes were calculated using the atlas of Paxinos and Watson (2007). Coordinates for the carbon-fiber recording electrode in the NAc, in millimetres, (referenced to bregma) were: anteroposterior (AP) = +1.2, mediolateral (ML) = +1.5, dorsoventral (DV) = -6.5 to 8.0; the stimulating electrode in the LHb AP -3.7, ML, ±0.8, DV -5.4 and the stimulating electrode in the VTA (interaural coordinates) AP= -2.2, ML= -0.6, DV= -2.0 to 1.2. Once a robust voltammetric response was obtained (determined by the size of the current at the peak oxidation potential of DA) the electrodes were maintained at these coordinates throughout the experiment.

**Electrochemistry**

Fast scan cyclic voltammetry was performed using 5μm-diameter carbon fiber electrodes. Voltammetric scans, stimulus waveform generation and timing, data collection were performed using Wireless Instantaneous Neurotransmitter Concentration System (Bledsoe et al., 2009). All carbon fiber electrodes were tested for stable background currents and responsiveness prior to each experiment sitting. Voltammetric scans from -0.4V to 1.3V and from 1.3V to -0.4V were performed at 300V/s in a triangle waveform which was repeated every 100ms, with the potential held at -0.4V between scans. The high scan-rates used produce a large background current (Heien & Wightman, 2006), which is removed by subtracting a series of cyclic voltammograms prior to stimulation. The resultant background-subtracted voltammogram provides a signature for the species oxidised or reduced at the CFM. DA at the carbon-fiber surface is oxidised (at ~ +0.6V versus Ag/AgCl) and the electroformed DA-o-quinone is then reduced back to DA (at ~ -0.3V versus Ag/AgCl). Changes in the resulting oxidative current are proportional to changes in DA concentration at the carbon fiber surface (Robinson & Wightman, 2006).

**Electrical Stimulation**

DA release was evoked with monophasic stimulation pulses at 125μA, 60Hz, 2ms pulse width for 23 pulses (Master 9 Pulse Stimulator and Iso-Flex Stimulation Pulse Isolator; AMPI,
Israel). These parameters were chosen to mimic endogenous transient DA release (Heien & Wightman, 2006). During the DBS phase of the experiments the brain regions were stimulated for 90 minutes at 100μA, 90μs pulse width, and 130Hz for the HF DBS. The stimulation parameters for the HF condition were chosen to approximate the charge density of that experienced by patients receiving DBS for TRD (Hamani, Diwan, Isabella, Lozano, & Nobrega, 2010) that takes into account the smaller volume of the rat brain and the size of the electrodes.

**Histological Verification of Electrode Placement**

Lesions were delivered with all electrodes still in place, by applying a DC current to the stimulating electrodes (100μA for 5sec) for histological verification (see Figure 1 for histology schematics). Animals were euthanized using a 1.0 ml urethane intracardial injection and the brains were removed immediately then fixed in a 10% formalin solution. Brain slices were prepared (40μm coronal sections) using a cryostat microtome (Cryostat HM500 Series) at -23°C, mounted on glass slides and viewed under a light microscope.

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**Figure 1.** Schematic representations of A: VTA stimulating electrode; B: HF LHb DBS; C: voltammetric recording electrode in the NAc. Red dots signify saline treated animals and yellow dots for ACTH-treated animals.
**Results**

**Attenuated DA release and potentiation in ACTH treated animals with LHb DBS**

Independent sample *t*-tests were conducted to examine meaningful differences in mean percent change of DA release in ACTH- vs. saline- treated animals. As hypothesized, these analyses revealed a restoration of DA function with DBS in animals with chronic ACTH administration, yet these effects did not translate into long-term synaptic plasticity. Table 1 summarizes the mean percent change and corresponding SD and SEM for control saline and ACTH groups.

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**Table 1. A summary table of percentage change observed for VTA-evoked DA release in the NAc prior to, following and post NAc, IL and LHb DBS.**

LHb –lateral habenula; DBS –deep brain stimulation; ACTH –adrenocorticotropic hormone; HF – high frequency; *n* = 6-7 for each group.

An independent samples *t*-test revealed in the LHb, HF DBS potentiation NAc DA release in both control and ACTH-treatment groups (pre-DBS vs. DBS), *t*(10) = 1.288, *p* > 0.05. VTA-evoked NAc DA efflux post-DBS relative to during DBS in control and ACTH pre-treated animals where the effects of DA signal was not sustained in ACTH animals, *t*(10) = 3.244, *p* < 0.01. VTA-evoked NAc DA efflux which was recorded 2 hours post-DBS, compared to during active DBS in control and ACTH pre-treated animals indicate evidence of long term plasticity only for healthy controls, *t*(10) = 4.331, *p* < 0.01 (Figure 2.).
Figure 2. Representative DA release and reuptake in healthy (saline treated) vs. antidepressant resistant animals. The ability for DA reuptake in ACTH-treated animals is possibly impaired, as signified by the decaying tail.
Below, Figure 3 represents an averaged time course of LHb DBS-induced long-term potentiation of NAc DA in healthy controls, whereas a recovered transient DA efflux in ACTH animals is decaying after the discontinuation of DBS.

![Graph showing transient VTA stimulation-evoked NAc dopamine efflux](image)

**Figure 3.** Averaged data points showing percent change in transient VTA stimulation-evoked NAc dopamine efflux prior to, during and following 90 minutes of high frequency (HF) LHb DBS in control and ACTH-treated animals.

**Discussion**

In support of previous DBS success in clinical practice, we show that high frequency stimulation is necessary to restore attenuated DA function in rats treated with chronic ACTH. The therapeutic effects of DBS, however, were not sustained once active stimulation of LHB has been stopped. These findings suggest that deficits in synaptic plasticity may be implicated in TRD, possibly inhibiting the capacity of enduring therapeutic responses to be induced and maintained. The results of this study indicate that modulating of DA neurotransmission in the NAc via LHb DBS may be involved in the therapeutic effects of DBS for TRD, possibly in the amelioration of the
anhedonic and amotivational symptoms of the depressive syndrome based on the function of these circuits.

With no active stimulation, ACTH animals' capacity for long term potentiation was absent. Plastic changes in the mesolimbic DA pathway may be how DBS reinstates treatment response in TRD patients. Neuroplasticity has been shown to be disrupted in patients suffering from MDD (Duman, 2002; Pittenger & Duman, 2008) and in animal models of stress (Goto & Grace, 2006). Long-term biochemical and neuroplastic changes in the mesolimbic DA pathway have been found to occur following acute and chronic stress in rats (Ortiz, Fitzgerald, Lane, Terwilliger, & Nestler, 1996; Saal, Dong, Bonci, & Malenka, 2003). Effectively, antidepressant treatment promotes neuroplasticity, possibly reversing the disruption during the pathological state (Duman, 2002; Pittenger & Duman, 2008).

Authors of clinical case report interpret their findings as likely resulting from inhibitory effects of LHb DBS, the suppression of LHb hyperactivity, which corresponds to the remission of depressive symptoms (Sartorius et al., 2010). LHb hyperactivity has been hypothesized as an etiologic factor in MDD, based on imaging studies. Relevant to current findings, decreased LHb metabolism was associated with increases in midbrain DA activity, established from amphetamine and apomorphine based rodent studies (Shumake & Gonzalez-Lima, 2013). This largely glutamatergic structure projects to the VTA, thereby exerting critical influence over VTA-NAc mesolimbic pathway. LHb has typically been found to have an inhibitory dopaminergic function over the NAc. Hence, an overactive LHb, observed in MDD patients and in the learned helplessness rodent model of depression, represses the activity of VTA and DRN. By depleting afferents from the basal ganglia which drives LHb hyperactivity, LHb DBS disinhibits the VTA and DRN (Veerakumar & Berton, 2015). Present findings of increase DA efflux following LHb DBS are consistent with existing data demonstrating that pharmacological inhibition of LHb increases DA release in the forebrain (Lecourtier, DeFrancesco, & Moghaddam, 2008).
Conversely, Stamatakis et al. (2013) suggest that the phasic DA release seen in the NAc in response to motivationally relevant stimuli, at least in part, could require activation of inhibitory afferents to LHb, thus disinhibiting midbrain dopaminergic neurons. Findings presented by this group, derived from optically evoked DA release and characterized by voltammetric methods, show that hybrid population of VTA neurons expressing dopaminergic and GABAergic markers send an inhibitory projection to the LHb and thus are able to directly inhibit LHb neurons, resulting in profound downstream effects on midbrain circuitry. In terms of our outcomes, phasic DA release, evoked by VTA in order to mimic those naturally occurring reward stimuli, may also work via similar mechanisms. VTA stimulation to evoke accumbal DA release could also provide inhibitory output to the LHb (thus further disinhibiting the VTA) where an overactive LHb (a largely excitatory structure) causes inhibition of phasic accumbal DA release. To make this concept clearer, LHb may be targeting midbrain GABAergic cells that normally inhibit the VTA DA neurons (and ~30% of cells in the VTA are GABAergic, and act as interneurons capable of suppressing adjacent DA neurons, see Creed et al. 2014 for a recent review). This also provide support for why pharmacological inhibition of the LHb would cause disinhibition of DA release in the forebrain. Mechanistic framework underscores the flexibility and complexity of the circuitry that impinges upon VTA dopaminergic neurons to promote motivated behavior, one of the core symptoms of depression (Stamatakis et al., 2013). Altogether, our results corroborating with previous literature highlights the bi-directional function of DA, depending on the target neurons and the state of the organism. Many support the diverse and differential role of DA which is sensitive to energy metabolism, stress and DA availability.

Firing patterns of mesolimbic dopamine neurons mediate an individual’s responses to chronic stress and antidepressant action. Similar to present findings, studies using a chronic social defeat model found abnormalities in the in vivo firing properties of VTA DA neurons which were tightly correlated with stress-induced behavioral abnormalities (Cao et al., 2010). Greater interest
of the mesolimbic DA system in the pathophysiology and treatment of depression have been gained, given the accumulating evidence of its role in hedonia and motivation (Friedman et al., 2007; Lammel et al., 2012; Nestler & Carlezon, 2006; Salamone & Correa, 2012). Future research should expand on the differential role of DA and corresponding behavioral adaptations.
References


Chapter 9. General Discussion

As introduced in earlier chapters, deep brain stimulation (DBS) has emerged as an alternative method for the treatment of refractory patients with depression and continues to hold great promise. Yet, our lack of understanding on its mechanisms of action are likely limiting our capacity to optimize this neuromodulation advancement for clinical practice, as illustrated by two recently failed clinical trials (Dougherty et al., 2015; Malone et al., 2009; Mayberg, Personal Communication, 2007). The series of papers presented in this thesis includes preclinical works aimed at providing insight into the therapeutic mechanisms of action of DBS in treatment resistant depression (TRD). Additionally, experiments were designed to model the neurobiology of antidepressant resistance, providing an opportunity to understand its distinct disease pathophysiology and how biological adaptations can reverse drug resistance.

From an experimental perspective, DBS can be used as an excellent probe to modulate neuronal activity in key regions and associative networks implicated in stress, stress response and antidepressant action (Benabid, 2015). The works presented here adopted this approach in combination with a number of complementary laboratory techniques such as gene expression profiling, immunoblotting, mitochondrial analysis and fast scan cyclic voltammetry (FSCV) to investigate biological mechanisms associated with treatment responsivity. Standardized behavioral assays were used throughout the chapters as an indicator of antidepressant-like response to various DBS targets. Our aims for each chapter were as follows: Chapter 4) to evaluate the effects of nucleus accumbens (NAc) DBS and adrenocorticotrophic hormone (ACTH) treatment on mitochondrial function and to characterize corresponding antidepressant-like and mania-like behaviors; Chapter 5) to establish lateral habenula (LHb) DBS efficacy in an animal model of tricyclic resistance and to quantify treatment modifications on calcium/calmodulin-dependent
protein kinase II alpha/beta (CaMKIIα/β), glycogen synthase kinase 3 alpha/beta (GSK3α/β), and AMP-activated protein kinase (AMPK) expression locally, and in the infralimbic (IL) cortex; Chapter 6) to interpret changes in endogenous gene expression modulated by IL DBS following forced swim test (FST) to identify acute, local neural responses to DBS in antidepressant-resistant vs. naïve animals; and Chapter 7) to examine phasic dopamine (DA) neurotransmission in the core of the NAc during high frequency (HF) stimulation of the IL, NAc and LHb, consistent with the role DA plays in emotion, motivation and TRD. Further, we monitored DA neurotransmission after discontinuing stimulation of these regions to observe neuroplastic processes in therapeutic responses of DBS. These studies have provided insight into the region- and/or network-specific mechanisms of DBS associated with induction of a therapeutic response in the antidepressant resistant state.

### Stress and the HPA axis

Cellular and systemic responses to stress are plastic and continue to adapt with the organism over time in response to changing environmental demands (Belda et al., 2008). How the system deals with the first stress exposure is particularly important as it instigates cellular adaptations evolutionarily pre-programmed to facilitate effective neurohormonal responses to future insults. For example, memory consolidation following an emotionally stressful event can prepare an organism for an adaptive response in the face of apparent threat, however, under certain circumstances, it also has the ability to trigger multiple pathological states (Valenti, Gill, & Grace, 2012). Long lasting effects of stress not only depend on stress type and chronicity, but also on the developmental stage of an organism (Lupien, McEwen, Gunnar, & Heim, 2009). Incidentally, the stress system plays both adaptive and maladaptive roles, either strengthening resilience or accelerating psychopathological processes (i.e. neuroprogression). Given that the hypothalamic pituitary adrenal (HPA) axis functions to promulgate stress and fine-tunes the metabolic requirements at the system and cellular level, animal models, such as the ACTH model of
antidepressant resistance, can help us to better understand biological characteristics of TRD. The HPA axis is a major participant in optimal stress reaction for survival (Gold, 2015) as well as treatment responsivity (Stetler & Miller, 2011), where it receives constraining inputs from the subgenual prefrontal cortex, a site implicated in stress appraisal and antidepressant action. To date, studies on the functional integrity of the HPA axis under various therapeutic interventions report conflicting results (Flores, Kenna, Keller, Solvason, & Schatzberg, 2006; Nemeroff & Owens, 2004; Papiol et al., 2007) and therefore, its involvement with antidepressant processes is incompletely understood. The model utilized in this series of experiments, chronic ACTH administration, however, indicates the HPA axis plays a critical role in the development of antidepressant non-response and in so doing, helps to fill this gap in clinical research.

**Overall Behavioral Outcomes**

The biological and behavioral reactions of an organism to stress, its stress coping responses, occur in the face of perceived environmental or systemic stressors and are thought to critically influence risk for and resilience against ill health. Our results of ACTH-induced tricyclic antidepressant resistance, replicated previous studies by Caldarone and Brunner (2009), Kitamura et al. (2008) and Walker et al. (2015). Depending on stress reactivity, recovery and the availability of adaptive coping resources, overcoming threatening or benign stimuli (through appraisal processes) may be indicative of the organism’s resiliency or susceptibility to mood disturbances. Brain regions such as the amygdala, hippocampus, prefrontal cortex, NAc and LHb are primary mediators of stress resiliency and vulnerability because of their roles in cognitive appraisal, and the regulation of behavioral, emotional and physiological responses to a given stressor. The aforementioned FST is a well validated tool, with a strong predictive validity for screening antidepressant effectivity, through the assessment of animals’ behavioral coping response (climbing, swimming, and immobility). It utilizes anthropomorphic concepts of different coping styles to identify animals that actively respond to a perceived stressor and those that demonstrate
passivity, previously referred to as ‘behavioral despair’. The robust active FST behavioral profile of animals receiving NAc, LHb and IL DBS indicates that these treatments are efficacious in otherwise tricyclic resistant animals. The outcomes further corroborate findings obtained in other preclinical depression models and clinical reports documenting the efficacy of DBS (Hamani et al., 2014; Meng et al., 2011; Riva-Posse et al., 2014). In these studies, animals receiving active stimulation in each brain region demonstrated clear antidepressant (active coping) responses relative to sham or surgery only animals under inescapable FST stress. These findings are promising; nevertheless FST results should be interpreted in light of its ability to evaluate antidepressant activity based on the reduction of immobility time (a test with predictive validity), rather than through a demonstrated reduction of anhedonia (i.e. sucrose preference test, considered strong face validity for depression, Nestler and Hyman (2010)). Of course, whether the differences in FST responses is due to the severity of the given stressor that it perceives or the inescapable nature, is impossible to discern.

Key findings from each experiment

Chapter 4: NAc DBS. There were two major findings in chapter 4. Firstly, mitochondrial function was associated with treatment resistant state vs. antidepressant action. Stress induces robust inhibition of mitochondrial energy generation (Gong, Chai, Ding, Sun, & Hu, 2011) and damages the mitochondrial ultrastructure. Changes in mitochondrial size, distribution and function are also observed in major depressive disorder (MDD) patients (Gardner & Boles, 2011). ACTH-treated animals’ capacity to create adenosine triphosphate (ATP) in response to the increased energy demand during inescapable situation was deficient. To our knowledge, this is the first preclinical study that directly implicates mitochondrial function in the underlying mechanisms of DBS. Secondly, NAc DBS obtained interesting behavioral results where both antidepressant-like and mania-like phenotypes were developed only in those with ACTH treatment and DBS electrode implantation. We observed that in ACTH-treated animals, electrode implantation prior to active DBS, elicited a mania-like phenotype in a sub-set of ACTH-treated animals. This was not observed in
the vehicle treated group. Here, the presentations of hyper-locomotor behavior, a failed response to imipramine and increased appetitive behaviors were accompanied by heightened mitochondrial efficiency. This contrasts the attenuated efficiency found in ACTH-treated animals and may reflect a biological overcompensation for this deficit. In other words, the ACTH-treated animals may have lacked the metabolic resources to meet the metabolic demands necessary to engage in an active behavioral response considered predictive of antidepressant response. In contrast, the hyperactive 'mania-like' animals demonstrated enhanced mitochondrial respiration activity. This was determined with the seahorse XP24 analyzer, which interrogates major energy producing pathways of the cell in brain tissue.

The current findings provide a crucial link between NAc DBS induced alterations in the medial prefrontal cortex (mPFC) mitochondrial function and the dysregulated HPA axis followed by chronic ACTH administration. Notably, DA and mitochondrial dysfunction are both implicated in neurodegeneration, established predominantly based on the Parkinsonism, schizophrenic and bipolar population data. It is widely thought that hyperactive DA levels underlie mania where increasing synaptic levels of DA (following amphetamine administration) correspond to defining manic-like features of bipolar disorder (BPD) in both humans and mice (Berk et al., 2007). Free radicals are produced during the metabolism of monoamines including DA, by monoamine oxidase A and B, both of which are located on the outer mitochondrial membrane (Manji et al., 2012). Mitochondria use several protective antioxidant molecules to interrupt or minimize this oxidative process, thereby reducing cytotoxicity from high levels of DA. Previously, increased oxidation has been observed in the DA enriched area of PFC in BPD patients (Kim, Andreazza, Yeung, Isaacs-Trepanier, & Young, 2014) and amphetamine increased markers of this oxidation in animals (Frey et al., 2006). Consequently, blocking DA receptors are among the most effective treatments for BPD patients (Cipriani et al., 2009). DA inhibits mitochondrial mobility through D2R agonist which subsequently decreased Akt-GSK3β signaling (Chen, Owens, & Edelman, 2008). A disturbance of
energy metabolism evidenced by behavioral and molecular results herein, may also reflect those changes in DA that have been previously observed in the clinical population. Levels of PFC DA have also been shown to be reduced with chronic ACTH administration (Walker et al. 2013). This was also demonstrated for transient NAc dopamine in the current series of studies (see Chapter 7). Additionally, the socially isolating conditions, as utilized herein, have been found to alter presynaptic DA transmission in rats (Lapiz et al. 2003). Mitochondrial and dopamine dysfunction may therefore be an important mediator of cycling mood states, with uncorrected metabolic deficits contributing to the neuroprogressive nature of MDD and BPD, eventuating in refractory depression states.

As an aside, a point should be made on possible pro-inflammatory reactions that likely occurred following electrode implantation and may have contributed to the observed mania-like behavioral phenotype. Others have shown that the electrode implantation surgery procedure alone can induce an antidepressant response in the chronic mild stress animal model of depression (Perez-Caballero et al., 2014). These authors further suggest that clinical treatment outcomes may be confounded by use of anti-inflammatories. Reduction in immobility time (attenuation observed with NAc electrode placement, although not statistically significant) observed for our ACTH animals, may be explained by acute pro-inflammatory reactions also. Inflammatory processes have been associated with treatment response where rats implanted with electrodes, irrespective of whether they received active stimulation, showed antidepressant-like behaviors (Perez-Caballero et al., 2014). This effect was due to regional inflammation, where it was temporally correlated with an increase of glial-fibrillary-acidic-protein immunoreactivity which was blocked by anti-inflammatory drugs. Furthermore, a retrospective study by the same group indicated that the early responses of MDD patients subjected to DBS was poorer when they received anti-inflammatory drugs in conjunction.
Chapter 5: LHb DBS. The critical role of energy regulation in our animal model of stress was further illustrated by the therapeutic mechanisms of LHb DBS. Immunoblotting quantified total protein expression, phosphorylated and total, 30 min post FST. Protein responses were expected to be distinctive, conditional upon ACTH vs. Saline administration separate to DBS treatment. DBS-facilitated local phosphorylation of CaMKIIα/β and GSK3α/β, with the normalization of AMPK activity in the IL was found. These effects were correlated with the adoption of active coping strategies in the FST. In clinical and preclinical trials of pharmacological and neuromodulatory interventions, decreased metabolic activity of the IL (or SCG in human trials) has consistently been associated with antidepressant efficacy (Mayberg et al., 2014; Riva-Posse et al., 2014). Similar to these previous findings, our results provide further support for attenuated metabolism of SCG and treatment response. This was indicated by reduced expression of AMPK in the IL following active stimulation, simultaneous to the activity-dependent phosphorylation of GSK3 and CAMKII in LHb.

The actions of these proteins also include management of cellular growth and survival. AMPK, where its activity is governed by cell's energy demand, mitigates dopamine dysfunction and is involved in maintaining mitochondrial homeostasis (Ng et al., 2012). As mentioned in the introduction, GSK3 mediates responses to lithium and affects dopamine activity. Furthermore, inhibitory phosphorylation of GSK3 and CaMKII coupled depolarization protects neurons from apoptosis (Song et al., 2010). To detail, the pro-survival effects of CaMKII has been found to be mediated by GSK3 phosphorylation and thus leading to inactivation. It has been well reported that depolarizing conditions sustain neuronal survival by causing influx of Ca²⁺ via L-type Ca²⁺ channels (Ghosh & Greenberg, 1995; West, Griffith, & Greenberg, 2002), which implicates Ca²⁺ as a necessary second messenger for survival signaling. When activated by elevated Ca²⁺, CaMKII has been documented to mediate this depolarization for neuronal survival. Therapeutic effects observed in our animal model of TRD correlated with the modifications in the phosphorylated and overall expression of these molecules.
Cellular mediators of calcium signaling, energy regulation and plasticity were identified as potential underlying mechanisms of LHb DBS in reinstating one’s treatment response. The differential role of LHb (which is activity dependent) and IL highlights distinctive network effects of DBS and provide interesting avenues for future application of these brain regions. The LHb has been reported to selectively target mPFC-projecting dopaminergic VTA neurons that produce aversion/avoidance in rodents (Lammel et al., 2012) and this LHb-mPFC-VTA may further be involved in stress-related cognitive disturbances in MDD (Mizoguchi et al., 2000). Present findings reinforce the importance of obtaining antidepressant actions via normalizing cellular and metabolic processes in the IL region of rats (SCG homologous) exerted by LHb stimulation.

Chapter 6: IL DBS. In Chapter 6, the investigation of gene expression proved a valuable tool for identifying candidate genes and altered molecular pathways in this stress diathesis model of treatment resistance and the restoration process. This study aimed to interpret changes in local gene expression modulated by IL DBS and after an exposure to the FST. Investigating the IL cortex is particularly significant due to its role in HPA axis regulation, cellular energetics (McKlveen, Myers, & Herman, 2015) as well as its connections to other depression relevant regions such as bed nucleus of the stria terminalis, NAc and LHb. Key sensors of energy demand, cell division/growth, protein synthesis and glucose/glycogen regulation were significantly altered in both ACTH and Saline-treated animals. Introducing an exogenous stressor, the FST, to ACTH animals resulted in decreased p53, yet activated glucose production pathways, possibly to compensate for the increasing energy demand and protecting against the deleterious effects of stress. Environmental stressors further altered WNT and B-catenin signal transductions leading to the inhibition of cell proliferation. This process is another major compensatory action in controlling for maladaptation’s that may contribute to presentation of depressive symptoms. Endogenous systematic stress on the other hand, induced up-regulation of metabolic pathways implicated in hypoxia, suggestive of oxygen deprivation within that region. Finally, a combination of endogenous (ACTH) and exogenous
(FST) stress led to altered gene and protein expressions in regulators of cell proliferation and apoptosis. Initiation of apoptosis is necessary to lessen the chances of senesce and DNA mismatch. Absence of appropriate apoptotic processes, therefore, facilitates the neuroprogression of mood disorders and prevents possible correction of maladaptations on a cellular level.

The manipulation of inescapable environmental stressor together with the administration of ACTH uncovered differential cellular/molecular mechanisms that the animals adopt, providing a snapshot of the molecular mechanisms contributing to antidepressant-resistance in this model. These molecular mechanisms, in turn, will inform important information in developing basic animal models for target discovery. Thereafter, target discovery should be based on casual relations, not phenomena, for translational and predictive treatment outcomes.

Chapter 7 & 8: FSCV recording of DA transmission following high frequency NAc, IL and LHb DBS. FSCV, given its temporal specificity, was an important tool for studying ventral tegmentum area (VTA) evoked NAc DA release and plasticity in chapter 7. Plasticity and stress is intimately linked and as discussed in Chapter 1, their relationship is crucial in either accelerating or slowing the onset of disease progression. High frequency DBS of each region modulated transient NAc DA efflux. DBS at these targets may thus serve to regulate the availability of DA release in response to stimuli. In healthy controls, an attenuation and long term depression (LTD) of phasic DA were observed following NAc and IL DBS. Uniquely, LHb DBS induced long term potentiation (LTP). As hypothesized, this DBS-induced long-term plasticity of VTA-evoked NAc DA efflux in ACTH animals was absent. Moreover, the ACTH-induced reduction of VTA-evoked phasic DA release was restored with LHb DBS. However, as LTP was absent, the signal recovered to its pre-DBS baseline levels soon after cessation of DBS. The findings from this study, thus suggest that the mesoaccumbens circuit is dysregulated in ACTH animals and that this may be a contributing factor for their resistance to antidepressants.
The deficit in synaptic plasticity induced by chronic pre-treatment with ACTH suggests inhibition of long-term plasticity may contribute to the progression of treatment resistance in depression. The DA system is not only modulated by stress and metabolic mediators on a cellular level, but at the systems level the output of DA release is contingent upon the interplay between metabolic hormones and the experience of stress. DA neurons are particularly sensitive to physiologic energy demands, changing the responsivity to stimuli, with important implications for psychological well-being and stress appraisal (Schellekens, Finger, Dinan, & Cryan, 2012).

Dysregulation of DA system function has been proposed to partially underlie the delayed responses to antidepressants (Dunlop & Nemeroff, 2007). A role for DA in antidepressant treatment non-response is suggested from data demonstrating that responders to SSRI s exhibit increased DA binding to striatal D2 receptors –with the degree of increased D2 receptor binding correlated with improvement in depressive symptoms (Moylan, Maes, Wray, & Berk, 2013). Repeated stress may lead to sensitization of the mesolimbic DA system via increased glucocorticoids (GR), as GR themselves may selectively facilitate DA transmission in NAc (Oswald et al., 2005). The DA system has been found to demonstrate complex responses to stressors and likely plays a major role in adaptive as well as maladaptive responses, particularly as they influence effort-related motivation and psychopathology (Salamone, Correa, Farrar, & Mingote, 2007; Valenti et al., 2012).

In addition, the FSCV outcomes suggest that pathway-specific dopaminergic output of the VTA is intricately regulated by long-range inputs to drive reward or aversion. Salamone and colleagues, through their works looking at DA (Salamone & Correa, 2012; Salamone et al., 2007), also emphasize this neurotransmitter as selective and dissociative where it acts on activational aspects of motivation. In other words, not only it is closely linked to motivation and reward, but it plays a crucial role in effort-related decision making and hedonic sensitivity. Previous findings, together with our results, reinforce the role of DA in emotional, behavioral and attentional aspects
of depressive phenotype. It underscores important therapeutic implications for DA, as the DA system is interlaced with stress and is implicated in the neuroprogressive nature of mood disorders (Berk et al., 2007).

**Potential DBS mechanisms and its implications as an alternative treatment**

DBS may be reinstating or reversing the cellular, molecular and system adaptations following stress by modulating: 1) activity dependent local cellular functions; 2) orthodromic vs antidromic inhibition or excitation of afferent regions; 3) inhibitory action; and lastly 4) modulating local and distal networks. The most plausible model of DBS mechanisms is stimulation-induced modulation of pathological network activity reflecting one or more of those actions listed above. Dr Paul Holtzheimer recently presented an important perspective, suggesting that DBS may help the brain to reinstate to a normal *dynamic* state (where this does not equate to a state of a 'healthy' brain), but enables patients to rehabilitate via a progressive process of recovery (SOBP 2015 conference). This rehabilitation may require a combination of adjunctive psychotherapy, social support and healthy lifestyle changes. In order to effectively treat these severely refractory patients, the field of psychiatric research needs to move away from circuit replacement theory but instead, towards fostering plasticity and neuronal remodeling (Bessa et al., 2009). Findings herein, together with the existing literature, demonstrate significant therapeutic responses by influencing inter-connected intermediary nodes and molecules that mediate plasticity, neuronal growth and energy regulation. Given the extreme treatment resistant forms of MDD the patients had, it is not surprising that these effects may only take place after a considerable time of active stimulation that may also differ from individual to individual.

**A need for understanding different etiologies**

As depression is heterogeneous, subpopulations of TRD exist. In Chapter 1, these concerns were raised and a subsequent review of previous literature made clear of their impact on diagnosis,
treatment effectivity and population statistics. The manner in which patients may enter a depressive episode changes across developmental trajectory and disease progression, which could incidentally account for much of the inconsistency in the neurobiological literature, in relation to, for example, the HPA activity, peripheral BDNF or the changes reported in structural and functional imaging data. It is also a distinct possibility that if the neurobiological mechanisms change across episodes and depression severity, then different antidepressant strategies might be necessary at these stages, and this might account largely for the clinical failure in TRD population (apart from treatment adherence, tolerability etc.).

Foremost factors behind individual variability in disease trajectory may be explained by genetic x environmental interactions, types of stressors, early life stress and comorbidities with medical or other psychological conditions. A short discussion of stress is important here given that our animal model utilizes disturbances in the stress axis as a basis for studying the antidepressant effects of DBS. Overlapping constitutional and experiential factors promote both vulnerability to depression and resistance to treatment (Willner, Scheel-Krüger, & Belzung, 2013). Differentiation exists for the effects of intense stressor vs. a combination of low levels of stress with a variety of factors that confer vulnerability to depression (Valenti et al., 2012). These differences accordingly cause cellular damages, dysregulated monoamines transmission to regional hyper/hypo-activity. Currently available antidepressants targeting monoamines, primarily repair a damaged hippocampus and are therefore ineffective in depression subtypes where it is assumed that stress has had only a minor precipitating role (perhaps vulnerability due to genetics). Antidepressants promote hippocampal neurogenesis but this is not a critical event for their mood-rectifying actions (Anacker & Pariante, 2011). Demonstrated by the present findings, the mesoaccumbens pathway and its associative regions play a crucial role in treatment responsivity that cannot be all accounted for by hippocampal processes.
All parts of the system can be narrowed down to specific symptoms of depression, with corresponding network activity (e.g. rumination and negative self-referential attributions reflect hyperactivity in amygdala and ventral prefrontal cortex regions whereas anhedonia has been linked to hypoactivity of NAc and dorsal prefrontal cortex). Current findings suggest that complex networks and interactions between signaling pathways will generate a wide variety of symptoms and severity, contingent upon the degree of dysfunction in each region (and their major molecular function), which in turn, depends upon the patients’ profile of vulnerability, precipitants and manner in which they are affected. It is also apparent that traditional ways of thinking about depression, based around a single brain region or a neurotransmitter are no longer adequate and the field of translational neuroscience and biological psychiatry are moving away from it.

Hippocampal function, for example has been heavily focused in studying the neurobiology of depression and antidepressant properties. As shown in our findings, a broader focus is essential because, according to our current understanding, the hippocampus may almost be incidental to the symptomatology of depression. Neurogenesis maintains an efficient level of functioning within the hippocampus (Airan et al., 2007), which is concerned primarily with contextual learning (Rudy, 2009). In the mPFC, synaptic remodeling and plasticity may underlie the action of antidepressant drugs. More direct involvement in the psychological (via bed nucleus of the stria terminalis, PFC, NAc and LHb) and physiological (PVN –stress axis) areas characterize the multifaceted depressive symptomatology and severity.

A new understanding could adopt a perspective that different routes can be taken in order to achieve the same therapeutic outcome –via correcting for an abnormality either critical in its physiological function or of several functions that are not critical (primary) in itself. Again, this highlights the notion that DBS or any other drug intervention may exert its antidepressant effects by modulating pathological network activity. In light of recent negative results (Dougherty et al., 2015), a lower response rate may be due to a systematic issue where individual DBS parameters
were established using a complicated process quickly within days and then left unchanged for the 4-months controlled period (Schlaepfer, 2015). This approach, which is often similar to administering first-line pharmacotherapies, is counterintuitive because MDD patients take lengthy (often years) treatment course to reach optimal antidepressant efficacy. It would be of great interest in the future to examine how antidepressants could promote recovery of key symptoms with or without the reversal of the original etiology of the disease. Other psychopharmacological and neuromodulatory methods (e.g., TMS) could also be formulated to encounter these tracts on a signaling to systems level.

An interaction between strong expectation of DBS effect x implantation of electrodes may explain no distinct separation on therapeutic outcomes between treatment and placebo. Previously, anticipation of treatment benefit in placebo groups have been found to affect outcomes with various antidepressants (Benedetti, Carlino, & Pollo, 2011). On the other hand, HDMRS or other depression scores used in clinical trials may not adequately represent the functional outcomes or emotional differences these patients experience. Changes in our expectations and/or addition of other measures could more appropriately investigate the results of DBS efficacy.

Also, the development of mechanistic/mathematical models could capture the inter-relationships that are so many and far too great for our minds to comprehend as to defy accurate predictions of the effects of any perturbation. Previously, Belzung and de Villemeur (2010) have presented such formal computational model of the hippocampal control of the HPA axis. Our gene expression data portrays a number of interrelated pathways that differentially or similarly respond to stress that are unique to the state of organism. Such models may help us better converge different cellular cascades and molecular interactions to use them for clinically meaningful, personalized and optimal treatment deliverance. These models could provide predictive outcomes that links specific depression etiologies to treatment options.
Finally, a concept that demands attention is a suggestion that what is important about depression is not so much its symptomatology as its chronicity: that is, the phenomenology of depression is a frequent natural response to adversity and what distinguishes people who become clinically diagnosable from those who do not, is that they become “stuck in a rut” and are unable to recover (Holtzheimer & Mayberg, 2011). The progressive nature of MDD consequently increases vulnerabilities to future depressive episodes, relapses and precipitates illness course leading to functional deterioration (Moylan, Maes, Wray & Berk, 2013).

**Limitations**

The work represented here should be interpreted in light of several limitations. First of all, a diverse set of behavioral paradigms could have been implemented to better characterize antidepressant-like effects and neuroplastic processes (novelty suppressed feeding, morris water maze etc.). Nevertheless, the experiments were carried out within the given time and available resources. More importantly, we were particularly interested in characterizing a treatment resistance phenotype where the FST was deemed sufficient in such a case. There is no perfect animal model for depression. However, each animal model offers a unique insight into a specific feature of this heterogeneous disorder.

Secondly, rats were housed in isolation from arrival to testing in chapters 4 & 5 to promote a depressive-like phenotype that should, in theory, enhance the appearance of TRD in combination with ACTH treatment. In experiments where socially-housed rats were used (chapters 6-8), the observed effects could have differed from the earlier experiments as those animals were not exposed to the social isolation paradigm. Nevertheless, the reader should take a note that there is an emphasis on the possible antidepressant-like effects of DBS using the ACTH model in chapters 4 & 5; whereas chapter 6 explored gene x environmental interactions in a broader sense. Chapters 7
& 8 on the other hand, specifically investigated the region of interest, frequency of stimulation and treatment on accumbal DA release.

A deep, inherent issue that lies with preclinical research, and with the perspective of a diathesis/stress model, is that animal models of depression are mostly models of a first depressive episode. Animal models of depression have not addressed the progressive vulnerability that develops over successive episodes of depression. Consequently, the preclinical literature has almost nothing to say about the mechanisms by which depression becomes progressively more autonomous to stress (Willner et al., 2013). Animal models of repeated depressive episodes may be the next step in preclinical research. Our experimental design presented a novel method in incorporating chronic ACTH administration (an endogenous stressor) over time to observe its continual effects. Corticosterone administration, could be a useful way of identifying systems of interest but preempts investigation of important elements to the stress system. Whereas, our exploratory approach (especially in LHb and IL DBS chapters) allowed us to capture novel therapeutic targets and corroborated with previous findings to build a more integrative picture on the roles of key molecules and brain regions to necessitate antidepressant effects.

Conclusions/Future directions

The experiments within this thesis have significant implications for studying behaviors that are relevant to both health and disease. We further need to study native patterns of cellular function or circuit projections. By doing so, we should be able to distinguish the mechanisms that ‘flip’ an individual from healthy to mental illness and what exacerbates that condition. At large, current experimental designs involve focusing on recreating or reflecting a disease state. It would be of great value to understand natural cell dynamics and mammalian behavior to identify factors that speeds up the transition from adaptive to maladaptive system. A problem with studying mechanisms and corresponding behavior by turning systems up or down is that we have
preconceived expectations and ‘chosen’ pathways that we think are involved for certain stress responses. Conversely, teasing out the intimately interconnected systems and studying them one by one, enables us to put together a whole picture. By separating the phenomena of interest from subjective emotional states, we may be able to better understand the complexity of the systems (see Figure 1).

Figure 1. We need to carefully redefine subjective human emotion state to animal behavior. Technical advances in the last decade has expanded our capacity to map neural pathways that are relevant across species. Implementation of appropriate animal models may provide us with new possibilities for establishing individualized, stage specific treatment options. Diagram included with permission from Hendriksen and Groenink (2015).

Stimulus and conditioned experience are not factors that fundamentally make us feel emotional. It is our capacity to have a conscious awareness of this experience and a cognitive processing that this is happening to ‘us’ that makes an event an emotionally relevant one. Such
higher executive functioning processes require cognition and language, which is also culturally bound and environmentally driven, rather than made up of innate survival responses—these innate autonomic reactions are only temporary.

In conclusion, current antidepressant and neuromodulatory methods improve abnormal mood states through multidimensional mechanisms that are themselves incompletely understood. Presented works in this thesis demonstrates that each pathway (bioenergetics, synaptic plasticity, dopamine, mitochondrial function and stress) represents valuable pieces that provide a more comprehensive and converging picture on factors mediating treatment responsivity. Not all have to be dysregulated or be severe in order to cause enough of a deficit to ultimately bring about treatment resistance in an individual. Furthermore, it may be that these cellular and molecular systems work in intricate orchestration which ultimately disturbs cellular patterns on a systems-wide level. We need to understand whether primary and/or secondary causes need to be targeted for effective treatment outcome. The initial etiology may not correctly represent the necessary target for recovery as the patients move through different stages in the neuroprogression of mood disorders.

Overall, the outcomes underscore the significance in understanding the underlying antidepressant properties that achieves treatment response in individuals of refractory state in TRD, using DBS to manipulate and probe different systems. Such an approach gave us insight into the consequences of serious neuroprogressive nature of this disorder, which not only contributes to the economic and societal burden but may lead to fatal consequences for its sufferers. Armed with this knowledge, we must work on preventative strategies and interventions that could take place during one’s ultra-risk prodromal stages. The following chapter, titled “Stress, inflammation, and cellular vulnerability during early stages of affective disorders: Biomarker strategies and opportunities for prevention and intervention” (Walker et al., 2014), highlights how some of the mechanisms identified in this thesis may also be relevant targets for prevention and intervention.
during the prodromal (not yet clinically diagnosable) stage of mood disorders. With successful intervention and earlier modulation of these systems during the critical neurodevelopmental stages, the neuroprogression of these illnesses may be hindered or even prevent the onset. Apart from many other factors that contribute to the makeup of TRD population, early intervention may be a fundamental, long-lasting step to reduce the growth of susceptible individuals falling into such a debilitating stage of the disease. Shifting the way we typically think of mental disorder as stages of neurodevelopment provides an optimum foundation for prevention-driven research.
References


Chapter 10. Stress, inflammation, and cellular vulnerability during early stages of affective disorders: biomarker strategies and opportunities for prevention and intervention

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Stress, inflammation, and cellular vulnerability during early stages of affective disorders: biomarker strategies and opportunities for prevention and intervention

Adam J. Walker1,2, Yesul Kim1,2, J. Blair Price1, Rajas P. Kale1,3, Jane A. McGillivray2, Michael Berk4,5,6,7 and Susannah J. Tye1,2,8*

1 Department of Psychiatry and Psychology, Mayo Clinic, Rochester, MN, USA
2 School of Psychology, Deakin University, Melbourne, VIC, Australia
3 School of Engineering, Deakin University, Geelong, VIC, Australia
4 School of Medicine, Deakin University, Geelong, VIC, Australia
5 Department of Psychiatry, University of Melbourne, Melbourne, VIC, Australia
6 Orygen Youth Health Research Centre, Melbourne, VIC, Australia
7 The Florey Institute of Neuroscience and Mental Health, Melbourne, VIC, Australia
8 Department of Psychiatry, University of Minnesota, Minneapolis, MN, USA

The mood disorder prodrome is conceptualized as a symptomatic, but not yet clinically diagnosable stage of an affective disorder. Although a growing area, more focused research is needed in the pediatric population to better characterize psychopathological symptoms and biological markers that can reliably identify this very early stage in the evolution of mood disorder pathology. Such information will facilitate early prevention and intervention, which has the potential to affect a person's disease course. This review focuses on the prodromal characteristics, risk factors, and neurobiological mechanisms of mood disorders. In particular, we consider the influence of early-life stress, inflammation, and allostatic load in mediating neural mechanisms of neuroprogression. These inherently modifiable factors have known neuroadaptive and neurodegenerative implications, and consequently may provide useful biomarker targets. Identification of these factors early in the course of the disease will accordingly allow for the introduction of early interventions which augment an individual's capacity for psychological resilience through maintenance of synaptic integrity and cellular resilience. A targeted and complementary approach to boosting both psychological and physiological resilience simultaneously during the prodromal stage of mood disorder pathology has the greatest promise for optimizing the neurodevelopmental potential of those individuals at risk of disabling mood disorders.

Keywords: prodrome, depression, bipolar, biomarker, stress, inflammation, cellular resilience, plasticity

INTRODUCTION

There is increasing appreciation for the need to both identify and treat mood disorders during their earliest stages (1). Although some dispute remains, maladaptive changes in mood and behavior first become evident during the prodromal period (2). However, the low specificity of these changes makes the prodromal stage difficult to definitively characterize prior to disease onset (3). Observable changes in mood and general physiologic functioning can include increases in sadness, anhedonia, irritability, anger, and anxiety, together with alterations in sleep and energy (4). Correlating these symptoms with prodromal biomarkers offers an exciting juncture whereby targeted interventions could be opportunistically employed to prevent neurodegenerative changes from accruing as the disease progresses (5). The potential to intervene during the prodromal stage of psychiatric illness through the detection and remediation of novel biomarkers has perhaps been best studied in schizophrenia, wherein most individuals experience a lengthy prodromal period prior to the full emergence of diagnosable psychotic symptoms (6). As an exemplar, low levels of nervonic acid appear to be a risk factor for conversion from high-risk to frank psychosis (7), and this risk of conversion may be reduced by targeted omega-3 fatty acid supplementation (8). Encouraging results from this work have renewed interest in the early detection of affective disorders, particularly bipolar disorder, with the hope that earlier and more targeted interventions might slow disease progression (3, 9–12). This can significantly impact neuroprogression and subsequent disease course for the individual (13). This concept of “neuroprogression” refers to the cumulative restructuring of the central nervous system which in turn mediates the development and persistence of psychiatric illness (14, 15). This process results from disturbances in inflammatory mediators, neurotrophins, oxidative stress, and energy regulation (14, 15).

BIOMARKER STRATEGIES FOR PRODROMAL MOOD DISORDERS

STRESS AND ALLOSTATIC LOAD

Stress sensitization and early detection

Stress is one of the best-studied mediators by which genetic vulnerabilities are translated into mood disorder pathology through the process of neuroprogression (16–18). Numerous studies have...
demonstrated that both depression and bipolar disorder are more prevalent in individuals who have experienced adverse early-life events. This is partly because such experiences prime future physiological and neural responses to stress, elicit a state of chronic inflammation (19), alter cellular mediators of plasticity and energy metabolism, and increase cellular “wear and tear” (20–22). Early-life stress (2) can be particularly deleterious because of its potential to influence the programming of the hypothalamic–pituitary–adrenal (HPA) axis (23) to induce persistent sensitization of neuroendocrine, autonomic, oxidative, and immune responses to stress. Over time these sensitized systems cumulatively contribute to the cellular and synaptic alterations underlying neuroprogression (21, 24–26). Specific examples include changes in reactivity of inflammatory cytokines [e.g., interleukin 6 (IL-6)] (25), alterations in markers for lipid peroxidation [e.g., 8-iso-prostaglandin F(2α)], oxidative damage to DNA (8-hydroxy-2′-deoxyguanosine) and RNA (8-hydroxyguanosine) (24), as well as altered cortisol, adrenocorticotropic hormone, and corticotrophin releasing factor responses (26). Identification of the state of physiologic and cellular resilience or sensitivity to stress may provide an important indicator of the level of neuroprogression and stress-mediated disease pathology for affective disorders, potentially prior to the initial manifestation of the mood episode (22).

One mechanism whereby HPA axis sensitization is likely to occur is through epigenetic regulation of stress response processes (21, 27). Evidence shows that exposure to various forms of stress result in multiple epigenetic changes in limbic regions as well as the HPA axis (21, 27). Interestingly, a recent study by Klendel and colleagues (18) found that only individuals who exhibited allele-specific DNA demethylation in functional glucocorticoid response elements of FK506 binding protein 5 (FKBP5), were prone to developing persistent cortisol dysregulation (18, 21). Further, this association was found to be dependent on an interaction effect with trauma in early life, suggesting that key developmental stages are directly related to stability of the observed effects across time (18). In another study, significant interactions between peripheral FKBP5 mRNA expression and disease progression were reported, suggesting that polymorphisms in the gene directly impact the extent of neuroendocrine dysregulation, and corresponding neuroprogression (28). The FKBP5 risk allele and corresponding levels of mRNA expression may represent useful biomarkers. These markers could be employed to identify individuals in the prodromal stages of stress-sensitive psychiatric disorders, such as major depression or bipolar disorder. Such detection would facilitate early intervention and could improve resilience and alleviate allostatic load in the prodromal individual.

**Early-life stress and accumulation of allostatic load**

Accumulation of allostatic load is a key mechanism through which early-life stress is thought to result in psychopathology (29). This is mediated via a series of enduring adaptive changes across a range of systems primed both to respond rapidly to challenge, as well as to restore homeostatic equilibrium (30). Adaptive allostatic mechanisms may fail when chronically challenged or when regulatory systems falter. This leads to a state of allostatic overload, which is thought to considerably impact the clinical course of mood disorders (31–33). Without sufficient opportunity for recovery, the brain and body are repeatedly exposed to molecular mediators of stress that can increase the level of cellular “wear and tear” (33). These mediators, which include metabolic factors, inflammatory cytokines, neurotrophins, and oxidative species, collectively impact an individual’s mental and physical resilience as outlined below [for more detailed reviews see Ref. (6, 34, 35)]. Both physiological (i.e., immune and/or metabolic) and psychological (i.e., bullying) stressors contribute significantly to allostatic load, and thus need to be considered together when assessing both risk and relative staging of mood disorder pathology (6, 34).

Enhancing an individual’s capacity to buffer the physiologic toll that accumulates through allostatic overload should be considered an important early intervention strategy. As allostatic load accumulates and attempts to maintain cellular homeostasis fail, cell danger signals are propagated and pro-apoptotic cell signaling pathways become increasingly engaged (36–39). This may play a role in medical comorbidities such as heart disease (40), as well as interfere with the therapeutic mechanisms of antidepressants and mood stabilizers to impair treatment efficacy (41–43). Internal stressors that activate the HPA axis and associated allostatic systems can limit an individual’s capacity for allostasis even prior to the onset of external stressors (36). For example, an endogenous load can build through the expression of homocysteine or inflammatory cytokines, limiting the capacity of adaptive responses in the face of subsequent stressors. Interventions that counter this load and reduce levels of proinflammatory mediators or interfere with their neuromodulatory actions could limit neuroprogression in both bipolar and unipolar depression, as well as enhance capacity for antidepressant efficacy (44–46).

**INFLAMMATORY PROFILE**

Stress during earlier life is not only associated with disruption of the HPA axis, but may also serve to sensitize proinflammatory responses to future insults (47–49). Inflammatory mechanisms are increasingly appreciated for their critical role in mood disorder pathophysiology, in particular via their regulation of neuronal excitability, synaptic transmission, synaptic plasticity and neuronal survival (41, 50, 51). Of specific interest are proinflammatory mediators, such as cytokines [i.e., interleukin 1, IL-6, and tumor necrosis factor alpha (TNF-α)] and C-reactive protein (CRP). CRP is often used as a biomarker for inflammation in studies due to its relationship with proinflammatory cytokines and role in the immune response. As demonstrated by Slopen and colleagues (49), individuals at ages 10 and 15 who reported adverse life events at critical stages between the ages of 1.5 and 8 years were found to have significantly increased levels of CRP and IL-6. These heightened concentrations were correlated with immune activation and depressive-like symptoms. Notably, increased CRP levels have been used previously to predict depression severity and recurrence rates in males (48, 52).

There is a growing literature supporting the use of inflammatory biomarkers as predictors of ensuing mood disorder pathology (22). Research to date has been focused on investigating the relationship between inflammatory cytokines and affective disorders in adults; however, their specific role in early onset/adolescent psychopathology is less well explored (53). Cytokines are thought to influence neurodevelopment during key
stages, such as adolescence, interacting with biological systems including those of stress hormones and gonadal hormones (53). As such, perturbation of inflammatory balance in adolescents may significantly contribute to neuroprogression and development of psychiatric illness (19, 53, 54). For example, elevated serum levels of TNF-α, IL-6, and interleukin-10 (IL-10) have been reported during the early stages of bipolar disorder (55), and CRP appears to be a biomarker of de novo depression risk (56).

As the mood disorder pathology progresses, an increasing number of proinflammatory cytokines are observed, including elevated levels of interferon gamma (IFN-γ) (22, 54, 55). Notably, increases in IFN-γ are associated with dysregulation of the tryptophan metabolite pathway via direct role in indoleamine 2,3-dioxygenase (IDO) activation. Activation of IDO is commonly found in later stages of mood disorders, and is a biomarker of depression-like behavior mediated by neural inflammation in animal models (48). Proinflammatory cytokines activate IDO, resulting in depletion of serotonin and augmentation of quinolinic acid (QUIN) metabolism over kynurenic acid (KYN). Tryptophan metabolites (kynurenine, KYNA, 3-hydroxykynurenine, and QUIN) act as neuromodulators to influence behavioral, neuroendocrine, and neurochemical aspects of depression (57–60). Consequently, this accumulation of QUIN facilitates neurodegeneration over neuroprotection, impacting mood disorder neuroprogression and resultant disability (61).

It is noteworthy to mention several other findings regarding altered inflammation in youth with psychiatric pathology. Increased mRNA and protein expression levels of IL-1β, IL-6, and TNF-α were reported in the anterior prefrontal cortex of adolescent suicide victims compared with normal control subjects (62). Elevated levels of inflammatory cytokines (among others: TNF-α, IL-1β, IL-6, and IFN-γ) were also observed in the serum of pediatric patients who experienced first-episode psychosis, in addition to increased leukocyte counts and evidence of blood–brain barrier damage (63). Quantification of inflammatory biomarkers (e.g., TNF-α, IL-6, IL-10, or CRP) may thus prove useful for detecting individuals at risk for developing a mood disorder. A recent study by Byrne and colleagues (64) suggests that levels of peripheral cytokines (e.g., IFN-γ) and CRP in salivary samples may correlate with serum samples in young people. Salivary assay may prove to be a simpler, less invasive method of estimating peripheral levels of inflammatory markers in adolescents (64). This provides one avenue whereby prodromal individuals could potentially be identified and their disease onset delayed.

DIMINISHED SYNAPTIC INTEGRITY

Homeostatic control of synaptic connections within key mood-related circuits plays a critical role in the etiology of mood disorders (65). Stress and inflammation as discussed in previous sections are implicated in disruption of synaptic signaling and integrity during the early stages of mood disorder pathogenesis. This is mediated in part through the inhibition of neurotrophic function, of which brain derived neurotrophic factor (BDNF) is the most thoroughly characterized. BDNF plays an important role in neuronal development, survival, and function, including activity-dependent synaptic plasticity (66). Synaptic plasticity is characterized by various processes, including synaptic remodeling, synaptogenesis, long-term potentiation, and long-term depression, all of which critically mediate the flow of electrochemical information throughout the central nervous system (67, 68). Stress, allostatic load, inflammation, antidepressants, and mood stabilizers exert major effects on signaling pathways that regulate cellular plasticity, suggesting these are critical neurobiological mediators of mood dysfunction and therapeutic intervention (69–72).

Glycogen synthase kinase-3 (GSK-3), part of the signaling cascade regulated by BDNF, plays an important role in synaptic homeostasis through regulation of synaptic deconsolidation (pruning) and glutamate receptor cycling (73). Increased GSK-3-mediated synaptic deconsolidation has been suggested to be an important factor contributing to reduced spine density in mood disorders (74). Additionally, levels of activated GSK-3 are increased in post-mortem brain tissue from individuals with unipolar and bipolar depression (74). In addition to BDNF, GSK-3 is deactivated by signals originating from numerous signaling pathways demonstrated to be dysregulated in mood disorders (e.g., Wnt and PI3K pathways), and is either the direct or downstream target of many mood stabilizer and antidepressant medications (75). GSK-3 activity is modulated by serotonin and dopamine, and is a critical node at the intersection of multiple neurotransmitter and cell signaling cascades (68). As a result, GSK-3 modulates not only synaptic plasticity but also apoptotic mechanisms and, in turn, plays a critical role in mediating cellular resilience (75). For this reason, GSK-3 has received much attention for its potential to be targeted as an early intervention strategy during the prodrome period.

IDENTIFYING IMPAIRED CELLULAR RESILIENCE

Stress, allostatic overload, and neuroinflammation function together to impair synaptic plasticity and cellular resilience. Disrupted plasticity along with increased cellular vulnerability contributes significantly to the pathophysiology of mood disorders and directly to the neuroprogressive nature of the disease course (3, 76). Some of the key mechanisms of disease progression affecting cellular resilience include: oxidative stress, decreased neurotrophic factor expression, reduced neurogenesis, impaired regulation of calcium, altered endoplasmic reticulum and mitochondrial function, together with dysregulated energy metabolism and insulin signaling. Each of these mechanisms are mediated by allostatic overload and neuroinflammation [for detailed reviews see Ref. (3, 36, 76–78)]. Together, these processes demonstrate that in addition to synaptic integrity, maintenance of cellular homeostasis is critical for facilitating cellular resilience and attenuating mood disorder pathogenesis (79), which is also likely to enhance the capacity for treatment response during later stages of the disorder (80).

Cellular vulnerability and resilience are mediated by apoptotic and anti-apoptotic intracellular signaling cascades, respectively. Apoptosis is important for the regulation of developmental processes and prevention of cancerous growths. Excessive apoptosis in neuronal systems, however, leads to neurodegeneration and certain cell populations are at increased risk of stress-mediated apoptotic cell death (80). Apoptosis is a tightly regulated and energy-dependent process, which coordinates programmed cell death in response to different stimuli (81). This can occur through stimulation of death receptor proteins...
[i.e., tumor necrosis factor (TNF) receptor] by cytokines of the TNF superfamily or in response to mitochondrial degradation. These stimuli result in activation of executioner caspases that function to coordinate cellular process necessary for apoptosis, including cessation of cell repair processes and cell cycle progression, cytoskeletal and nuclear disassembly, and flagging the cell for phagocytosis (82). Distinct classes of antidepressants and mood stabilizers have been demonstrated to facilitate cellular resilience to prevent progression of pro-apoptotic processes, and novel treatments are currently being developed to target these specific mechanisms (83). Biomarkers that characterize the level of neuronal vulnerability relative to resilience may prove useful as biomarkers of prodromal mood disorder pathology. This has been demonstrated for later stages of bipolar disorder (84), however more studies are needed to determine the utility of such cell danger biomarkers during the mood disorder prodrome (22).

**OPPORTUNITIES FOR PREVENTION AND INTERVENTION**

**IDENTIFYING VULNERABILITIES AND BUILDING RESILIENCE AT THE CELLULAR LEVEL**

Identification of individuals at risk of developing a mood disorder, or those in the prodromal stage, provides a potential opportunity to target these mechanisms for neuroprotective interventions that enhance cellular resilience, maintain synaptic plasticity and boost psychological resilience (Figure 1) (85). One of the longest held notions of brain plasticity is that certain critical periods or windows exist in development, during which circuitry is consolidated for lifetime functionality. Recently, there is a rising consensus that developmentally induced plasticity can, to an extent, be reversed by “re-opening” those windows of plasticity (86). Hyman and Nestler (87) have underscored the importance of shifting the brain into an “adaptive state” to necessitate the antidepressant response. Their theory of “initiation and adaption” is exemplified by psychotropic drugs wherein primary molecular targets that initiate alterations in brain function activate homeostatic mechanisms that return the system to an adaptive and treatment responsive state (87). Plasticity and cellular resilience are thus necessary for the efficacy of antidepressants and mood stabilizing treatments. McGorry and colleagues (6, 88) and others (89) have demonstrated this concept with pre-psychotic interventions, and repeatedly emphasized the need to take advantage of the “windows of opportunity” present within the prodromal stages of psychiatric disease (6, 88, 89). During this stage, the course of the disease remains theoretically plastic and amenable to intervention (90). Previous literature indicates that once risk or prodromal symptoms of mood disorders are identified, there is some (91), but not unequivocal (92) evidence that early intervention in adolescents can significantly reduce mood-related symptoms and incidence of fully diagnosable psychiatric disorders such as depression (93–95). Neuroprotective pharmacotherapies together with appropriate psychotherapy may reduce the risk of neuropsychiatric disease progression in young people which, together with allostatic load reducing behavioral interventions, may significantly slow the trajectory of the disease course into adulthood (6, 36, 96). Such interventions may include reducing lifestyle mediators of allostatic load (19, 97).

![Figure 1](image-url)
coping, and immune change in response to stress (100, 101). Moreover, it has been found to be protective against the development of depressive symptoms in later life (102). Its potential role in buffering against the negative emotional consequence of adverse events has led to a view of optimism as an index of resilience (103). Optimists may also choose lifestyles that promote physical as well as mental health, thereby reducing other aspects of allostatic load.

Healthy lifestyle, similar to optimism, provides a solid foundation for adaptation, and increases available resources for buffering the neurodegenerative effects of stress. Specifically, previous literature highlights the importance of healthy diet, adequate sleep, avoidance of smoking, and sufficient exercise (104). A population-based study reported higher emotional well-being among physically active youths, independent of social class and health status (105). Across a 2-year period, Motl and colleagues (106) found changes in physical activity were inversely related to a change in depressive symptoms. Levels of physical activity in childhood can modulate the risk of adult depression (107). Exercise modulates many of the core biomarkers of neuroprogression, including inflammation, oxidative stress, and neurotrophins (108). Poor eating habits and sleep have been linked to the manifestation of toxic stress and unhealthy growth in pediatrics by disrupting the architecture of the plastic, adaptive brain (109). There is now extensive evidence that poor diet quality is a risk for adolescent depression (110), and new data suggests that maternal diet influences the mental health of offspring (111). Similarly, smoking increases the risk of mood and anxiety disorders, and appears to influence similar biological pathways (112, 113). Parents and care givers of younger children need to be informed of the potential impact that a healthy lifestyle can have in mitigating mood-related symptoms and problematic behaviors. Low-risk interventions such as those aforementioned are critical for enhancing both psychological and biological resilience to stress. When such perspectives and lifestyle health behaviors are consolidated early in childhood and adolescence, the cumulative effect may be meaningful (103).

CONCLUSION

Early intervention offers the possibility of altering the trajectory of mood disorder pathology. In so doing, we may curtail the progressive nature of the illness, both through neuroprotection and prevention of peripheral health. Prevention and intervention treatments should go beyond stabilizing mood to include various and complementary strategies for reducing allostatic load, perhaps through psychoeducation and lifestyle-related interventions, including effective stress management. The combination of these techniques with specific pharmacotherapies may significantly improve functional outcomes by both reducing cellular insults and enhancing resilience. In so doing, this optimizes the capacity for maintenance of synaptic integrity and cellular resilience, which must be aggressively targeted as a therapeutic strategy during the prodromal stage of mood disorder pathology (90). This neuroprotective approach not only slows neuroprogression associated with the disease, but lays a foundation for more treatment-responsive outcomes during later stages.

AUTHOR CONTRIBUTIONS

Adam J. Walker, Yesul Kim, J. Blair Price, Rajas P. Kale, Jane A. McGillivray, Michael Berk, and Susannah J. Tye each made contributions to the writing of this manuscript.

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REFERENCES


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Early stage affective disorders


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This article was submitted to Neuropsychiatric Imaging and Stimulation, a section of the journal Frontiers in Psychiatry. Copyright © 2014 Walker, Kim, Price, Kale, McGillivray, Berk and Tye. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction which does not comply with these terms.
1a. Acclimatization & Surgery + Post-Op 21d of ACTH or Vehicle IPs begin OFT FST-t FST Phase 2 7d of HF DBS begin OFT FST-t FST

DAYS 1 14 15 16 IMIPRAMINE CHALLENGE 21 22 DBS EFFICACY

1b. ACTH DBS Electrode Tract DBS Electrode Tract
Figure 1.
Figure 3.

- For p/CaMKIIα, $r^2 = 0.76$, $p = 0.05$.
- For p/CaMKIIβ, $r = 0.58$, $p = 0.13$.
- For p/GSK3α, $r^2 = 0.91$, $p = 0.01$.
- For p/GSK3β, $r^2 = 0.83$, $p = 0.03$.
- For p/AMPK, $r^2 = 0.70$, $p = 0.07$. 

Note: The figure shows scatter plots for each of the phosphorylation states with corresponding correlation coefficients and p-values.
Figure 4.

- **p/CaMKII**
  - p = 0.01
  - $r^2 = 0.72$

- **p/CaMKIIβ**
  - p = 0.007
  - $r = 0.78$

- **p/GSK3α**
  - p = 0.008
  - $r^2 = 0.78$

- **p/GSK3β**
  - p = 0.027
  - $r^2 = 0.65$

- **p/AMPK**
  - p = 0.01
  - $r^2 = 0.73$
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Table 3

Linear regression analyses for the LHb region

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New Table 1:

Primer sequences for RT-PCR analysis of the target genes identified through functional gene enrichment.

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<td><a href="mailto:ysk@deakin.edu.au">ysk@deakin.edu.au</a></td>
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If there are multiple authors, give a full description of HDR thesis author’s contribution to the publication (for example, how much did you contribute to the conception of the project, the design of methodology or experimental protocol, data collection, analysis, drafting the manuscript, revising it critically for important intellectual content, etc.)

My contributions toward this book chapter publication includes structural outline of the chapter, design of the figures, drafting of the manuscript, critical evaluation of intellectual content and overall edits.

**I declare that the above is an accurate description of my contribution to this paper, and the contributions of other authors are as described below.**

<table>
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<th>Signature and date</th>
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</table>

## 4. Description of all author contributions

<table>
<thead>
<tr>
<th>Name and affiliation of author</th>
<th>Contribution(s) (for example, conception of the project, design of methodology or experimental protocol, data collection, analysis, drafting the manuscript, revising it critically for important intellectual content, etc.)</th>
</tr>
</thead>
</table>
| Katheryn M. O’Connor                                               | Design of the figures  
Department of Psychiatry & Psychology, Mayo Clinic, Rochester MN, United States  
Drafting of the manuscript |
| Susannah J. Tye                                                    | Conception of the book chapter  
Department of Psychiatry & Psychology, Mayo Clinic, Rochester MN, United States  
Preparation and critical evaluation of the manuscript |
5. Author Declarations

I agree to be named as one of the authors of this work, and confirm:

i. that I have met the authorship criteria set out in the Deakin University Research Conduct Policy,

ii. that there are no other authors according to these criteria,

iii. that the description in Section 4 of my contribution(s) to this publication is accurate,

iv. that the data on which these findings are based are stored as set out in Section 7 below.

If this work is to form part of an HDR thesis as described in Sections 2 and 3, I further

v. consent to the incorporation of the publication into the candidate’s HDR thesis submitted to Deakin University and, if the higher degree is awarded, the subsequent publication of the thesis by the university (subject to relevant Copyright provisions).

<table>
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<th>Name of author</th>
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<td>Susannah J. Tye</td>
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6. Other contributor declarations

I agree to be named as a non-author contributor to this work.

<table>
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<th>Name and affiliation of contributor</th>
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This form must be retained by the executive author, within the school or institute in which they are based.

If the publication is to be included as part of an HDR thesis, a copy of this form must be included in the thesis with the publication.
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<td><a href="mailto:ysk@deakin.edu.au">ysk@deakin.edu.au</a></td>
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<td>Department of Psychiatry and Psychology, Mayo Clinic (200 1st St SW, Rochester, MN 55902, USA)</td>
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HDR thesis author’s contribution to the publication include majority of behavioural data collection, conducted all of the data analysis, interpretation of the results, drafting of the manuscript and revising it critically for significant intellectual content.

I declare that the above is an accurate description of my contribution to this paper, and the contributions of other authors are as described below.

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</thead>
</table>
| Sean McGee                             | Conception of the project  
Molecular and Medical Research  
Strategic Research Centre, School of Medicine, Faculty of Health, Deakin University  
Design of methodology and experimental protocol  
Review and interpretation of results  
Revision of the manuscript |

| Juliane K. Czeczor | Mitochondrial data collection & analysis  
Molecular and Medical Research  
Strategic Research Centre, School  
Revision of the manuscript |
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<td>Behavioural data collection, Revision of the manuscript</td>
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<tr>
<td>Rajas Kale</td>
<td>School of Engineering, Faculty of Science Engineering &amp; Built Environment, Deakin University</td>
<td>Behavioural data collection, Revision of the manuscript</td>
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<tr>
<td>Shari Sutor</td>
<td>Translational Neuroscience Lab, Department of Psychiatry &amp; Psychology, Mayo Clinic, Minnesota, USA</td>
<td>Behavioural data collection, H&amp;E Staining, Revision of the manuscript</td>
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<tr>
<td>Ken Walder</td>
<td>Molecular and Medical Research Strategic Research Centre, School of Medicine, Faculty of Health, Deakin University</td>
<td>Conception of the project, Design of methodology and experimental protocol, Review and interpretation of results, Revision of the manuscript</td>
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<td>Abbas Kouzani</td>
<td>School of Engineering, Faculty of Science Engineering &amp; Built Environment, Deakin University</td>
<td>Conception of the project, Design of methodology and experimental protocol, Revision of the manuscript</td>
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<td>Michael Berk</td>
<td>IMPACT Strategic Research Centre, School of Medicine, Faculty of Health, Deakin University</td>
<td>Conception of the project, Design of methodology and experimental protocol, Review and interpretation of results, Revision of the manuscript</td>
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<tr>
<td>Susannah J. Tye</td>
<td>Translational Neuroscience Lab, Department of Psychiatry &amp; Psychology, Mayo Clinic, Minnesota, USA</td>
<td>Principle Investigator of the project – overseeing the different stages of the experiment, Conception of the project, Design of methodology and experimental protocol, Review and interpretation of results, Revision of the manuscript</td>
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<th>Yes / No</th>
<th>If Yes, please complete Section 3 If No, go straight to Section 4.</th>
</tr>
</thead>
</table>

## 3. HDR thesis author’s declaration

<table>
<thead>
<tr>
<th>Name of HDR thesis author if different from above. (If the same, write “as above”)</th>
<th>School/Institute/Division if based at Deakin</th>
<th>Thesis title</th>
</tr>
</thead>
<tbody>
<tr>
<td>As above</td>
<td>School of Psychology, Deakin University</td>
<td>Antidepressant mechanisms of deep brain stimulation for treatment resistant depression</td>
</tr>
</tbody>
</table>

If there are multiple authors, give a full description of HDR thesis author’s contribution to the publication (for example, how much did you contribute to the conception of the project, the design of methodology or experimental protocol, data collection, analysis, drafting the manuscript, revising it critically for important intellectual content, etc.)

HDR thesis author’s contribution to the publication include conception of the project (with the guidance from the principle investigator –Dr. Susannah Tyte), the design of experimental protocol, data collection and subsequent analysis, drafting of the manuscript and finally critical revisions for important intellectual content and significance of the findings to the relevant field.

I declare that the above is an accurate description of my contribution to this paper, and the contributions of other authors are as described below.

<table>
<thead>
<tr>
<th>Signature and date</th>
<th>____________________________</th>
<th>10.29.15</th>
</tr>
</thead>
</table>

## 4. Description of all author contributions

<table>
<thead>
<tr>
<th>Name and affiliation of author</th>
<th>Contribution(s) (for example, conception of the project, design of methodology or experimental protocol, data collection, analysis, drafting the manuscript, revising it critically for important intellectual content, etc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chunling Hu&lt;br&gt;Department of Psychiatry and Psychology, Mayo Clinic, Rochester, MN USA</td>
<td>Molecular data collection&lt;br&gt;Critical revision and preparation of the manuscript</td>
</tr>
<tr>
<td>Linda Byrne&lt;br&gt;School of Psychology, Deakin University</td>
<td>Critical revision and preparation of the manuscript</td>
</tr>
<tr>
<td>Mark A. Frye</td>
<td>Critical revision and preparation of the manuscript</td>
</tr>
</tbody>
</table>
5. Author Declarations
I agree to be named as one of the authors of this work, and confirm:

i. that I have met the authorship criteria set out in the Deakin University Research Conduct Policy,

ii. that I have no other authors according to these criteria,

iii. that the description in Section 4 of my contribution(s) to this publication is accurate,

iv. that the data on which these findings are based are stored as set out in Section 7 below.

If this work is to form part of an HDR thesis as described in Sections 2 and 3, I further

v. consent to the incorporation of the publication into the candidate’s HDR thesis submitted to Deakin University and, if the higher degree is awarded, the subsequent publication of the thesis by the university (subject to relevant Copyright provisions).

Name of author | Signature* | Date
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Chunling Hu | Signature Redacted by Library | 10.27.15
Linda Byrne |  | 
Mark A. Frye | Signature Redacted by Library | June 11 2015
Susannah J. Tye | Signature Redacted by Library | June 11 2015

6. Other contributor declarations
I agree to be named as a non-author contributor to this work.

Name and affiliation of contributor | Contribution | Signature* and date
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* If an author or contributor is unavailable or otherwise unable to sign the statement of authorship, the Head of Academic Unit may sign on their behalf, noting the reason for their unavailability, provided there is no evidence to suggest that the person would object to being named as author.

7. Data storage
The original data for this project are stored in the following locations. (The locations must be within an appropriate institutional setting. If the executive author is a Deakin staff member and data are stored outside Deakin University, permission for this must be given by the Head of Academic Unit within which the executive author is based.)

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Yesul Kim

completed a twelve-month appointment,
from February 4, 2013, through
January 31, 2014,
as a Research Trainee in the
Department of Psychiatry and Psychology

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Donna Cahill
Temporary Professional Personnel Office

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